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Delivered a talk on my work entitled "Homology modelling of Lactate dehydrogenase of Trichomonas vaginalis by using swiss-model & what if" At 1st Chandigarh Science Congress. March 10-11, 2007, Chandigarh, India

MANUSCRIPTS UNDER PREPARATION:

Kumar A, and Mukhopadhyay T; Identification of a novel small molecule drug that selectively kills prostate cancer cells.

Kumar A, and Mukhopadhyay T; A novel small molecule targeting the p53–MDM2 interaction to induce apoptosis in lung cancer cell line (a Bioinformatics approach).

Kumar A, and Mukhopadhyay T; SRD5A2 role in the cell migration and apoptosis in DU145 and PC3.
Synthesis, antiproliferative, acute toxicity and assessment of antiandrogenic activities of some newly synthesized steroidal lactams

Neelima Dhingra, T.R. Bhardwaj, Neeraj Mehta, Tapas Mukhopadhyay, Ashok Kumar

1. Introduction

Benign prostatic hyperplasia (BPH) is the nonmalignant enlargement of the prostate gland with an increase in numbers of both epithelial and stromal cells within the periurethral transition zone of the prostate, resulting in the constriction of prostatic urethra [1]. The prevalence increases to 50% by the age of 60 years and to 90% by the age of 85 years [2].

Abnormal increase in the number of cells in prostate may result not only from increased cell proliferation but also from decreased level in programmed cell death (apoptosis) [3]. Cells die in response to developmental signals and the process is characterized by number of biochemical changes. Any influence between the physiological process of cell proliferation and cell death may lead to change in prostate size with the subsequent development of abnormalities in the gland [4]. So it is reasonable to assume that cytotoxic agents are able to induce apoptosis, cause significant decrease in proliferation rate and are useful for the treatment of disease that involve abnormal or uncontrolled cell proliferation.

Nature treasure has an abundant source of cytotoxic agents obtained from various plant sources like Paclitaxel [5], Thapsia garganica [6] and extract of Vitex agnus-castus fruit [7]. Number of semi-synthetic derivatives like vinblastine [8], doxorubicin A [9], fluorouracil carbazoles [10], certain derivatives of quinoline [11] have also been reported as therapeutic agents for the treatment of symptomatic BPH. Various synthetic derivatives of suberoylanilide hydroxamic acid [12], 2-arylthiazolidine-4-carboxylic acids [13] etc. has been reported to possess significant cytotoxic property. Though treatment with standard cytotoxic agents does provide some palliative relief but are associated with systemic toxicity.

The management of BPH has undergone a rapid evolution over the past decade to aid men with lower urinary tract symptoms attributed to bladder outlet obstruction. Although not fully defined, the sources of symptoms in patient with BPH appear to be both static and dynamic component [14,15]. Treatment of clinical BPH aims to improve symptoms, prevent urinary tract infections, avoid renal insult, relief obstruction and improve bladder emptying. Great strides in the development of antiandrogen have fueled this evolution.
The biological basis of androgen ablation therapy lies in the observation that the embryonic development of the prostate is dependent on the androgen [16]. Steroidal 5α-reductase is a NADPH dependent enzyme that catalyzes the irreversible conversion of 4-ene-3-oxosteroid i.e. testosterone (7) to the corresponding 5α-H-3-oxosteroid i.e. dihydrotestosterone (DHT) (Fig. 1) [17]. Two isozymes of 5α-reductase have been cloned, expressed and characterized based on difference in chromosomal localization, tissue expression pattern and biochemical properties [18,19]. Therefore, 5α-reductase inhibitors represent one of the mainstay interventions in the treatment of BPH. During the last two decades a number of non-steroidal [20] and steroidal compounds [21,22] have been prepared as competitive or non-competitive inhibitors of 5α-reductase. Of these, 4-aza steroids were found to possess comparatively high inhibitory activity as exemplified by Finasteride (MK-906) (1) (Fig. 2) [23]. Finasteride was the first 5α-reductase inhibitor clinically approved in 1992 in U.S. for the treatment of BPH. It has demonstrated its biochemical efficacy with an 80% reduction of intraprostatic DHT and a 28% reduction in prostate size in patients with BPH and this compound is currently used for the treatment of BPH [24,25]. A series of other 17-substituted 4-azasteroids were also studied for 5α-reductase inhibitory activity. This led to the development of Dutasteride (2), by Glaxo Smith Kline in 2002, a new dual inhibitor able to reduce the DHT level by 85% [26].

The 5α-reductase inhibitory activity of these azasteroids is considered to be attributed by the lactam in ring A of the steroidal nucleus that mimics intermediate transition state [27]. Other inhibitors which look promising are the steroidal SK& F 105 687 (3) [27] and turosteride (4) [28] and progesterone ester (5) [29]. Recently our laboratory has reported 3D-QSAR SOMFA studies focused on refining the molecular architecture of new steroidal inhibitors of human 5α-reductase for the management of BPH [30,31].

Thus aiming at the discovery of potent, selective anti-proliferative agent with reduced toxicity and active 5α-reductase inhibitor, steroid molecules were utilized as biological vector for chemotherapeutic agents. Given the significance of lactam in some of clinically proved drugs (1,2) and ester group in 5 with increased antiandrogenic activity, we reasoned to synthesize compounds with general structure having lactam in ring A and various esters at 3β position of androsten nucleus. The synthesized compounds
were evaluated for antiproliferative activity and effect on serum androgen level.

2. Results and discussion

2.1. Chemistry

For the syntheses of compounds 13–22, 3β-hydroxy-17a-aza-o-homo-5-androsten-17-one (12) was used as starting material. The 12 was synthesized from commercial available (25R)-5-spirosten-3β-ol (Diosgenin) (6) according to the literature as shown in Fig. 3 [32-34]. Esters 13–22 of 3β-hydroxy-17a-aza-o-homo-5-androsten-17-one (12) were prepared by treating 3β-hydroxyl function with various acids in dichloromethane in presence of dicyclohexylcarbodiimide (DCC) [35].

2.2. In vitro antiproliferative activity using cell lines DU-145

Compounds were tested for antiproliferative activity using DU-145 cell line as described by Mosamann [36]. All the compounds were tested at five different concentrations in the culture medium and Finasteride (1) was used as reference drug. The percentage viable cells and percentage growth inhibition value are presented in Fig. 4 and Table 1. Linear regressed line was drawn to calculate the concentration required to cause 50% inhibition in cell growth (IC50) (Table 2). The conclusions summarized in the following paragraph are based on the significance (P < 0.001).

In comparison to Finasteride, 17a-aza-o-homo-17-one based compounds reported in this paper revealed a general reduction in the level of cellular cytotoxicity. 17-Oxo-17a-aza-o-homo-5-androsten-3β-yl 4-nitrobenzoate (15) displayed a better cytotoxicity comparable with that of Finasteride in DU-145 cells. Compound 16 with amino group at para position instead of nitro exhibited similar activity as 15. More in the terms of p-substitution, groups like -OH (17), -Cl (19) and -CH3 (20) have been found to increase the potency, suggesting that para-position of phenyl ring can tolerate wide variety of groups. At low concentrations, compound 17, 19 and 20 showed strong activities and maintained a relatively high activity up to 2.0 µg/mL as comparable to Finasteride but there was no further significant increase in growth.

![Fig. 3. Synthesis of 17-oxo-17a-aza-o-homo-5-androsten-3β-yl esters (13–22).](image-url)
inhibition. This loss in potency may be due to saturation of cells.
Unsubstituted benzoxyloxy ester 14 was found to be 19 fold less active and the concentration required to cause 50% growth inhibition will be more than reference drug. Introduction of spacer like -CH2- and -OCH2- separating the phenyl ring and carboxylic acid furnished the compounds 21 and 22, but no significant increase in the percentage growth inhibition was observed. However, 18 has not shown any growth inhibition in prostate cells, further suggesting that unsubstituted (benzoate) and p-substituted benzene at 3j) of 17a-aza-D-homo-5-androsten-17-one plays an important role in providing potency and selectivity.

2.3. In vitro cytotoxicity using mouse macrophages (acute toxicity)

In vitro cytotoxicity using DU-145 cells in the preliminary evaluation of anti-cancer drugs enables us to select most potent compound, but cytotoxic agents however frequently exhibit unspecific toxicity. Nevertheless the ability to selectively kill the target cell remains a highly desirable property of potential new therapeutic cytotoxic agents. In this study, we have demonstrated the applicability of red dye uptake (MTT) assay using mouse macrophages (Ballo C) for in vitro toxicity testing of newly synthesized compounds [37]. The assay quantifies the viable cells after 24 h incubation of cells with five different concentrations. Fig. 5 demonstrated a direct and proportional relation between cell number and concentration. The results obtained from MTT assay were statistically significant (P < 0.001) and linear equation obtained allowed us to determine toxicity index (LC50). The summarized data is presented in Table 1.

Data from this study clearly indicated that compounds 13, 17 and 19 with high LC50 values were not toxic to mouse macrophages. We found that low doses of 17 upon 24 h of exposure induced more than 20% of cell mortality and further there was not significant decrease in the number of viable cell over the range of five concentrations, whereas the viability of cells were hardly reduced to 60% with 5 µg/mL of 13 and 19. The prepared analogue 14 was less toxic to mouse macrophages showing LC50 of 43.65 µM.

Acute toxicity of the compounds 15, 16, 20 and 21 was comparable to Finasteride while the toxicity of compounds 18 and 22 was about 1.5–4 times higher than that of reference drug.

2.4. In vivo effect on steroid androgen level

Enzyme involved in the biosynthesis and metabolism of testosterone are attractive target for designing and development of the drugs to be useful in treatment of BPH as indicated in Fig. 1. Intact male rats (Sprague Dawley, 200–250 g) were used in the designed study in which various compounds were compared for in vivo effect on serum androgen level, as judged by the their ability to attenuate the conversion of T into DHT (Fig. 2). ELSA for T was found to be suitable for determination in serum of rats since the cross reactive DHT levels were extra low in male. The procedure measure T equally well and method met all the requirements of precision, accuracy, sensitivity and selectivity [38].

Serum T level were increased in Finasteride treated rats (1.26 ± 0.02 ng/mL compared with 0.742 ± 0.07 ng/mL control rats). It is apparent from Fig. 6 that almost all the ester derivatives of 3β-hydroxy-17a-aza-D-homo-5-androsten-17-one have increased the serum T level as compared to control. The effect on potency of different substratats at para-position of phenyl ring is in approximate order of -NO2 (15) > -NH2 (16) > -Cl (20) > -OH (17) > -Cl(19) except for -OCH3 (18) where no significant change in concentration of serum T has been found. Analogues 13 and 18 with poor antiproliferative activity showed satisfactory increased level of T up to 0.8 ± 0.029 and 1.05 ± 0.068 ng/mL. It is worth mentioning that 17a-aza-D-homo-5-androsten-17-one, steroids substituted at 3β with benzoate or p-substituted benzoate offer the optimal A ring substitution pattern for good antiandrogenic activity. This increased biological activity of 17a-aza-D-homo-5-androsten-17-one steroids may be because of formation of product like transition state. These results are consistent with earlier observations of increased activity of 4-aza steroids as 5α-reductase inhibitor [39].
Table 2
Inhibitory concentrations of the investigated compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finasteride</td>
<td>3.9</td>
</tr>
<tr>
<td>13</td>
<td>N.aNd</td>
</tr>
<tr>
<td>14</td>
<td>N.aNd</td>
</tr>
<tr>
<td>15</td>
<td>9.5</td>
</tr>
<tr>
<td>16</td>
<td>5.2</td>
</tr>
<tr>
<td>17</td>
<td>7.1</td>
</tr>
<tr>
<td>18</td>
<td>5.7</td>
</tr>
<tr>
<td>19</td>
<td>8.3</td>
</tr>
<tr>
<td>20</td>
<td>7.5</td>
</tr>
<tr>
<td>21</td>
<td>7.1</td>
</tr>
<tr>
<td>22</td>
<td>N.aNd</td>
</tr>
</tbody>
</table>

Nd = not determined.

4. Experimental section

4.1. Chemistry

The melting points were determined on Veego melting point apparatus and are uncorrected. Proton nuclear magnetic resonance (1H NMR) spectra were obtained using Bruker AC-300F, 300 MHz and Bruker AC-400F, 400 MHz spectrometer for solutions in deuteriochloroform, deuterated dimethylsulfoxide and are reported as ppm, downfield from tetramethylsilane (TMS) as internal standard. The spin multiplicities are indicated by the symbols, s (singlet), d (doublet), t (triplet), q (quartlet), m (multiplet) and br (broad). Infrared (IR) spectra were obtained with Perkin-Elmer 882 Spectrometer and XRL FT-IR model using a potassium bromide pellets (in cm⁻¹). The ultraviolet spectra were recorded on Perkin-Elmer, Lambda 15 spectrophotometer. Elemental analyses were carried out on a Perkin-Elmer 2400 CHN elemental analyzer. Reactions were monitored and the homogeneity of the products was checked by TLC. Plates for thin layer chromatography (TLC) were prepared with silica gel G and activated at 110°C for 30 min. Silica gel G60 F aluminum sheets plates were used for final monitoring. The plates were developed by exposure to iodine vapour. Anhydrous sodium sulphate was used as drying agents. All the solvents were dried and freshly distilled prior to use according to standard procedure.

4.1.1. General procedure for preparation of 13-22

To a stirred solution of 3-hydroxy-17a-aza-5-androsten-17-one (12) (0.5 g, 1.6 mmol) and dicyclohexylcarbodiimide (DCC) (0.34 g, 1.6 mmol) in anhydrous dichloromethane (30.0 mL) was added acid (1.6 mmol) and the mixture was stirred for 48 h at room temperature. Disappearance of the starting material and completion of the reaction were confirmed by TLC. The precipitated dichlorohexylurea (DCU) was filtered and solvent removed under vacuum. The resulting residue was crystallized from ethyl acetate: petroleum ether (60:80).

Table 3
Acute toxicity of the investigated compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>LC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finasteride</td>
<td>28.2</td>
</tr>
<tr>
<td>12</td>
<td>N.d</td>
</tr>
<tr>
<td>13</td>
<td>94.0</td>
</tr>
<tr>
<td>14</td>
<td>43.6</td>
</tr>
<tr>
<td>15</td>
<td>27.8</td>
</tr>
<tr>
<td>16</td>
<td>25.1</td>
</tr>
<tr>
<td>17</td>
<td>75</td>
</tr>
<tr>
<td>18</td>
<td>8.4</td>
</tr>
<tr>
<td>19</td>
<td>100.5</td>
</tr>
<tr>
<td>20</td>
<td>22.8</td>
</tr>
<tr>
<td>21</td>
<td>26</td>
</tr>
<tr>
<td>22</td>
<td>11.7</td>
</tr>
</tbody>
</table>

Nd = not determined.

Fig. 5. Toxicity of the compounds to mouse macrophages (Balb C). Cell viability was determined based on the MTT assay. Each point represents a mean value and SEM of 3 independent experiments. *p < 0.001 are significantly different compared to Finasteride according to one-way ANOVA followed by Tukey’s test.

3. Conclusions

In this paper various 3β esters of androsten having lactam in ring α are prepared and evaluated for antiproliferative effect on well established prostate cancer cells, acute toxicity on mouse macrophages and in vivo effect on serum androgen level.

Structure activity relationship from antiproliferative study revealed that 17α-aza-5-homo-5-androsten-17-one steroids possess significant antiproliferative activity. With respect to substitution, p-substituted benzoxyloxy (15, 16) were by far the most effective antiproliferative agents. However, the exact mechanism of action these compounds remain to be elucidated. The present study indicates that compounds possess a potential for development into anti-cancer drugs that may prove to be effective against prostate cells.

Among the compounds tested for acute toxicity using mouse macrophages, none of the compound has been found to be toxic to the normal cells even at high concentration except few of them. Thus preliminary evaluation in vitro cytotoxicity and toxicity studies enabled us to screen or select potent compounds with reduced toxicity such as 13, 15, 16, 17 and 20.

As the compounds were potent in vitro on the prostate cancer cells it was worthwhile to pursue with investigation of in vivo effect on serum androgen level by measuring the serum T level in rat model. Some of the selected compounds (15, 16, 17 and 20) showed potent antiandrogenic activity. It is noteworthy that steroid structural requirement for good antiandrogenic activity and antiproliferative activity are similar.

In vitro and in vivo experiments have been found to be very encouraging and that may prove as potential lead for the development of compounds to be used in the treatment of BPH with their control action towards prostate with their dual action by controlling the growth of prostate with their cytotoxicity activity towards prostate cell and by decreasing the DHT level without having negative effect on normal cells.
4.11.1. 17-Oxo-17α-aza-D-homo-5-androsten-3β-yl 4-nitrobenzoate (15). The compound (0.27 g, 1.6 mmol) was used to prepare 17-oxo-17α-aza-D-homo-5-androsten-3β-yl 4-nitrobenzoate compound (16) (0.22 g, 44.0%) by above described method: mp 172–175 °C; IR (KBr, cm–1): 3440, 2940, 1730, 1680, 1240; 1H NMR (400 MHz, CDCl3): δ 1.26 (s, 3H, 19-CH3), 1.45 (s, 3H, 18-CH3), 4.08 (m, 1H, 3α-H), 6.14 (br, 1H, 6-vinyl), 7.36 (1H, NH), 7.50 (d, J = 6.2, 2H, 3-CH and 5-CH aromatic) and 8.07 ppm (d, J = 8.0, 2H, 2-CH and 6-CH aromatic); 13C NMR (400 MHz, CDCl3): δ 171.45 (NHCO), 169.75 (COO), 154.87 (ArC-4), 141.06 (C-5), 129.68 (2ArCH), 120.06 (ArC-1), 113.37 (2ArCH), 120.32 (C-6), 70.77 (C-3), 53.63 (CH3-0), 24.54 (C-19) and 20.38 ppm (C-18). Anal. Calcd. for C26H33 N04 (%): N, 3.31. Found: N, 3.03.

4.11.1.7. 17-Oxo-17α-aza-D-homo-5-androsten-3β-yl 4-chlorobenzoate (19). The compound (0.29 g, 16 mmol) was used to prepare 17-oxo-17α-aza-D-homo-5-androsten-3β-yl 4-chlorobenzoate (19) (0.3 g, 60.0%) by above described method: mp 172–175 °C; IR (KBr, cm–1): 3320, 2950, 1722, 1680, 1240; 1H NMR (400 MHz, CDCl3): δ 1.26 (s, 3H, 19-CH3), 1.45 (s, 3H, 18-CH3), 4.09 (m, 1H, 3α-H), 6.14 (br, 1H, 6-vinyl), 7.36 (1H, NH), 7.50 (d, J = 6.2, 2H, 3-CH and 5-CH aromatic) and 8.07 ppm (d, J = 7.2, 2H, 2-CH and 6-CH aromatic); 13C NMR (400 MHz, CDCl3): δ 171.23 (NHCO), 168.41 (COO), 156.31 (ArC-4), 141.09 (C-5), 131.91 (2ArCH), 128.83 (2ArCH), 126.90 (ArC-1), 120.30 (C-6), 70.70 (C-3), 56.95 (CH3-0), 24.54 (C-19) and 20.32 ppm (C-18). Anal. Calcd. for C26H33 N04 Cl (%): N, 3.20. Found: N, 3.02.

4.11.1.8. 17-Oxo-17α-aza-D-homo-5-androsten-3β-yl 4-methylbenzoate (20). The compound (0.32 g, 64.0%) was prepared using 4-methoxybenzoic acid (0.32 g, 16 mmol) by above described method: mp 165–172 °C; IR (KBr, cm–1): 3320, 2950, 1722, 1680, 1240; 1H NMR (400 MHz, CDCl3): δ 1.26 (s, 3H, 19-CH3), 1.45 (s, 3H, 18-CH3), 4.09 (m, 1H, 3α-H), 6.14 (br, 1H, 6-vinyl), 7.36 (1H, NH), 7.50 (d, J = 6.2, 2H, 3-CH and 5-CH aromatic) and 8.07 ppm (d, J = 7.2, 2H, 2-CH and 6-CH aromatic); 13C NMR (400 MHz, CDCl3): δ 171.18 (NHCO), 167.44 (COO), 150.19 (ArC-4), 141.05 (C-5), 133.80 (2ArCH), 123.47 (2ArCH), 120.07 (C-6), 70.46 (C-3), 24.91 (C-19) and 20.07 ppm (C-18). Anal. Calcd. for C26H33 N04 Cl (%): N, 3.17. Found: N, 3.52.
(m, 1H, 3α.-H), 5.36 (br, 1H, 6-vinylic) and 6.97 ppm (m, 5H, aromatic); 13C NMR (400 MHz, CDCl3): δ 170.54 (NHCO), 168.89 (COO), 151.65 (ArC-1), 140.75 (C-5), 129.75 (ArCH), 121.23 (ArC-4), 120.96 (C-6), 114.33 (2ArCH), 104.00 (s, C-18), 40.88 (s, C-19) and 20.38 ppm (C-18). Anal. Calcd. for C27H35NO3 (%): N, 3.32. Found: N, 3.48.

4.1.1.10. 17-Oxo-17a-aza-D-homo-5-androsten-3-yl phenoxyacetate (22). Phenoxycetic acid (0.24 g, 16 mmol) was used in above mentioned method to get the 17-oxo-17a-aza-D-homo-5-androsten-3-yl phenoxyacetate (22), 0.25 g, 50% yield; mp 192–195 °C; IR (KBr, cm⁻¹): 3270, 2930, 1735, 1700, 1420; 1H NMR (400 MHz, CDCl3): δ 10.88 (s, 1H, 18-CH), 10.4 (s, 3H, 19-CH3), 6.71 (m, 1H, 3α-H), 4.60 (s, 2H, OCH2); 5.41 (br, 1H, 6-vinylic) and 6.57 ppm (m, 5H, aromatic); 13C NMR (400 MHz, CDCl3): δ 170.67 (NHCO), 168.12 (COO), 151.65 (ArC-1), 139.75 (C-5), 129.70 (ArCH), 121.23 (ArC-4), 120.74 (C-6), 114.33 (2ArCH), 72.57 (C-3), 64.78 (OCH2COO–), 25.49 (C-19) and 20.57 ppm (C-18). Anal. Calcd. for C27H35NO3 (%): N, 3.20. Found: N, 3.48.

4.2. Biological evaluation

4.2.1. In vitro antiproliferative activity using cell lines DU-145

4.2.1.1. Chemical and biochemicals. All the chemicals were of reagent grade and were used without purification. Dulbecco’s modified eagle medium (DMEM), fetal bovine serum, sodium dihydrogen phosphate, disodium hydrogen phosphate and dimethyl sulfoxide were purchased from Hi Media (Mumbai). Finasteride was obtained as a gift sample from Cipla, Mumbai (India) and was of analytical grade (assay 99.9%). MTT for assay was obtained from Sigma-Aldrich Chemicals.

4.2.1.2. Cell culture and animals. Human prostate cancer cell line, DU-145 was procured from National Center for Cell Science (Pune, India) and cell line were grown in DMEM media supplemented with 10% heat inactivated fetal bovine serum, 100 μg/mL streptomycin and 100 μg/mL penicillin in a highly humidified atmosphere of 95% air with 5% CO2 at 37 °C in NUAIRE incubator.

Albino mice (laca strain) weighing 20–25 g of either sex and Sprague Dawley rats were procured from Central Animal House, Panjab University, Chandigarh. Animals were housed under standard conditions and allowed to free access to both food and water available ad libitum until used.

4.2.1.3. Samples. All synthesized steroidal compounds were dissolved in ethanol and diluted to appropriate concentration: 0.01, 0.5, 1.0, 2.0, 5.0 μg/mL from the two stock solutions of 1 mg/mL and 0.001 μg/mL Stocks were maintained at room temperature.

4.2.1.4. MTT assay. Newly synthesized compounds were evaluated for their growth inhibitory activity using MTT assay. This assay quantifies the viable cells by observing the reduction of tetrazolium salt, MTT to formazan crystals by the live cells. Based on the absorbance of the cell sample after the test is carried out, viable cells can be measured.

DU-145 cell line was used and cells were grown as described above. Cells were cultured at a density of 5 × 104 cells/well in 96 well plates at 37 °C in 5% CO2 atmosphere and were allowed to attach for 24 h. The cells were treated in triplicate with graded concentration of sample and reference drug Finasteride at 37 °C for 48 h. A 20 μl aliquot of MTT solution was added directly to all the appropriate wells. Following 4 h of incubation at 37 °C, the media was removed and formazan crystals, which results from the reduction of MTT by active cell were dissolved in 100 μL DMSO and vigorously mixed to dissolve the reacted dye. The absorbance of each well was read on ELISA plate reader (Merrick) at 570 nm. The spectrometer was calibrated to zero absorbance using culture medium without cells. The relative cell viability (%) related to control well containing cell culture medium without drug was calculated by [A] test/[A] control × 100.

% Growth Inhibition = [OD]control- [OD] test/[OD]control × 100

% Cell Viability = [A]test/[A] control × 100

4.2.2. In vitro cytotoxicity using mouse macrophages (acute toxicity)

4.2.2.1. MTT assay. Cells (mouse macrophages) were used as normal cells and plated at a density of 5 × 104 cells/well in 96 plates at 37 °C in 5% CO2. Cells were exposed in graded concentration of compounds at designated various concentration. Each concentration was tested in triplicate wells. After 48 h fresh MTT (20 μg/1 mg/mL) was added directly to all the wells and culture was incubated for 4 h at 37 °C. During this incubation, MTT was converted into a water insoluble formazan complex by metabolic activity of viable cells. Formazan crystal were taken and dissolved in 100 μL of DMSO, which give light pink color. The absorbance of each well was read on ELISA plate reader at 570 nm. The spectrometer was calibrated to zero absorbance using culture medium without cells. The relative cell viability (%) related to control well containing cell culture medium without drug was calculated by [A] test/[A] control × 100.
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References

17-Oximino-5-androsten-3β-yl esters: synthesis, antiproliferative activity, acute toxicity, and effect on serum androgen level

Neelima Dhingra · Tilak Raj Bhardwaj · Neeraj Mehta · Tapas Mukhopadhyay · Ashok Kumar · Manoj Kumar

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Abstract The 17-oximino-5-androsten-3β-yl esters (10a–10j) were synthesized from commercially available (25R)-5-Spirosten-3β-ol (Diosgenin) (4) as starting material. The synthesized compounds were evaluated for their antiproliferative activity against prostate specific cancer cell line DU-145, acute toxicity, and effect on serum androgen level and were compared with Finasteride used as positive control. Some of the compounds exhibited better cytotoxicity and antiandrogenic activity than the reference control. The detailed synthesis, spectroscopic data, and biological evaluation for the synthesized compounds are reported.

Keywords Dihydrotestosterone · 5-Alpha reductase enzyme · Benign prostatic hyperplasia · Steroids · Androgen

Abbreviations

BPH Benign prostatic hyperplasia
T Testosterone
DHT Dihydrotestosterone
DCC Dicyclohexylcarbodiimide
DU-145 Prostate cancer cell line

Introduction

Benign prostatic hyperplasia is the nonmalignant enlargement of the prostate gland with increase in numbers of both epithelial and stromal cells within the periurethral transition zone of the prostate, resulting in the constriction of prostatic urethra (Bullock and Andriole, 2006). The prevalence increases to 50% by the age of 60 years and to 90% by the age of 85 years (Berry et al., 1984).

Abnormal increase in the number of cells in prostate may result not only from increased cell proliferation but also from decreased level in programmed cell death (apoptosis) (Isaacs and Coffey, 1989). Number of available cytotoxic agents are able to induce apoptosis, and thus, can cause significant decrease in proliferation rate and are useful for the treatment of disease that involves abnormal or uncontrolled cell proliferation (Perez-Stable, 2006; Jakobsen et al., 2001; Brady et al., 2002; Garsky et al., 2001; Gediya et al., 2005; Gududuru et al., 2005). Treatments with standard cytotoxic agents do provide some palliative relief, but are associated with system toxicity.

On the other hand, excessive production of dihydrotestosterone has been implicated in this pathological condition. Steroidal 5α-reductase is an NADPH-dependent enzyme that catalyzes the irreversible conversion of 4-en-3-oxosteroid, i.e., testosterone (T) to the corresponding 5α-H-3-oxosteroi d, i.e., dihydrotestosterone (DHT) (Fig. 1) (Bruchovsky et al., 1996). Two isozymes of 5α-reductase have been
Metabolism of Testosterone to 5α-Dihydrotestosterone

**Fig. 1** Mechanism of 5α-reductase enzyme

cloned, expressed, and characterized based on difference in chromosomal localization, tissue expression pattern, and biochemical properties (Bruchosky et al., 1996; Andersson and Russell, 1990). Therefore, 5α-reductase inhibitors represent one of the mainstay interventions in the treatment of benign prostatic hyperplasia. During the past two decades a number of non-steroidal (Occhiato et al., 2004) and steroidal compounds (Chen Li et al., 1994; Kenny et al., 1997) have been prepared as competitive or non-competitive inhibitors of 5α-reductase.

Hartmann synthesized number of pregnenolone (1, 2a-2e) (Fig. 2)-based steroids bearing an oxime group connected directly or via a spacer to the steroidal D ring, where the oxime group is capable to form a coordinate bond with heme iron of enzyme (Hartmann et al., 2000). On the other hand, progesterone esters (3a, 3b) (Fig. 3) synthesized in Mexico laboratories exhibited high antiandrogenic activity (Cabeza et al., 2001).

Taking into consideration the 5α-reductase inhibitory activity of the reported oximes (1, 2a-2e) and importance of the ester group in 3a and 3b, it was considered of interest to introduce both the oxime and ester functionalities in the steroidal androstane skeleton. It is expected that such molecules will suitably bind with the enzyme. The literature has not mentioned much about the evaluation of cytotoxicity along with 5α-reductase inhibitory activity. Thus, in this study, we prepared a series of 17-oximino-5α-androsten-3β-ol ester steroids, and all the synthesized compounds were evaluated for antiproliferative activity, acute toxicity, and their effect on serum androgen levels.

**Results and discussion**

**Chemistry**

For the syntheses of compounds (10a-10j), 17-oximino-5α-androsten-3β-ol (9) was used as starting material. The 9 was synthesized from commercially available (25R)-5-spirosten-3β-ol (Diosgenin) (4) according to the literature (Scheme 1) (Mason and Kepler, 1945; Hershberg, 1948; Regan and Hayes, 1956). Representative esters (10a-10j) were prepared by treating 3β-hydroxy function with various acids in dichloromethane in the presence of dicyclohexylcarbodiimide (DCC). In the esterification reaction, DCC acts as dehydrating agent which forms an O-acylurea called acid-DCC complex, similar to an acid anhydride or acyl halide. This is followed by attack of alcohol on carboxylic carbon of acid-DCC complex, as a nucleophilic catalyst to give esters and dicyclohexyl urea as side product (March, 2001).

**Biological evaluation**

**Antiproliferative activity on DU-145**

 Newly synthesized compounds were evaluated for their antiproliferative activity in comparison to reference drug Finasteride at five different concentrations using prostate specific cancer cell line DU-145 (Mosmann, 1983). The percentage of viable cells and percentage growth inhibition values are presented in Fig. 4 and Table 1. Linear
Regression line was drawn to calculate the concentration required to cause 50% inhibition in cell growth (IC50) (Table 2). The conclusions summarized in the following paragraph are based on the significance (P < 0.001).

All the compounds (10a–10j) evaluated at National Centre for Human Genome Studies and Research, Panjab University, Chandigarh for antiproliferative activity against DU-145 cell line demonstrated a general reduction in the level of cellular cytotoxicity. Antiproliferative activity was optimal with unsubstituted analog (phenyl ring) and its para-substituted analogs. Compounds 10b–10e, 10g, 10h, and 10j were found to be more active with over 80%
growth inhibition at concentration of 5.0 µg/ml relative to Finasteride (78% at 5.0 g/ml), whereas compounds 10a (4.8 µm) and 10i (6.5 µm) showed somewhat lower activity than that of reference Finasteride. Structure activity relationship from present antiproliferative study demonstrated that unsubstituted phenyl analog 10b and compounds 10d, 10e, and 10h with an electron donating moiety at para position found to be more potent than reference Finasteride. On the other hand, analogs 10c, 10g with electron withdrawing substituents demonstrated relatively less cytotoxicity. However, compounds 10a and 10i were mainly inactive. 17-Oximino-5-androsten-3β-y1 4-methoxybenzoate (10f) showed and maintained strong activity up to 2.0 µg/ml as comparable to Finasteride, but further there was no significant increase in growth inhibition.

In *in vitro* cytotoxicity using mouse macrophages (*acute toxicity*)

In *in vitro* cytotoxicity test using cancer cell lines in the preliminary evaluation of cytotoxic agents enables us to select most potent compound, but cytotoxic agents, however, frequently exhibit unspecific toxicity. Nevertheless, the ability to selectively kill the target cell remains a highly desirable property of potential new therapeutic cytotoxic...
Table 2 Inhibitory concentration of the investigated compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (µM)</th>
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</thead>
<tbody>
<tr>
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<td>3.9</td>
</tr>
<tr>
<td>9</td>
<td>Nd*</td>
</tr>
<tr>
<td>10a</td>
<td>4.8</td>
</tr>
<tr>
<td>10b</td>
<td>3.8</td>
</tr>
<tr>
<td>10c</td>
<td>3.07</td>
</tr>
<tr>
<td>10d</td>
<td>3.7</td>
</tr>
<tr>
<td>10e</td>
<td>2.9</td>
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<tr>
<td>10f</td>
<td>5.4</td>
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<tr>
<td>10g</td>
<td>3.4</td>
</tr>
<tr>
<td>10h</td>
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<tr>
<td>10i</td>
<td>6.5</td>
</tr>
<tr>
<td>10j</td>
<td>6.3</td>
</tr>
</tbody>
</table>

* Nd not determined

Table 3 Acute toxicity of the investigated compounds

<table>
<thead>
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<th>Compound</th>
<th>LC₅₀ (µL)</th>
</tr>
</thead>
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<tr>
<td>9</td>
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<tr>
<td>10a</td>
<td>28.0</td>
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<tr>
<td>10b</td>
<td>89.4</td>
</tr>
<tr>
<td>10c</td>
<td>19.5</td>
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<tr>
<td>10d</td>
<td>29.4</td>
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<tr>
<td>10e</td>
<td>22.0</td>
</tr>
<tr>
<td>10f</td>
<td>14</td>
</tr>
<tr>
<td>10g</td>
<td>24.0</td>
</tr>
<tr>
<td>10h</td>
<td>22</td>
</tr>
<tr>
<td>10i</td>
<td>11.8</td>
</tr>
<tr>
<td>10j</td>
<td>11.5</td>
</tr>
</tbody>
</table>

* Nd not determined

In vitro toxicity of newly synthesized compounds was tested with Red dye uptake (MTT) assay (Valasinas et al., 2001). The assay quantifies the viable cells, after 24 h incubation of cells with five different concentrations. Figure 5 demonstrated a direct and proportional relation between cell number and concentration. The results obtained from MTT assay were statistically significant (P < 0.001) and linear equation obtained allowed us to determine toxicity index (LC₅₀). The summarized data have been presented in Table 3.

Concerning the toxicity of the compounds toward mouse macrophages, the results of our study clearly indicated that compounds 10b and 10j with high LC₅₀ values were non-toxic to mouse macrophages. Acute toxicity of the compounds 10a, 10d, 10e, 10g, and 10h was comparable to Finasteride while the toxicity of compounds 10c, 10i, and 10f was about 1.5 times higher than that of reference drug.

In vivo (effect on serum androgen level)

Enzymes involved in the biosynthesis and metabolism of testosterone are attractive target for designing and development of the drugs to be useful in treatment of benign prostatic hyperplasia (BPH) as indicated Fig. 1. Intact male rats (Sprague-Dawley, 200–250 g) were used in the designed study in which various compounds were compared for in vivo 5α-reductase inhibitory potency, as judged by their ability to attenuate the conversion of testosterone into dihydrotestosterone (DHT) (Gerhard, 2002; Hartmann et al., 2000). ELISA for T were found to be suitable for determination in the serum of rats since the cross-reactive DHT levels were quite low in males. The procedure measures T equally well, and method met all the requirements of precision, accuracy, sensitivity, and selectivity (Stahl et al., 1984).

The results of the various administrated compounds on the serum concentration level of testosterone have been presented in Fig. 6. All the compounds except for 10e and 10f have shown significant increase in serum testosterone level as compared to the control. Ester derivatives 10d and 10h with electron releasing group at α-position of phenyl ring have been found to possess increased activity. While the presence of electron withdrawing moiety at this position causes the loss of activity (10c and 10g). Significant decrease in activity has been found in the compound 10a and 10j with an extra methylene group.

Conclusion

Antaandrogenic activity of the newly synthesized esters of 17-oximino-5-androsten-3β-ol, together with acute toxicity and cytotoxicity, supports the fact that esterification of the hydroxy group at position 3 with p-substituted aromatic acid gives compounds better antiproliferative and antian- drogenic activity.
Experimental section

Chemistry

The melting points were determined on Veego melting point apparatus and are uncorrected. 1H-NMR spectra were obtained using Brucker AC-300F, 300 MHz and Brucker AC-400F, 400 MHz spectrometer for solutions in CDCl3, DMSO-d6 and are reported in parts per million (ppm), downfield from tetramethylsilane (TMS) as internal standard. The spin multiplicities are indicated by the symbols, s (singlet), d (doublet), t (triplet), q (quartlet), m (multiplet), and br (broad). Infrared (IR) spectra were recorded on Perkin-Elmer 882 Spectrum and RXI, FT-IR model using potassium bromide pellets (in cm⁻¹). The ultraviolet spectra were recorded on Perkin-Elmer, Lambda 15 spectrophotometer. Elemental analyses were carried out on a birkhAuser.

General procedure for preparation of compounds 10a–10j

To a stirred solution of 17-oximino-5-androsten-3-ol (9) (0.5 g, 1.6 mmol) and dicyclohexylcarbodiimide (DCC) (0.34 g, 1.6 mmol) in anhydrous dichloromethane (30.0 ml) was added acid (1.6 mmol) and the mixture was stirred for 48 h at room temperature. Disappearance of the starting material and completion of the reaction were confirmed by TLC. The precipitated dicyclohexylurea (DCU) was filtered, and solvent was removed under vacuum. The resulting residue was crystallized from ethyl acetate: petroleum ether (60:80).

17-Oximino-5-androsten-3β-yl chloroacetate (10a) 17-Oximino-5-androsten-3β-yl chloroacetate (10a) (0.24 g, 48.0%), was prepared by method as described above using chloroacetic acid (0.16 g, 1.6 mmol): mp 96–100°C; IR (KBr): 3490, 2940, 2820, 1750, 1700, and 1200 cm⁻¹; 1H-NMR (400 MHz, CDCl3): δ 1.01 (s, 3H, 18-CH3), 1.05 (s, 3H 19-CH3), 4.03 (s, CD3COO), 7.07 (m, 1H, 3α-H), and 5.40 ppm (br, 1H, 6-vinylic); Anal. Calcd for C26H34N203: Cl: N, 6.63. Found: N, 6.79.

17-Oximino-5-androsten-3β-yl 4-hydroxybenzoate (10d) The compound 10d (0.34 g, 1.6 mmol) was used to prepare 17-oximino-5-androsten-3β-yl 4-hydroxybenzoate compound (10c) (0.34 g, 64.0%) by above described method: mp 166–170°C; IR (KBr): 3300, 2930, 1700, 1650, and 1235 cm⁻¹; 1H-NMR (400 MHz, CDCl3): δ 3.85 (m, 1H, 3α-H), 5.40 (br, 1H, 6-vinylic), and 6.84–7.28 (m, 5H, aromatic); Anal. Calcd for C26H33N03: Cl: N, 3.44. Found: N, 3.74.

17-Oximino-5-androsten-3β-yl 4-aminobenzoate (10d) 4-Nitrobenzoic acid (0.2 g, 1.6 mmol) by above described method: mp 166–170°C; IR (KBr): 3300, 2930, 1700, 1650, and 1235 cm⁻¹; 1H-NMR (400 MHz, CDCl3): δ 0.89 (s, 3H, 18-CH3), 1.1 (s, 3H 19-CH3), 4.07 (m, 1H, 3α-H), 5.40 (br, 1H, 6-vinylic), and 6.84–7.28 (m, 5H, aromatic); Anal. Calcd for C26H34N203: Cl: N, 6.19. Found: N, 6.19.

17-Oximino-5-androsten-3β-yl 4-nitrobenzoate compound (10c) (0.34 g, 68.0%) by above described method: mp 166–170°C; IR (KBr): 3300, 2930, 1700, 1650, and 1235 cm⁻¹; 1H-NMR (400 MHz, CDCl3): δ 0.89 (s, 3H, 18-CH3), 1.03 (s, 3H 19-CH3), 3.85 (m, 1H, 3α-H), 5.4 (br, 1H, 6-vinylic), 6.65 (d, J = 6.8, 2H, 3-CH, and 5-CH aromatic), 7.87 (d, J = 7.0, 2H, 2-CH, and 6-CH aromatic); Anal. Calcd for C26H33N03: Cl: N, 3.69. Found: N, 3.52.

17-Oximino-5-androsten-3β-yl 4-nitrobenzoate compound (10c) (0.34 g, 68.0%) by above described method: mp 166–170°C; IR (KBr): 3300, 2930, 1700, 1650, and 1235 cm⁻¹; 1H-NMR (400 MHz, CDCl3): δ 0.89 (s, 3H, 18-CH3), 1.03 (s, 3H 19-CH3), 3.85 (m, 1H, 3α-H), 5.4 (br, 1H, 6-vinylic), 6.65 (d, J = 6.8, 2H, 3-CH, and 5-CH aromatic), 7.87 (d, J = 7.0, 2H, 2-CH, and 6-CH aromatic); Anal. Calcd for C26H33N03: Cl: N, 3.69. Found: N, 3.52.
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\[ \delta \text{ 0.89 (s, 3H, 18-CH\textsubscript{3}), 1.05 (s, 3H, 19-CH\textsubscript{3}), 2.17 (s, 1H, NOH), 4.18 (m, 1H, 3\textsuperscript{a}-H), 6.35 (br, 1H, 6-vinylic), 7.46 ppm (d, } J = 8.1, 2H, 3-CH, and 5-CH aromatic), and 7.46 ppm (d, } J = 7.9, 2H, 2-CH, and 6-CH aromatic); \] Anal. Calcd for C\textsubscript{27}H\textsubscript{35}NO\textsubscript{3}; N, 3.32. Found: N, 3.32.

**17-Oximino-5-androsten-3\textsuperscript{a}-yl 4-methoxybenzoate (10j)** 4-Methoxybenzoic acid (p-anisic acid) (0.24 g, 1.6 mmol) was used to prepare 17-oximino-5-androsten-3\textsuperscript{a}-yl 4-methoxybenzoate compound (10j) (0.31 g, 62.0%) by above described method: mp 167–170°C; IR (KBr): 3320, 2935, 1730, and 1250 cm\textsuperscript{-1}; \textsuperscript{1}H-NMR (400 MHz, CDC\textsubscript{13}): \delta 0.77 (s, 3H, 18-CH\textsubscript{3}), 0.84 (s, 3H 19-CH\textsubscript{3}), 3.78 (s, 3H, –OCH\textsubscript{3}), 4.06 (m, 1H, 3\textsuperscript{a}-H), 5.97 (br, 1H, 6-vinylic), 6.83 (d, } J = 7.9, 2H, 2-CH, and 6-CH aromatic); and 7.46 ppm (d, } J = 8.4, 2H, 2-CH, and 6-CH aromatic); Anal. Calcd for C\textsubscript{27}H\textsubscript{35}NO\textsubscript{4}; N, 3.20. Found: N, 3.07.

**17-Oximino-5-androsten-3\textsuperscript{a}-yl 4-chlorobenzoate (10g)** 4-Chlorobenzoic acid (0.22 g, 1.6 mmol) was used to prepare 17-oximino-5-androsten-3\textsuperscript{a}-yl 4-chlorobenzoate (10g) (0.3 g, 60.0%) by above described method: mp 118–122°C; IR (KBr): 3200, 2940, 1750, 1650, and 1220 cm\textsuperscript{-1}; \textsuperscript{1}H-NMR (400 MHz, CDCl\textsubscript{3}): \delta 0.89 (s, 3H, 18-CH\textsubscript{3}), 1.02 (s, 3H, 19-CH\textsubscript{3}), 1.05 (s, 3H, 18-CH\textsubscript{3}), 3.65 (s, 3H, –OCH\textsubscript{3}), 4.65 (m, 1H, 3\textsuperscript{a}-H), 5.45 (br, 1H, 6-vinylic), and 7.29 (m, 5H, aromatic); Anal. Calcd for C\textsubscript{27}H\textsubscript{33}NO\textsubscript{4}; N, 3.07. Found: N, 3.07.

**Biological evaluation**

**Chemicals and biochemicals**

Reagent grade chemicals were used without purification. Dulbecco’s modified eagle medium (DMEM), fetal bovine serum, sodium dihydrogen phosphate, disodium hydrogen phosphate, and dimethyl sulfoxide were purchased from Hi Media (Bombay). Finasteride was obtained as a gift sample from Cipla, Bombay (India) and was of analytical grade (assay 99.9%). MTT for assay was purchased from Sigma-Aldrich Chemicals.

**Cell culture and animals**

Human prostate cancer cell line, DU-145, was procured from National Centre for Cell Science (Pune, India), and cells were grown in DMEM supplemented with 10% heat inactivated fetal bovine serum, 100 μg/ml streptomycin, and 100 μg/ml penicillin in a highly humidified 5% CO\textsubscript{2} at 37°C in NUAIRE incubator.

Albino mice (laca strain) weighing 20–25 g of either sex and Sprague–Dawley rats were procured from Central Animal House, Panjab University, Chandigarh. Animals were housed under standard conditions and allowed to free access to both food and water available ad libitum until used.

**Samples**

All steroids were dissolved in ethanol and diluted to appropriate concentration: 0.01, 0.5, 1.0, 2.0, and 5.0 μg/ml from the two stock solutions of 1 mg/ml and 0.001 μg/ml. Stocks were maintained at room temperature.

**In vitro antiproliferative activity on DU-145 (MTT assay)**

Newly synthesized compounds were evaluated for their growth inhibitory activity using MTT assay. This assay quantifies the viable cells by observing the reduction of tetrazolium salt, MTT, to formazan crystals by the live cells. Based on the absorbance of the cell sample after the test is carried out, cell viable can be measured.
For this purpose DU-145 cell line was used, and cells were grown as described above. Cells were cultured at a density of $5 \times 10^4$ cells/well in 96-well plates at 37°C in 5.0% CO$_2$ atmosphere and were allowed to attach for 24 h. The cells were treated in triplicate with graded concentration of sample and reference drug Finasteride at 37°C for 48 h. A 20 µl aliquot of MTT solution was added directly to all the appropriate wells. Following 4 h of incubation at 37 µl, the media were removed and formazan crystals, which results from the reduction of MTT by active cell were dissolved in 100 µl DMSO and vigorously mixed to dissolve the reacted dye. The absorbance of each well was read on Elisa plate reader (Merck) at 570 nm. The spectrometer was calibrated to zero absorbance using culture medium without cells. The relative cell viability (%) related to control well containing cell culture medium without drug was calculated by $\frac{[A]_{test}}{[A]_{control}} \times 100$.

% Growth inhibition

$= \frac{[OD]_{control} - [OD]_{test}}{[OD]_{control}} \times 100$

$[OD]_{test} = $ absorbance test sample

$[OD]_{control} = $ absorbance control sample

In vitro cytotoxicity using mouse macrophages

[acute toxicity (MTT assay)]

Cells (mouse macrophages) were used as normal cells and plated at a density of $5 \times 10^4$ cells/well in 96 plates at 37°C in 5% CO$_2$. Cells were exposed to graded concentration of compounds at designated various concentrations. Each concentration was tested in triplicate wells. After 48 h, fresh MTT 20 µl (1 mg/ml) was added directly to all the wells and culture was incubated for 4 h at 37°C. During this incubation, MTT was converted into a water insoluble formazan complex by metabolic activity of viable cells. Formazan crystals were taken and dissolved in 100 µl of DMSO, which gives light pink color. The absorbance of each well was read on Elisa plate reader (Merck) at 570 nm. The spectrometer was calibrated to zero absorbance using culture medium without cells. The relative cell viability (%) related to control well containing cell culture medium without drug was calculated by $\frac{[A]_{test}}{[A]_{control}} \times 100$.

% Cell viability

$= \frac{[A]_{test}}{[A]_{control}} \times 100$

$[A]_{test} = $ absorbance test sample

$[A]_{control} = $ absorbance control sample

In vivo (effect on serum androgen levels)

In order to measure the serum androgen level, all the compounds were suspended in mixture of olive oil and ethanol (95:5) and administered once intraperitoneally equimolar to 40 mg/kg body weight of Finasteride. Animals were given corresponding amount of vehicle only. Animals were divided into 3 groups: vehicle (control), Finasteride (standard), and treated (test sample), and each group consists of 5 animals. Sprague–Dawley rats were treated with Finasteride and equimolar dose of compounds. After 6 h of treatment, blood was withdrawn by cardiac puncture under diethyl ether anesthesia and serum was separated from cells by centrifugation. Plasma testosterone values were obtained by ELISA plate reader at 450 nm and are given in ng/ml [38, 39].

Elisa

The aliquots of 50 µl of each of standards, control, and unknown (serum samples) were added to testosterone antibody-coated wells. 100 µl of HRP-testosterone conjugate was added to all the wells, and the plates were shaken gently on a shaker for proper mixing of the reagents. Following 4 h of incubation at 37°C, the incubation mixture was removed. The wells were washed with phosphate buffer for 5-6 times (200 µl each time), followed by addition of 100 µl of H$_2$O$_2$ substrate in each of the wells. The plates were further incubated at 37°C for 20 min. At the end of incubation, reaction was stopped by using 100 µl of 0.5 M H$_2$SO$_4$ as stopping reagent. The absorbance of each well was read on Elisa plate reader at 450 nm [40, 41].

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References


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