Arch Pharm Res Vol 34, No 7, 1055-1063, 2011
DOI 10.1007/s12272-011-0702-2

Archives of Pharmacal Research
www.springer.com/12272

Synthesis, Antiproliferative Activity, Acute Toxicity and Assessment of the Antiandrogenic Activities of New Androstane Derivatives

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(Received December 23, 2009/Revised July 30, 2010/Accepted August 8, 2010)

A number of 17-oxo-5-androsten-3β-yl esters (**9a-9f**) and 3β-alkoxy-5-androsten-17-ones (**11a-11e**) were synthesized from commercially available (25*R*)-5-spirosten-3β-ol (Diosgenin) (**4**) as starting material. The synthesized compounds were evaluated for their antiproliferative activity against the prostate-specific cancer cell line DU-145, acute toxicity and effect on serum androgen levels, and compared with finasteride as positive control. Some of the compounds exhibited better cytotoxicity and antiandrogenic activity than the reference control. The detailed synthesis, spectroscopic data and biological activity of the synthesized compounds are reported.

Key words: Five alpha reductase enzyme, Benign prostatic hyperplasia, Androstane derivatives, Androgens, Prostate 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 periurethal transition zone of the prostate, resulting in the constriction of prostatic urethra (Bullock and Andriole, 2006). The prevalence of this condition increases to 50% by the age of 60 and to 90% by the age of 85 years (Lowe, 2005).

An abnormal increase in the number of cells in the prostate may result not only from increased cell proliferation but also from a decreased level of programmed cell death (apoptosis) (Bruckheimer and Kyprianou, 2000). Cells die in response to development signals, and the process is characterized by a number of biochemical changes. Any influence between the physiological process of cell proliferation and cell death may lead to a change in prostate size, with the subsequent development of abnormalities in the gland (Djavan et al., 2002). Therefore, it is reasonable to assume that cytotoxic agents are able to induce apoptosis and cause a significant decrease in proliferation rate. Hence, they are useful for the treatment of diseases that involve abnormal or uncontrolled cell proliferation.

There is an abundance of natural cytotoxic agents which can be obtained from various plant sources, such as *Paclitaxel* (Perez-Stable, 2006), *Thapsia garganic* (Jakobsen et al., 2001) and the extract of Vitex agnus–Castus fruit (Weisskopf et al., 2005). A number of semi-synthetic derivatives like vinblastine (Brady et al., 2002), doxorubicin A (Garsky et al., 2001), fluoroindolocarbazoles (Balasubramanian et al., 2004), and certain derivatives of quinoline (Gomez-Monterrey et al., 2003) have also been reported as therapeutic agents for the symptomatic treatment of BPH. Various synthetic derivatives of suberoylanilide hydroxamic acids (Gediya et al., 2005), such as 2-arylthiazolidine-4-carboxylic acids (Gududuru et al., 2005) have also been reported to possess significant cytotoxic properties. Even though treatments with standard cytotoxic agents do provide some palliative relief, they are often associated with system toxicity.

The management of BPH has undergone a rapid

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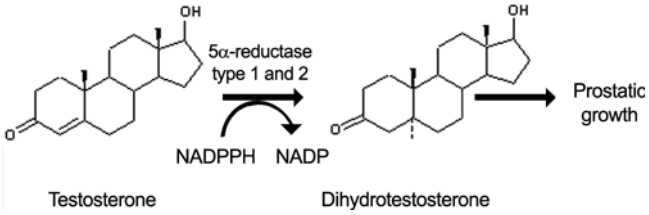

Fig. 1. Androgen dependent growth of prostate

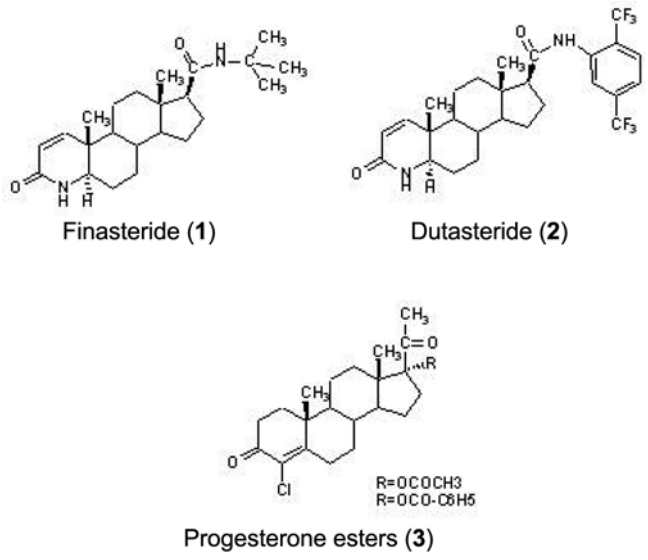

Fig. 2. Structure of potent reported compounds

evolution over the past decade to aid male subjects with lower urinary tract symptoms attributed to bladder outlet obstruction. Although not fully defined, symptoms in patients with BPH appear to obstruct adenoma (static or mechanical component) and/or contract smooth muscles under α-receptor mediated sympathetic stimulation (dynamic component) (Sraddha and Kourals, 2005; Lepor, 2009). Treatment of clinical BPH aims to reduce symptoms, relieve obstruction and improve bladder emptying, prevent urinary tract infections and avoid renal insult. The development of α–blockers and antiandrogens has contributed significantly to the management of BPH.

The biological basis of androgen ablation therapy lies in the observation that the embryonic development of the prostate is dependent on androgen (dihydrotestosterone) (Smith and Carson, 2009). Steroidal 5α-reductase is a NADPH dependent enzyme that catalyzes the irreversible conversion of 4-en-3-oxosteroid, i.e. testosterone (T) to the corresponding 5α-H-3-oxosteroid, i.e. dihydrotestosterone (DHT) (Fig. 1) (Georgianna and John, 1997). Two isozymes of 5α-reductase have been cloned, expressed and characterized based on differences in chromosomal localization, tissue expression patterns and biochemical properties (Andersson and Russell, 1990; Bruchovsky et al., 1996). Therefore, 5α-reductase inhibitors represent one of the primary interventions in the treatment of benign prostatic hyperplasia. During the last two decades a number of non-steroidal (Occhiato et al., 2004) and steroidal compounds (Li et al., 1995; Kenny et al., 1997) have been prepared as competitive or non-competitive inhibitors of 5a-reductase. Of these, 4-aza steroids were found to possess comparatively high inhibitory activity as exemplified by finasteride (**1**) (Fig. 2) (Weisser et al., 1994). Finasteride was the first 5a-reductase inhibitor clinically approved in 1992 in the USA for the treatment of benign prostatic hyperplasia. Its biochemical efficacy was demonstrated with an 80% reduction of intraprostatic DHT and 28% reduction in prostate size in patients with BPH and this compound is currently used for the treatment of benign prostatic hyperplasia (Brooks et al., 1994; Bratoeff et al., 1999). Several other 17–substituted 4-azasteroids were also studied for 5α-reductase inhibitory activity, which led to the development of dutasteride (**2**) by Glaxo Smith Kline in 2002. This was a new dual inhibitor able to reduce dihydrotestosterone levels by 85% (Tarter and Vaughan, 2006).

Progesterone esters (3) were found to exhibit high antiandrogenic activity (Cabeza et al., 2001). This phenomenon could be explained by taking into consideration that ester moiety forms covalent linkage with the enzyme in addition to compete with the binding site, thus irreversibly inhibiting enzymatic activity. A number of steroidal esters have been synthesized in our laboratories as antiproliferative and antiandrogens (Dhingra, 2008). Studies on these compounds have shown significant antiproliferative activity and increases in the serum androgen (T) level. Furthermore, Li et al. reported that 5α-reductase could be best inhibited by the compounds having structural similarity to natural substrates (Li et al., 1995).

Since the esters described above showed high biological activity and substrate (T) like analogues could better inhibit the enzyme, we describe the synthesis and biological activity of 17-oxo-5-androsten-3β-yl esters in this study. For structure activity relationships based on the reports by Bakshi and coworkers (Bakshi et al., 1996) on alkoxy steroidal derivatives on the antiproliferative activity on DU-145 cell line, we extended the study to include 3β-alkoxy-5-androsten-17-one derivatives.

MATERIALS AND METHODS

The melting points were determined on Veego melting point apparatus and are uncorrected. Proton (^{1}H) nuclear magnetic resonance (^{1}H-NMR) spectra were obtained using Brucker AC-300F, 300 MHz and Brucker AC-400F, 400 MHz spectrometer for solutions in deuteriochloroform, deuterated dimethylsulfoxide and are reported in parts per million (ppm), using 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 Spectrum and RXI, FT-IR model using potassium bromide pellets (in cm^{-1}). The ultraviolet spectra were recorded on a 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 were used for final monitoring. The plates were developed by exposure to iodine vapour. Anhydrous sodium sulphate was utilized as drying agents. All the solvents were freshly distilled and dried prior to use according to standard procedures.

General procedure for preparation of (9a-9f)

To a stirred solution of 3β-hydroxy-5-androsten-17-one (**8**) (0.5 g, 2.0 mmol) in dry pyridine (10.0 mL), freshly prepared acid chloride (2.0 mmol) was added and the mixture was stirred for 12 h at room temperature. Completion of the reaction was confirmed by TLC. The reaction mixture was poured into cold water (50.0 mL). The precipitated material was filtered, washed, dried and recrystallized from acetone to obtain the desired product.

17-Oxo-5-androsten-3β-yl benzoate (9a)

17-Oxo-5-androsten-3β-yl benzoate (**9a**) was prepared by the method described above using acid chloride of benzoic acid. Yield 76.0%; m.p. 78-80°C; IR (KBr, cm^{-1}): 2950, 1740, 1720, 1270; ^{1}H-NMR ($CDCl_3$) δ ppm: 1.03 (s, 3H, 18-C*H*$_3$), 1.1 (s, 3H, 19-C*H*$_3$), 4.86 (m, 1H, 3α-*H*), 5.45 (br, 1H, 6-vinylic), 7.43 (t, *J* = 7.5 Hz, 3H, 3-C*H*, 4-C*H* and 5-C*H* aromatic), 8.04 (d, *J* = 9.0 Hz, 2H, 2-C*H* and 6-C*H* aromatic); Calculated for $C_{26}H_{32}O_3$: C, 79.56; H, 8.22. Found: C, 79.39; H, 8.62.

17-Oxo-5-androsten-3β-yl 4-nitrobenzoate (9b)

Acid chloride of 4-nitrobenzoic acid was used to prepare 17-Oxo-5-androsten-3β-yl 4-nitrobenzoate compound **9b** by above described method. Yield 74.0%; m.p. 92-94°C; IR (KBr, cm^{-1}): 2940, 1725, 1270; ^{1}H-NMR ($CDCl_3$) δ ppm: 0.92 (s, 3H, 18-C*H*$_3$), 1.03 (s, 3H, 19-C*H*$_3$), 4.91 (m, 1H, 3α-*H*), 5.47 (br, 1H, 6-vinylic), 8.20 (d, *J* = 9.0 Hz, 2H, 3-C*H* and 5-C*H* aromatic) and 8.28 (d, *J* = 9.0 Hz, 2H, 2-C*H* and 6-C*H* aromatic); Calculated for $C_{26}H_{31}NO_5$: N, 3.20. Found: N, 3.60.

17-Oxo-5-androsten-3β-yl 4-methoxybenzoate (9c)

The compound **9c** was obtained using 4-methoxybenzoyl chloride by above described method. Yield 72.0%; m.p. 97-105°C; IR (KBr, cm^{-1}): 2940, 1740, 1705, 1250, 1024; ^{1}H-NMR ($CDCl_3$) δ ppm: 0.90 (s, 3H, 18-C*H*$_3$), 1.09 (s, 3H, 19-C*H*$_3$), 3.86 (s, 2H, C*H*$_3$O-), 4.83(m, 1H, 3α-*H*), 5.44 (br, 1H, 6-vinylic), 6.92 (d, *J* = 9.0 Hz, 2H, 3-C*H* and 5-C*H* aromatic) and 7.95 (d, *J* = 9.0 Hz, 2H, 2-C*H* and 6-C*H* aromatic); Calculated for $C_{27}H_{34}O_4$: C, 76.74; H, 8.11. Found: C, 76.44; H, 8.57.

17-Oxo-5-androsten-3β-yl 4-chlorobenzoate (9d)

4-Chlorobenzoic acid was used to obtain 17-Oxo-5-androsten-3β-yl 4-chlorobenzoate (**9d**) by above described method. Yield 70.0%; m.p. 142-148°C; IR (KBr, cm^{-1}): 2940, 1780, 1720, 1270; ^{1}H-NMR ($CDCl_3$) δ ppm: 0.90 (s, 3H, 18-C*H*$_3$), 1.09 (s, 3H, 19-C*H*$_3$), 4.65 (m, 1H, 3α-*H*), 5.39 (br, 1H, 6-vinylic), 7.40 (d, *J* = 9.0 Hz, 2H, 3-C*H* and 5-C*H* aromatic) and 7.97 (d, *J* = 9.0 Hz, 2H, 2-C*H* and 6-C*H* aromatic); Calculated for $C_{26}H_{31}O_3Cl$: C, 73.14; H, 7.32. Found: C, 72.66; H, 6.82.

17-Oxo-5-androsten-3β-yl 4-methylbenzoate (9e)

The compound **9e** was prepared using acid chloride of 4-methylbenzoic acid (*p*-toluic acid) by above described method. Yield 80.0%; m.p. 87-90°C; IR (KBr, cm^{-1}): 2940, 1770, 1710, 1225; ^{1}H-NMR ($CDCl_3$) δ ppm: 0.89 (s, 3H, 18-C*H*$_3$), 1.09 (s, 3H, 19-C*H*$_3$), 2.45 (s, 3H, -C*H*$_3$), 4.84 (m, 1H, 3α-*H*), 5.45 (br, 1H, 6-vinylic), 7.31 (d, *J* = 6.0 Hz, 2H, 3-C*H* and 5-C*H* aromatic) and 8.03 (d, *J* = 9.0 Hz, 2H, 2-C*H* and 6-C*H* aromatic); Calculated for $C_{27}H_{34}O_3$: C, 79.76; H, 8.43. Found: C, 80.11; H, 7.87.

17-Oxo-17a-aza-D-homo-5-androsten-3β-yl phenoxyacetate (9f)

Acid chloride of phenoxyacetic acid was used in above mentioned method to get the 17-Oxo-5-androsten-3β-yl phenoxyacetate **9f.** Yield 68.0%; m.p. 135-137°C; IR (KBr, cm^{-1}): 2940, 1735, 1220; ^{1}H-NMR ($CDCl_3$) δ ppm: 0.88 (s, 3H, 18-C*H*$_3$), 1.04 (s, 3H, 19-C*H*$_3$), 4.60 (s, 3H, OC*H*$_2$), 4.73 (m, 1H, 3α-*H*), 5.40 (br, 1H, 6-vinylic) and 7.29 (m, 5H, aromatic); Calculated for $C_{27}H_{34}O_4$: C, 76.74; H, 8.11. Found: C, 77.28; H, 8.67.

Preparation of the compounds (11a-11e)

17-Oxo-5-androsten-3β-yl *p*-toluenesulphonate (10)

p-Toluenesulphonyl chloride (1.3 g, 7.0 mmol) was added to the solution of 3β-hydroxy-5-androsten-17-one (**8**) (1.0 g, 3.0 mmol) in pyridine (10.0 mL). The reaction mixture was heated on a steam bath for 8 h, cooled and poured into ice-cold water (100.0 mL). The precipitated product was filtered, washed thoroughly with water and then used for the rest of the reaction (0.6 g, 60.0%) m.p. 120-125°C; IR (KBr, cm^{-1}): 2940, 1735, 1334 and 1173.

General procedure for preparation of 11a-11e

The tosylate **10** was prepared fresh for each subsequent reaction (on account of its instability) and then treated with the appropriate alcohol. The completion of the reaction was monitored by TLC and the solution was concentrated to induce crystallization. The crystallized material was filtered, washed and dried to obtain the desired ethers.

3β-Methoxy-5-androsten-17-one (11a)

Yield 65.0%; m.p. 100-105°C; IR (KBr, cm^{-1}): 2940, 1735, 1367, 1008; ^{1}H-NMR (400MHz, $CDCl_3$) δ ppm: 0.91 (s, 3H, 18-C*H*$_3$), 1.03 (s, 3H, 19-C*H*$_3$), 3.76 (m, 1H, 3α-*H*), 4.5 (s, 3H, -OC*H*$_3$) and 5.40 (br, 1H, 6-vinylic); Calculated for $C_{20}H_{30}O_2$: C, 179.42; H, 10.00. Found: C, 80.00; H, 10.32.

3β-Ethoxy-5-androsten-17-one (11b)

Yield 41.6%; m.p. 108-115°C; IR (KBr, cm^{-1}): 2940, 1735, 1374, 1009; ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 0.91 (s, 3H, 18-C*H*$_3$), 1.02 (s, 3H, 19-C*H*$_3$), 1.20 (t, J = 7.5, 3H, -C*H*$_3$CH$_2$), 3.16 (m, 1H, 3α-*H*), 3.5 (q, J = 9.0 Hz, 2H, CH$_3$C*H*$_2$-) and 5.37 (br, 1H, 6-vinylic); Calculated for $C_{21}H_{32}O_2$: C, 79.70; H, 10.19. Found: C, 79.62; H, 9.68.

3β-(1-Methylethoxy)-5-androsten-17-one (11c)

Yield 60.0%; m.p. 142-145°C; IR (KBr, cm^{-1}): 2940, 1735, 1372, 1135; ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 0.89 (s, 6H, 18-C*H*$_3$, 19-C*H*$_3$), 1.02 (d, J = 9.0 Hz, 6H, (C*H*$_3$)$_2$-), 3.75 (m, 1H, 3α-*H*), 4.5 (m, 1H, C*H*(CH$_3$)$_2$) and 5.40 (br, 1H, 6-vinylic); Calculated for $C_{22}H_{34}O_2$: C, 79.95; H, 10.37. Found: C, 79.39; H, 10.79.

3β-(1,1-Dimethylethoxy)-5-androsten-17-one (11d)

Yield 46.6%; m.p. 140-145°C; IR (KBr, cm^{-1}): 2945, 1735, 1370, 1007; ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 0.88 (s, 3H, 18-C*H*$_3$), 1.05 (s, 3H, 19-C*H*$_3$), 2.17 (s, 9H, -(C*H*$_3$)$_3$C-), 3.76 (m, 1H, 3α-*H*) and 5.40 (br, 1H, 6-vinylic); Calculated for $C_{23}H_{36}O_2$: C, 80.18; H, 10.53. Found: C, 79.84; H, 9.82.

3β-Cylcohexoxy-5-androsten-17-one (11e)

Yield 50.0%; m.p. 142-145°C; IR (KBr, cm^{-1}): 2940, 1735, 1375, 1035; ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 0.88 (s, 3H, 18-C*H*$_3$), 1.05 (s, 3H, 19-C*H*$_3$), 3.76 (m, 1H, 3α-*H*), 4.45 (m, 1H, C*H*(CH$_2$)$_2$) and 5.40 (br, 1H, 6-vinylic); Calculated for $C_{25}H_{38}O_2$: C, 81.03; H, 10.34. Found: C, 81.64; H, 9.70.

Biological evaluation

Chemicals and biochemicals

All the chemicals were of reagent grade and 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. Finasteride was obtained as a gift sample from Cipla, and was of analytical grade (assay 99.9%). MTT for the assay was obtained from Sigma-Aldrich.

Cell culture and animals

Human prostate cancer cell line, DU-145, was procured from National Centre for Cell Science and 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% CO_2 at 37°C in a 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, India. Animals were housed under standard conditions and allowed free access to both food and water, available *ad libitium* until used.

Samples preparation for *in vitro* study

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

In vitro antiproliferative activity using cell line DU-145 (MTT assay)

Newly synthesized compounds were evaluated for their growth inhibitory activity using MTT assay. This assay quantifies 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, cell viability can be measured.

For this purpose, the DU-145 cell line was used and cells were grown as described above. Cells were cultured at a density of 5×10^3 cells/well in 96 well

plates at 37°C in 5.0% CO_2 atmosphere and allowed to attach for 24 h. The cells were treated in triplicate with a graded concentration of the sample and the reference drug finasteride at 37°C for 48 h. A 20 µL aliquot of MTT solution was added directly to all the wells. Following 4 h of incubation at 37°C, the media was removed and formazan crystals, which result from the reduction of MTT by active cells, were dissolved in 100 µL DMSO and vigorously mixed. The absorbance of each well was read on an ELISA plate reader (Merck) at 570 nm. The relative cell viability (%) related to the control was calculated by [A] test / [A] control × 100.

% Growth Inhibition
$= [OD]_{control} - [OD]_{test} / [OD]_{control} \times 100$
$[OD]_{test}$ = 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 and plated at a density of 5×10^3 cells/well in 96 well plate at 37°C in 5% CO_2. Cells were exposed to graded concentration of compounds. Each concentration was tested in triplicate. 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. The MTT assay was carried out as described earlier and the absorbance of each well was read on ELISA plate reader at 570 nm.

Effect of steroids on serum androgen levels

In order to measure the serum androgen level, all the compounds were suspended in a mixture of olive oil and ethanol (95:5) and administered once intraperitoneally equimolar to 40 mg/kg body weight of finasteride. Control animals were given a corresponding amount of vehicle only. Animals were divided into 3 groups; vehicle (control), finasteride (standard), treated (test sample) and each group consisted of 5 animals. Sprague-Dawley rats were treated with finasteride and an 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 presented as ng/mL (Hartmann et al., 2000; Vogel, 2002).

ELISA assay for plasma testosterone determination

Aliquots of 50 µL of each standard, control and unknown (serum samples) sample 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 5-6 times (200 µL each time), followed by addition of 100 µL of H_2O_2 substrate in each of the wells. The plates were further incubated at 37°C for 20 min. At the end of incubation, the reaction was stopped by 100 µL of 0.5 M H_2SO_4 as the stopping reagent. The absorbance of each well was read on an ELISA plate reader at 450 nm (Rassaie et al., 1992).

RESULTS AND DISCUSSION

Chemistry

3β-Hydroxy-5-androsten-17-one (**8**) was used as starting material for the syntheses of esters (**9a-9f**) and ethers (**11a-11e**). The **8** was synthesized from commercially available (25*R*)-5-spirosten-3β-ol (Diosgenin) (**4**) according to reported methods (Velgova and Kohout, 1985; Baraua et al., 1998; Li et al., 2005). Reaction of 3β-Hydroxy-5-androsten-17-one (**8**) with a solution of appropriate acid chlorides of various acids in acetone provided esters in good yield as shown in Fig. 3.

3β-Hydroxy-5-androsten-17-one (**8**) was converted to the tosylate **10** by treating with *p*-toluenesulphonyl chloride. Refluxing of **10** with various alcohols gave the corresponding 3β-alkoxy-5-androsten-17-ones (**11a-11e**) in moderate to good yield.

Biological activity

Antiproliferative activity using cell line DU-145

Compounds (**9a-9i**) and (**11a-11e**) were tested for antiproliferative activity using DU-145 cells as described by Mosmann et al. (Mosmann, 1983). All the compounds were tested at five different concentrations in the culture medium and finasteride (**1**) was used as positive control. The percentage of viable cells is presented in Fig. 4 and Fig. 5. Linear regressed line was drawn to calculate the concentration required to cause 50% inhibition in cell growth (IC_{50}) (Table I).

As shown in Table I, IC_{50} values obtained with steroidal esters (**9a-9f**) and steroidal ethers (**11a-11e**) were comparatively higher than those obtained with finasteride, thus indicating that finasteride has a higher cytotoxicity toward prostate cancer cell line activity than the steroids described in this paper. On the other hand, compound **9d** and **11c** with an IC_{50} value of 8.7 µm and 9.7 µm had higher antipro-

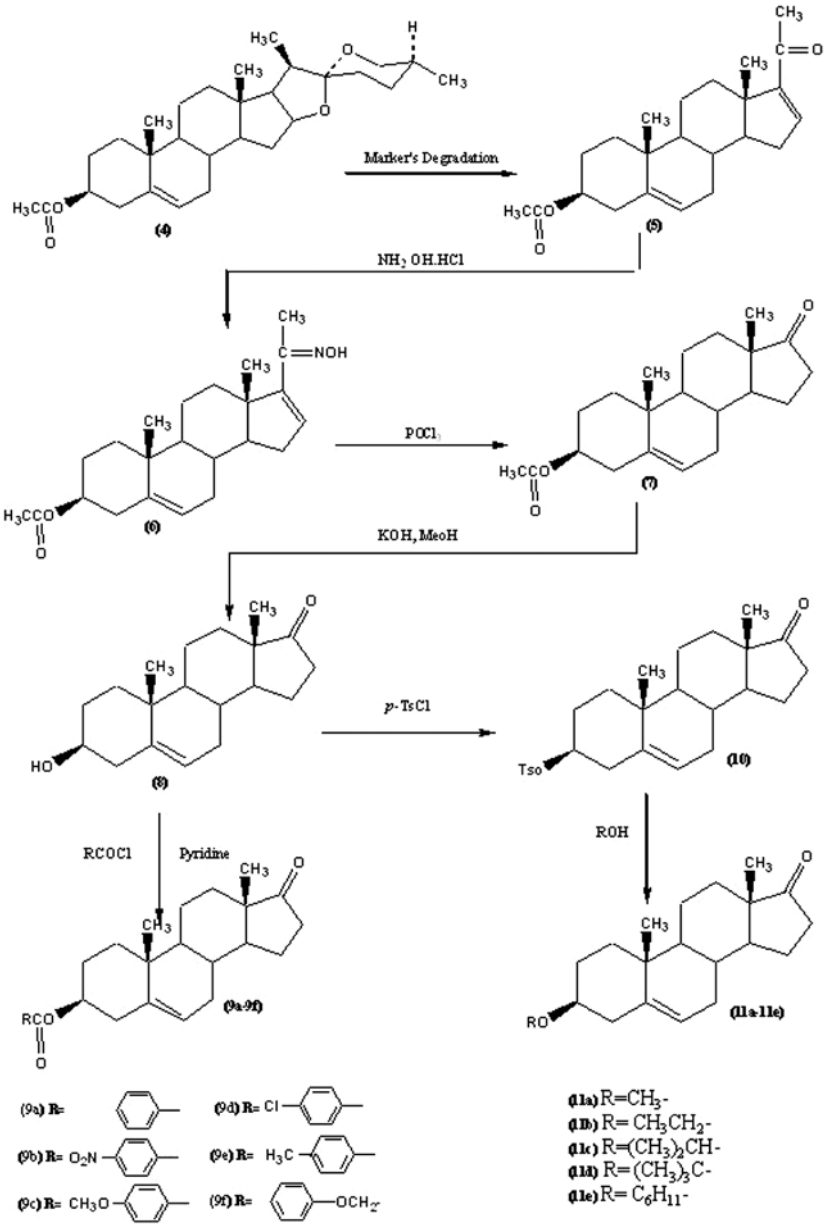

Fig. 3. Synthesis of 17-oxo-5-androsten-3β-yl esters (**9a-9f**); 3β-alkoxy-5-androsten-17-one derivatives (**11a-11e**)

liferative activity compared to the rest of the synthesized analogues.

In vitro cytotoxicity using mouse macrophages (acute toxicity)

In vitro cytotoxicity tests using DU-145 cells in the preliminary evaluation of anticancer drugs enabled us to select the most potent compounds, 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 (Balb C) for *in vitro* toxicity testing of newly synthesized compounds (Valasinas et al., 2001). The assay quantifies the viable cells, after 24 h incubation of cells with five different concentrations. The results obtained from MTT assay were statistically significant ($p < 0.001$) and linear equation obtained allowed us to determine the toxicity index (LC_{50}). The summarized data is presented in Table II.

The data generated indicate that **9c** with high LC_{50} exerted a statistically significant chemoselectivity for the cancerous cells. The analogue **9d** was less toxic to mouse macrophages showing LC_{50} of 43.65 μm. Thus

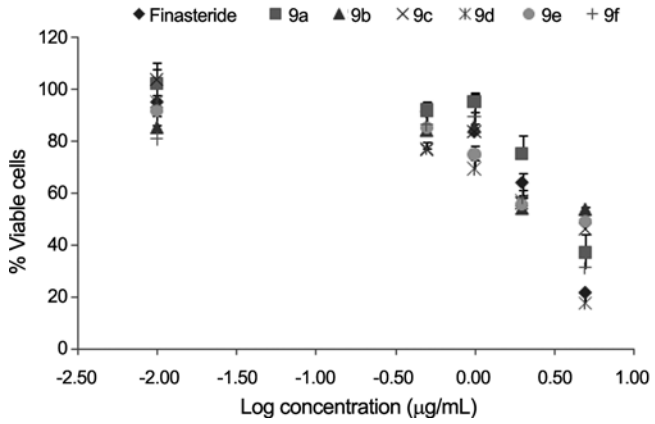

Fig. 4. Log dose-response relationship with regard to cytotoxicity of the compounds (**9a-9f**) on the number of living cells (DU-145) relative to the control. Each point represents a mean ± S.E.M. of 3 independent experiments. Linear regressed line was drawn to calculate the IC_{50}. ANOVA followed by Tukey's was applied. Data significantly different from the reference drug ($p < 0.001$).

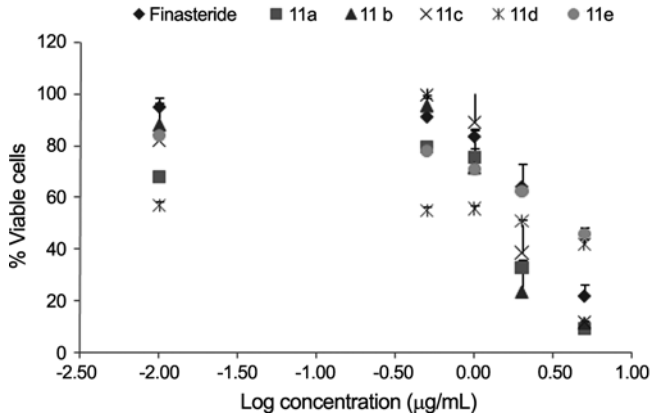

Fig. 5. Log dose-response relationship with regard to cytotoxicity of the compounds (**11a-11e**) on the number of living cells (DU-145) relative to the control. Each point represents a mean ± S.E.M. of 3 independent experiments. Linear regressed line was drawn to calculate the IC_{50}. ANOVA followed by Tukey's was applied. Data significantly different from the reference drug ($p < 0.001$).

in particular **9c** and **9d** are clearly lead molecules due to the potencies displayed towards DU-145 cells coupled to their cancerous cell selectivity. Acute toxicity of the compounds **9a, 9f, 11c** and **11e** was comparable to finasteride while the toxicity of compounds **9b, 11a, 11b, 11d** and **9e** was about 1.5-4 times higher than that of the reference drug.

In vivo effect on serum androgen level

Enzymes involved in the biosynthesis and metabolism of testosterone are attractive targets for the design and development of drugs which may be useful in the treatment of benign prostatic hyperplasia (BPH) as indicated in Fig. 1.

Table I. Antiproliferative effects and IC_{50} of the compounds against the prostate cancer cell line DU-145

Compound	IC_{50} (μm)
Finasteride	3.9
9a	11.4
9b	12.4
9c	11.2
9d	8.7
9e	11.9
9f	11.9
11a	10.4
11b	10.4
11c	9.7
11d	10.2
11e	14.8

Table II. Cytotoxic effects and LC_{50} of the compounds against mouse macrophages

Compound	LC_{50} (μm)
Finasteride	28.2
9a	22.7
9b	11.3
9c	157
9d	46
9e	2.5
9f	29.4
11a	18.4
11b	19.08
11c	26.4
11d	17.2
11e	22.5

Intact male rats (Sprague-Dawley, 200-250 g) were used in the designed study in which various compounds were compared *in vivo* for 5α-reductase inhibitory potency, as judged by their ability to attenuate the conversion of testosterone into dihydrotestosterone (DHT). ELISA for T was found to be suitable for determination in serum of rats since the cross reactive DHT levels were very low in males. The procedure measures T equally well and this method met all the requirements for precision, accuracy, sensitivity and selectivity (Stahl et al., 1984). The results of the study on the effect of various administrated compounds on the serum concentration of testosterone are presented in Fig. 6.

Effects of the compounds on serum testosterone level (Fig. 6) indicated that almost all the esters of 17-oxo-5-androsten-3β-ol (**9a-9f**) increased serum testosterone concentration very effectively despite their moder-

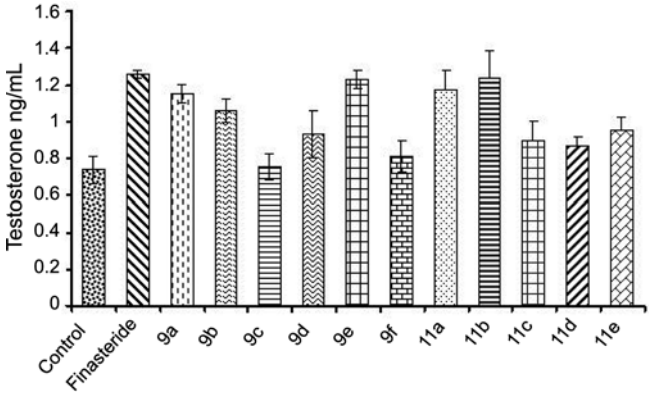

Fig. 6. Effect of compounds on serum level of testosterone. Results are mean ± S.E.M. of five experiments, and significant ($p < 0.05$) as compared to control.

ate antiproliferative activity compared to the control. These were benzoate (**9a**) *p*-nitro (**9b**), *p*-chloro (**9d**) and *p*-methyl (**9e**). The compound **9c** and **9f** were almost similar to **9e** except for having additional 'O' showed no inhibition of the enzyme, suggesting the limited steric group preference at position 4 of the phenyl ring. Compounds which have increased testosterone concentration probably did so by competing with the natural substrate (testosterone). However, in the class of 3β-alkoxy-5-androsten-17-one derivatives, analogues have shown moderate (**11d**, 0.87 ± 0.05) to excellent (**11b**, 1.24 ± 0.14) activity. Compounds **11a** and **11b** with methyl and ethyl group were found to have increased activity, whereas branched chain alkoxy derivatives (**11c**, **11d**) showed comparatively poor inhibition of 5α-reductase, indicating the preference of enzymes for linear alkyl group at this position. The 6 h post dose serum T levels were 0.954 ± 0.069 ng/mL for the compound **11e** (cyclohexyl group).

This work describes the preparation and evaluation of several steroidal esters and alkoxy derivatives. Among the newer derivatives, 17-Oxo-5-androsten-3β-yl 4-chlorobenzoate (**9d**) and 3β-(1-Methylethoxy)-5-androsten-17-one (**11c**) showed promising antiproliferative activity toward prostate cancer cell lines with IC_{50} values 8.7 and 9.7 μm respectively. Screening of the acute toxicity of these compounds further identified **9d** and **11c** as less toxic compounds. In conclusion, it has been shown that the potency, selectivity, and low toxicity of these compounds make them valid leads for synthesizing new compounds that possess better activity.

ACKNOWLEDGEMENTS

The grants received from the University Grants Commission, New Delhi and Council of Scientific and Industrial Research, New Delhi are gratefully acknowledged. We are thankful to Mr Dewank, Archer Chem, Mumbai, for the reference sample of finasteride.

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