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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

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 (25*R*)-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
MTT	[3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
DMEM	Dulbecco's modified eagle medium

N. Dhingra · T. R. Bhardwaj · N. Mehta · M. Kumar (✉)
University Institute of Pharmaceutical Sciences,
Panjab University, Chandigarh 160-014, India
e-mail: manoj_uips@pu.ac.in

T. R. Bhardwaj
I.S.F. College of Pharmacy, Ferozepur Road,
Moga 142001, India

T. Mukhopadhyay · A. Kumar
National Centre for Human Genome Studies and Research,
Panjab University, Chandigarh 160-014, India

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-oxosteroid, i.e., dihydrotestosterone (DHT) (Fig. 1) (Bruchoksky *et al.*, 1996). Two isozymes of 5α-reductase have been

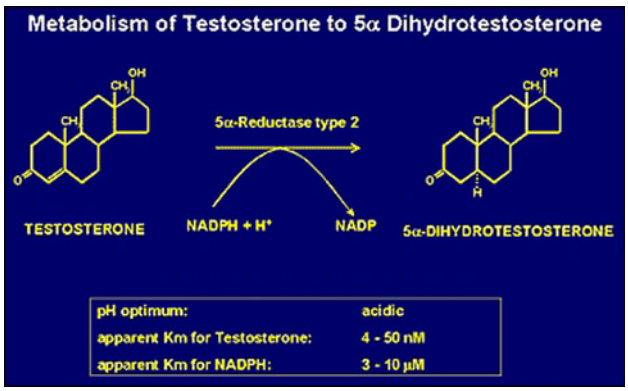


Fig. 1 Mechanism of 5α-reductase enzyme

cloned, expressed, and characterized based on difference in chromosomal localization, tissue expression pattern, and biochemical properties (Bruchoksky *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

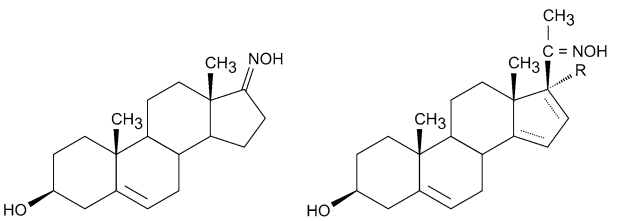


Fig. 2 Structures of some potent pregnenolone oximes

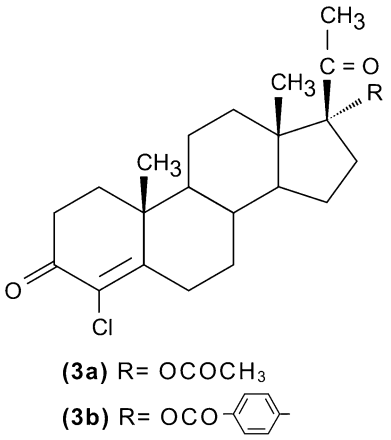


Fig. 3 Structures of some potent progesterone esters

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-5-androsten-3β-yl 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

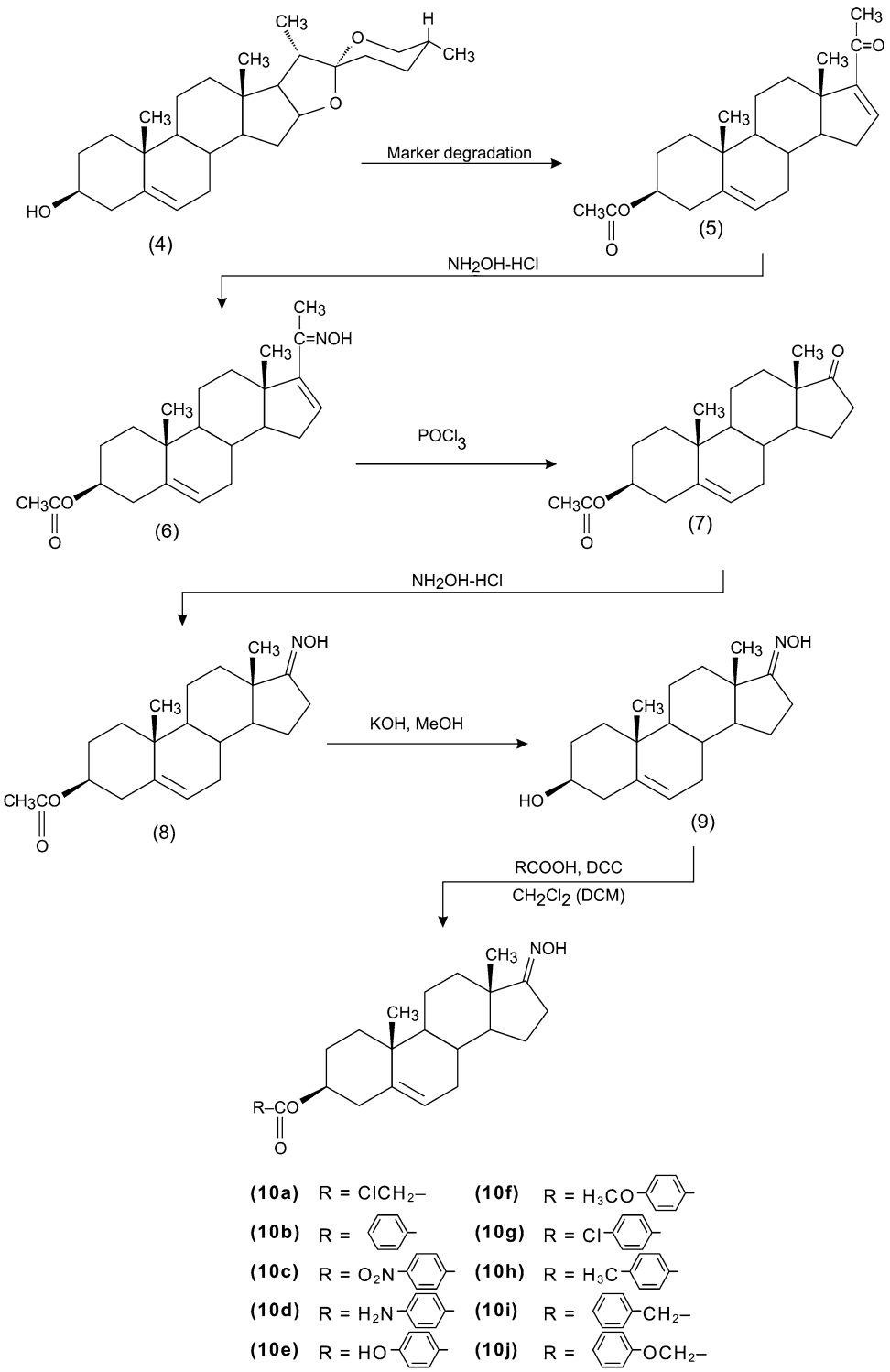
For the syntheses of compounds (**10a**–**10j**), 17-oximino-5-androsten-3β-ol (**9**) was used as starting material. The **9** was synthesized from commercially available (25*R*)-5-spirosten-3β-ol (Diosgenin) (**4**) according to the literature (Scheme 1) (Mason and Kepler, 1945; Hershberg, 1948; Regan and Hayes, 1956). Representative esters (**10a**–**10j**) were prepared by treating 3β-hydroxyl 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

Scheme 1



regressed line was drawn to calculate the concentration required to cause 50% inhibition in cell growth (IC_{50}) (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%

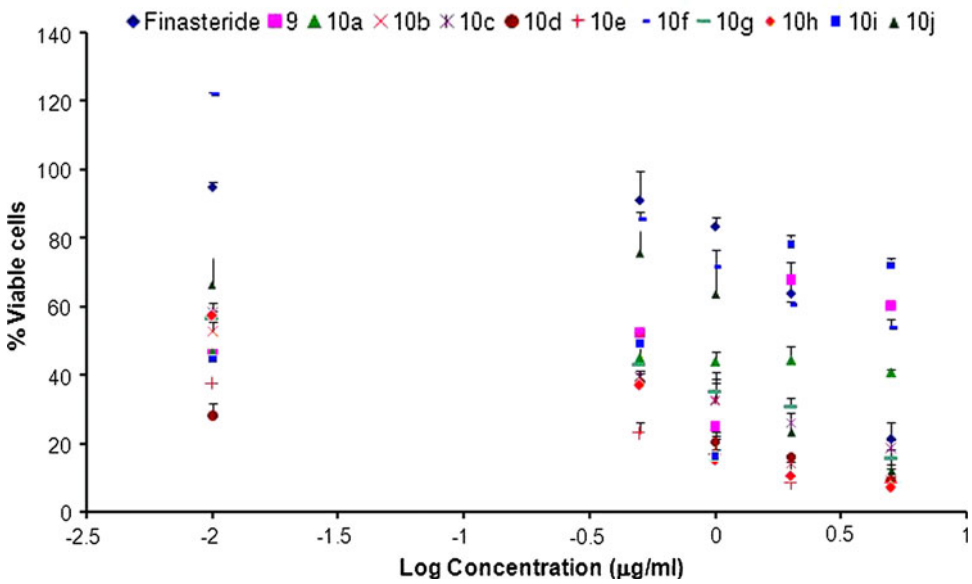


Fig. 4 Log dose–response relationship with regard to cytotoxicity of the compounds on the number of living cells (DU-145) relative to the control. Each point represents a mean ± SEM of 3 independent experiments. Linear regressed line was drawn to calculate the IC_{50}. ANOVA followed by Tukey's was applied.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

Table 1 Antiproliferative activity of the compounds 9, 10a–10j

Compound	% Growth inhibition (mean ± SEM)[a]				
	0.01 µg/ml	0.5 µg/ml	1.0 µg/ml	2.0 µg/ml	5.0 µg/ml
Finasteride	5.00 ± 1.58	8.83 ± 8.40	16.48 ± 2.49	36.0 ± 8.95	78.51 ± 4.63
9	N_i^b	N_i^b	N_i^b	N_i^b	N_i^b
10a	53.0 ± 0.48	55 ± 4.23	56 ± 1.22	56.0 ± 0.75	59.23 ± 2.43
10b	47.02 ± 1.67	48.86 ± 1.66	67.62 ± 1.62	85.87 ± 4.08	91.10 ± 2.10
10c	41.50 ± 0.71	60.51 ± 4.90	67.26 ± 3.70	74.03 ± 0.77	81.53 ± 1.79
10d	71.82 ± 4.24	62.10 ± 11.39	79.54 ± 7.76	83.89 ± 6.70	91.02 ± 1.25
10e	62.65 ± 2.46	76.89 ± 5.94	83.08 ± 0.75	91.39 ± 1.50	91.54 ± 0.19
10f	30.68 ± 3.52	51.43 ± 3.22	59.23 ± 2.77	65.56 ± 0.51	69.46 ± 3.76
10g	43.41 ± 0.77	57.1 ± 5.96	64.90 ± 7.40	69.17 ± 7.48	84.47 ± 0.74
10h	42.60 ± 2.21	62.99 ± 4.78	84.99 ± 3.99	89.55 ± 0.96	92.94 ± 0.13
10i	N_i^b	N_i^b	N_i^b	N_i^b	N_i^b
10j	33.59 ± 7.66	24.25 ± 6.37	36.19 ± 9.85	76.52 ± 5.58	87.81 ± 1.11

[a] Antiproliferative effect is expressed as a percentage of control and is mean ± SEM of triplicate measurements

[b] *Ni* no significant inhibition

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-yl 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 vitro cytotoxicity using mouse macrophages (acute toxicity)

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

Compound	IC_{50} (μm)
Finasteride	3.9
9	Nf[a]Nd[b]
10a	4.8
10b	3.8
10c	3.07
10d	3.7
10e	2.9
10f	5.4
10g	3.4
10h	2.3
10i	6.5
10j	6.3

[a] *Ni* not significant inhibition

[b] *Nd* not determined

Table 3 Acute toxicity of the investigated compounds

Compound	LC_{50} (μm)
Finasteride	28.2
9	Nd[a]
10a	28.0
10b	89.4
10c	19.5
10d	29.4
10e	22.0
10f	14
10g	24.0
10h	22
10i	11.8
10j	89

[a] *Nd* not determined

agents (Cholody *et al.*, 2005). 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 (LC_{50}). 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 LC_{50} 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.

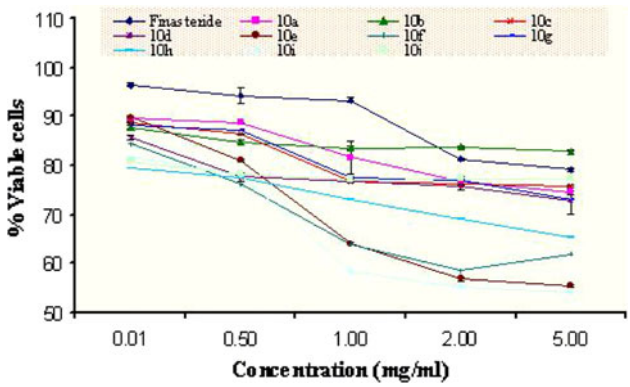


Fig. 5 Toxicity of the compounds to mouse macrophages (Balb C). Cell viability was determined based on the MTT assay. Each point represents a mean value and SEM of 3 independent experiments. * $P < 0.001$ are significantly different compared to Finasteride according to the one-way ANOVA followed by Tukey's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

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 *p*-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

Antiandrogenic 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 antiandrogenic activity.

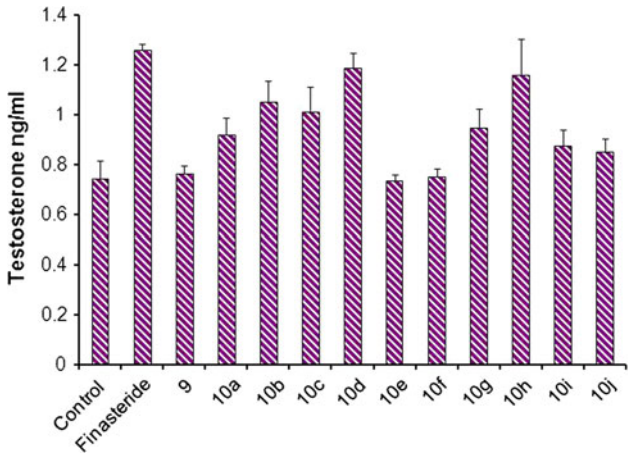


Fig. 6 Effect of compounds on serum level of testosterone. Results are mean ± SEM of five experiments. * $P < 0.05$ significant as compared to control, [a] $P < 0.05$ significant as compared to Finasteride

Experimental section

Chemistry

The melting points were determined on Veego melting point apparatus and are uncorrected. ^{1}H-NMR spectra were obtained using Brucker AC-300F, 300 MHz and Brucker AC-400F, 400 MHz spectrometer for solutions in $CDCl_3$, 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 obtained with 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 Perkin-Elmer 2400 CHN elemental analyzer. The 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° for 30 min. Silica gel G60 F aluminum sheets were used for final monitoring. The plates were developed by exposure to iodine vapor. Anhydrous sodium sulfate was utilized as drying agents. All the solvents were freshly distilled and dried prior to use according to standard procedure.

*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}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.01 (s, 3H, 18-CH_3), 1.05 (s, 3H 19-CH_3), 4.03 (s, $ClCH_2COO$), 4.70 (m, 1H, 3α-H), and 5.40 ppm (br, 1H, 6-vinylic); Anal. Calcd for $C_{21}H_{30}NO_3Cl$: N, 3.69. Found: N, 3.52.

*17-Oximino-5-androsten-3β-yl benzoate (**10b**)* The compound **10b** (0.32 g, 64.0%) was prepared using benzoic 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}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.89 (s, 3H, 18-CH_3), 1.1 (s, 3H 19-CH_3), 4.07 (m, 1H, 3α-H), 5.40 (br, 1H, 6-vinylic), and 6.84–7.28 (m, 5H, aromatic); Anal. Calcd for $C_{26}H_{33}NO_3$: N, 3.44. Found: N, 3.74.

*17-Oximino-5-androsten-3β-yl 4-nitrobenzoate (**10c**)* 4-Nitrobenzoic acid (0.27 g, 1.6 mmol) was used to prepare 17-oximino-5-androsten-3β-yl 4-nitrobenzoate compound (**10c**) (0.34 g, 68.0%) by above described method: mp 185–188°C; IR (KBr): 3300, 2930, 1700, 1650, and 1234 cm^{-1}, ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.89 (s, 3H, 18-CH_3), 1.03 (s, 3H 19-CH_3), 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 $C_{26}H_{32}N_2O_5$: N, 6.19. Found: N, 5.64.

*17-Oximino-5-androsten-3β-yl 4-aminobenzoate (**10d**)* 17-Oximino-5-androsten-3β-yl 4-aminobenzoate (**10d**) (0.28 g, 56.0%) was prepared by method described above using 4-aminobenzoic acid (0.22 g, 1.6 mmol): mp 165–168°C; IR (KBr): 3330, 2930, 1700, 1630, and 1240 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.93 (s, 3H, 18-CH_3), 1.05 (s, 3H 19-CH_3), 2.17 (1H, NOH), 4.08 (m, 1H, 3α-H), 6.20 (br, 1H, 6-vinylic), 7.22 (d, $J = 8.3$, 2H, 3-CH, and 5-CH aromatic), and 8.26 ppm (d, $J = 8.4$, 2H, 2-CH, and 6-CH aromatic); Anal. Calcd for $C_{26}H_{34}N_2O_3$: N, 6.63. Found: N, 6.79.

*17-Oximino-5-androsten-3β-yl 4-hydroxybenzoate (**10e**)* The compound **10e** (0.31 g, 62.0%) was prepared using 4-hydroxybenzoic acid (0.23 g, 1.6 mmol) by above described method: mp 179–182°C; IR (KBr): 3310, 2930, 1710, 1680, and 1235 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$):

δ 0.89 (s, 3H, 18-CH_3), 1.05 (s, 3H 19-CH_3), 2.17 (1H, NOH), 4.18 (m, 1H, 3α-H), 6.35 (br, 1H, 6-vinylic), 6.83 (d, $J = 8.1$, 2H, 3-CH, and 5-CH aromatic), and 7.46 ppm (d, $J = 8.4$, 2H, 2-CH, and 6-CH aromatic); Anal. Calcd for $C_{26}H_{33}NO_4$: N, 3.31. Found: N, 3.32.

*17-Oximino-5-androsten-3β-yl 4-methoxybenzoate (**10f**)* 4-Methoxybenzoic acid (*p*-anisic acid) (0.24 g, 1.6 mmol) was used to prepare 17-oximino-5-androsten-3β-yl 4-methoxybenzoate compound (**10f**) (0.31 g, 62.0%) by above described method: mp 122–125°C; IR (KBr): 3340, 2935, 1735, 1670, and 1250 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.77 (s, 3H, 18-CH_3), 0.84 (s, 3H 19-CH_3), 3.78 (s, 3H, –OCH_3), 4.06 (m, 1H, 3α-H), 5.97 (br, 1H, 6-vinylic), 6.83 (d, $J = 7.9$, 2H, 3-CH, and 5-CH aromatic), and 7.46 ppm (d, $J = 8.0$, 2H, 2-CH, and 6-CH aromatic); Anal. Calcd for $C_{27}H_{35}NO_4$: N, 3.20. Found: N, 3.07.

*17-Oximino-5-androsten-3β-yl 4-chlorobenzoate (**10g**)* 4-Chlorobenzoic acid (0.26 g, 1.6 mmol) was used to obtain 17-oximino-5-androsten-3β-yl 4-chlorobenzoate (**10g**) (0.3 g, 60.0%) by above described method: mp 167–170°C; IR (KBr): 3310, 2930, 1740, 1650, and 1235 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.03 (s, 3H, 18-CH_3), 1.04 (s, 3H 19-CH_3), 2.17 (1H, NOH), 4.15 (m, 1H, 3α-H), 5.40 (br, 1H, 6-vinylic), 7.50 (d, $J = 7.0$, 2H, 3-CH, and 5-CH aromatic), and 7.97 ppm (d, $J = 7.2$, 2H, 2-CH, and 6-CH aromatic); Anal. Calcd for $C_{26}H_{32}NO_3Cl$: N, 3.17. Found: N, 3.25.

*17-Oximino-5-androsten-3β-yl 4-methylbenzoate (**10h**)* The compound **10h** (0.29 g, 58.0%),was prepared using 4-methylbenzoic acid (*p*-toluic acid) (0.22 g, 1.6 mmol) by above described method: mp 135–139°C; IR (KBr): 3310, 2930, 1700, 1650, and 1235 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.89 (s, 3H, 18-CH_3), 1.03 (s, 3H 19-CH_3), 2.41 (1H, NOH), 4.08 (m, 1H, 3α-H), 6.25 (br, 1H, 6-vinylic), 7.20 (d, $J = 7.9$, 2H, 3-CH, and 5-CH aromatic), and 7.43 ppm (d, $J = 8.0$, 2H, 2-CH, and 6-CH aromatic); Anal. Calcd for $C_{27}H_{35}NO_3$: N, 3.32. Found: N, 3.77.

*17-Oximino-5-androsten-3β-yl 4-phenylacetate (**10i**)* 17-Oximino-5-androsten-3β-yl phenylacetate (**10i**) (0.29 g, 58.0%) was prepared by method as described above using phenylacetic acid (0.22 g, 1.6 mmol): oily residue; IR (KBr): 3320, 2935, 1730, and 1250 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.91 (s, 3H, 18-CH_3), 1.05 (s, 3H 19-CH_3), 3.65 (s, 2H, –CH_2), 4.65 (m, 1H, 3α-H), 5.45 (br, 1H, 6-vinylic), and 7.32 (m, 5H, aromatic); Anal. Calcd for $C_{27}H_{35}NO_3$: N, 3.37. Found: N, 3.39.

*17-Oximino-5-androsten-3β-yl phenoxyacetate (**10j**)* Phenoxyacetic acid (0.24 g, 1.6 mmol) was used in above-mentioned method to get the 17-Oxo-17a-aza-D-homo-5-androsten-3β-yl phenoxyacetate (**10j**) (0.27 g, 54.0%): mp 118–122°C; IR (KBr): 3200, 2940, 1750, 1650, and 1220 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.88 (s, 3H, 18-CH_3), 1.02 (s, 3H, 19-CH_3), 4.60 (s, 2H, –OCH_2), 4.7 (m, 1H, 3α-H), 5.40 (br, 1H, 6-vinylic), and 7.29 (m, 5H, aromatic); Anal. Calcd for $C_{27}H_{35}NO_4$: N, 3.20. Found: N, 3.60.

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_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 5×10^3 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 $[A]_{test}/[A]_{control} \times 100$.

$$\% \text{ Growth inhibition} = [\text{OD}]_{control} - [\text{OD}]_{test}/[\text{OD}]_{control} \times 100$$

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

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

In vitro cytotoxicity using mouse macrophages [acute toxicity (MTT assay)]

Cells (mouse macrophages) were used as normal cells and plated at a density of 5×10^3 cells/well in 96 plates at 37°C in 5% CO_2. 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 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 $[A]_{test}/[A]_{control} \times 100$.

$$\% \text{ Cell viability} = [A]_{test}/[\text{A}]_{control} \times 100$$

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

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

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. Control 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_2O_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_2SO_4 as stopping reagent. The absorbance of each well was read on Elisa plate reader at 450 nm [40, 41].

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