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Synthesis and biological evaluation of 3-tetrazolo steroidal analogs: Novel class of 5α-reductase inhibitors

Saurabh Aggarwal [a], Manoj Kumar Mahapatra [a], Rajnish Kumar [a], Tilak R. Bhardwaj [a,b], Rolf W. Hartmann [c,d], Jörg Haupenthal [c], Manoj Kumar [a,*]

[a] University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh 160014, India
[b] School of Pharmacy & Emerging Sciences, Baddi University of Emerging Sciences & Technology, Baddi, Himachal Pradesh, India
[c] Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Campus 81, D-66123 Saarbrücken, Germany
[d] Department of Pharmacy, Pharmaceutical and Medicinal Chemistry, Saarland University, Campus C23, Saarbrücken, Germany

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ABSTRACT

In the present study, a series of steroidal tetrazole derivatives of androstane and pregnane have been prepared in which the tetrazole moiety was appended at C-3 and 17a-aza locations. 3-Tetrazolo-3,5-androstadien-17-one (**6**), 3-tetrazolo-19-nor-3,5-androstadien-17-one (**10**), 3-tetrazolo-3,5-pregnadien-20-one (**14**), 17a-substituted 3-tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (**26**–**31**) and 3-(2-acetyltetrazolo)-17a-aza-D-homo-3,5-androstadien-17-one (**32**) were synthesized from dehydroepiandrosterone acetate (1) through multiple synthetic steps. Some of the synthesized compounds were evaluated for their in vitro 5α-reductase (5AR) inhibitory activity by measuring the conversion of [^{3}H] androstenedione in human embryonic kidney (HEK) cells. In vivo 5α-reductase inhibitory activity also showed a significant reduction (p <0.05) in rat prostate weight. The most potent compound **14** showed 5AR-2 inhibition with IC_{50} being 15.6 nM as compared to clinically used drug finasteride (40 nM). There was also a significant inhibition of 5AR-1 with IC_{50} 547 nM compared to finasteride (453 nM).

1. Introduction

Androgens plays a vital role in benign prostatic hyperplasia (BPH) and cancerous growth in prostate by acting through a member of steroid nuclear receptor super family, that is, androgen receptor.[1] It directs the transcription of genes responsible for growth and maintenance of prostate, for which androgenic pathway has emerged as a target of therapeutic intervention in both benign and cancerous diseases. 5α-Reductase (5AR), a NADPH-dependent and membrane bound enzyme, catalyzes the conversion of testosterone (T) to more potent dihydrotestosterone (DHT) (Fig. 1).

BPH is the most common benign tumor affecting over 40% of men above the age of 70.[2] DHT stimulates several growth factors that drive cellular proliferation in the human prostate. Therefore, inhibition of 5AR has been considered as a legitimate therapeutic target and there is a greater thrust for development of newer inhibitors. There are two main isozymes (5AR-1 and 5AR-2) with a different tissue distribution pattern and with distinct biochemical and pharmacological properties.[3] A third type of isozyme has been detected and described in hormone refractory prostate cancer (HRPC) and is ubiquitous in mammals, being found also in non-androgenic tissues such as pancreas and brain.[4,5] The most commonly used 5AR inhibitor in BPH treatment is finasteride, a steroidal compound inhibiting mainly 5AR-2. Finasteride was the first 5AR inhibitor approved in U.S. for the treatment of BPH.[6,7] The long term pharmacotherapy with finasteride has generally been well tolerated.[8] However, its limited activity and side effects which are related with sexual function (libido, impotence, and ejaculatory disorder) have prompted to look for newer 5AR inhibitors.[9] Epristeride, a carboxy steroid, is also a potent inhibitor of 5AR-2 while a weak inhibitor of 5AR-1. Earlier we have also reported a series unsaturated carboxysteroids (17a-substituted 17-oxo-17a-aza-D-homo-3,5-androstadien-3-oic acids) and their 5AR inhibitory potency.[10] The mechanism of 'inverted action' or 'back binding', as proposed by McDonald et al. has been observed in 17a-Aza-D-Homo-steroids which have also exhibited significant 5AR inhibitory activity. Thus it can be considered that 17a-aza steroids might be exhibiting same type of mechanism of action as 4-azasteroids.[11]

* Corresponding author. Mobile: +91 9417455011.
E-mail address: manoj_uips@pu.ac.in (M. Kumar).

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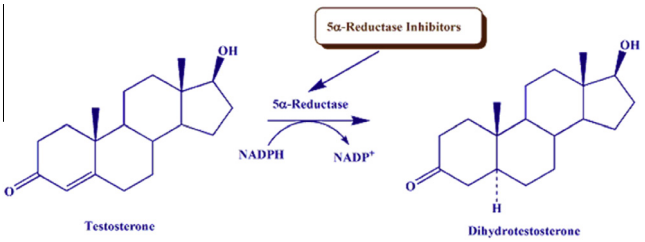


Figure 1. Formation of dihydrotestosterone by 5α-reductase.

Bioisosteres are those chemical substituents or groups with analogous physical or chemical properties which imparts broadly similar biological properties to the chemical compound. Tetrazoles are the most commonly used metabolism-resistant bioisostere of the carboxylate moiety, for which medicinal chemists paid a specific attention toward tetrazole group.[12] Tetrazoles, after getting ionized at physiological pH, shows a planar structure like their carboxylate anion. Tetrazole anions are 10 times more lipophilic than carboxylate anions but the interesting fact is both of them have similar acidity (pK_a 4.5–4.9 and 4.2–4.4 for tetrazole and carboxylate anion, respectively) which accounts for high membrane permeability of tetrazoles.[13] Moreover, tetrazoles are resistant to many biological metabolic degradation pathways which causes increased half life. Matta and co-workers computational study has given evidence that anions of tetrazole and carboxyl group shows bioisosterism, due to similarity in the geometrical arrangement of four local minima in their respective electrostatic potentials (a quartet of minima).[14]

Planarity in certain part of steroidal ring A seems to be a prerequisite for the steroidal nucleus to enter into the active site of the enzyme, as suggested by different literatures.[15–18] Planarity has been provided to certain extent by sp^2 hybridized carbon of C-1 and C-2 in case of Finasteride, Dutasteride, and conjugated double bonds at C-3 and C-5 in Epristeride (Fig. 2).

QSAR studies from our laboratory also indicated that a bulky group at 17 position will lead to favorable activity.[19] A tetrazole is an isostere of carboxylic group, hence it was envisaged to synthesize molecules having tetrazole group at C-3 mimicking the charge replacement of carboxylate ion and having various alkyl substituents at 17a-nitrogen of D-ring lactam. This can also be accomplished by enlarging 5 membered D-ring to 6 membered lactam and substituting hydrogen with bulky groups. Therefore it was envisioned to synthesize epristeride related analogs having 3-tetrazolo-3,5-diene moiety in androstane. In addition compounds would have 17-oxo-17a-aza-D-homo lactam in ring D instead of ring A as in finasteride.

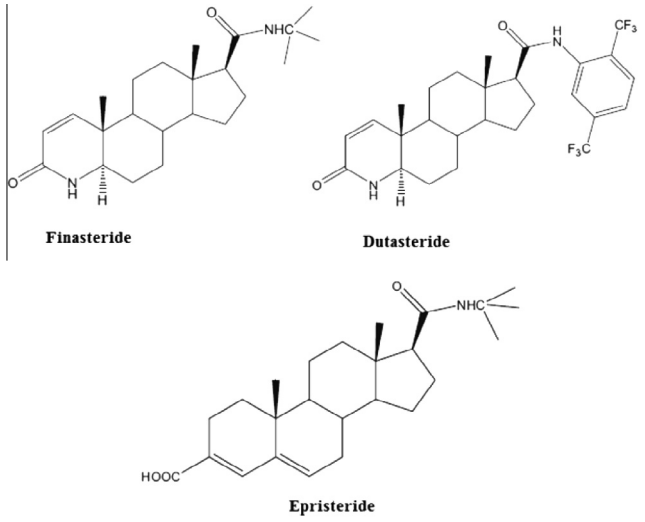


Figure 2. Structures of active 5α-reductase inhibitors.

Guarna et al. synthesized a novel class of compounds 19-nor-10-azasteroids which were found to be better inhibitors of the 5AR enzyme.[20,21] Therefore, it was also envisaged to synthesize molecule not having 19-methyl group and having rings A and B mimicking that of epristeride. The present paper describes the synthesis of a novel class of steroidal compounds which were evaluated in vitro for 5AR inhibition against both 5AR-1 and 5AR-2, DU-145 cancer cell lines and in vivo by prostate weighing method.

2. Results and discussion

2.1. Chemistry

2.1.1. Synthesis of 3-tetrazolo-3,5-androstadien-17-one (6)

17-Oxo-5-androsten-3β-yl acetate (dehydroepiandrosterone) (**1**) was hydrolyzed by refluxing with methanolic potassium hydroxide to obtain 3β-hydroxy-5-androsten-17-one (**2**). Oppenauer oxidation of **2** gave 4-androstene-3,17-dione (**3**). Compound **3** on reaction with phosphorous tribromide in glacial acetic acid yielded 3-bromo-3,5-androstadien-17-one (**4**). The bromo compound (**4**) was refluxed with cuprous cyanide in dimethylformamide to yield 3-cyano-3,5-androstadien-17-one (**5**). The cyano compound (**5**) when treated with sodium azide in presence of triethylamine hydrochloride gave the desired 3-tetrazolo-3,5-androstadien-17-one (**6**) (Scheme 1).

2.1.2. Synthesis of 3-tetrazolo-19-nor-3,5-androstadien-17-one (10)

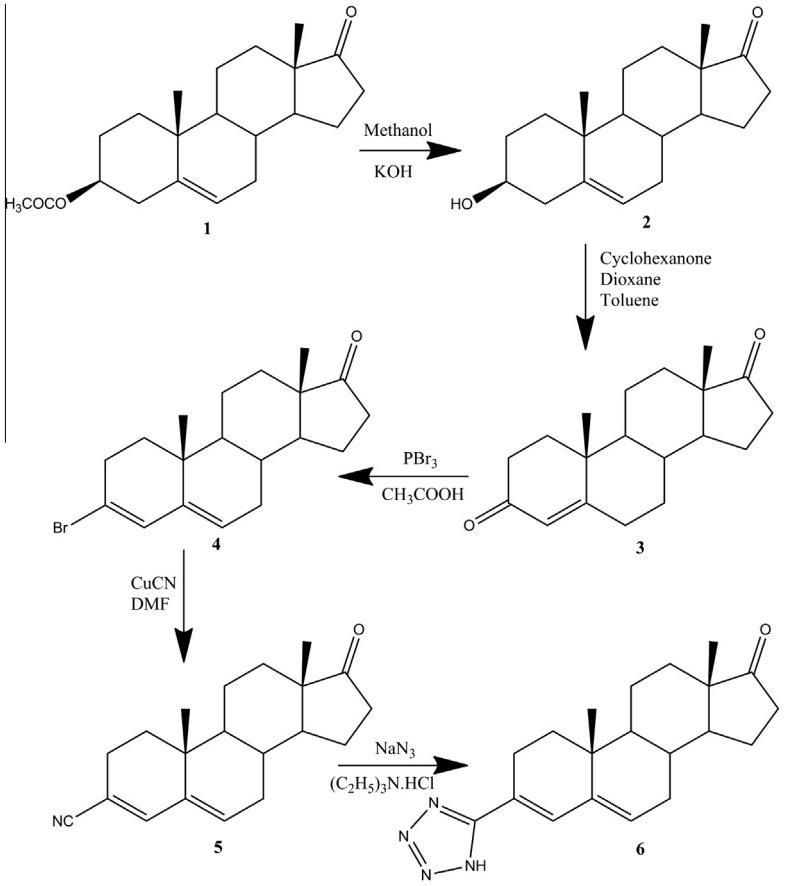
19-Nor-4-androsten-3,17-dione (**7**) was converted to its bromo derivative (**8**) by treating with phosphorous tribromide in glacial acetic acid. The bromo derivative on refluxing with cuprous cyanide in dimethylformamide gave 3-cyano-19-nor-3,5-androstadien-17-one (**9**). The cyano compound (**9**) on reaction with sodium azide in presence of triethylamine hydrochloride gave 3-tetrazolo-19-nor-3,5-androstadien-17-one (**10**) (Scheme 2).

2.1.3. Synthesis of 3-tetrazolo-3,5-pregnadien-20-one (14)

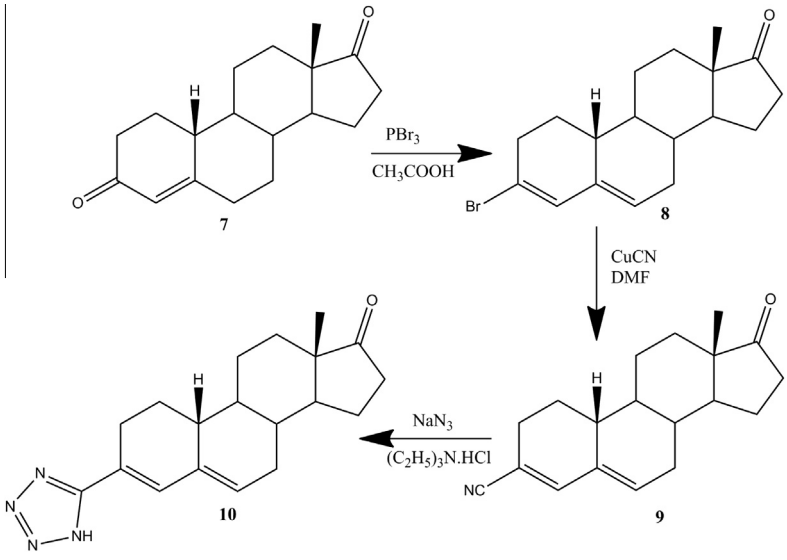
4-Pregnene-3,20-dione (**11**) was treated with phosphorous tribromide in glacial acetic acid to yield 3-bromo-3,5-pregnadien-20-one (**12**). Compound **12** was converted to its cyano derivative (**13**) by refluxing with cuprous cyanide in dimethylformamide. The cyano derivative was treated with sodium azide and triethylamine hydrochloride to yield 3-tetrazolo-3,5-pregnadien-20-one (**14**) (Scheme 3).

2.1.4. Synthesis of 3-cyano-17a-aza-D-homo-3,5-androstadien-17-one (20)

17-Oxo-5-androsten-3β-yl acetate (**1**) was converted to its oxime (**15**) by refluxing in ethanol with hydroxylamine hydrochloride and sodium acetate trihydrate. 17-Oximino-5-androsten-3β-yl acetate (**15**) on Beckmann's rearrangement with thionyl chloride gave 17-oxo-17a-aza-D-homo-5-androsten-3β-yl acetate (**16**). The lactam (**16**) was hydrolyzed by refluxing with methanolic potassium hydroxide to obtain 3β-hydroxy-17a-aza-D-homo-5-androsten-17-one (**17**). Oppenaeur oxidation of **17** gave 17a-aza-D-homo-4-androstene-3,17-dione (**18**) which on reacting with phosphorous tribromide in glacial acetic acid gave 3-bromo-17-a-aza-D-homo-3,5-androstadien-17-one (**19**). The bromo derivative was refluxed with cuprous cyanide in dimethylformamide to yield 3-cyano-17a-aza-D-homo-3,5-androstadien-17-one (**20**) (Scheme 4).



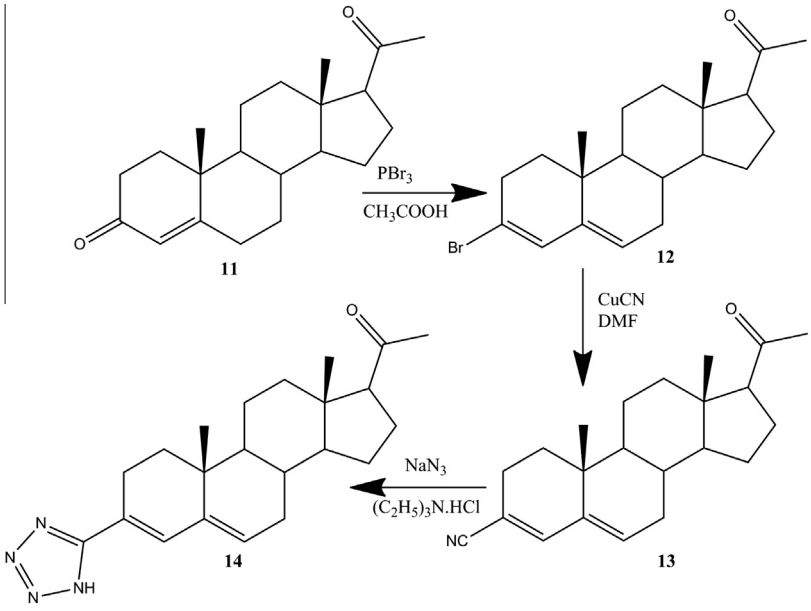
Scheme 1. Synthesis of 3-tetrazolo-3,5-androstadien-17-one.



Scheme 2. Synthesis of 3-tetrazolo-19-nor-3,5-androstadien-17-one.

2.1.5. Synthesis of *N*-substituted 3-tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one derivatives (26–31)

The cyano compound (**20**) was treated with sodium azide in presence of triethylamine hydrochloride to form 3-tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (**26**). 3-Cyano-17a-aza-D-homo-3,5-androstadien-17-one (**20**) was also treated with methyl iodide, ethyl bromide, allyl bromide, benzyl chloride and acrylonitrile respectively in presence of sodium hydride in tetrahydrofuran to get 17a-methyl-3-cyano-17a-aza-D-homo-3,5-androstadien-17-one (**21**), 17a-ethyl-3-cyano-17a-aza-D-homo-3,5-androstadien-17-one (**22**), 17a-allyl-3-cyano-17a-aza-D-homo-3,5-androstadien-17-one (**23**), 17a-benzyl-3-cyano-17a-aza-D-homo-3,5-androstadien-17-one (**24**) and 17a-cyanoethyl-3-cyano-17a-aza-D-homo-3,5-androstadien-17-one (**25**) respectively. Compounds **21**–**25** on reaction with sodium azide gave 17a-methyl-3-tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (**27**), 17a-ethyl-3-tetrazolo-17a-



Scheme 3. Synthesis of 3-tetrazolo-3,5-pregnadien-20-one.

aza-D-homo-3,5-androstadien-17-one (**28**), 17a-allyl-3-tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (**29**), 17a-benzyl-3-tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (**30**) and 17a-tetrazoloethyl-3-tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (**31**), respectively (Scheme 5).

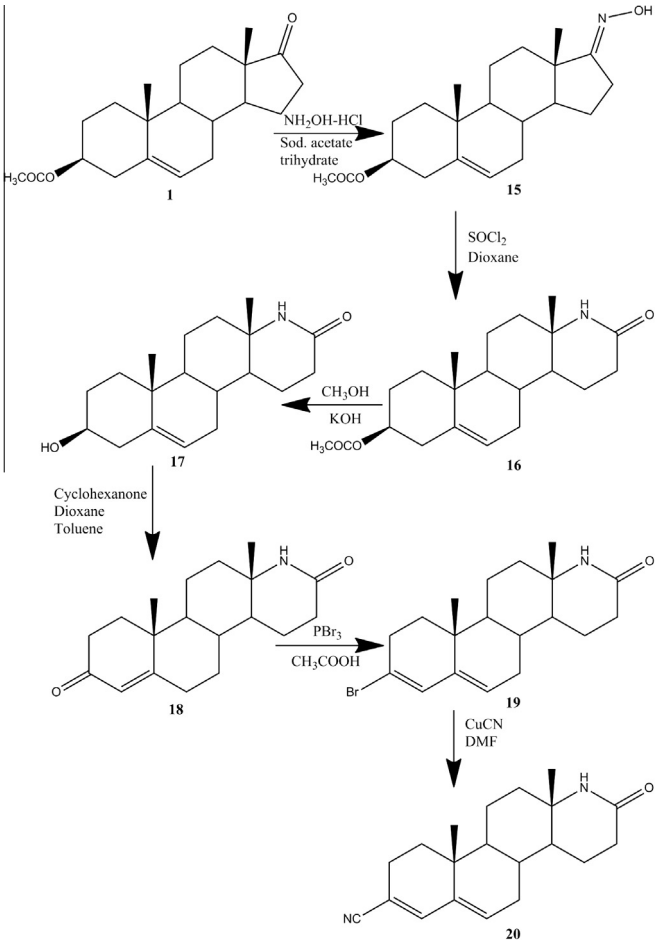
2.1.6. Synthesis of 3-(2-acetyltetrazolo)-17a-aza-D-homo-3,5-androstadien-17-one (32)

3-Tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (**26**) was treated with acetic anhydride in pyridine to obtain 3-(2-acetyltetrazolo)-17a-aza-D-homo-3,5-androstadien-17-one (**32**) (Scheme 6).

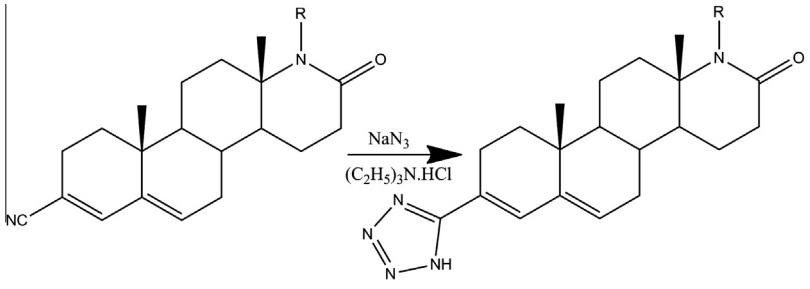
2.2. In vitro human 5AR inhibitory activity (HEK 293 cells method)

Human embryonic kidney cell line (HEK293) lacking endogenous 5α-reductase activity has replaced the conventional BPH microsomal assay and the DU-145 cell free assay. The whole cell system seemed to be more close to the in vivo situation and offers faster and selective results as compared to the use of microsomal enzymes. The main disadvantages of the earlier assay methods were reported in our previous report.[10]

Previously we have reported a series of unsaturated 3-carboxysteroids which were active against both 5AR-1 and 5AR-2. Since tetrazoles are isosteres of the carboxylic acids, some 3-tetrazolo steroidal derivatives were prepared from their cyano precursors and some of them were evaluated for their inhibitory potency against 5AR-1 and 5AR-2 at 10 μM and 2 μM concentrations. Compound **6** was found to be a potent dual inhibitor of 5α-reductase showing 100% inhibition for 5AR-2 isozyme at both 10 μM and 2 μM concentration with IC_{50} being 83.8 nM. It showed around 81.1% inhibition for 5AR-1 isozyme at 10 μM. Similarly, compound **10** has exhibited around 80% inhibition for both 5AR-1 and 5AR-2 isozyme at 10 μM concentration, while it also showed about 60% inhibition at 2 μM concentration against 5AR-2 isozyme. Compound **14** showed 100% inhibition for 5AR-2 isozyme at 10 μM and 2 μM concentration with IC_{50} being 15.6 nM indicating that the compound is twice more potent than finasteride. It has shown around 95% inhibition for 5AR-1 isozyme at 10 μM with IC_{50} being 547 nM. Compound **27** showed 91.2% inhibition at 10 μM and around 84.3% inhibition against 5AR-2 at 2 μM concentration with IC_{50} of 273.8 nM. Compound **29** showed 100% inhibition at 10 μM and 95% inhibition at 2 μM concentration against 5AR-2 with IC_{50}



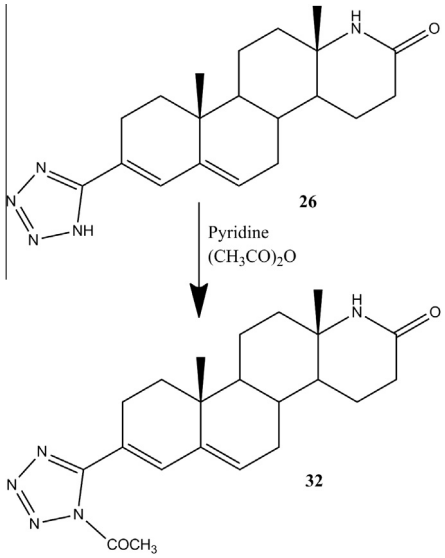
Scheme 4. Synthesis of 3-cyano-17a-aza-D-homo-3,5-androstadien-17-one.



(20) R= -H
(21) R= -CH_3
(22) R= -C_2H_5
(23) R= -$CH_2CH{=}CH_2$
(24) R= -$CH_2C_6H_5$
(25) R= -CH_2CH_2CN

(26) R= -H
(27) R= -CH_3
(28) R= -C_2H_5
(29) R= -$CH_2CH{=}CH_2$
(30) R= -$CH_2C_6H_5$
(31) R= -CH_2CH_2-tetrazol-5-yl

Scheme 5. Synthesis of compounds **26**–**31**.



Scheme 6. Synthesis of 3-(2-acetyltetrazolo)-17a-aza-D-homo-3,5-androstadien-17-one.

being 157 nM. Finasteride, a clinically used drug, was used as the standard in the assay. It showed IC_{50} value of 453.0 nM for 5AR-1 enzyme while for 5AR-2 enzyme the value was 40 nM in the present assay. Compounds **28**, **30**–**32** could not be evaluated because of the stability challenges for these molecules. The in vitro 5AR inhibition data have been summarized in Table 1.

2.3. In Vitro evaluation against DU-145 prostate cancer cell line

Human prostate carcinoma cell lines have been available since 1977 and represent a good experimental model to assess new hormonal therapies. There are many prostate cancer cell lines available out of which DU145 is androgen-independent cell line.[22] Finasteride, a selective 5AR-2 inhibitor, has been reported to reduce the proliferation rate in vitro of DU145 prostate cancer cell line although they being 'hormone-independent' suggesting that the local formation of even small amounts of DHT in prostate neoplastic tissues may play some role also in the pathogenesis of androgen-dependent prostate cancer.[23] There are also some reports that DU145 cancer cell line expresses mRNA for and expresses functional 5AR-1 activity.[24] Finasteride has been evaluated for reducing the risk of prostate cancer in a clinical trial, that is, the Prostate Cancer Prevention Trial (PCPT). Therefore, 5AR-1 inhibitors can be considered as a treatment strategy against prostatic cancer whereas inhibition of 5AR-2 may not be an appropriate therapeutic modality for the treatment of prostatic cancer.

Some of the synthesized compounds, based on their in vitro and in vivo activity, were evaluated for their antiproliferative potential on DU-145 cells as described by Mosamann.[25] All the compounds were tested at seven different concentrations in the culture medium and finasteride was used as reference drug. Linear regression line was drawn to calculate the concentration required to cause 50% inhibition in cell growth (IC_{50}) and the results for DU-145 have been reported in Table 2.

Some of the synthesized compounds (**10**, **27**, **29**) were found to be weakly active against DU-145 cell line probably due to the fact that the compounds were 5AR-2 active, that is, they inhibit synthesis of DHT and hence were unable to show effect on androgen independent cell lines. 3-Tetrazolo-19-nor-3,5-androstadien-17-one (**10**) exhibited nearly same activity as that of Finasteride on DU-145 cell line while there was no response to cell lines in case of 3-tetrazolo-3,5-androstadien-17-one (**6**), 3-tetrazolo-3,5-pregnadien-20-one (**14**) and 17a-aza-3-tetrazolo-D-homo-3,5-androstadien-17-one (**26**). *N*-Methyl derivative (**27**) and *N*-allyl derivative (**29**) however showed anti-proliferative activity on cell lines similar to that of Finasteride (Table 2).

2.4. In vivo inhibitory activity (change in rat prostate weight in mature male rats)

Despite of the histological and biochemical differences, rat prostate is commonly used as the prostate growth model because of its similar growth pattern with that of humans. Only active compounds (based on their in vitro 5α-reductase inhibitory activity) along with standard drug finasteride were selected for in vivo activity.[26–28] The results of the activity have been given in Table 3. The percentage decrease in the organ weights (ventral prostate, dorsal prostate, vas deferens and epididymis) has been reported in Table 4. In other organs the decrease was not significant. None of the tested compounds induced any mortality.

Animals treated (2 weeks) with Finasteride or a 5AR inhibitor were expected to have prostate weights significantly smaller than those of control animals. Consistent with this expectation, Finasteride produced a statistically significant decrease in ventral and dorsal prostate where it reduced the weight to around 44% as compared to control.

3-Tetrazolo-3,5-androstadien-17-one (**6**) significantly reduced the weight of ventral prostate (35.20%), vas deferens (59.11%)

Table 1
In vitro activity data of synthesized compounds against 5AR-1 and 5AR-2

Compound code	Activity code	5AR-1% inhibition at 10 μM	5AR-1% inhibition at 2 μM	5AR-2% inhibition at 10 μM	5AR-2% inhibition at 2 μM	IC_{50} of 5AR-2 (nM)
6	SAR-43	81.1 ± 2.5	38.7 ± 5.8	100.0 ± 0.0	100.0 ± 0.0	83.8 ± 17.4
10	SAR-47	79.7 ± 5.0	36.2 ± 6.6	79.7 ± 5.0	60.6 ± 2.1	n.d.
14	SAR-49	95.0 ± 7.1	82.6 ± 0.0 (IC_{50}-547.0 nM)	100.0 ± 0.0	100.0 ± 0.0	15.6 ± 4.7
26	SAR-44	9.6 ± 10.0	n.d.	100.0 ± 0.0	90.6 ± 8.4	220.1 ± 66.2
27	SAR-45	6.4 ± 9.0	n.d.	91.2 ± 2.8	84.3 ± 1.6	273.8 ± 45.2
29	SAR-46	31.7 ± 0.9	n.d.	100.0 ± 0.0	94.7 ± 7.6	157.0 ± 27.6
Finasteride	—	—	IC_{50}-453 nM	—	—	40 nM

n.d.—not determined; activity of compounds **28** and **30**–**32** couldn't be performed due to stability challenges.

Table 2
In vitro activity of synthesized compounds against DU-145 cell line

Compound code	Activity code	IC_{50} (μg/ml)	IC_{50} (μM)
6	SAR-43	N.I.	N.I.
10	SAR-47	174.40	536.34
14	SAR-49	N.I.	N.I.
26	SAR-44	N.I.	N.I.
27	SAR-45	143.20	389.12
29	SAR-46	195.10	495.50
Finasteride	—	204.20	548.00

N.I.—no siginificant inhibition; activity of compounds **28** and **30**–**32** couldn't be performed due to stability challenges.

and epididymis (36.25%) thereby exhibiting strong inhibition of 5α-reductase as displayed in vitro activity. It also reduced the weights of dorsal prostate (15.25%) and seminal vesicles but the reduction was not significant. However, its 19-nor counterpart (**10**) displayed weak inhibition in vivo as the reduction in the weights of organs, that is, ventral and dorsal prostate, epididymis and vas deferens were not significant. This indicated that loss of methyl group has lead to a reduction in activity in vivo. Other organs like liver, adrenal and testis have miniscule effect on weight which was not statistically significant.

3-Tetrazolo-3,5-pregnadien-20-one (**14**) however, emerged as one of the potent 5AR inhibitor in vitro as well as in vivo. It showed statistically significant reduction of ventral prostate weight (35.40%), epididymis (31.76%), vas deferens (57.10%) and seminal vesicles at a dose of 1 mg/kg. Dorsal prostate weight was also reduced (17.78%) but was not significant. Hence, substitution of tetrazole moiety at 3rd position led to the formation of more potent 5α-reductase inhibitor. Probably pregnane type moiety along with the tetrazole one affords better binding of the compounds at the active site of the enzyme. Effects on other organ weights like liver, adrenal and testis were not significant.

The unsubstituted compound (**26**) although caused reduction in the weights of ventral prostate, dorsal prostate, epididymis but the effect was significant only on vas deferens (37.09%). On N-alkylation the methyl (**27**) and allyl (**29**) derivatives formed, caused significant reduction in the weight of ventral prostate (29.94% and 33.61%, respectively) while the *N*-allyl derivative also showed significant reduction in the weight of vas deferens (37.07%). But these compounds didn't show any significant effect on other organs such as liver, adrenal, testis and seminal vesicles. Not much effect on the body weight was seen. Hence, it can be concluded that N-alkylation leads to the potent 5AR inhibitors in vitro as well as good inhibitory activity in vivo.

3. Conclusion

In the present study a series ten steroidal compounds consisting of 3-tetrazolo-3,5-androstadien-17-ones (**6**), 3-tetrazolo-19-nor-3,5-androstadien-17-ones (**10**), 3-tetrazolo-3,5-pregnadien-20-one (**14**), 17a substituted 3-tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (**26**–**31**) and 3-(2-acetyltetrazolo)-17a-aza-D-homo-3,5-androstadien-17-one (**32**) have been synthesized as a novel class of 5ARIs through multistep synthesis. 3-Tetrazolo-3,5-pregnadien-20-one (**14**) emerged as a potent dual inhibitor for 5AR-1 and 5AR-2 isozymes as it showed 5AR-2 inhibition with IC_{50} being 15.6 nM as compared to finasteride (40 nM) and a significant 5AR-1 inhibition with IC_{50} 547 nM as compared to finasteride being 453 nM. These results provide support to the hypothesis that having tetrazole group at C-3 mimicks the charge replacement of carboxylate ion and may provide a potential lead for design and development of newer class of 5AR inhibitors.

4. Experimental

4.1. Chemistry

The synthetic procedure of different intermediates 3-cyano-3,5-androstadien-17-one (5), 3-cyano-19-nor-3,5-androstadien-17-one (9), 3-cyano-3,5-pregnadien-20-one (13), 3-cyano-17a-aza-D-homo-3,5-androstadien-17-one (20), 17a-methyl-3-cyano-17a-aza-D-homo-3,5-androstadien-17-one (21), 17a-ethyl-3-cyano-17a-aza-D-homo-3,5-androstadien-17-one (22), 17a-allyl-3-cyano-17a-aza-D-homo-3,5-androstadien-17-one (23), 17a-benzyl-3-cyano-17a-aza-D-homo-3,5-androstadien-17-one (24) and 17a-cyanoethyl-3-cyano-17a-aza-D-homo-3,5-androstadien-17-one (25) were described in detail in our previous report.[10] The synthesis of the novel compounds are described in the following subsections.

The preparation of the tetrazole compounds from their respective cyano derivatives followed a similar procedure. The cyano compound (5 mmol) and sodium azide (0.84 g, 13.0 mmol) were dissolved in toluene (10 ml) and added to the solution of freshly prepared triethylamine hydrochloride formed by passing dry hydrogen chloride gas in triethylamine (2 ml) in toluene. The mixture was heated at 110–120 °C for 20–42 h with continuous stirring. After completion of the reaction, the mixture was cooled and extracted with water (3 × 20 ml). To the aqueous extract, conc. hydrochloric acid was added drop wise to precipitate the tetrazole. Any differences from the standard procedure have been noted below in the individual compound synthesis.

4.1.1. 3-Tetrazolo-3,5-androstadien-17-one (6)

3-Cyano-3,5-androstadien-17-one (**5**) (1.47 g, 5.0 mmol) and sodium azide (0.84 g, 13.0 mmol) were dissolved in toluene (10 ml) and added to the solution of freshly prepared triethylamine hydrochloride formed by passing dry hydrogen chloride gas in triethylamine (2 ml) in toluene. The mixture was heated at 110–120 °C for 20 h with continuous stirring. After completion of the reaction the mixture was cooled and extracted with water (3 × 20 ml). To the aqueous extract concentrated hydrochloric acid

Table 3
Effect of once daily 14-day oral treatment with compounds on body weight and organ weights in adult male rats

Compound tested	Body weight gain (g)% ± SE	Ventral prostate mg/100 g ± SE	Dorsal prostate mg/100 g ± SE	Seminal vesicles mg/100 g ± SE	Testis mg/100 g ± SE	Adrenal mg/100 g ± SE	Liver g/100 g ± SE	Vas deferens mg/100 g ± SE	Epididymis mg/100 g ± SE
Control	9.412 ± 1.632	59.494 ± 4.733	50.325 ± 7.519	175.439 ± 12.833	1054.099 ± 55.648	14.762 ± 0.600	2.994 ± 0.254	67.613 ± 3.248	187.356 ± 5.961
Finasteride	8.032 ± 2.748	32.866 ± 4.371*	27.519 ± 6.206*	105.51 ± 19.006	1058.022 ± 37.041	16.575 ± 1.704	3.5097 ± 0.12	57.843 ± 5.253	160.371 ± 17.802
Dutasteride	11.914 ± 2.747	35.858 ± 7.239*	29.211 ± 4.508*	118.275 ± 6.869	1067.385 ± 42.275	14.208 ± 1.473	3.209 ± 0.192	52.802 ± 4.551	155.415 ± 6.302
Testosterone	3.767 ± 1.597	70.923 ± 4.572	45.124 ± 4.875	167.305 ± 27.917	1108.457 ± 69.965	13.969 ± 1.349	3.292 ± 0.088	55.097 ± 7.961	171.193 ± 22.858
6; SAR-43	18.382 ± 0.315	38.551 ± 2.719*	42.651 ± 5.372	95.251 ± 22.228	1090.046 ± 88.751	17.986 ± 1.492	3.257 ± 0.109	27.646 ± 3.158*	119.445 ± 20.601
10; SAR-47	21.51 ± 2.668*	56.124 ± 1.804*	46.695 ± 1.762	188.757 ± 14.106*	1350.787 ± 59.1	19.76 ± 0.696	3.457 ± 0.059	59.232 ± 2.964	180.908 ± 11.739
14; SAR-49	8.328 ± 1.894	38.433 ± 1.709*	41.379 ± 3.368	78.265 ± 6.407*	844.538 ± 78.239	17.762 ± 0.744	3.649 ± 0.174	29.009 ± 2.205*	127.85 ± 12.045*
26; SAR-44	15.498 ± 3.349	44.744 ± 2.133	31.548 ± 2.092	183.148 ± 11.137	1152.287 ± 42.188	16.421 ± 0.355	3.215 ± 0.091	42.532 ± 4.169*	166.584 ± 6.219
27; SAR-45	15.456 ± 1.070	41.684 ± 2.707*	44.593 ± 4.466	199.778 ± 19.449	1233.129 ± 32.282	16.362 ± 0.672	3.328 ± 0.078	59.796 ± 5.271	170.447 ± 8.044
29; SAR-46	19.229 ± 1.148	39.499 ± 1.200*	41.802 ± 2.169	141.816 ± 8.288	1318.403 ± 31.837	24.261 ± 0.488	3.548 ± 0.092	42.548 ± 1.944*	169.924 ± 4.467

Activity of compounds **28** and **30–32** couldn't be performed due to stability challenges.
* p <0.050: Dunnett's t-test (control used as reference group); n = 5 rats for each treatment group.

was added drop wise to precipitate the tetrazole. The product was filtered, washed and dried under vacuum to afford 3-tetrazolo-3,5-androstadien-17-one (**6**) (1.20 g, 71.4%): mp 230–232 °C; UV_{max} (MeOH): 274.0 nm ($\log\varepsilon$ 4.40); IR (KBr, cm^{-1}): 2946, 2862, 1733, 1625 and 1547; ^1H NMR (DMSO-d_6): δ 0.89 (s, 3H, 18-CH_3), 1.00 (s, 3H, 19-CH_3), 5.79 (br s, 1H, 6-vinylic), 6.98 (br s, 1H, 4-vinylic) and 15.8 ppm (br s, NH); ^{13}C NMR (DMSO-d_6): δ 219.26 (C-17), 155.38 (C-N), 32.57 (C-1), 22.42 (C-2), 118.47 (C-3), 132.00 (C-4), 140.33 (C-5), 128.64 (C-6), 30.49 (C-7), 30.73 (C-8), 51.05 (C-9), 34.37 (C-10), 19.83 (C-11), 35.22 (C-12), 46.97 (C-13), 47.57 (C-14), 21.27 (C-15), 30.97 (C-16), 13.25 (C-18) and 18.68 (C-19); Mass (APCI): 339.60 $[M+1]^+$.

4.1.2. 3-Tetrazolo-19-nor-3,5-androstadien-17-one (10)

A solution of 3-cyano-19-nor-3,5-androstadien-17-one (**9**) (1.40 g, 5.0 mmol) and sodium azide (0.84 g, 13.0 mmol) in toluene (10 ml) and added to the freshly prepared triethylamine hydrochloride prepared by passing dry hydrogen chloride gas in triethylamine (2 ml) in toluene. The reaction mixture was heated at 110–120 °C for 24 h with continuous stirring. After completion of the reaction the mixture was cooled and extracted with water (3 × 20 ml). Tetrazole was precipitated by adding concentrated hydrochloric acid drop wise to the aqueous layer. The solid product was filtered and dried under vacuum to get 3-tetrazolo-19-nor-3,5-androstadien-17-one (**10**) (1.10 g, 68.3%); mp 265–267 °C; UV_{max} (MeOH): 272.8 nm ($\log\varepsilon$ 4.45); IR (KBr, cm^{-1}): 3116, 2875, 1734, 1628 and 1543; ^1H NMR (DMSO-d_6): δ 0.92 (s, 3H, 18-CH_3), 5.88 (br s, 1H, 6-vinylic), 7.05 (br s, 1H, 4-vinylic) and 15.84 ppm (br s, NH); ^{13}C NMR (DMSO-d_6): δ 219.26 (C-17), 155.45 (C-N), 35.20 (C-1), 25.64 (C-2), 120.36 (C-3), 132.40 (C-4), 135.67 (C-5), 128.85 (C-6), 26.18 (C-7), 35.59 (C-8), 50.21 (C-9), 40.20 (C-10), 21.16 (C-11), 30.09 (C-12), 47.05 (C-13), 43.18 (C-14), 25.21 (C-15), 30.97 (C-16), and 13.22 (C-18); Mass (APCI): 325.33 $[M+1]^+$.

4.1.3. 3-Tetrazolo-3,5-pregnadien-20-one (14)

3-Cyano-3,5-pregnadien-20-one (**13**) (1.61 g, 5.0 mmol) and sodium azide (0.84 g, 13.0 mmol) were dissolved in toluene (10 ml) and added to the freshly prepared triethylamine hydrochloride prepared by passing dry hydrogen chloride gas in triethylamine (2 ml) in toluene. The reaction mixture was heated at 110–120 °C for 24 h with continuous stirring. After completion of the reaction the mixture was cooled and extracted with water (3 × 20 ml). Tetrazole was precipitated by adding concentrated hydrochloric acid dropwise to the aqueous layer. The solid product was filtered and dried under vacuum to get 3-tetrazolo-3,5-pregnadien-20-one (**14**) (0.89 g, 66.9%); mp 240–242 °C; UV_{max} (MeOH): 270.4 nm ($\log\varepsilon$ 4.32); IR (KBr, cm^{-1}): 3424, 2938, 1698, 1631 and 1546; ^1H NMR (DMSO-d_6): δ 0.61 (s, 3H, 18-CH_3), 0.96 (s, 3H, 19-CH_3), 2.09 (s, 3H, 21-CH_3), 5.76 (br s, 1H, 6-vinylic) and 6.95 (br s, 1H, 4-vinylic); ^{13}C NMR (DMSO-d_6): δ 208.02 (C-20), 155.57 (C-N), 62.64 (C-17), 32.63 (C-1), 20.51 (C-2), 118.49 (C-3), 140.23 (C-4), 131.98 (C-5), 129.18 (C-6), 32.39 (C-7), 31.50 (C-8), 56.15 (C-9), 34.25 (C-10), 22.47 (C-11), 37.97 (C-12), 43.37 (C-13), 47.29 (C-14), 22.20 (C-15), 23.83 (C-16), 12.95 (C-18), 18.69 (C-19) and 31.10 (C-21); Mass (APCI): 339.60 $[M+1]^+$.

4.1.4. 3-Tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (26)

3-Cyano-3,5-pregnadien-17a-aza-D-homo-3.5-androstadien-17-one (**20**) (1.55 g, 5.0 mmol) and sodium azide (0.84 g, 13.0 mmol) were dissolved in toluene (5 ml) and added to the freshly prepared triethylamine hydrochloride prepared by passing dry hydrogen chloride gas in triethylamine (2 ml) in toluene. The reaction mixture was heated at 110–120 °C for 35 h with continuous stirring. After completion of the reaction the mixture was cooled and

Table 4
Percentage decrease in organ weight after treatment with synthesized compounds

Compound code	Ventral prostate	Dorsal prostate	Vas deferens	Epididymis
Finasteride	44.76	45.32	14.45	14.40
6; SAR-43	35.20	15.25	59.11	36.25
10; SAR-47	5.66	7.21	12.40	3.44
14; SAR-49	35.40	17.78	57.10	31.76
26; SAR-44	24.79	37.31	37.09	11.09
27; SAR-45	29.94	11.39	11.56	9.03
29; SAR-46	33.61	16.94	37.07	9.30

extracted with water (3 × 20 ml). Tetrazole was precipitated by adding concentrated hydrochloric acid dropwise to the aqueous layer. The solid product was filtered and dried under vacuum to get 3-tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (**26**) (0.70 g, 61.4%); mp 265–267 °C; UV_{max} (MeOH): 269.2 nm (log ε 4.34); IR (KBr, cm^{-1}): 3172, 2942, 2770, 1660, 1631, 1550 and 1237; ^1H NMR (DMSO-d_6): δ 0.92 (s, 3H, 19-CH_3), 1.16 (s, 3H, 18-CH_3), 5.78 (br s, 1H, 6-vinylic), 6.98 (br s, 1H, 4-vinylic) and 7.51 ppm (1H, NH); ^{13}C NMR (DMSO-d_6): 170.82 (C-17), 155.84 (C-N), 119.10 (C-3), 132.24 (C-4), 140.41 (C-5), 129.16 (C-6), 32.80 (C-1), 22.88 (C-2), 31.03 (C-7), 32.05 (C-8), 47.95 (C-9), 34.91 (C-10), 20.05 (C-11), 39.07 (C-12), 53.77 (C-13), 47.35 (C-14), 20.92 (C-15), 31.48 (C-16), 19.00 (C-18) and 22.09 (C-19); Mass (APCI): 354.27 $[M+1]^+$.

4.1.5. 17a-Methyl-3-tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (27)

17a-Methyl-3-cyano-17a-aza-D-homo-3,5-androstadien-17-one (**21**) (1.62 g, 5.0 mmol) and sodium azide (0.84 g, 13.0 mmol) were dissolved in toluene (5 ml) and added to the freshly prepared triethylamine hydrochloride prepared by passing dry hydrogen chloride gas in triethylamine (2 ml) in toluene. The reaction mixture was heated at 110–120 °C for 42 h with continuous stirring. After completion of the reaction the mixture was cooled and extracted with water (3 × 20 ml). Tetrazole was precipitated by adding concentrated hydrochloric acid dropwise to the aqueous layer. The solid product was filtered and dried under vacuum to get 17a-methyl-3-tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (**27**) (1.19 g, 65.0%); mp 260–262 °C; UV_{max} (MeOH): 270.4 nm (log ε 4.46); IR (KBr, cm^{-1}): 3098, 2945, 2755, 1606 and 1539; ^1H NMR (DMSO-d_6): δ 0.95 (s, 3H, 19-CH_3), 1.20 (s, 3H, 18-CH_3), 2.85 (s, 3H, *N*-CH_3), 5.78 (br s, 1H, 6-vinylic), 6.98 (br s, 1H, 4-vinylic) and 16.26 ppm (br s, 1H, NH); ^{13}C NMR (DMSO-d_6): 168.91 (C-17), 154.79 (C-N), 118.50 (C-3), 131.87 (C-4), 139.82 (C-5), 128.83 (C-6), 32.27 (C-1), 22.37 (C-2), 31.05 (C-7), 31.98 (C-8), 48.36 (C-9), 34.30 (C-10), 19.02 (C-11), 36.58 (C-12), 58.10 (C-13), 46.08 (C-14), 20.80 (C-15), 31.34 (C-16), 17.48 (C-18), 18.41 (C-19) and 26.11 (*N*-CH_3); Mass (APCI): 368.27 $[M+1]^+$.

4.1.6. 17a-Ethyl-3-tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (28)

17a-Ethyl-3-cyano-17a-aza-D-homo-3,5-androstadien-17-one (**22**) (1.69 g, 5.0 mmol) and sodium azide (0.84 g, 13.0 mmol) were dissolved in toluene (10.0 ml) and added to the freshly prepared triethylamine hydrochloride prepared by passing dry hydrogen chloride gas in triethylamine (2 ml) in toluene. The reaction mixture was heated at 110–120 °C for 35 h with continuous stirring. After completion of the reaction the mixture was cooled and extracted with water (3 × 20 ml). Tetrazole was precipitated by adding concentrated hydrochloric acid dropwise to the aqueous layer. The solid product was filtered and dried under vacuum to get 17a-ethyl-3-tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (**28**) (1.30 g, 68.4%); mp 220–222 °C; UV_{max} (MeOH): 272.0 nm (log ε 4.42); IR (KBr, cm^{-1}): 2942, 2741, 2677, 1598 and 1462; ^1H NMR (DMSO-d_6): δ 0.97 (s, 3H, 19-CH_3), 1.18 (s, 3H, 18-CH_3), 1.13 (t, 3H, CH_3-CH_2-N), 3.16 (dq, 1H, CH3C*H*H-N), 3.67 (dq, 1H, CH3CH*H*-N), 5.79 (br s, 1H, 6-vinylic) and 7.02 ppm (br s, 1H, 4-vinylic); ^{13}C NMR (DMSO-d_6): 169.34 (C-17), 129.51 (C-3), 132.11 (C-4), 140.09 (C-5), 128.86 (C-6), 32.56 (C-1), 22.61 (C-2), 31.40 (C-7), 32.32 (C-8), 48.93 (C-9), 34.60 (C-10), 19.28 (C-11), 37.08 (C-12), 59.29 (C-13), 46.40 (C-14), 21.02 (C-15), 31.74 (C-16), 15.42 (C-18), 18.65 (C-19), 35.95 (*N*-CH_2-CH_3) and 19.04 (*N*-CH_2-CH_3); Mass (APCI): 382.24 $[M+1]^+$.

4.1.7. 17a-Allyl-3-tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (29)

17a-Allyl-3-cyano-17a-aza-D-homo-3,5-androstadien-17-one (**23**) (1.75 g, 5.0 mmol) and sodium azide (0.84 g, 13.0 mmol) were dissolved in toluene (10.0 ml) and added to the freshly prepared triethylamine hydrochloride prepared by passing dry hydrogen chloride gas in triethylamine (2 ml) in toluene. The reaction mixture was heated at 110–120 °C for 30 h with continuous stirring. After completion of the reaction the mixture was cooled and extracted with water (3 × 20 ml). Tetrazole was precipitated by adding concentrated hydrochloric acid dropwise to the aqueous layer. The solid product was filtered and dried under vacuum to get 17a-allyl-3-tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (**29**) (0.95 g, 51.1%); mp 255–257 °C; UV_{max} (MeOH): 270.4 nm (log ε 3.97); IR (KBr, cm^{-1}): 2933, 1622 and 1457; ^1H NMR (DMSO-d_6): δ 0.94 (s, 3H, 19-CH_3), 1.17 (s, 3H, 18-CH_3), 3.74 (dd, 1H, *N*-H*H*C), 4.19 (dd, 1H, *N*-*H*HC), 5.03 (dd, 1H, *N*-CH_2-CH=C*H*H), 5.09 (dd, 1H, *N*-CH_2-CH=CH*H*), 5.83 (ddq, 1H, *N*-CH_2-*C*H=CHH), 5.76 (br s, 1H, 6-vinylic) and 6.98 ppm (br s, 1H, 4-vinylic); ^{13}C NMR (DMSO-d_6): 169.26 (C-17), 118.48 (C-3), 131.87 (C-4), 139.76 (C-5), 128.64 (C-6), 36.76 (C-1), 22.36 (C-2), 31.03 (C-7), 32.11 (C-8), 48.52 (C-9), 34.24 (C-10), 19.07 (C-11), 42.97 (C-12), 59.23 (C-13), 46.03 (C-14), 20.71 (C-15), 32.25 (C-16), 18.37 (C-18), 18.93 (C-19), 135.79 (*N*-CH_2*C*H=CH_2), 114.89 (*N*-CH_2CH=*C*H_2) and 45.53 (*N*-*C*H_2CH=CH_2); Mass (APCI): 394.40 $[M+1]^+$.

4.1.8. 17a-Benzyl-3-tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (30)

17a-Benzyl-3-cyano-17a-aza-D-homo-3,5-androstadien-17-one (**24**) (1.00 g, 2.5 mmol) and sodium azide (0.41 g, 6.5 mmol) were dissolved in toluene (5.0 ml) and added to the freshly prepared triethylamine hydrochloride prepared by passing dry hydrogen chloride gas in triethylamine (1 ml) in toluene. The reaction mixture was heated at 110–120 °C for 30 h with continuous stirring. After completion of the reaction the mixture was cooled and extracted with water (3 × 20 ml). Tetrazole was precipitated by adding concentrated hydrochloric acid dropwise to the aqueous layer. The solid product was filtered and dried under vacuum to get 17a-benzyl-3-tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (**30**) (0.58 g, 52.2%); mp 260–262 °C; UV_{max} (MeOH): 274.4 nm (log ε 4.32); IR (KBr, cm^{-1}): 3401, 2942, 1606 and 1449; ^1H NMR (DMSO-d_6): δ 0.91 (s, 3H, 19-CH_3), 1.23 (s, 3H, 18-CH_3), 5.00 (dd, 1H, PhCH*H*-N), 4.26 (dd, 1H, PhC*H*H-N), 5.78 (br s, 1H, 6-vinylic), 6.95 (br s, 1H, 4-vinylic) and 7.22 ppm (m, 5H, aromatic protons); ^{13}C NMR (DMSO-d_6): 169.72 (C-17), 129.02 (C-3), 131.67 (C-4), 139.77 (C-5), 129.60 (C-6), 32.20 (C-1), 22.35 (C-2), 31.05 (C-7), 31.06 (C-8), 48.57 (C-9), 34.25 (C-10), 19.34 (C-11), 37.02 (C-12), 59.50 (C-13), 45.94 (C-14), 20.67 (C-15), 32.25 (C-16), 18.39 (C-18), 18.79 (C-19), 125.91, 126.27, 127.82, 139.94 (aromatic carbons) and 43.71 (*N*-$CH_2C_6H_5$); Mass (APCI): 444.30 $[M+1]^+$.

4.1.9. 17a-Tetrazoloethyl-3-tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (31)

17a-Cyanoethyl-3-cyano-17a-aza-D-homo-3,5-androstadien-17-one (**25**) (0.91 g, 2.5 mmol) and sodium azide (0.41 g, 6.5 mmol) were dissolved in toluene (10.0 ml) and added to the freshly prepared triethylamine hydrochloride prepared by passing dry hydrogen chloride gas in triethylamine (1 ml) in toluene. The reaction mixture was heated at 110–120 °C for 30 h with continuous stirring. After completion of the reaction the mixture was cooled and extracted with water (3 × 10 ml). Tetrazole was precipitated by adding concentrated hydrochloric acid dropwise to the aqueous layer. The solid product was filtered and dried under vacuum to get 17a-tetrazoloethyl-3-tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (**31**) (0.63 g, 62.3%); mp 250–255 °C; UV_{max} (MeOH): 274.0 nm (log ε 4.45); IR (KBr, cm^{-1}): 3400, 2929, 1624, 1456 and 1259; ^1H NMR (DMSO-d_6): δ 0.95 (s, 3H, 19-CH_3), 1.18 (s, 3H, 18-CH_3), 3.41 (m, 1H, CH2-C*H*H-N), 3.87 (m, 1H, CH2-CH*H*-N), 2.97 (m, 1H, CH*H*-CH_2-N), 2.71 (m, 1H, C*H*H-CH_2-N), 5.77 (br s, 1H, 6-vinylic) and 6.98 ppm (br s, 1H, 4-vinylic); ^{13}C NMR (DMSO-d_6): 170.19 (C-17), 156.04 (C-N), 154.18 (*N*-CH_2CH_2-C-N–), 119.40 (C-3), 131.92 (C-4), 140.32 (C-5), 128.97 (C-6), 32.78 (C-1), 23.99 (C-2), 31.57 (C-7), 32.49 (C-8), 48.90 (C-9), 34.81 (C-10), 21.24 (C-11), 37.00 (C-12), 59.91 (C-13), 46.59 (C-14), 22.90 (C-15), 31.98 (C-16), 18.89 (C-18), 19.07 (C-19), 46.35 (*N*-CH_2CH_2C-N–) and 19.43 (*N*-CH_2CH_2C-N–); Mass (APCI): 430.35 $[M-18]^+$.

4.1.10. 3-(2-Acetyltetrazolo)-17a-aza-D-homo-3,5-androstadien-17-one (32)

3-Tetrazolo-17a-aza-D-homo-3,5-androstadien-17-one (**26**) (1.06 g, 3.0 mmol) was dissolved in pyridine (10.0 ml) and acetic anhydride (5.0 ml) was added to it. The solution was refluxed for 5 h. The excess of acetic anhydride was treated with methanol after cooling to room temperature was poured into crushed ice. The resulting precipitate was filtered, washed thoroughly with water and dried to yield 3-(2-acetyltetrazolo)-17a-aza-D-homo-3,5-androstadien-17-one (**32**) (0.75 g, 63.0%); mp 220–222 °C; UV_{max} (MeOH): 283.6 nm (log ε 4.45); IR (KBr, cm^{-1}): 3423, 2933, 1685 and 1631; ^1H NMR (DMSO-d_6): δ 0.88 (s, 3H, 19-CH_3), 1.09 (s, 3H, 18-CH_3), 2.45 (s, 3H, *N*-$COCH_3$), 5.75 (br s, 1H, 6-vinylic), 6.77 (br s, 1H, 4-vinylic) and 7.34 ppm (br s, 1H, NH); ^{13}C NMR (DMSO-d_6): 170.29 (C-17), 162.41 (C-N), 118.34 (C-3), 132.05 (C-4), 139.84 (C-5), 129.60 (C-6), 32.15 (C-1), 21.29 (C-2), 30.52 (C-7), 31.53 (C-8), 47.46 (C-9), 34.44 (C-10), 19.56 (C-11), 38.58 (C-12), 53.26 (C-13), 46.82 (C-14), 20.41 (C-15), 31.06 (C-16), 18.51 (C-18), 21.59 (C-19), 10.54 (*N*-CO-**C**H_3) and 164.76 (*N*-**C**O-CH_3); Mass (APCI): 410.31 $[M+15]^+$.

4.2. Biological activity

4.2.1. In vitro human 5α-reductase inhibitory activity

HEK-I and HEK-II served cell lines were used as a source of 5AR enzyme. Human embryonic kidney cell line (HEK293) lacking endogenous 5AR activity was transfected with the cDNAs encoding for both 5AR-1 and 5AR-2 by inserting them into pRcCMV (Cytomegalovirus promoter of the eukaryotic expression vector). Single cell clones were selected having substantially high enzymatic activity and were established as permanent cell lines.

4.2.2. Cell culture

The adherent fibroblastoid HEK293 cell line was obtained from DSMZ, Braunsch-weig, Germany (DSM ACC 305) and maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS), 0.25% sodium hydrogen carbonate, 100 units penicillin/ml, and 100 μg streptomycin/ml. The cells were grown in a humidified 95% O_2–5% CO_2 atmosphere at 37 °C in 175 cm^2 tissue culture flasks. Every 3–4 days they were split at a ratio of 1:6. For transfection experiments cells were used at passage number 8–10. Tissue culture reagents were from c.c.pro (Neustadt/W., Germany), except Geneticin selective antibiotic (G418 sulfate), which was from Calbiochem (Bad Soden, Germany).[28]

4.2.3. Construction of 5AR expression plasmids

The *Not* I-insert of the plasmid ph5α45 is a full length human cDNA encoding the 5AR-1 isoenzyme. It was inserted downstream of the Cytomegalovirus (CMV) promoter of the eucaryotic expression vector pRcCMV (Invitrogen, Groningen, Netherlands). This vector carries an additional neomycin resistance. The new construct which encoded human 5AR-1 was named pRcCMV-I and used for transfection.

The *Sal* I/*Not* I-insert of the plasmid pBS-76-1 correspond to the full length human cDNA encoding the 5AR-2 isoenzyme. It was first inserted by the *Sal*I/*Not*I-sites into pUC21-vector and recleaved by Hind III and X*ba*I. By this strategy a 5′-Hind III-and a 3′-X*ba*I-site was added to the 5AR-2 encoding DNA fragment, by which it was inserted into the expression vector pRcCMV. The resulting plasmid (pRcCMV-II) was used for transfecting HEK293 cells.[28]

4.2.4. Transfection procedure

One day before the transfection experiment 1×10^7 HEK293 cells were seeded in 100 mm culture dishes. By this procedure the culture is approximately 70% confluent on the day of transfection. The liposomal transfecting reagent Roti®-Fect was used for transfecting cells either with pRcCMV-I or pRcCMV-II following the manufacturers recommendations. The optimal DNA/reagent ratio was 10 μg plasmid and 20 μl Roti®-Fect reagent.

4.2.5. Selection of stable clones

Initially the concentration at which G418 sulfate inhibits the growth of untransfected HEK293 cells was determined. Therefore, varying concentrations of G418 sulfate (50 μg/ml-1000 μg/ml) were added to adherent HEK293 cells seeded in 24-well tissue culture plates at a density of 200,000 cells/well. After 6 days incubation at 37 °C, viable cells were determined using the trypan blue exclusion test. At a dose of 400 μg/ml G418 sulfate 50% of the cells were killed. Two days after transfection the growth medium was replaced by medium containing 500 μg/ml G418 sulfate. During the following 3 weeks of incubation untransfected cells subsequently died. To remove cell debris the medium was replaced every four days. Stable cell clones could be identified by phase contrast microscopy at the end of the second week. Single cell clones were picked and transferred into 60 rnm culture plates for further analysis.

Using these cell lines, selected synthesized compounds as well as the clinically used drug finasteride were tested, by measuring the conversion of [^3H] androstenedione.[25,26] HPLC reversed phase technique was used to quantify the reaction products. The details regarding preparation of solutions and HPLC analysis have been discussed in our previous report.[10]

4.2.6. Inhibition assay

Lysates were obtained after harvesting and resuspending 80% confluent cells in homogenate buffer (300 mM saccharose + 5 mM Tris–HCl + 0.1 mM EDTA) followed by homogenization by using ultrasonication. Suspensions of both cell lines (HEK-I and HEK-II) were used for all the assays. A master mix of 124.7 μl tris buffer, 50 μl androstenedione solution (500 nM) and 50 μl regenerating system (NADP/glucose/glucose-6-P-dehydrogenase = 1:2:1) was made and mixed with 0.3 μl ^3H labeled androstenedione. 25 μl of the compound solution (in 2% DMSO) to be tested was added to this solution to get a final test volume of 250 μl. 250 μl of the cell suspension was added to 250 μl of the master mix to make a total volume of 500 μl and incubated at 37 °C for 30 min. The reaction was stopped by addition of 750 μl of ether. The steroids were

extracted, dried and resuspended in 35 μl of methanol followed by radioactivity HPLC based detection. The amount of converted tritiated androstenedione was measured for each synthesized compound to determine the inhibitory activity of the compounds.[29,30]

4.2.7. In vivo 5AR inhibitory activity

Male wistar rats were used in the study in accordance with a protocol (6598/PS dated 21.08.2010) approved by the Institutional Animal Ethical Committee (IAEC) at the central animal house facilities of Panjab University, Chandigarh, India. The experiments were conducted as per CPCSEA (Committee for Prevention, Control and Supervision of Experimental Animals) guidelines. All rats were housed under standard conditions with free access to food and water. The control diet for the rats contained 50.0% nitrogen free extract, 20% protein, 8% fat, 4% cellulose, 1–1.5% of each mineral and vitamin mix. The control diet was obtained from Ashirwad Industries (Chandigarh, India). Finasteride was procured from Dr Reddy's Laboratories, Hyderabad as gift sample. Mature male Wistar rats were domesticated for a month and oral administration was started after 9 weeks. Following this varying amounts of drug suspension at a dose of 5 ml/kg was administered orally once daily for 14 consecutive days. All the test and reference compounds were suspended in 0.50% carboxymethylcellulose sodium salt (Himedia) solution. Rats were weighed and sacrificed by ether anaesthesia on the 15th day after 24 h of last dosing. The following organs were identified, removed and after removal of adhering fat and connective tissue weighed: ventral prostate, dorsal prostate, seminal vesicles, testes, epididymis, vas deferens, liver and adrenal glands. Organ weights were recalculated (mg/100 g body weight), that is, dividing the weight of the tissue by the body weight so as to remove variation due to the body weights among the groups. All weighing of the organs were made on Shimadzu AW 220 balance. Animals were divided into groups of 5 animals each. The following groups were taken in the study: Control (animals receiving only vehicle, i.e., 0.50% CMC), others were being Finasteride (animals receiving 5 ml/kg Finasteride) and synthesized compounds (animals receiving 5 ml/kg compound). Results are expressed as mean ± standard error of mean (SEM) of 5 animals per group. Statistical significance of differences between groups was determined by one-way analysis of variance (ANOVA) followed by Dunnett's test.[31,32] For the statistical determination, statistical computerized software SIGMASTAT 3.5 was used. A probability (P) value of less than 0.05 indicates a statistically significant difference between the treatment groups.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.12.048.

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