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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Anti-androgenic curcumin analogues as steroid 5-alpha reductase inhibitors

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Abstract Anti-androgen can be used in the treatment of benign prostatic hyperplasia, acne, hirsutism, and androgenic alopecia. For the search of anti-androgenic activity through steroid 5-alpha reductase (S5αR) inhibition mechanism, 12 natural analogs from plant origins, i.e., curcumin (**1**) demethoxycurcumin (**2**), and bisdemethoxycurcumin (**3**) isolated from *Curcuma longa* Linn., compounds **18**, **20**, **21**, **22**, **24**, and **25** isolated from *Curcuma comosa* Roxb., amide analogs **29–31** obtained from *Bougainvillea spectabilis* Willd. together with 21 synthesized analogs were evaluated for S5αR inhibitory activity using liquid chromatography–mass spectrometry assay. The results showed that compounds **1**, **2**, **4**, **5**, **6**, **7**, and **9** possessed S5αR inhibitory activity and compounds **1**, **4**, and **5** were the most potent (IC_{50} of 13.4 ± 0.4, 15.3 ± 3.1 and 8.9 ± 0.9 μM, respectively). This suggests that the unsaturated enone moiety in the chain linked between two aromatic rings of curcumin analog was imperative to the activity. Moreover, the *m*-methoxyl and *p*-hydroxyl substitutions in aromatic region of 1,6-heptadiene-3,5-dione linker were necessary. The cytotoxic effect on androgen-dependent cell, human dermal papilla was investigated to obtain safety information profile. We found that 1,6-heptadiene-3,5-dione linker was important for safety. This work stated that anti-androgen activity of curcumin analogs was through S5αR inhibition mechanism and the information might lead to further design of new curcumin analogs with improved potency and safety.

Keywords Curcumin analogues · Steroid 5-alpha reductase · Anti-androgen · Dermal papilla

Abbreviations

S5αR	Steroid 5α-reductase
DHT	Dihydrotestosterone

Introduction

Testosterone and dihydrotestosterone (DHT) are androgenic hormones that are pivotal to expression of secondary sex characteristic in men and in women. An important source of DHT is by steroid 5-alpha-reductase (S5αR) using nicotinamide adenine dinucleotide phosphate (NADPH) to reduce testosterone. There are three major isoforms of S5αR, i.e.,

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type I-III which are site specifically located within the target cells (Azzouni et al. 2012). DHT is 10-fold more potent than testosterone with higher affinity to the single androgen receptor (Russell and Wilson 1994; Azzouni et al. 2012). DHT over-production causes several androgen dependent diseases such as acne, hirsutism, androgenic alopecia (AGA), benign prostate hyperplasia (BPH), and prostate cancer (Cilotti et al. 2001). BPH increases with age: incidence 50% at 50 years old and up to 80–90% at 70 years in autopsy studies (Roehrborn 2005). AGA also increases with age affecting almost 50% of men and reduces their self-esteem and quality of life (Rhodes et al. 1998; Sinclair 1998).

Currently, S5αR inhibitors are first-line treatment androgen-dependent disorders. There are only two of these, finasteride and dutasteride, which have wide-spread regulatory approval for BPH and male AGA and these are based on steroid structures. However, their use is accompanied by sexual dysfunction and reduced libido (Traish et al. 2011; Traish et al. 2014). To circumvent these side effects, non-steroidal S5αR inhibitors are being sought (Inami et al. 1997; Occhiato et al. 2004).

Curcuma longa L. (Zingiberaceae family) has been used in Asian traditional medicine to treat many diseases including liver ailments, peptic ulcer, biliary disorders, flatulence, and skin diseases (Luthra et al. 2001). Its principle components, curcuminoids, include curcumin (**1**) and two minor compounds, demethoxycurcumin (**2**) and bisdemethoxycurcumin (**3**) (Araujo and Leon 2001). Curcuminoids are pharmacologically active as antioxidants, anti-inflammatories, antimicrobials, and anticancers (Maheshwari et al. 2006; Anand et al. 2008; Gupta et al. 2013). Recently, curcumin analogues were reported their cytotoxic toward prostate cancer cell lines (Fuchs et al. 2009; Piantino et al. 2009), and were action as androgenic receptor antagonists (Ohtsu et al. 2002; Lin et al. 2006). However, alternative mechanism for anti-androgenic activity of curcumin analogs through S5αR inhibition mechanism has not been identified yet which ultimately may have the potential of more selectively targeting androgen-dependent disorders. Here, we aimed to investigate the structure–activity relationships (SAR) of curcumin analogs for their S5αR inhibitory activity thereby providing useful lead compounds to develop more potent S5αR inhibitors. Moreover, the potential S5αR inhibitors from curcumin analogs were chosen and evaluated the safety profile on androgen-dependent cell, human dermal papilla.

Material and methods

General experiment

Liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) equipment; separation used an auto sampling Agilent 1200 Infinity Series high performance liquid chromatography (HPLC) system with two 108 vial trays or two 96-well plates (Agilent Technologies, Santa, Clara, CA, USA) and an analytical reversed phase Phenominex Luna® C18(2) (150 × 4.6 mm, 5 µm particle size) column and a guard column (Phenomenex C18, 4 × 3 mm, 5 µm particle size). The HPLC was connected with an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS (Agilent Technologies) equipped with a dual electrospray ionization detector. The mass data was analyzed using Agilent MassHunter Qualitative Analysis software version B06.00. Testosterone and dihydrotestosterone were purchased from Sigma Aldrich (MO, USA) and NADPH was from OYC (Tokyo, Japan). The BCA protein assay was purchased from Pierce (Rockford, IL, USA) and hydroxylamine was from Carlo Erba Reagent (Milan, Italy).

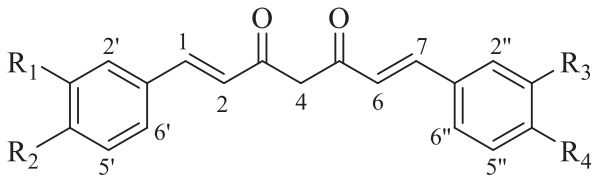
Curcumin analogs preparations

The curcuminoid are comonly known which constituents, **1** (curcumin), **2** (demethoxycurcumin), and **3** (bisdemethoxycurcumin) were isolated from rhizome of *C. longa* (Changtam et al. 2010) Compound **4** was synthesized by the demethylation reaction of compound **1** as described previously (Changtam et al. 2010). Compounds **5–16**, the 1,4,6-heptatriene-3-one linkage analogs, were synthesized by aldol condensation of substituted cinnamones and substituted cinnamaldehydes in base-catalyzed condition (Chuprajob et al. 2014). Compound **17** was synthesized by dehydration of hexahydrocurcumin as described in literature (Changtam et al. 2010). Compounds, **18**, **20**, **21**, **22**, **24**, and **25** were isolated from rhizome of *Curcuma comosa* Roxb. (Zingiberaceae) and compounds **19**, **23** were semi-synthesized using acetylation provided as described in literatures (Suksamrarn et al. 2008; Sornkaewa et al. 2015). The compounds with the related structure in aromatic ring, i.e., vanillic acid (**26**), ferulic acid (**27**), and piperine (**28**) were purchased from Sigma-Aldrich (MO, USA) while the phenolic amides (**29–31**) were isolated from bark of *Bougainvillea spectabilis* Willd. The spectroscopic data of **29–31** was in agreement with previous reported (Holzbach and Lopes 2010; Frerot et al. 2015). All analogs were tested on S5αR inhibitory activity using validated LC-MS assay.

Enzyme preparation

Androgen dependent LNCaP cells (CRL-1740™, American Type Culture Collection, VA, USA) provided the source of S5αR. Briefly, the LNCaP cells were cultured at 37 °C under 5% CO_2 humidified atmosphere and ≥80% confluent cells harvested, washed and centrifuged at 1900 g for 10 min The cell pellets were collected and re-suspended in tris-HCl buffer pH 7.4 (two volume of pellets) then

Table 1 Inhibition of S5αR by the 1,6-heptadiene-3,5-dione linkage series



Compounds **1-4**

Compound	R_1	R_2	R_3	R_4	% Inhibition at 300 µM	IC_{50} (µM)
1	OCH_3	OH	OCH_3	OH	104.0 ± 0.3	13.4 ± 0.4
2	H	OH	OCH_3	OH	101.4 ± 0.2	22.5 ± 0.6
3	H	OH	H	OH	22.2 ± 1.2	>300
4	OH	OH	OH	OH	107.2 ± 1.4	15.3 ± 3.1

homogenized by sonication. The protein concentration of homogenized cells was measured using Pierce bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). The final total protein was not less than 75 µg in S5αR inhibitory assay.

Steroid 5 alpha reductase inhibitory assay using LC-MS

The enzymatic assay, steroid 5-alpha inhibitory activity assay using LC-MS (Srivilai et al. 2016) was mimic biological conversion of testosterone to DHT by S5αR and NADPH co-factor. In briefly, the enzymatic assay was performed in 96-well plates following a validated assay. This used dihydrotestosterone formation quantitated by LC-MS to measure S5αR activity. The final enzymatic reaction volume was 200 µL and composed of 34.74 µM testosterone, 1 mM NADPH, homogenized crude enzyme (equivalent to 75 µg protein) and the volume was adjusted to 200 µL by adding tris-HCl buffer pH 7.4. The reaction was initiated by incubation at 37 °C. After 60 min, the reaction was quenched by added 300 µL of hydroxylamine (10 mg/mL) in 80% (v/v) ethanol and incubated at 60 °C for 60 min which completely hydroxylamine-derivatised all the produced DHT making it suited to MS quantification. Then, the 96-well plate was centrifuged at 1700 *g* for 10 min, and the supernatant collected and the derivatised-DHT quantitated by LC-MS. Two control samples were used; C1 which contained the complete reaction mixture but quenched before enzymatic incubation, and the control (C2) in which was enzymatic reaction was quenched at 60 min after enzymatic incubation. The 10 µL of test substance was added to replace that volume of tris-HCl buffer pH 7.4 likewise the blank solvent (dimethyl sulfoxide (DMSO)) that used to dissolve test substance, in the same volume was added to replace buffer in C1 and C2. The DHT production was measured using LC-MS. The extracted ion chromatogram (EIC) of derivatized-DHT (*m/z* [M + H] +,306.2428), the area under curve at retention time 6.95 min was used to express enzymatic inhibition:

$$S5\alpha R\,\text{inhibition} = [1 - (\text{Sample} - C1)/(C2 - C1)] \times 100\%$$

The known S5αR inhibitor, finasteride was used as the positive control.

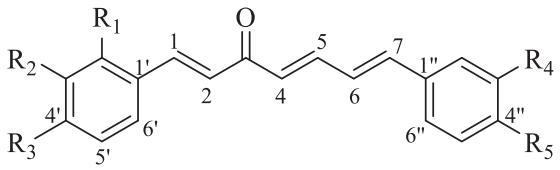
Cytotoxicity study on cell base model

Cytotoxicity of curcuminoid analogs on human follicle dermal papilla cells; Primary human follicle dermal papilla cells (PromoCell GmbH, Heidelberg, Germany) were cultured in follicle dermal papilla cell growth medium (PromoCell GmbH,) to which was added fetal bovine serum (4% v/v), bovine pituitary extract (0.4% v/v), basic fibroblast growth factor (1 ng/mL) (PromoCell GmbH, Heidelberg, Germany). The cells were seeded into 96-well plates, and incubated for 24 h at 37 °C under a 5% CO_2 humidified atmosphere. The medium was then removed and the test compounds in the culture medium were added for another 24 h. Then, 10 µL of 5 mg/mL MTT reagent was added and incubated for 2 h. The medium was removed and the formazan produced in the viable cells was solubilized by adding DMSO:EtOH (1:1 v/v). The absorbance at 595 nm was measured using microplate reader and the % cell viability was determined by comparing the absorbance with the control (non-treated).

Data analysis

The %S5αR inhibition was plotted against log10 [curcumin analogs] and the half maximum inhibitory concentration (IC_{50}) was caluculated using Graph-Pad Prism Software version 6 (San Diego, USA). Cytotoxicity was calculated as half maximal effectiveness concentration (EC_{50}) and the

Table 2 S5αR inhibitory activities of 1,4,6-heptatriene-3-one linkage series



Compounds **5-16**

Compound	R_1	R_2	R_3	R_4	R_5	% Inhibition at 300 μM	IC_{50} (μM)
5	H	OCH_3	OH	OCH_3	OH	102.41 ± 1.17	8.92 ± 0.86
6	H	H	OH	H	OH	85.92 ± 1.35	93.51 ± 3.67
7	H	H	OH	OH	H	92.94 ± 0.13	88.57 ± 2.30
8	H	OH	H	OH	H	42.78 ± 1.04	>100
9	H	OCH_3	OH	OH	H	87.67 ± 0.71	78.8 ± 3.8
10	H	OH	OCH_3	OH	OCH_3	22.06 ± 0.55	>300
11	H	OH	OCH_3	OH	H	28.11 ± 1.84	>300
12	H	H	H	H	H	3.05 ± 0.45	ND[a]
13	H	NO_2	H	H	H	19.80 ± 1.77	>300
14	Cl	H	H	H	H	5.30 ± 3.65	>300
15	H	H	OCH_3	H	H	23.30 ± 4.90	>300
16	OH	H	H	OC_3H_3	H	19.80 ± 4.23	>300

[a] ND; not determined

maximum concentration of curcumin analogs at 2000 and 1000 μM which supposed to be 100% cell death or complete the cell growth inhibition was used for EC_{50} calculation. In addition to the mean, the standard deviation were all calculated from at least triplicate of experiment.

Results and discussion

From preliminary study, we found that *C. longa* ethanolic extract inhibited S5αR ($IC_{50} = 9.0 \pm 1.2$ μg/mL), which accorded with a similar action of *C. longa* extract (Jang et al. 2007). The main chemical constituent of *C. longa*, curcuminoids which was the mixture of curcumin; demethoxycurcumin; bisdemethoxycurcumin (65:25:10, the % by weight, Sigma-Aldrich, MO, USA), was also evaluated and showed 3-fold higher potency than the extract ($IC_{50} = 3.7 \pm 0.3$ μg/mL). The result implied that curcuminoids might be responsible for S5αR inhibitory activity. The known S5αR inhibitor, finasteride was used as the positive control and exhibited the $IC_{50} = 0.76 \pm 0.03$ μM which was in agreement with the previous work using similar enzyme origin from prostate cancer cell (LNCaP) (Seo et al. 2002; Lazier et al. 2004). The LNCaP cells provided mainly type S5αR I and III isoforms (Negri-Cesi et al. 1998; Godoy et al. 2011). In this study, we, therefore, expanded to investigate and identify the anti-androgen activity through the S5αR inhibitory mechanism in particular constituents of curcuminoids and their analogs.

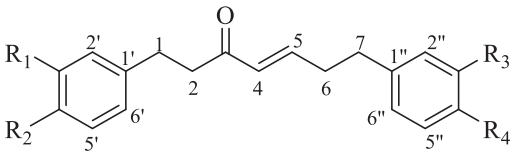
1,6-heptadiene-3,5-diones

The first series was on the diketone linkage between aromatic rings. The modifications were focused in substitution on the aromatic rings as shown in Table 1. Curcumin (**1**) had an IC_{50} of 13.4 ± 0.4 μM but removing the 3′-methoxyl giving **2**, reduced S5αR inhibitory potency by ~2-fold. Removing the other methoxyl at meta position in **2**, dramatically reduced the S5αR inhibitory activity with $IC_{50} >$ 300 μM (**3**). Replacing both methoxyl groups in **1** by hydroxyl groups yielding compound **4** with a similar potency suggesting that either methoxyl or hydroxyl groups at the C-3′ and 3″ position are crucial for S5αR inhibition. Thus these meta-position substitutions might cause H-bond interaction or act as bulky groups at the S5αR binding site.

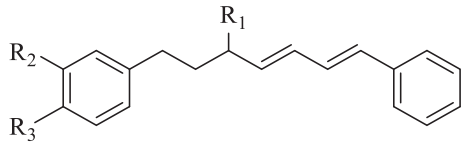
1,4,6-heptatriene-3-one

In this series, the importance of the 3,5 ketones in the linkage was demonstrated when compared with the first series. The effect of substitutions in aromatic regions of 1,4,6-heptatriene-3-one is shown in Table 2. The monoketone analogues **5** and **6** showed the similar substitution on the aromatic of compound **1** and **3**, respectively. The IC_{50}s

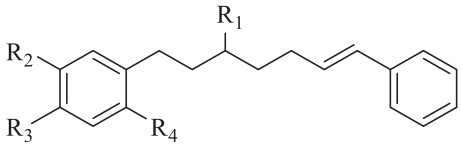
Table 3 S5αR inhibitory activities of some curcumin analogues with different degree of unsaturated linkage between aromatic rings and miscellaneous compounds



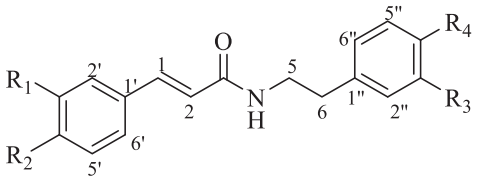
Compound **17**



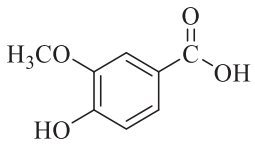
Compound **18**,**19**, **20**, **21**



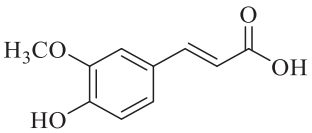
Compound **22**, **23**, **24**, **25**



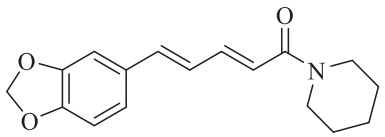
Compound **29**-**31**



26 = no activity



27 = no activity



28 = no activity

Compound	R_1	R_2	R_3	R_4	% Inhibition at 300 μM	IC_{50} (μM)
17	OCH_3	OH	OCH_3	OH	48.90 ± 2.47	>100
18	=O	H	OH	H	5.12 ± 1.13	>300
19	=O	OH	OAc	H	22.37 ± 3.96	>300
20	-OH	H	H	H	0.0	ND[a]
21	-OH	H	OH	H	0.0	ND[a]
22	=O	H	OH	H	7.74 ± 3.75	>300
23	=O	H	OAc	H	24.11 ± 4.53	>300
24	-OH	H	OH	H	0.0	ND[a]
25	-OH	OH	OH	H	0.0	ND[a]
29	OCH_3	OH	OCH_3	OH	46.45 ± 2.73	ND[a]
30	OCH_3	OH	H	OH	29.00 ± 2.68	ND[a]
31	OCH_3	OH	H	OCH_3	40.00 ± 4.20	ND[a]

[a] ND; not determined

of both **5** and **6** improved compared with **1** and **3**. The 1,4,6-heptatriene-3-one linker might give more favorable conformation of the molecule for S5αR binding than 1,6-heptadiene-3,5-dione linker. Moving one site of hydroxyl substitution in aromatic region on reduced ketone site at 4′′-position in **6** to 3′′-position of **7** did not alter the inhibitory activity while moving a 4′-hydroxyl group of **7** to 3′ position in **8**, the activity decreased to more than 100 μM. The electron donating property at para-position on ketone site might be required to add the electron density to pi-system so that the carbonyl group could be more nucleophilic and suitable for binding. Another possibility is that the para-hydroxyl substitution in aromatic ring moiety which was found in **5**, **6**, **7**, and **9** might interact to the S5αR

as an H-bond donor. A methoxy substitution at 3′ of **7** to yield **9** slightly increased inhibition. The result suggests that the 3′-methoxyl group is required for the inhibitory activity, however the importance is less than hydroxyl group at para-position (especially on ketone site). This conclusion is supported by reversion of the hydroxyl and methoxyl groups in **5** to **10.** The activity was clearly eliminated and the same result was found in **11**. Only core skeleton of 1,4,6-heptatriene-3-one linkage (**12**) without any substitution on aromatic ring showed no activity. Changing substitution groups to 3′-nitro in **13**, 2′-chloro in **14**, 4′-methoxyl in **15** and 2′-hydroxyl-3″-o-propargryl in **16**, did not affect the inhibitory activity. All these results support the idea that the proper substitute group in aromatic part is needed for S5αR inhibitory activity and the suitable linker might improve the activity.

The last series was miscellaneous groups with different degree of unsaturated linkage or with additional amide linkage between aromatic rings. All structures and inhibitory activities are shown in Table 3. The analogs with 1,6-heptadiene-3,5-dione linker (**1**) and 1,4,6-heptatriene-3-one linker (**5**) showed strong activity so their unsaturated-enone linker might be imperative to the activity. Reducing the unsaturated enone linker of **5** to 4-heptene-3-one linker in **17** eliminated the activity >12 fold. In addition, reducing unsaturation in the linker but remaining 3-one in **18, 19, 22, 23** the activity was dramatically decreased. In the series of 3-hydroxyl-4,6-heptadiene and 3-hydroxyl-6-heptene, there were no activity found in the analogues (**20, 21, 24, 25**). This data suggests that the conjugate bonds might involve in electron transfer in the molecule and at least one carbonyl at the linkage between both aromatic is needed in curcuminoid analogues to make the appropriate conformation of the molecule for S5αR binding.

Furthermore, we also investigated small molecules that possess meta-methoxy and para-hydroxy substitutions in the aromatic ring like **1** with acid side chain which were vanillic acid (**26**) and ferulic acid (**27**). However, both compounds showed no activity. This might suggest that not only the aromatic portion with meta-methoxy and para-hydroxy substitution but also the proper side chain and functional group are essential for the activity. This was confirmed by the fact that piperine (**28**) with shorter unsaturated enone and different substitutions in aromatic ring showed no S5αR inhibitory activity. Another hypothesis was that an enone in a linkage might be a catalytic binding site similar to testosterone substrate. The increasing of nucleophilicity in testosterone by changing the enone to an amide in the steroid A ring created the 4-azasteroid drugs, finasteride, and dutasteride, with potent activity. Therefore, three natural compounds **29–31** with amide in the linkage were studied. Compounds **29** and **30** had similar substitution on the aromatic ring as in **1** and **2**, respectively, but the % inhibitory activity of both amide analogs was much lower compared to the curcumin analogues. Changing the substitution on the ring in **31** also resulted in decreased activity. The result implies that testosterone and curcumin analogues might have different binding characteristics or different binding pockets to S5αR. In case of curcuminoid analogues, the unsaturated bonds are required to turn the molecule in a proper conformation or act as pi-system to transfer electron to carbonyl oxygen for binding with S5αR. The SAR of obtained from our study can be summarized as follows: (i) the proper substituents in aromatic region of curcumin analogues is needed for activity, especially at meta-position and para-position, (ii) the unsaturated enone linker between aromatic rings is imperative to activity, (iii) at least one keto group in the unsaturated linker of curcumin analogues is required.

Table 4 Cytotoxicity on primary human follicle dermal papillary cells of the curcumin analogues with high S5αR inhibitory activity

Compound No.	Cytotoxicity, EC_{50} (μM)
1	>100
2	>100
4	>100
5	19.0 ± 6.1
6	48.0 ± 8.0
7	21.4 ± 5.6
9	22.7 ± 4.0

Data are means ± SD from triplicate experiments

The analogues which were effective as S5αR inhibitors could be the potential anti-androgen for hair loss treatments, nevertheless, the toxicity of the compounds should be concerned. Therefore, the active compounds in this study were screened for cytotoxicity using human dermal papilla cell. Compounds **1**, **2** and **4** showed cytotoxic EC_{50}s > 100 μM whereas compounds **5**, **6**, **7**, and **9** had EC_{50}s (20–50 μM) within the potential therapeutic range (Table 4) and this was possibly due to the absence of one keto group. This implies that 1,6-heptadiene-3,5-dione linkage is important for safety profile of curcuminoid analogs. Additional models might be needed to confirm the safety profiles of curcuminoid analogues.

Conclusion

In this study, we have identified and investigated the SAR of curcuminoid analogues on anti-androgenic activity through S5αR inhibition mechanism in various linkers and substitutions on aromatic region. Compounds **1**, **4**, and **5** showed high inhibitory activity in micro-molar range. The SAR data provides pointers for further refinements with greater S5αR inhibitory activity and safety. Compounds **1**

and **4** had low cytotoxicity and are promising candidates to treating androgenic alopecia.

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Conflict of interest The authors declare that they have no competing financial interests.

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