

Investigations regarding the WADA classification of the supplement ingredient androsta-1,4,6-triene-3,17-dione

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Abstract

In scientific literature androsta-1,4,6-triene-3,17-dione (ATD) was reported to effectively reduce estrogen biosynthesis by irreversible aromatase inhibition. Up to now no preparation was approved for medical use. Since 2010 WADA has explicitly included ATD in its list of substances prohibited for use in sports. But sanctions on an athlete strongly depend on the classification of the administered drug. As anabolic agents are considered as “Non-Specified Substances” while aromatase inhibitors are judged as “Specified Substances” this assignment is particularly important for the valuation of adverse analytical findings.

Thus, we have investigated the ability of ATD and its main metabolite 17OHAT to interact with the androgen receptor (AR). Using a yeast AR transactivation system ATD and 17OHAT could be identified to be moderate AR agonists. In addition 17OHAT was also found to be a functional AR antagonist. To investigate potential androgenic/anabolic effects of ATD *in-vivo*, a rodent Hershberger assay was performed. Neither anabolic nor androgenic properties of ATD were detected. Additionally blood plasma concentrations of testosterone were determined using ELISA and compared between the administration groups. In line with already published data demonstrating that ATD increases testosterone levels in men, our results support the classification of ATD as aromatase inhibitor. However, whether ATD and 17OHAT may also act as an antiandrogen, and how ATD effects testosterone levels has to be investigated in future studies.

Introduction

In scientific literature androsta-1,4,6-triene-3,17-dione (ATD) was reported to effectively reduce estrogen biosynthesis by irreversible aromatase inhibition [1,2]. Additionally it was shown to alter the 5 α -reductase activity at least in the rodents [3]. Up to now no preparation was approved for medical use. However, several preparations containing ATD appeared on the market labelled as dietary supplements, e.g. “Novedex XT” of the brand name “Gaspari Nutrition”. Thus, ATD can be easily accessed, and was already detected in athletes urines [4]. Since 2010 WADA has explicitly included ATD in its list of substances prohibited for use in sports (class S4. Hormone Antagonists and Modulators, particularised class S4.1. Aromatase Inhibitors). But sanctions on an athlete strongly depend on the classification of the administered drug. As anabolic agents are considered as “Non-Specified Substances” while aromatase inhibitors are judged as “Specified Substances” this assignment is particularly important for the valuation of adverse analytical findings. In a previous investigation the urinary elimination of ATD was studied [5,6]. Several reduced metabolites were detected including 17 β -hydroxyandrosta-1,4,6-trien-3-one (17OHAT), which was reported to represent the main metabolite of ATD. Thus, in the present study we have investigated the ability of ATD together with 17OHAT to interact with the androgen receptor (AR) in a yeast transactivation assay. Sohoni and Sumpter introduced such an assay for the detection of androgenic substances [7]. Recently it was demonstrated that it can be used to detect several steroids due to their binding to the androgen receptor [8-10]. We have also determined the anabolic and androgenic properties of ATD *in-vivo* in the rat and metabolism was studied with special respect to testosterone.

Materials and Methods

Chemicals

ATD was purchased from Steraloids (Wilton, USA) and its main metabolite 17OHAT was synthesized in our laboratory as described elsewhere [5]. All other substances and reagents were obtained from Sigma-Aldrich (Deisenhofen, Germany) or Tocris (Ellisville, USA).

Yeast Androgen Receptor Transactivation System

For the *in-vitro* assessment of the androgenicity a concentration dependent assay of ATD and its main metabolite 17OHAT was performed in a yeast androgen receptor transactivation assay. Cells were cultured as reported previously [11].

The yeast *Saccharomyces cerevisiae* cells are stably transfected with an AR construct and an expression plasmid carrying androgen-responsive sequences controlling the expression of the reporter gene *LacZ* encoding the enzyme β -galactosidase. The β -galactosidase activity, corresponding to the receptor binding capacity, catalyzed the enzymatic hydrolysis of chlorophenol red β -D-galactopyranoside. This was detected at 565 nm using a colorimetric assay. Within the assay for androgenicity of ATD and 17OHAT, the compounds were dissolved in DMSO and analyzed in a concentration dependent assay (10^{-4} - 10^{-10} M). 5α -Dihydrotestosterone (DHT) served as reference compound. Co-incubation of ATD and 17OHAT at different concentration levels with constant 10^{-8} M DHT was used to test for antagonistic properties.

Rodent Hershberger Assay

Castrated male Wistar rats were treated for 12 days once a day s.c. with ATD (1 mg/kg BW/day), TP (1 mg/kg BW/day, positive control) or vehicle (negative control) only. All animal handling and experimental conditions were according to the Institutional Animal Care and Use Committee guidelines, regulated by the German federal law for animal welfare, therefore the experiments were only performed after a detailed proposal was applied and accepted. The animals were housed under controlled conditions of illumination (12 h light / 12 h dark), temperature ($20\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ with relative humidity 50 – 80 %), and had *ad libitum* access to the diets and water. After necropsy the wet weights of the prostate, seminal vesicle, and the muscle levator ani were determined.

Additionally blood plasma was taken from the rats using heparin tubes during necropsy. The plasma concentrations of testosterone (T) were determined using EIA and compared between the administration groups. After extraction of the specimen with diethyl ether a heterologous competitive ELISA was performed using 96-well flat bottomed polystyrene microtiter plates (Greiner Labortechnik, Germany), a 'second antibody' coating technique and testosterone-horseradish peroxidase (HRP) as a label as described by Sarkar et al. [12].

Results and Discussion

Yeast Androgen Receptor Transactivation System

Using a yeast AR transactivation system, ATD and 17OHAT could be identified to be moderate AR agonists with transactivation potencies ~10times lower than the natural AR ligand DHT. In addition 17OHAT was found to be a functional AR antagonist (*Figure 1*).

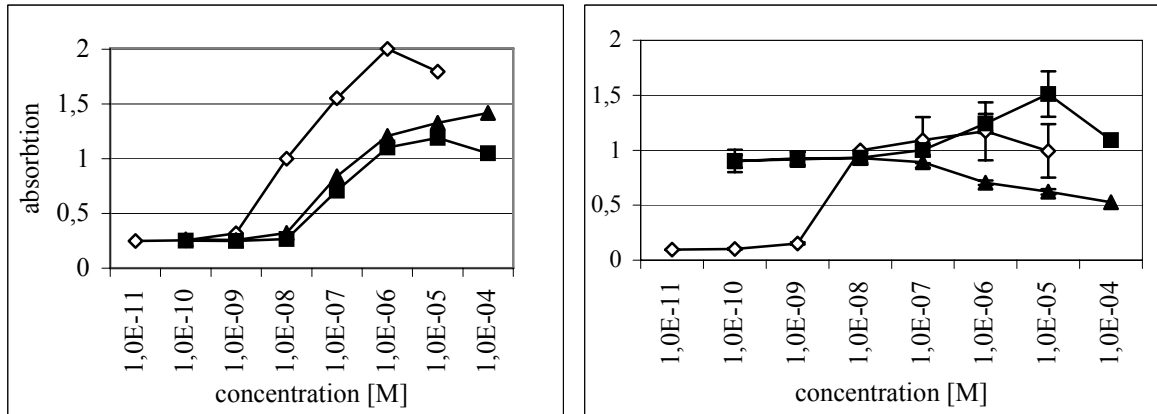


Figure 1: Dose dependent stimulation of β -Gal expression by DHT (\diamond , reference), ATD (\blacksquare), 17OHAT (\blacktriangle) in androgen sensitive yeast reporter gene assay, left: demonstrating binding to androgen receptor, right: co-incubation with 10^{-8} M DHT to test for antagonistic properties

Rodent Hershberger Assay

To investigate potential androgenic/anabolic effects of ATD *in-vivo* a rat Hershberger assay was performed: Orchiectomised rats were treated for 12 days s.c. (1 mg/kg BW/day) with ATD. Vehicle-treated orchiectomised and testosterone propionate (TP) treated rats (1 mg/kg BW/day) served as reference groups. The administration of ATD neither resulted in a significant stimulation of the prostate nor the seminal vesicles weights (Figure 2 A and B) serving as measure for the androgenic properties. The anabolic effect is investigated via the wet weight of the levator ani muscle (Figure 2 C). No stimulation effect on the lev. ani weights was observed. Thus, the administration of ATD neither results in anabolic nor androgenic effects. Analogous results were already published for the administration of 17OHAT [3].

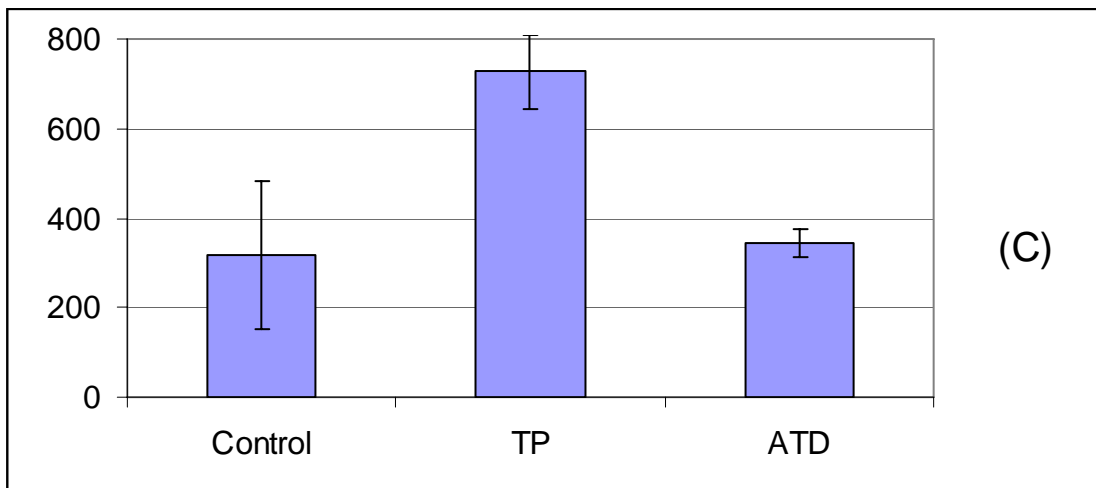
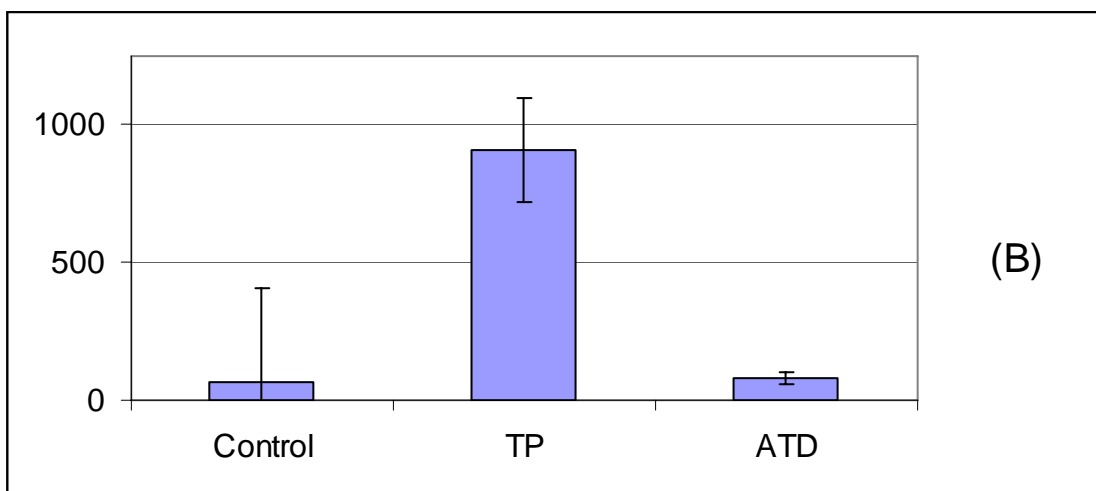
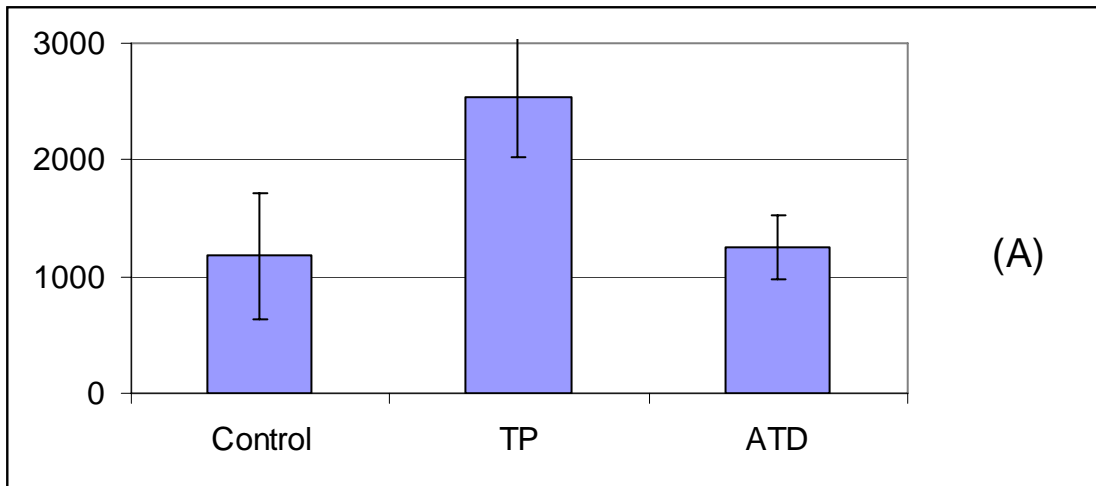


Figure 2: Effects of ATD on tissue wet weight (in mg/kg BW) of the prostate (A), seminal vesicle (B), and levator ani (C), in orchietomized rats. Control = untreated animals; TP= Testosterone propionate, s.c. treatment (1 mg/kg BW/day); ATD = Androsta-1,4,6-triene-3,17-dione s.c. treatment (1 mg/kg BW/day)

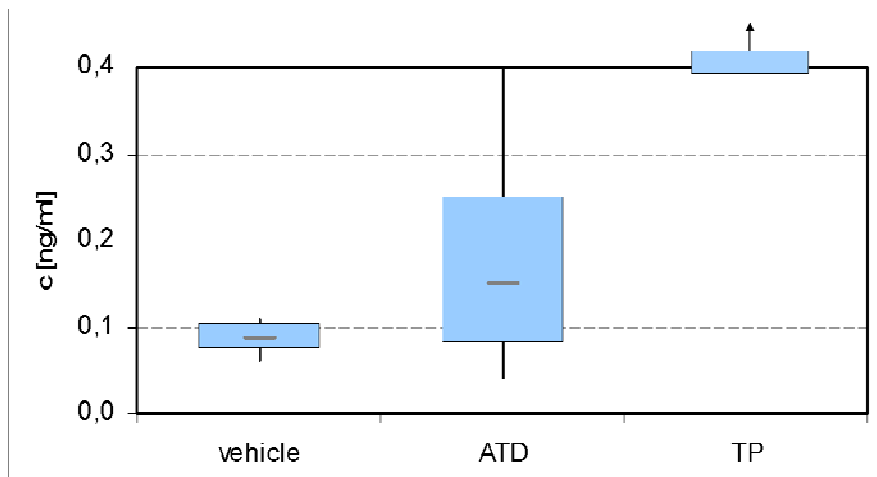


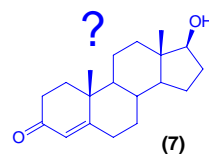
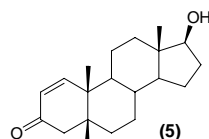
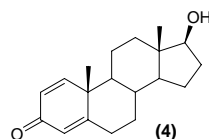
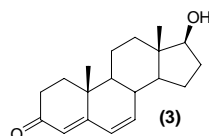
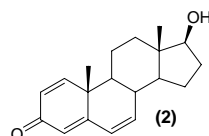
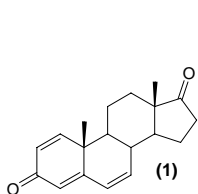
Figure 3: Plasma Testosterone concentrations determined in rats s.c. dosed with ATD, TP or vehicle only

Metabolism

The concentrations of testosterone in the rat plasma were determined using ELISA and compared between the administration groups. Slightly but not significantly increased plasma concentrations of T were detected in the ATD dosed rats (Figure 3).

Following an administration of four and eight weeks of the product Novedex XT (dietary supplement containing ATD) in humans Willoghby et al. [13] reported a significant increase of free and total plasma testosterone concentration, while LH levels remained unchanged. Several metabolites were identified in the urine following ATD administration in men Figure 4. Additionally a significant decrease in the urinary steroid profile ratio of androsterone (AND)/T was observed together with increases in the ratio of T/epiT, 5 α -/5 β -androstane-3 α ,17 β -diol and AND/etiocholanolone [5]. These data indicate either the formation of T as metabolite or its accumulation due to the inhibition of the aromatase.

Unconjugated



Sulfate

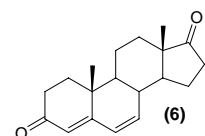


Figure 4: Chemical structures of ATD (1) and its urinary metabolites (2)-(7), adapted from [5]

Conclusion

ATD and its 17OH metabolite moderately bind to the androgen receptor in the yeast assay. ATD acts as receptor agonist, 17OHAT as functional antagonist. However *in-vivo* neither anabolic nor androgenic effects on the wet weights are detected for ATD and 17OHAT in the rat.

In line with already published data [5,13] demonstrating that ATD increases testosterone levels in men, our results support the classification of ATD as aromatase inhibitor. However whether ATD and 17OHAT may also act as an antiandrogen, and how ATD effect testosterone levels has to be investigated in future studies.

Acknowledgements

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