Toxicology Letters 292 (2018) 39–45

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Androgen- and estrogen-receptor mediated activities of 4-hydroxytestosterone, 4-hydroxyandrostenedione and their human metabolites in yeast based assays

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ARTICLE INFO

Keywords:
Saccharomyces cerevisiae
Formestane
4-Hydroxytestosterone
Doping
Molecular modeling

ABSTRACT

4-Hydroxyandrost-4-ene-3,17-dione, also named formestane, is an irreversible aromatase inhibitor and therapeutically used as anti-breast cancer medication in post-menopausal women. Currently, no therapeutical indication led to approval of its 17-hydroxylated analog 4-hydroxytestosterone, an anabolic steroid. However, it is currently investigated in a clinical trial for breast cancer. In context with sports doping, aromatase inhibitors are administered to reduce estrogenic side effects of misused anabolic substances or their metabolites. Therefore, both substances are prohibited in sports by the World Anti-Doping Agency (WADA). Analysis of urinary phase I and phase II metabolites showed similar results for both compounds. In the current investigation, 4-hydroxyandrost-4-ene-3,17-dione, 4-hydroxytestosterone and seven of their described urinary metabolites as well as 2α-hydroxyandrostenedione were tested in the yeast androgen screen and the yeast estrogen screen. Androgenic effects were observed for all tested substances, except for one, which showed anti-androgenic properties. With regard to the yeast estrogen screen, estrogenic effects were observed for only two metabolites at rather high concentrations, while six out of the ten substances tested showed anti-estrogenic properties. In terms of the strong androgenic effect observed for 4-hydroxytestosterone (10^{-8} M), 4-hydroxyandrost-4-ene-3,17-dione (10^{-8} M) and two more urinary metabolites, the yeast androgen assay may also be used to trace abuse in urine samples.

1. Introduction

Anabolic agents are mainly consumed by athletes to boost their muscle mass, for muscle adaptation and in muscle regeneration processes and/or to enhance their performance (Diel et al., 2008; Kicman and Gower, 2003; Parr and Schänzer, 2010). 4-Hydroxytestosterone (4,17β-dihydroxyandrost-4-en-3-one, 4HOT) is an anabolic steroid that is advertised for muscle building purposes, although there is no therapeutical approval so far, 4OHT is investigated in a phase II first in-human trial as treatment of triple-negative AR positive breast cancer (Parr et al., 2004; Vetter and Thürlimann, 2017). 4OHT is online advertised and marketed for oral self-administration. In comparison, 4-hydroxyandrost-4-ene-3,17-dione (4-hydroxyandrostenedione, formestane, 4HOA) is an irreversible aromatase inhibitor, structurally related to androstenedione, the natural substrate of the enzyme aromatase. Because of this aromatase restraining activity, 4HOA is used in breast cancer treatment in post-menopausal women (Brodie et al., 1977; Dowsett, 1994; Dowsett et al., 1992; Wiseman and Goa, 1996;

Abbreviations: DHT, dihydrotestosterone; DMSO, dimethyl sulfoxide; E2, 17β-estradiol; OD, optical density; WADA, World Anti-Doping Agency; YES, yeast estrogen screen; YAS, yeast androgen screen; 4HOA, 4-hydroxyandrost-4-ene-3,17-dione; 4HOT, 4-hydroxytestosterone; 2αHOA, 2α-hydroxyandrost-4-ene-3,17-dione; 3α,4αDHO5αA, 3α,4α-dihydroxy-5α-androstan-17-one; 3α,4βDHO5αA, 3α,4β-dihydroxy-5α-androstan-17-one; 3αHO5βA, 3α-hydroxy-5β-androstane-4,17-dione; 3β,4βDHO5αA, 3β,4β-dihydroxy-5α-androstan-17-one; 3β,17βDHO5αA, 3β,17β-dihydroxy-5α-androstan-4-one; 3α,17βDHO5βA, 3α,17β-dihydroxy-5β-androstan-4-one; 3βHO5αA, 3β-hydroxy-5α-androstane-4,17-dione

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https://doi.org/10.1016/j.toxlet.2018.04.026
Received 26 January 2018; Received in revised form 16 April 2018; Accepted 23 April 2018
Available online 24 April 2018
0378-4274/

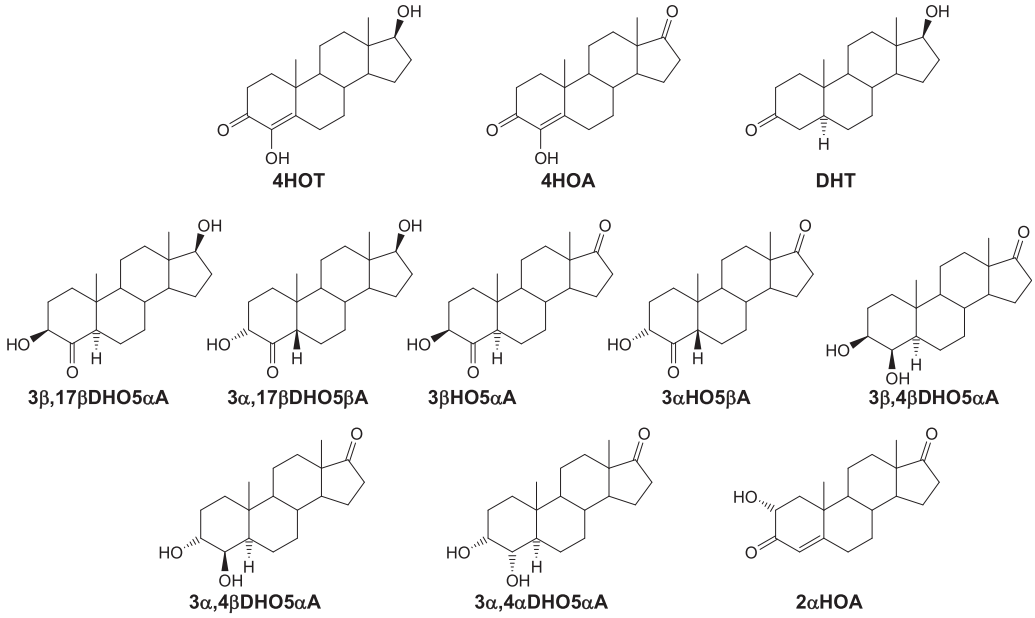

Fig. 1. Chemical structures of formestane (4HOA), 4-hydroxytestosterone (4HOT), 2α-hydroxyandrost-4-ene-3,17-dione (2αHOA), 3β-hydroxy-5α-androstane-4,17-dione (3βHO5αA) and 3α-hydroxy-5β-androstane-4,17-dione (3αHO5βA), 3β,4β-dihydroxy-5α-androstan-17-one (3β,4βDHO5αA), 3α,4β-dihydroxy-5α-androstan-17-one (3α,4βDHO5αA), 3α,4α-dihydroxy-5α-androstan-17-one (3α,4αDHO5αA), 3β,17β-dihydroxy-5α-androstan-4-one (3β,17βDHO5αA), 3α,17β-dihydroxy-5β-androstan-4-one (3α,17βDHO5βA).

Zweifel et al., 2017). Aromatase inhibitors are also used in context of doping in sports to reduce estrogenic side effects of the abuse of anabolic substances and at the same time are also discussed to increase endogenous androgen levels. Consequently, both substances, 4HOT and 4HOA, are included on the list of prohibited substances and methods of the World Anti-Doping Agency WADA (World Anti-Doping Agency, 2018). 4HOT and 4HOA are structurally closely related (structure formulae in Fig. 1) and, due to their small structural differences, both substances have a similar metabolism (Kohler et al., 2007; Parr et al., 2005; Poon et al., 1991, 1992). The metabolism of substances consists normally of two phases. Phase I metabolism is manifold and assembles for example oxidation, reduction and hydrolysis of the substances. Differently, the phase II metabolism comprises the conjugation for example with glucuronic or sulfuric acid, resulting in more soluble products. The phase I metabolism of 4HOT and 4HOA mainly results in reductively produced urinary metabolites. Phase II metabolism including sulfonation and glucuronidation has also been described to be similar after oral application of 4HOT and 4HOA. The urinary metabolites of 4HOT and 4HOA were analyzed following single oral administration trials. The results of this trial was the following: It was shown that after the intake of a single oral dose of 100 mg of 4HOA by male volunteers, 4HOA-glucuronide was detectable in the urine for about 66 h. In addition, 4HOT was identified as metabolite of 4HOA and excreted as glucuronide and sulfate for about 26 h. Further metabolites were detected and identified as 3α-hydroxy-5β-androstan-4,17-dione (3αHO5βA), 3β-hydroxy-5α-androstane-4,17-dione (3βHO5αA), 3β,17β-dihydroxy-5α-androstan-4-one (3β,17βDHO5αA) and 3α,17β-dihydroxy-5β-androstan-4-one (3α,17βDHO5βA) 3α,4α-dihydroxy-5α-androstan-17-one (3α,4αDHO5αA), 3β,4β-dihydroxy-5α-androstan-17-one (3β,4βDHO5αA) and 3α,4β-dihydroxy-5α-androstan-17-one (3α,4βDHO5αA) (Kohler et al., 2007; Lønning et al., 2001; Parr et al., 2005; Poon et al., 1991, 1992). Following these reports, we examined the androgenic and estrogenic properties of a number of the detected metabolites utilizing the yeast androgen (YAS) and estrogen screening (YES) assay (Diel et al., 2007; Kretzschmar et al., 2010). The intention of this study was to characterize the androgenic and estrogenic properties of the reported metabolites and to additionally assess whether the yeast androgen screen is able to detect androgenic activities of the androgen 4-hydroxytestosterone which is often misused for doping purposes as already described for other substances (Zierau et al., 2008). Beside that, we tested the effects of the aromatase inhibitor formestane in the yeast estrogen and androgen screening assay. The reference compounds of the metabolites of both substances 3α,4αDHO5αA, 3α,4βDHO5αA, 3αHO5βA, 3β,4βDHO5αA, 3β,17βDHO5αA, 3α,17βDHO5βA and 3βHO5αA as well as the related 2α-hydroxyandrost-4-ene-3,17-dione (2αHOA) were also included in the YAS and YES studies.

2. Materials and methods

2.1. Substances

Dihydrotestosterone (DHT, purity ≥ 97.5%) and estradiol (E2, purity ≥ 98%) were obtained by Sigma-Aldrich (Munich, Germany). 4-Hydroxyandrostenedione (4HOA) was purchased from Thinker Chemical ((purity > 99.5%) Hangzou, China). 2α-Hydroxyandrost-4-ene-3,17-dione (2αHOA, purity ≥ 96%), 4 hydroxytestosterone (4HOT, purity ≥ 98%) and the metabolites 3α,4α-dihydroxy-5α-androstan-17-one (3α,4αDHO5αA, purity ≥ 99.5%), 3α,4β-dihydroxy-5α-androstan-17-one (3α,4βDHO5αA, purity ≥ 97%), 3α-hydroxy-5β-androstane-4,17-dione (3αHO5βA, purity ≥ 98%), 3β,4β-dihydroxy-5α-androstan-17-one (3β,4βDHO5αA, purity ≥ 99%), 3β,17β-dihydroxy-5α-androstan-4-one (3β,17βDHO5αA, purity ≥ 97%), 3α,17β-dihydroxy-5β-androstan-4-one (3α,17βDHO5βA, purity ≥ 92%), 3β-hydroxy-5α-androstane-4,17-dione (3βHO5αA, purity ≥ 98%) were synthesized at the Center for Preventive Doping Research, German Sport University Cologne, Germany as described elsewhere (Kohler et al., 2007; Parr et al., 2005).

2.2. Yeast androgen receptor reporter gene assay

Yeast cells of the yeast androgen receptor reporter gene assay also called yeast androgen screen (YAS, (Sohoni and Sumpter, 1998)) were cultured as described previously (Zierau et al., 2008). The yeast strain is stored as cryo cultures in glycerol. For the assessment of the androgenity, the substances were dissolved and diluted in DMSO and used in a concentration-dependent assay, from 10^{-11} to 10^{-5} M for DHT (positive control) and from 10^{-10} to 10^{-4} M for 2αHOA, 4HOT, 4HOA, and the seven metabolites. To test for anti-androgenic properties of the substances, 5×10^{-9} M or 10^{-8} M DHT was added to the yeast medium prior to the concentration-dependent analysis of the test substances.

The YAS contained a stably transfected human AR construct as well as an expression plasmid carrying an androgen-responsive element