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A novel combined approach to detect androgenic activities with yeast based assays in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*

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ABSTRACT

We describe the construction and validation of novel test systems for detecting androgenic activities using a combination of the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. By applying the reporter enhanced Green Fluorescent Protein (EGFP) the incubation time could be reduced to only 24 h if compared to the classical β-galactosidase reporter (48 h). Both yeast systems were validated by analyzing the effects of seven androgens as well as five anti-androgens. One androgen (stanozolol) could be detected ten times more sensitive in *S. cerevisiae* than in *S. pombe*. Three of the five anti-androgenic substances showed no or only a slight effect in both yeast assays. The other two anti-androgens could be detected much better in *S. pombe*. Additionally, we could show that both yeast assays tolerated 10% urine within the media and still were capable to detect dihydrotestosterone at a concentration of 10^{-8} M suggesting the use of the assays for applied doping pre-screening.

In summary, the novel androgen-sensitive yeast assays have a large potential for various applications, e.g. as pre-screening in doping analysis or cattle feeding. A combination of both assays, exploiting these two phylogenetic very different yeasts, allows detection of the activity of a wide range of androgenic substances.

1. Introduction

The capacity to detect endocrine active substances has a high relevance in the fields of toxicology and pharmacy, as well as in drug discovery. For example a number of different methods is used to analyze endocrine properties of environmental pollutants, to detect the abuse of endocrine substances (e.g. in doping control or cattle-mast), or to find new substances for therapeutic purposes. The routinely most often used analytical methods are mass spectrometry and mammalian cell-based assays (Blankvoort et al., 2001; Dai et al., 1996; Korner et al., 1999; Sonneveld et al., 2005; Willemsen et al., 2004). In addition a number of yeast-based assays to detect the activity of endocrine active substances exists (Gaido et al., 1997; Sohoni and Sumpter, 1998; Hahn et al., 2006; Michelini et al., 2005; Sanseverino et al., 2005; Eldridge et al., 2007). Yeast-based assays need no specialized equipment and no complex pre-treatment of the samples (Wolf et al., 2010), and hence offer a multiplicity of distinct advantages, like robustness, low costs and easy handling. Additionally, the lack of mammalian receptors and the use of media without intrinsic steroids are beneficial compared to mammalian cell systems. In general, yeast cells exhibit robustness towards toxic effects of test chemicals or solvents (Beck et al., 2008). Furthermore, the chemical structure of the substances to be detected can be unknown. This is the biggest advantage compared to mass spectrometry, because it allows the detection of xenobiotics, newly constructed endocrine substances like the so-called designer-steroids and still unknown metabolites of ingested substances. However, the yeast-based systems do not allow to identify the activity of an individual compound within a complex mixture of endocrine substances. It is also known that many drugs that easily

Abbreviations: EGFP, Green Fluorescent Protein; hERα, human estrogen receptor α; *hAR1*, human androgen receptor gene; *ESR1*, estrogen receptor α gene; ARE, androgen responsive element; ERE, estrogen responsive element; WADA, world anti-doping agency; 8-PN, 8-prenylnaringenin; 6-DMAN, 6-(1,1-dimethylallyl)naringenin; DHT, dihydrotestosterone; SARM, selective androgen receptor modulator; GC, gas chromatography; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; PGK, phosphoglycerate kinase; DMSO, dimethyl sulfoxide.

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enter mammalian cells, do not freely penetrate the yeast cell wall (Parenteau et al., 2005).

The so far established yeast-based assays exploit either colorimetric (Gaido et al., 1997; Hahn et al., 2006; Sohoni and Sumpter, 1998) or luminescent (Eldridge et al., 2007; Michelini et al., 2005; Sanseverino et al., 2005) reporter systems. Yeasts used for the assays are Saccharomyces cerevisiae (S. cerevisiae) (Sohoni and Sumpter, 1998), *Schizosaccharomyces pombe* (*S. pombe*) (Jiao et al., 2008), and *Arxula adeninivorans* (Hahn et al., 2006), a dimorphic yeast of the family of *Saccharomycetaceae*. The recombinant yeast strain used by Hahn et al. (2006) co-expresses the human estrogen receptor α (hERα) and a reporter gene, coding for a phytase which is secreted into the media. Another well-established and often used yeast system to analyze endocrine properties of substances are the estrogen and androgen sensitive yeast assays developed by Sohoni and Sumpter (1998). The respective yeast cells harbor a chromosomally integrated copy of either the human androgen receptor gene (*hAR1*) or estrogen receptor α gene (*ESR1*). In addition, cells express a plasmid-borne derivative of the *lacZ*-gene under the control of a promoter containing androgen or estrogen responsive elements (AREs or EREs, respectively). *lacZ* expression is induced by binding of the receptor-dimer/ligand-complex to the responsive elements of the artificial promoter. The expressed β-galactosidase contains a signal peptide leading to secretion of the protein into the media. In turn it splits chlorophenol red-β-D-galactopyranoside in the media resulting in a color-change from yellow to red. The color intensity is proportional to the endocrine activity of the tested substances. The bioassays developed by Sohoni and Sumpter are used to screen for endocrine active substances or to assess the endocrine activity of specific compounds, e.g. in wastewater. The androgen bioassay can also be applied to detect the abuse of endocrine substances (Zierau et al., 2008b). The relevance of this application is evident in view of the laboratory statistics of the world anti-doping agency in 2008 (WADA-statistics, 2008), wherein 59% of all reported findings detected the misuse of prohibited anabolic agents.

Recently we used the androgen assay of Sohoni and Sumpter (1998) for the detection of substances in urine samples, which – due to the robustness of the yeasts – can be directly added to the yeast media without any special pre-treatment (Zierau et al., 2008b). In order to establish the assay in doping pre-screening analysis we set up to improve the system by using EGFP as a reporter. Additionally, we utilized fission yeast *S. pombe* to potentially allow the detection of a wider range of androgenic substances because phylogenetic studies demonstrated that *S. pombe* and *S. cerevisiae* are phylogenetically as far apart from each other as humans (Sipiczki, 2000). *S. pombe* was already used in an estrogenic bioassay by Jiao et al. (2008) to detect xenoestrogens in the aquatic environment (Jiao et al., 2008). Until now no assay for the detection of androgenic substances with *S. pombe* exists.

We cloned the *hAR1* gene into the *S. pombe* expression vector pJR1-3XL. The gene is under the control of the strong *nmt1* promoter that allows thiamine-repressible expression of *hAR1*. *EGFP* was used as a reporter. To validate the assays, the detection of several known androgenic doping substances listed in the WADA statistics was analyzed: boldenone, mesterolone, metandienone, metenolone, nandrolone, oxandrolone and stanozolol (WADA-statistics, 2008). Boldenone is a 1,2-dehydro-derivative of testosterone. In Germany, it is only approved for the use in veterinary medicine, but it is often misused in bodybuilding. Mesterolone was developed 1932 from Schering AG and merchandised as Proviron. It was the first androgenic compound to treat hormonal-related diseases in male patients. In bodybuilding, it is misused as estrogen antagonist to avoid gynecomasty caused by anabolic steroids. Metandienone is a 17-methylsteroid and highly liver toxic. Metenolone was also designed by Schering AG and merchandised as Primobolan. Its effects are only weakly androgenic but highly anabolic. Nandrolone is an also naturally occurring anabolic steroid. In male humans, the relation of testosterone to nandrolone is normally 50:1. Nandrolone has a considerably higher anabolic activity than testosterone and a low conversion rate to estrogens. Therefore, it is often misused in sports. Oxandrolone, first synthesized in 1962, is an anabolic 17-methylsteroid. This substance is approved, e.g. in the USA for the treatment of alcoholic hepatitis, of Turner syndrome and of weight loss caused by HIV. Stanozolol is a synthetic testosterone derivative. In veterinary medicine, it is used to stimulate appetite and to increase weight and muscle mass. At the Olympic Games in 1988 it became prominent after doping abuse of Ben Johnson (Boudreau and Konzak, 1991).

Additionally, we tested flutamide, nilutamide, bicalutamide, 8-prenylnaringenin (8-PN) and 6-(1,1-dimethylallyl)naringenin (6-DMAN) in the two systems to validate the yeast assays to screen for anti-androgenic effects. Flutamide, nilutamide and bicalutamide are known as strong anti-androgens. Flutamide is an orally available non-steroidal compound primarily used to treat early stages of prostate cancer. Nowadays, it has been largely replaced by bicalutamide due to a better profile regarding potential side-effects (Schellhammer et al., 1997). Nilutamide is an anti-androgenic compound used for the treatment of advanced prostate cancer. It blocks hAR, preventing its interaction with testosterone. 8-PN and 6-DMAN are extremely potent flavonoidal phytoestrogens. 8-PN found in hop *Humulus lupulus* (L.) exhibits estrogenic effects and low anti-androgenic properties, whereas 6-DMAN from the leaves of the African tree *Monotes engleri* (Gilg.) has estrogenic and anti-androgenic properties (Zierau et al., 2008a). Therefore, we included the examination of the effects of these substances of plant origin in our yeast assays. By the combination of the androgenic assay with an anti-androgenic approach, in which a defined concentration of dihydrotestosterone (DHT) is added in parallel to the substances, it would be possible to detect selective androgen receptor modulator (SARM)-like effects. This would lead to a signal in both, the androgenic and the anti-androgenic assay, as demonstrated by Bovee et al. (2008).

2. Materials and methods

2.1. Substances

The anabolic androgenic steroids were provided by the Department of Biochemistry of the German Sports University (Cologne, Germany). 6-(1,1-Dimethylallyl)naringenin (6-DMAN) and 8-prenylnaringenin (8-PN) were synthesized from naringenin as described previously (Gester et al., 2001). The purity of the used compounds was assessed to be >99% by gas chromatography (GC) and high performance liquid chromatography (HPLC). 6-(1,1-Dimethylallyl)naringenin and 8-prenylnaringenin were used as racemic mixtures. Flutamide, nilutamide and dihydrotestosterone (DHT) were obtained by Sigma-Aldrich (Munich, Germany), and bicalutamide by Interpharma (Prague, Czech Republic).

The urine was collected from 10 randomly chosen men, mixed and sterile filtrated with a 0.2 μm filter. The experimental conditions were in accordance with the Institutional Ethic Committee guidelines of the German Sport University Cologne.

2.2. Plasmids for S. cerevisiae

To construct the reporter plasmid carrying the reporter gene *EGFP* under the control of an androgen dependent promoter the vector p426*ADH* (Mumberg et al., 1995) was used. The alcohol dehydrogenase promoter of the vector was removed by restriction digestion with the enzymes *Sac*I and *Spe*I. After digestion with *Sac*I the 3′-overhanging end of the *Sac*I site was treated with nuclease S1 to generate blunt ends. Afterwards the plasmid was digested with *Spe*I to clone the polymerase chain reaction (PCR) product of the phosphoglycerate kinase (*PGK*) promoter. The *PGK* promoter containing three androgen response elements (ARE) was amplified using primers yPGKforDraI_2 (tatata TTTAAA caccctcatactattatcaggg) and yPGKrevSpeI_2 (gg ACTAGT agatcttttgtatttgttgtaaaaag) and the expression plasmid of the yeast strain constructed by Sohoni and Sumpter (1998) as a template. The PCR product was cut with *Dra*I and *Spe*I and ligated into the digested vector. The resulting plasmid p426*PGK* was used for cloning of the *EGFP* PCR product that was amplified with the forward primer EGFPforSpeI (tatatt ACTAGT aaaa atggtgagcaagggcgag) and the reverse primer EGFPrevBamHI (tatata GGATCC ttacttgtacagctcgtccatgcc) from