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SteroCheck^{TM} : A Bioassay for the Determination of Agonistic and Antagonistic Steroid Hormone Activities

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ABSTRACT

The conventional way in doping analysis of hormonal steroids is measuring of the amount/concentration of such substances in samples of interest. However, a prerequisite for this physico-chemically based measurement is knowledge of the chemical structure or, at least, the molecular weight of the steroids to be determined with the consequence that unknown molecules escape detection, *e.g.* the case "tetrahydrogestrinone (THG)" in the fall 2003. We now present a molecular-biological assay which enables the highly sensitive determination of steroid hormone activities without the necessity of knowing the chemical nature of either a single compound or substance mixtures responsible for these activities. This *in vitro* bioassay, the *SteroCheck*^{TM} (patent pending), reflects the *in vivo* mechanism of action of either androgen, estrogen, corticoid or gestagen agonistic and/or antagonistic active molecules. The principle in brief: transfection with a specific recombinant reporter gene construct of suitable steroid-sensitive human cell lines, which express - due to the inducible ability of the hormonal active substance(s) - a protein normally not occurring in vertebrates. The extent of the induced protein, *e.g.* the enzyme luciferase (luc), which is easily to determine, is directly proportional to the hormonal activity of the compound(s) present in the sample of interest.

Using the androgen version of *SteroCheck*^{TM} in test solutions with testosterone (T) and 5α-dihydrotestosterone (DHT), an explicit dose-response relationship "luc expression *vs.* T/DHT concentration" was shown with a detection limit < 10 pM. The extent of *total* androgenity, as determined in sera of women and men, reflects the considerably different levels of circulating androgens in both genders. Both the non-steroidal strong and the steroidal moderate androgen-*antagonists* flutamide and lilopristone (Lp) inhibit T or DHT-induced luc

expression; however, in the absence or at low concentrations of T/DHT, Lp acts as a strong androgen-*agonist*.

Comparative tests with the pure "prohormones" gestrinone, norethisterone, norandrostendione and their respective counterparts THG, norethandrolone, nandrolone, resulted in: 1) *all* of them reveal pronounced androgenic activity, 2) particularly at low concentration (3 nM), the metabolites exhibit two- to four-times higher androgenity than their precursors. "Spiking" the sera of women and men with these substances (30 nM) results in considerable changes in total androgenity; nandrolone and norethandrolone, *e.g.,* dramatically enhance the androgenic activity.

Using the glucocorticoid (GC) version of *SteroCheck*^{TM} in order to check GC activity of particular substances, some unexpected findings were obtained. The well-known GC-antagonists cortexolone, progesterone, medroxyprogesterone acetate (MPA, used in endocrine therapy), or the synthetic estrogen and gestagens ethinylestradiol and ethynodiol (components of oral contraceptives), were shown to be GC-agonists or GC-antagonists, respectively.

The estrogen, GC and androgen versions of *SteroCheck*^{TM} are at present in the process of optimization for commercially available and ready-to-use kits to determine with high sensitivity agonistic, antagonistic or mixed steroidal hormone activities of known and/or unknown substances in any sample of interest.

INTRODUCTION

The conventional way in doping analysis of steroidal hormones is determination of the amount/concentration of the respective substances in samples of interest. However, a pre-requisite for this physico-chemical measurement is knowledge of the chemical structure or, at least, the molecular weight of the steroids to be determined with the consequence that un-known molecules escape detection, *e.g.* THG in 2003.

Here, we present a molecular-biological assay which enables an extremely sensitive determination of steroid hormone like activities without the necessity of knowledge of the chemical nature of either a single compound or substance mixtures responsible for these activities. This *in vitro* bioassay, called *SteroCheck*^{TM} (patent pending), reflects the *in vivo* mechanism of action of androgenic active substances. This bioassay offers two beneficial aspects: a) it analyzes whether substances of interest, such as the so-called prohormones, possess any androgenic activity and should thus be prohibited and b) it may be used for the screening of athletes' blood and urine samples for androgenic compounds not yet included in the list of prohibited substances, *i.e.* any new designer steroids.

In addition to the androgen version, further variants of *SteroCheck*^{TM} have been designed for analysis of estrogenic, corticoidal [1] or gestagenic agonistic and/or antagonistic active molecules/compounds.

EXPERIMENTAL

No details can be supplied as yet since the patent for *SteroCheck*^{TM} is pending at present.

The principle in brief: transfection with a specific steroid/ligand-sensitive reporter gene construct of suitable steroid-responsive human cell lines, which express - due to the inducible ability of the hormonal active substance(s) - a protein normally not occurring in vertebrates, *i.e.* the easily measurable enzyme luciferase (luc). The amount and, consequently, the activity of the induced luciferase are directly proportional to the extent of the hormonal activity of the compound(s) present in the sample of interest.

The inducible luciferase (luc) reporter plasmid construct and the constitutive hAR expression plasmid were generously provided by R. Lanz, Baylor College of Medicine, Houston. The constitutive Renilla-luc reporter plasmid (phRL-null) and *Dual-Luciferase Reporter Assay System*^{TM} were purchased from Promega. Nortestosterone, norethandrolone, norandrostenedione, gestrinone, THG and norethisterone were generously provided by W. Schänzer, DSHS Cologne, Germany. The androgens T and DHT, triphenyltin (TPT), dibutyltin (DBT), tributyltin (TBT), monobutyltin (MBT), prochloraz (Pro), p,p'-dichlordiphenyldichlorethan (DDE), diuron (Diu), linuron (Lin), fenarimol (Fen), vinclozolin (Vin), methyltestosterone (MT), flutamide (Flut), clomiphene (Clo), finasteride (Fin) or letrozole (Let) were purchased from Sigma. Flutamide and lilopristone were kind gifts from W. Elger, Entec, Jena, Germany.

RESULTS AND DISCUSSION

Using the androgen version of *SteroCheck*^{TM} in test solutions with defined concentrations of testosterone (T) and 5α-dihydrotestosterone (DHT), an explicit dose-response relationship "luc expression *vs.* T or DHT concentration" with an extremely low detection limit < 10 pM could be demonstrated (Fig. 1).

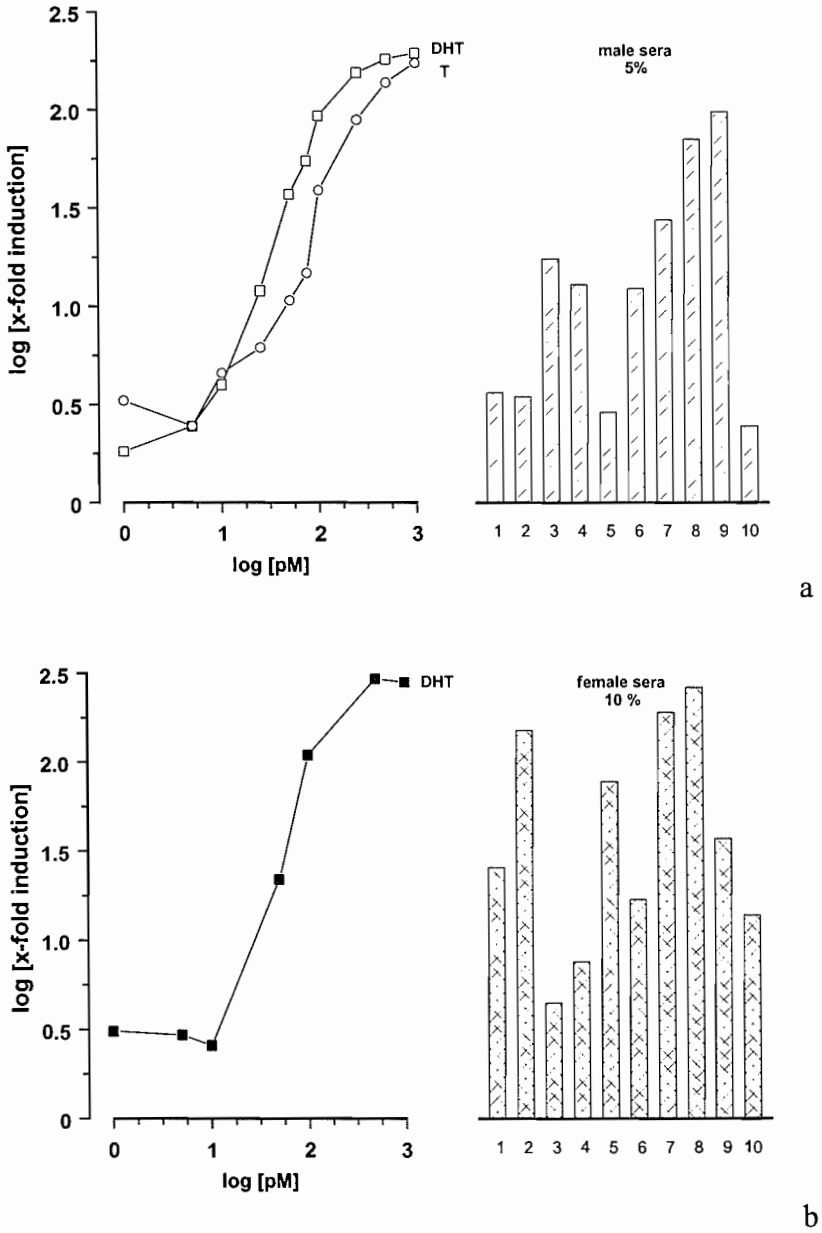


Fig. 1: Luciferase (luc) activity in test solutions and human sera, given as x-fold above control without androgens (y = 0), induced by various concentrations of testosterone and DHT (left), and by androgens present in diluted male (a) serum or female (b) serum (right). Test solutions and sera were diluted in cell culture medium.

The extent of total androgenicity, as determined in sera of men and women, diluted in cell culture medium (5% and 10%, respectively), reflects the different levels of circulating androgens in both genders. The induction of luc-expression can be inhibited by 1 µM flutamide, a strong non-steroidal androgen-antagonist. Flutamide alone does not display any androgenic activity. 1 µM lilopristone (Lp), a steroidal glucocorticoid and gestagen antagonist, also inhibits androgen induced luc expression; however, in the absence or at low concentrations of T or DHT, Lp acts as a strong androgen agonist (Fig. 2). The same phenomenon has

been observed with the well-known glucocorticoid and gestagen antagonist RU 486 (*Mifepristone*^{TM}) (data not shown).

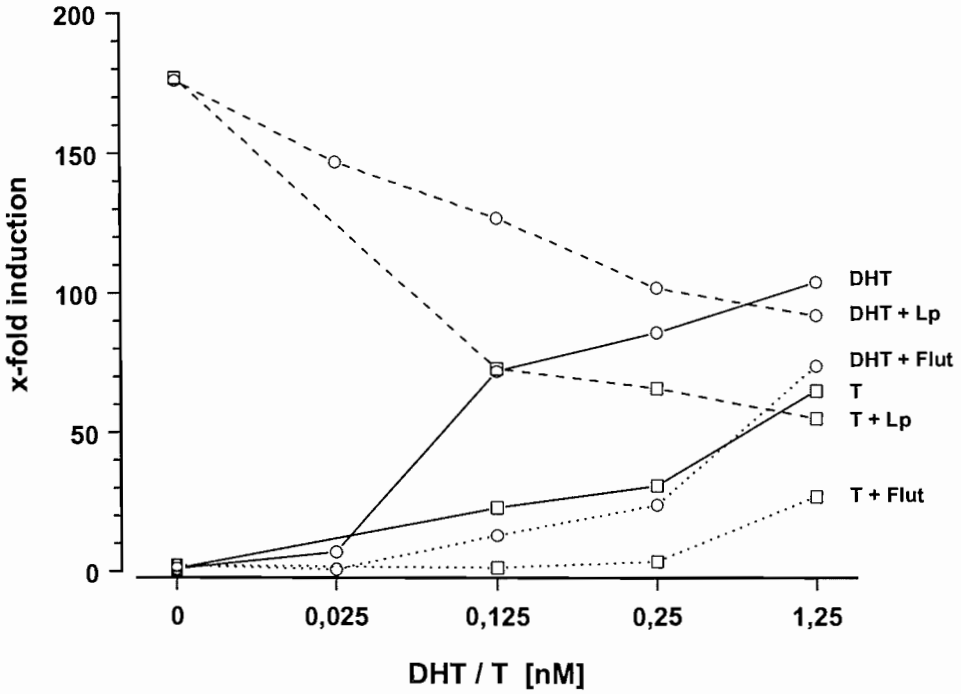


Fig. 2: Luc activity in test solutions, given as x-fold above control without androgens (y = 1). Cells were incubated with various concentrations of testosterone or DHT, without or with 1 µM flutamide (Flut) or lilopristone (Lp).

Three selected anabolic-androgenic steroids, THG, norethandrolone, nandrolone (nortestosterone), and their respective prohormones, gestrinone, norethisterone, norandrostenedione, were tested using *SteroCheck*^{TM}. All of them, *i.e.* even the prohormones, revealed pronounced androgenic activity (Fig. 3 a-d). Thus, for the first time it could be demonstrated that the so-called prohormones not only act as anabolic precursors in humans by being converted to highly active androgens, but that, in addition and independent of these metabolisms, they themselves exert androgenic activity.

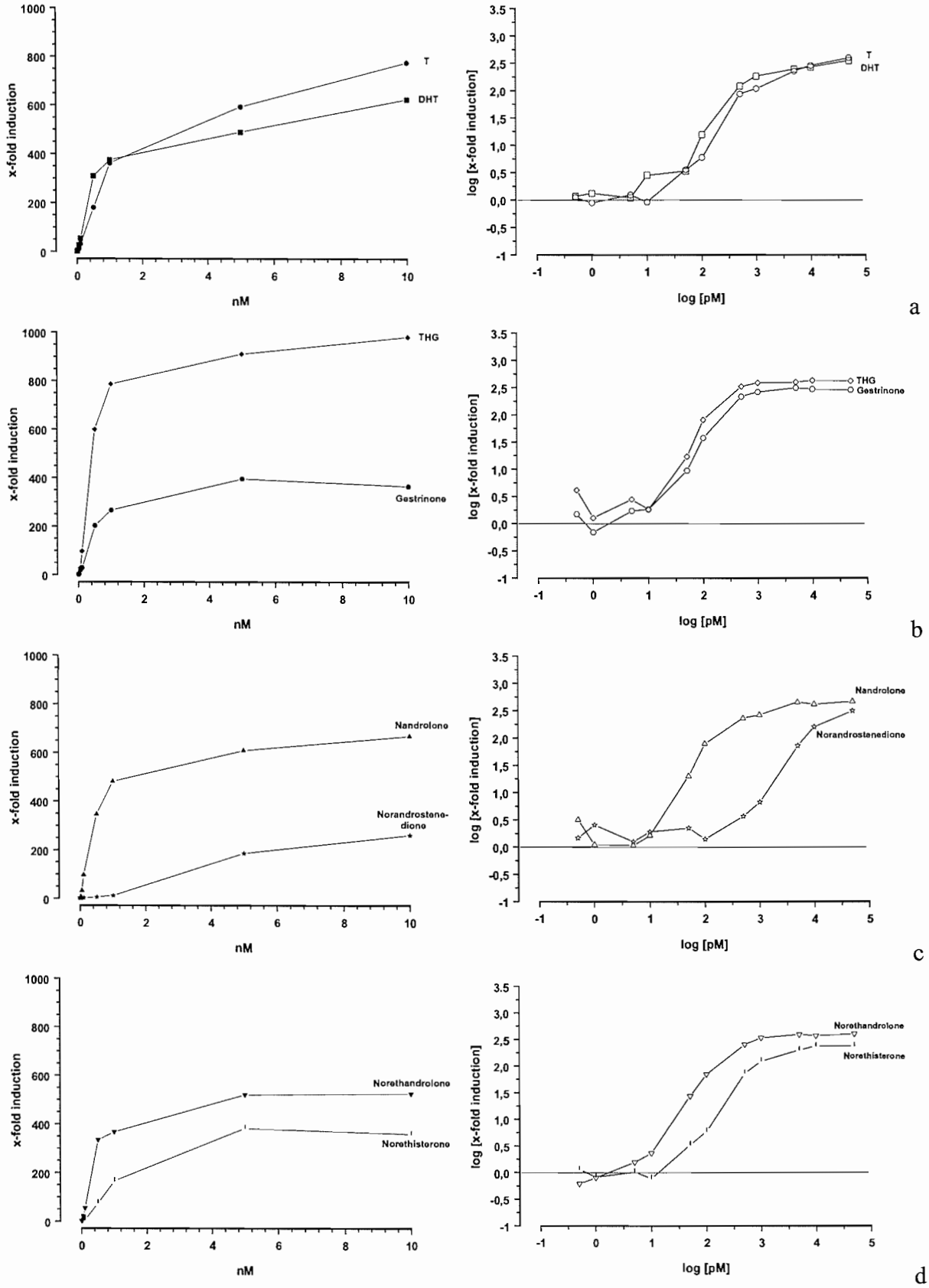


Fig 3: Luc activity in test solutions, given as x-fold above control without hormones (y = 1). Cells were incubated with various amounts of (a) DHT, testosterone, (b) THG, gestrinone, (c) nandrolone (nortestosterone), norandrostenedione, (d) norethandrolone and norethisterone. For reasons of convenience, the results are given both as linear and logarithmic diagrams.

Concernig THG, our findings have been corroborated by other authors using yeast cells ("yeast cell assay") [2]. However, *SteroCheck*^{TM} works with human cell lines for the following reasons: the purpose of this bioassay is assessment of hormonal activities relevant for humans. Therefore, the cell lines used as "biomeasurement system" should be a) typical representatives of androgen, estrogen, corticoidal or gestagen target cells and b) phylogenetically as closely related to humans as possible. At present, there exists no doubt that, apart from nuclear receptors and their cofactors, plasma membrane (PM) located receptors together with G-proteins are also decisively and even initially involved in the steroid hormone signal pathway. This has been clearly proven for androgens, estrogens and glucocorticoids [1]. Thus, the hormone activities measured using mammalian cell lines are the result of activating the steroid signal cascade, including PM- and nuclear receptors, by the inducing substances. In contrast, no proof of PM-receptors exists to date for yeast cells. Even in case of successful expression of human PM-receptors, yeast cannot provide the subsequent signal cascade.

"Spiking" the sera of women and men with these substances (30 nM) resulted in considerable changes in total androgenicity. Nandrolone and norethandrolone, *e.g.,* dramatically (women) or moderately (men) enhance the androgenic activity; the difference may depend on the extent of androgenic activity in the serum alone. At present, there is no explanation why spiking of the male serum with norandrostenedione, gestrinone, THG or norethisterone causes an antagonistic effect (Fig. 4).

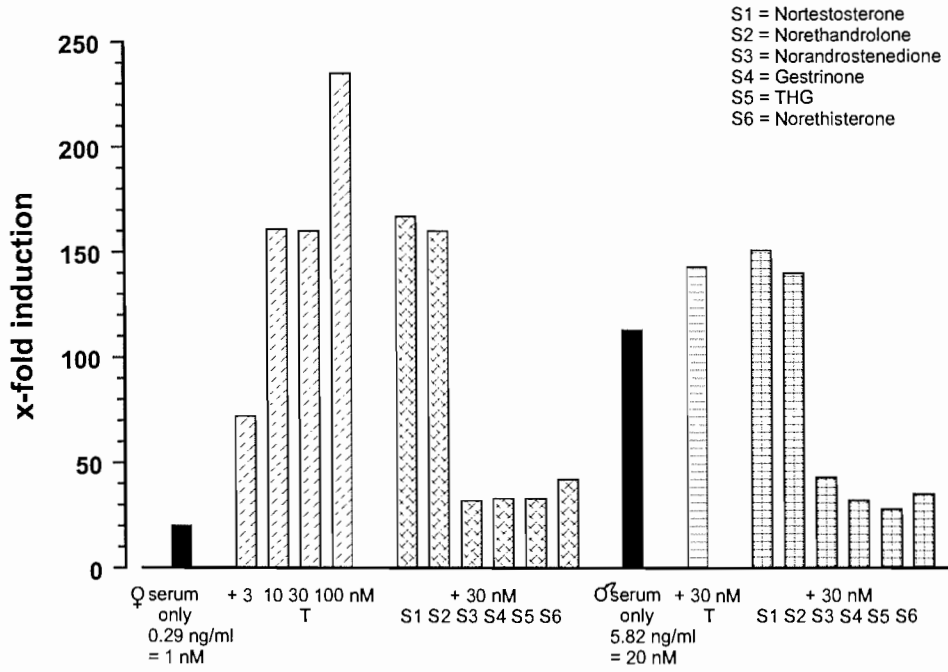
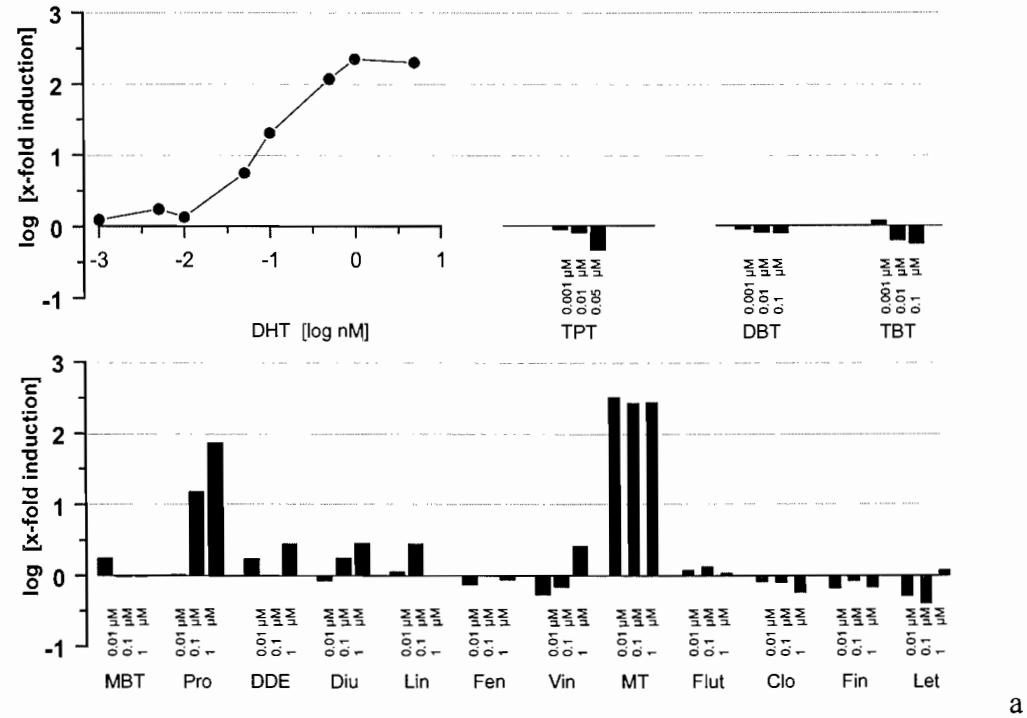
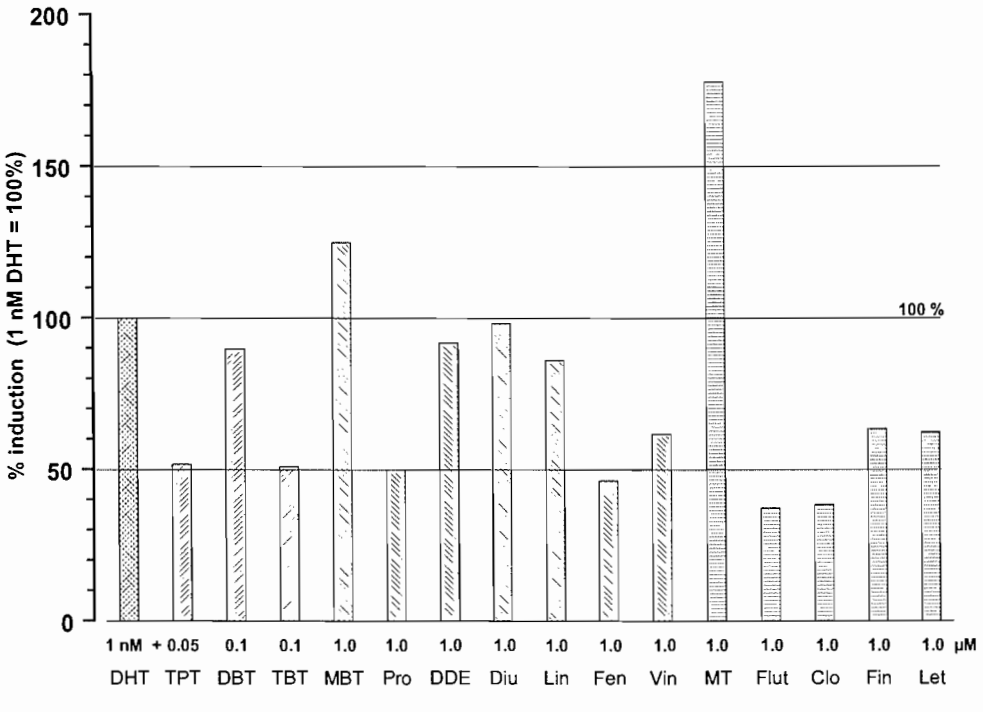


Fig. 4: Luc activity in human sera, given as x-fold above control without hormones (y = 1), induced by androgens in serum alone or serum spiked with testosterone (3 – 100 nM) or with 30 nM of nortestosterone (nandrolone), norethandrolone, norandrostenedione, gestrinone, THG or norethisterone, Determination of serum total testosterone concentration was performed using "*Immolite 2000*", DPC, Germany).



a



b

Fig. 5: Luc activity in test solutions. (a) Androgenic agonismus: cells were incubated with triphenyltin (TPT), dibutyltin (DBT), tributyltin (TBT), monobutyltin (MBT), prochloraz (Pro), p,p'-dichlordiphenyldichlorethan (DDE), diuron (Diu), linuron (Lin), fenarimol (Fen), vinclozolin (Vin), methyltestosterone (MT), flutamide (Flut), clomiphene (Clo), finasteride (Fin) or letrozole (Let). These are selected endocrine disruptors (ED), synthetic androgens and anti-androgens, anti-estrogens and inhibitors of the sex hormone metabolizing enzymes 5α-reductase 2 and aromatase. Luc activity is given as x-fold above control without test compounds (y = 0). (b) Androgenic antagonism: cells were incubated with 1 nM DHT alone (100%), or plus one of the above mentioned substances at different concentrations. For all substances the highest sub-toxic concentrations were chosen. Luc activity is given as % of 1nM DHT alone.

There is an increasing amount of non-steroidal substances with androgenic activity termed Specific Androgen Receptor Modulators (SARM's). Screening of various herbicides, fungicides, pesticides and selected organotin compounds, suspected of acting as endocrine disruptors (ED), resulted in pronounced androgenic activity of the widely used fungicide prochloraz, and in minor activity of monobutyltin, DDE, diuron, linuron and vinclozolin. DHT and methyltestosterone were used as positive controls (Fig. 5a).

SteroCheck^{TM} can also be used to test substances for antagonistic activity. A quantity as low as 50 nM of triphenyltin (TPT) inhibited the DHT-induced luc expression by about 50%. Also, 50% inhibition was reached with tributyltin (TBT), prochloraz and fenarimol, but with 100 nM and 1 µM, respectively. However, in the presence of DHT, prochloraz acted as a strong androgen-antagonist (Fig. 5b), a phenomenon which also applies to lilopristone and RU 486. In addition, this fungicide was proven to be a strong aromatase inhibitor, comparable to the known specific P_{450}-aromatase inhibitor Letrozole [3]. Therefore, it is a further candidate for "The Prohibited List" by the WADA.

CONCLUSION

The androgen version of *SteroCheck*^{TM} presented here, enables the highly sensitive analysis of known or unknown compounds/substances or mixtures of these with agonistic as well as antagonistic androgenic activity. It was not designed to determine the amount/concentration of known anabolic-androgenic steroids (AAS), but instead to detect and quantify androgenic activity in samples of interest (serum, urine). In summary, it provides a screening method for substances not detected *via* the conventional GC/MS.

The androgen, estrogen and GC versions of *SteroCheck*^{TM} are at present in the process of optimization for commercially available and ready-to-use kits for the determination of the respective steroidal hormone activities.

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