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Characterisation of gene expression patterns in 22RV1 cells for determination of environmental androgenic/antiandrogenic compounds☆

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Abstract

Alteration of androgen receptor function due to hormonally active compounds in the environment, may be responsible for impaired reproductive function in aquatic wildlife. Based on human prostate carcinoma 22RV1 cells, a cell culture expression system was established to test effects of putative androgenic/antiandrogenic compounds on endogenous gene expression. 22RV1 cells were shown to express human androgen receptor, but not human progestin (hPR) or human oestrogen receptor (hER) α and β. Six androgen-regulated genes (ARGs) were chosen to determine androgenic/antiandrogenic action using highly sensitive real-time RT-PCR. Results showed that gene expression is altered in a time-dependent manner. After stimulation of cells by DHT (10 nM), synthetic androgen R1881 (1 nM), or organic pesticides (difenoconazole, fentinacetate, tetramethrin) *TMPRSS2* mRNA expression was down-regulated by the factor 0.6 after 24 h of DHT treatment. Similar results were obtained when cells were assayed for mRNA expression of *PSA* after fentinacetate and R1881 stimulation. In contrast, *TMPRSS2* expression was up-regulated by the factor 0.9 when cells were stimulated by tetramethrin. Final goal of the work is a sensitive determination of differential gene expression by different compounds under study, achievement of substance-specific expression patterns and function related analysis of potential androgens/antiandrogens.

Keywords: 22RV1 cells; Androgen receptor; Androgen-regulated genes; Gene expression; Real-time RT-PCR

1. Introduction

Recent adverse trends in health and sexual development of aquatic wildlife animals may be linked to the presence of endocrine disruptors, i.e. substances that interfere with the endocrine system, in the environment [1,2]. The concept that environmental pollutants might have harmful effects on reproduction, is not based on theory, but is rather derived from observations of wildlife biologists in the field [3]. Even impairment of sexual development and reproduction in humans was proposed, although decrease of sperm counts during the past 50 years [4] could not be confirmed in worldwide studies [5]. The increase of reproductive organs pathology is commonly accepted for the last decades [6–9]. These diseases might be hormone dependent, but till now a causal relation between xenohormones and disorders observed could not be proven. Substances with androgenic or antiandrogenic effects can be characterised as natural steroids originating from human or animal metabolism [10], as phytooestrogens and as synthetic androgens. From the first group, we selected DHT as a reference steroid. Synthetic androgens include medically used compounds in hormone replacement therapy or growth promoters in farm animals [11,12] as well as illegally used synthetic androgens for human or animal doping. For our tests, we used synthetic androgen R1881. The last group of androgenic/antiandrogenic substances includes chemicals or metabolites of chemicals showing hormonal activity as an unrequested side effect. Three of the most suspicious endocrine disrupting chemicals used in plant protection are included in our evaluation. Difenoconazole and fentinacetate [13] are used as fungicides; the third—tetramethrin—as an insecticide. Though known androgenic or antiandrogenic substances can be detected at concentrations in the ppt range by means of sensitive methods like GC–MS, LC–MS or EIA [14,15], no statement on hormonal activity or effects on gene expression can be made using these methods. To predict the influence of these compounds on man or the environment, in vitro systems and animal-based in vivo assays are available. In vitro assays are based either on measurement of direct binding to the isolated receptor, on induction of a reporter gene regulated through the androgen responsive element (ARE) or on cell

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Table 1
Primer sequences, product length and melting temperature of six androgen-regulated genes and housekeeping gene

	PSA	*PSM*	*AR*	*NKX3.1*	*TMPRSS2*	*PMEPA1*
Forward Primer (bp)	29–46	1195–1214	252–272	568–586	1104–1123	895–918
Reverse Primer (bp)	107–187	1345–1364	558–578	740–761	1235–1254	1017–1040
Product length (bp)	159	170	326	261	151	146
Product melting temperature (°C)	90.80	83.37	86.33	88.49	89.00	89.20

Selected genes are as follows: Prostate-specific antigen (*PSA* accession no. X14810) [41] belonging to kallekrein gene family is a common tumour marker in cancer diagnosis, prostate-specific membrane antigen (*PSM* accession no. M99487) is a cell surface marker in the prostate [42], homeobox gene *NKX3.1* (accession no. U860669) [43] is involved in cell growth and differentiation, *TMPRSS2* (accession no. AF270487) supports normal cell growth and morphology [44], androgen receptor (*AR* accession no. M3423) [45], and *PMEPA1* (accession no. AF224278) [46] is involved in calcium binding.

proliferation assays. Competitive receptor assays have been established to measure binding affinities of different substances [16]. Recently, Bauer et al. [17] succeeded in establishing a test system on the basis of recombinant human AR. These assays can be performed on whole cells or cell homogenates [18,19].

In recombinant reporter-gene assays after co-transfection of cells, the ligand-activated receptors initiate transcription of a reporter gene [20–23], e. g. luciferase. Activity of the reporter gene is directly related to the transcriptional activation by the test compound. Both, yeast [24–26] and mammalian cells are available. On cellular level, proliferation activity of cells bearing naturally or recombinantly expressed AR is used as test criterium. A negative proliferative effect of androgens can be found in AR positive breast cancer cells [27–29]. It can also be detected in AR transfected breast or prostate cancer cells [30,31].

The assay described herein is based on induction or repression of six marker genes selected from a collection of previously identified androgen-regulated genes described, e.g. in human prostate carcinoma cell line LNCaP. Choice-criteria for androgen-regulated genes (ARGs) were as follows: genes should have an ARE in the promoter region, biological function should be known and genes should originate from different functional groups. All genes we choose herein match these requirements. For overview see Table 1. Transcriptional alterations were assayed by real-time polymerase chain reaction. In the present study, we show that a cell-based endogenous gene expression assay is very sensitive and can be used to assay androgen/antiandrogen-like effects of various putative androgenic/antiandrogenic chemicals. Characteristic gene expression patterns could be achieved for all substances under study.

2. Materials and methods

2.1. Reagents

Dihydrotestosterone (DHT, 5α-androstan-17β-ol-3-one) was received from Sigma–Aldrich (Germany). R1881 (17β-hydroxy-17-methylestra-4, 9, 11-trien-3-one) as a gift from Schering AG (Germany). Difenoconazole and tetramethrin were obtained from Dr. Ehrensdorfer (Germany), fentinacetate from Riedel de Haën (Germany). Charcoal (Norit A), and dextran (research grade, MG 65000-73000) were purchased from Serva (Germany). All pesticides were dissolved in ethanol p.a. (final ethanol percentage of less than 0.1%).

2.2. Cell culture

Human prostate carcinoma cell line 22RV1 [32] was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany, ACC 438) and routinely cultured in 40% RPMI 1640 medium, 40% Dulbecco's MEM supplemented with 20% heat-inactivated foetal bovine serum (FBS) plus 100,000 units/l penicillin and 100 mg/l streptomycin (Sigma, Germany). Steroids were removed from FBS essentially as described by Darbre et al. [33]. FBS was incubated with 0.5% activated charcoal and 0.05% dextran T-70 for 30 min at 55 °C the charcoal particles were removed by centrifugation at 4 °C for 20 min at 4.500 × *g*. This step was repeated and stripped serum was sterile filtered and stored in aliquots at −20 °C. All media were obtained from Gibco-BRL (USA). Cells were split 1:6 every 7 days and cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Cell treatment

Cells were seeded in medium containing 20% charcoal-stripped FBS for 72 h before treatment with steroids or pesticides and allowed to form a confluent monolayer. DHT (10 nM) or synthetic steroid R1881 (1 nM) was added and cells were harvested at 0, 6 and 24 h after stimulation for RNA extraction. Same procedure was carried out with other ligands: difenoconazole (100 nM), fentinacetate (100 nM) and tetramethrin (50 nM). Control cultures were continuously grown in steroid-depleted untreated medium for the same intervals of time.

2.4. RNA isolation and reverse transcription reaction

Total RNA was isolated from 22RV1 cells using peqGold TriFast™ (peqLab Biotechnologie GmbH, Germany)

with the guanidinium-isothiocyanate method [34] according to the manufacturer's instruction. Synthesis of first strand cDNA was performed by using 1000 ng of total RNA and 200 U MMLV-reverse transcriptase (Promega, USA) according to the manufacturer's protocol. RT reaction was carried out in 40 μl reaction volume in a gradient cycler (Tgradient, Biometra®, Germany).

2.5. Determination of nucleic acid concentration

All measurements of nucleic acid concentration were performed at 260 nm (OD_{260}) in a spectrophotometer (BioPhotometer®, Eppendorf, Germany) with 220–160 nm Uvettes® (Eppendorf).

2.6. Human oestrogen (hERα and β) and human progestin (hPR) receptor expression analysis in the cell line 22RV1

Total RNA from untreated cells was extracted and reverse-transcribed as mentioned above. Expression of receptors was investigated using following primers: progestin 5′-GAGAGCTCATCAAGGCAATTG-3′ (sense) and 5′-CACCATCCCTGCCAATATCTTG-3′ (antisense) producing a 227 bp product; hERα primers 5′-AGGGAAGCTCCTATTTGCTCC-3′ (sense) and 5′-CGGTGGATGTGGTCCTTCTCT-3′ (antisense) yielding a 234 bp product; hERβ 5′-GCTTCGTGGAGCTCAGCCTG-3′ (sense) and 5′-AGGATCATGGCCTTGACACAGA-3′ (antisense), generating a 262 bp product. Subsequently, PCR products were subjected to gel-electrophoresis on 1.5% agarose gels containing 0.5 μg/ml ethidium bromide.

2.7. Real-time PCR quantification

Quantification of genes of interest was carried out in LightCycler® (Roche Diagnostic, Germany) using LightCycler® DNA Master SYBR® Green I technology [35]. Mastermixes for each PCR run were prepared as follows: 6.4 μl water, 1.2 μl $MgCl_2$ (4 mM), 0.2 μl Forward Primer (20 pmol), 0.2 μl Reverse Primer (20 pmol) and 1 μl Fast Start DNA Master SYBR® Green I (Roche Diagnostics, Germany) mix 9 μl of mastermix and 1 μl (25 ng) of reverse-transcribed total RNA. Each sample was subjected to 40 cycles of PCR consisting of 15 s at 95 °C for denaturation, 10 s at corresponding annealing temperature and 20 s at 72 °C for elongation. Primers for all six androgen-regulated genes were designed using HUSAR-software (DKFZ, Heidelberg) and synthesised by MWG Biotech (Germany). For primer sequences and product length see Table 1.

Fluorescence data report was computed directly with LightCycler® software 3.5 (Roche Diagnostics, Germany). For the determination of crossing-points (CP) "Second Derivative Maximum" method was applied [36]. Data analysis was performed using relative quantification software REST© [37]. Relative expression (E) was automatically calculated according to equation: $E = 2^{\Delta CP}$. For depiction of up/down regulation $2 \log E$ was determined to set gene expression in control samples to zero.

2.8. Statistics

All data were statistically processed in SigmaPlot® 2000 (SPSS Inc., USA) and SigmaStat® 2.0 (Jandel Corporation, USA).

3. Results

3.1. Characterisation of 22RV1 cell line

Using real-time RT-PCR 22RV1 cells showed expression of human androgen receptor only. Neither mRNA of human oestrogen receptor α and β (hERα and β) nor mRNA of human progestin receptor (hPR) could be detected (data not shown).

3.2. Primer specificity, real-time RT-PCR efficiencies, intra- and inter-assay variation

After real-time RT-PCR all primer pairs proved to generate amplicons showing one single melting peak with high

Table 2
Characterisation of real-time LightCycler® PCR

	NKX3.1	*PMEPA1*	*TMPRSS2*	*PSA*	*PSM*	*AR*	Ubiquitin
Start template	mRNA	mRNA	mRNA	mRNA	mRNA	mRNA	mRNA
PCR efficiency	1.90	1.92	1.98	2.07	2.13	1.99	1.99
Quantification limit (ng)	0.2	0.04	0.04	0.2	0.04	0.04	0.04
Quantification range (ng)	25–0.2	25–0.04	25–0.04	25–0.2	25–0.04	25–0.04	25–0.04
Test linearity correlation	22RV1 total RNA ($r = 1.00$)	22RV1 total RNA ($r = 1.00$)	22RV1 total RNA ($r = 0.98$)	22RV1 total RNA ($r = 0.98$)	22RV1 total RNA ($r = 0.99$)	22RV1 total RNA ($r = 0.92$)	22RV1 total RNA ($r = 0.95$)
Intra-assay variation (%, $n = 3$)	0.5	0.4	1.0	0.3	0.8	1.6	0.7
Inter-assay variation (%, $n = 3$)	4.4	3.5	2.3	2.6	3.2	4.9	1.8

Intra-assay (test precision) and inter-assay variation (test variability), $n = 3$. Calculation of test precision and test variability is based on the variation of crossing points (CP) from the CP mean value.

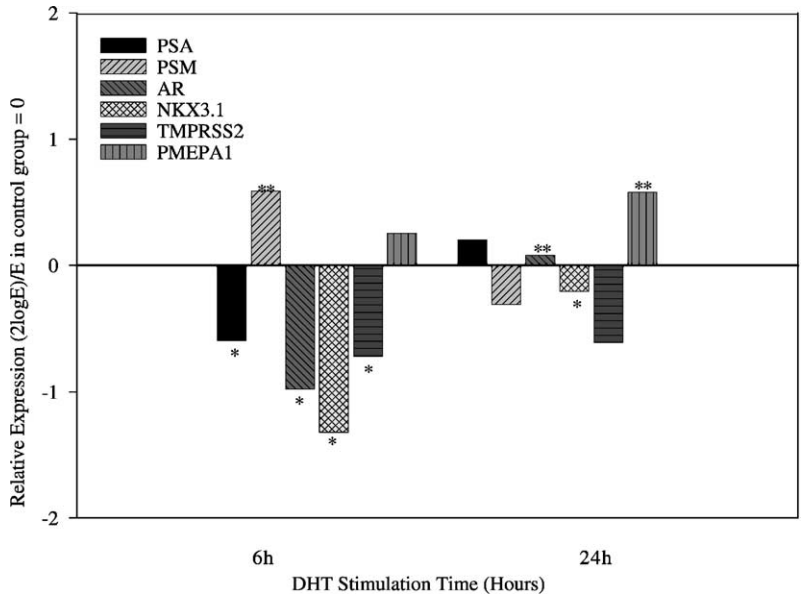

Fig. 1. Time response of DHT (10 nM) induction of ARGs mRNA in 22RV1 cells. $^{*}P < 0.01$, $^{**}P < 0.05$ indicates significant differences between treatment groups.

specificity and high sensitivity. Length of desired product was confirmed by high-resolution gel-electrophoreses, (for overview, see Table 2). After 40 cycles of real-time RT-PCR no primer–dimer formation could be observed. PCR efficiencies were calculated from the given slopes in LightCycler® Software 3.3 (Roche Diagnostic, Germany) (Table 2). To confirm accuracy and reproducibility of real-time PCR the intra-assay precision was determined in replicates of three within one LightCycler® run. Inter-assay variation was investigated in three different experimental runs performed on 3 days. Calculation of test precision and test variability is based on the CP variation from the CP mean value (Table 2).

3.3. Screening for androgen-regulated genes in 22RV1 cells

To test the different candidate marker genes, we exposed 22RV1 cells to either increasing concentrations of test compound for 24 h or a fixed concentration for different time periods. The candidate genes were then assayed for dose-

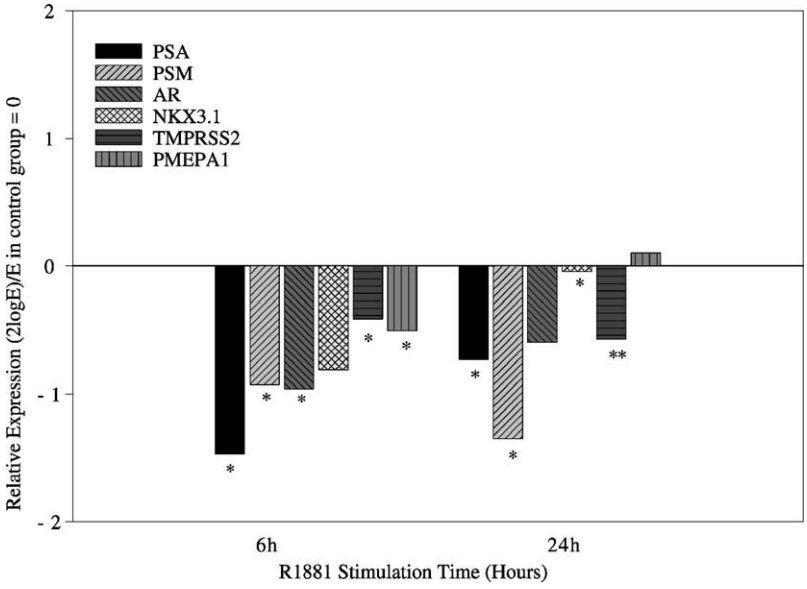

Fig. 2. Time response of R1881 (1 nM) induction of ARGs mRNA in 22RV1 cells. $^{*}P < 0.01$, $^{**}P < 0.05$ indicates significant differences between treatment groups.

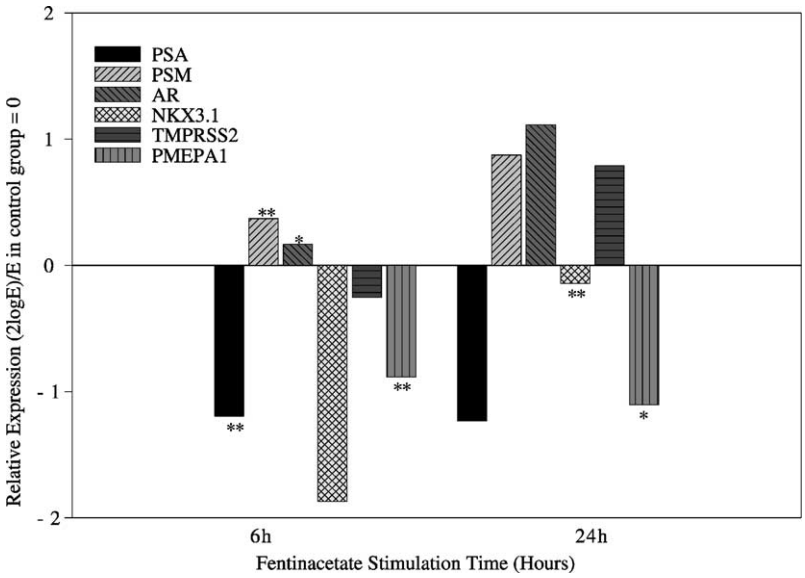

Fig. 3. Time response of fentinacetate (100 nM) induction of ARGs mRNA in 22RV1 cells. $^{*}P < 0.01$, $^{**}P < 0.05$ indicates significant differences between treatment groups.

and time-dependent effects on mRNA expression. In this paper, we present treatment dependent expression profiles for six representative marker genes after 6 and 24 h of treatment. Dose- and time-settings presented here were chosen according to optimal viability of treated cells and maximal alteration of gene expression. Lower dosages did not lead to significant changes of gene induction/repression. Higher doses, especially of pesticides proved to be cytotoxic in vitro. Concentration of DHT and R1881 were adopted from current literature [41,44,46].

3.4. Analysis of androgen-regulated gene expression in different treatment groups

Our experimental conditions 10 nM DHT treatment of 22RV1 cells for 6 and 24 h revealed consistent up/down regulation of six prostate relevant androgen-regulated genes: *PSA*, *PSM*, *AR*, *NKX3.1*, *TMPRSS2*, *PMEPA1* (Fig. 1). ARGs expression was also determined after 6 and 24 h of R1881 (1 nM) treatment (Fig. 2). After 6 h of culture treatment, all genes appeared to be down-regulated. *PMEPA1*

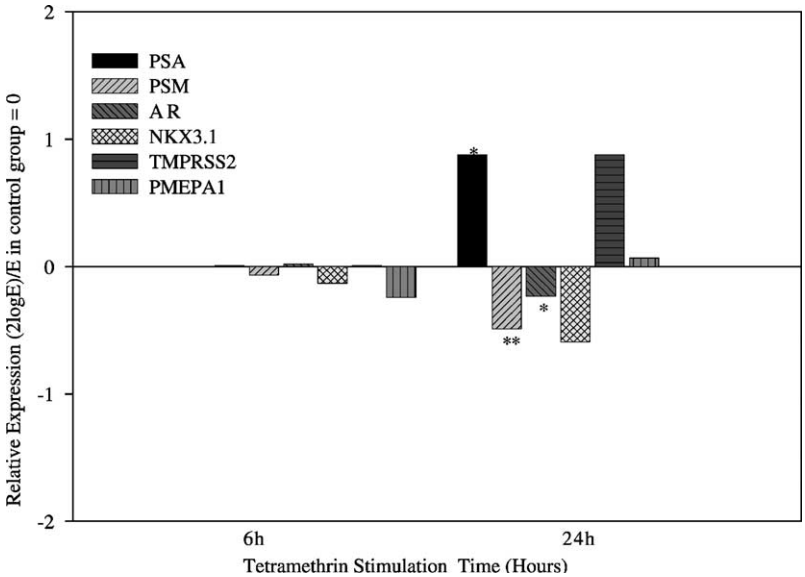

Fig. 4. Time response of tetramethrin (50 nM) induction of ARGs mRNA in 22RV1 cells. $^{*}P < 0.01$, $^{**}P < 0.05$ indicates significant differences between treatment groups.

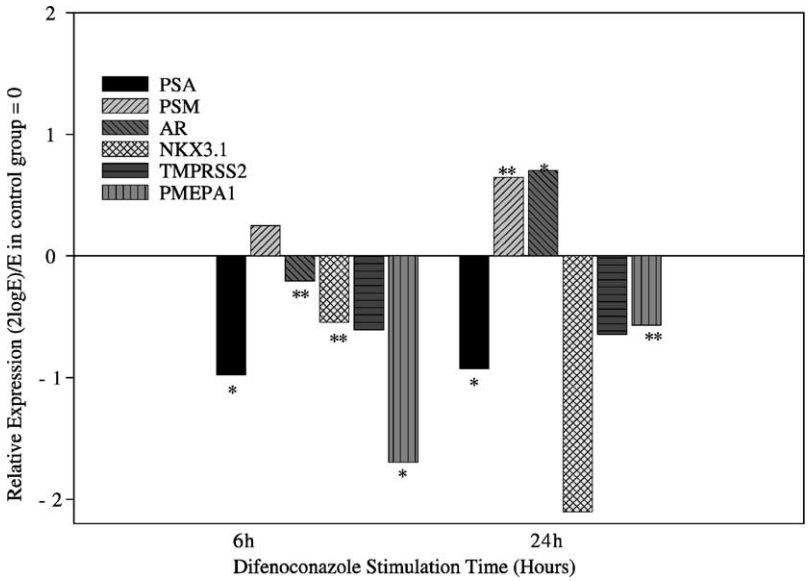

Fig. 5. Time response of difenoconazole (100 nM) induction of ARGs mRNA in 22RV1 cells. $^{*}P < 0.01$, $^{**}P < 0.05$ indicates significant differences between treatment groups.

gene expression was up-regulated after 24 h whereas all other genes remained down-regulated (Fig. 2). Homeobox *NKX3.1* gene was down-regulated by the factor 1.9 after 6 h in response to fentinacetate (Fig. 3). After 24 h, there was less than factor 0.1 increase in *NKX3.1* mRNA levels.

All six ARGs had no statistically significant increase at least 6 h after tetramethrin (Fig. 4) treatment. After 24 h, we detected induction of *PSA* by the factor 0.7 and of *TMPRSS2* (0.9) by tetramethrin. Prolonged treatment with 100 nM difenoconazole produced a further increase of *NKX3.1* transcription with a 0.5 and 2.1 increase of mRNA after 6 and 24 h, respectively (Fig. 5). For complete depiction of gene expression pattern see Figs. 1–5.

4. Discussion

The herein described assay is based on human prostate carcinoma 22RV1 that is an androgen-dependent, serially transplantable nude mouse xenograft derived from a primary human prostate cancer. Transplanted 22RV1 tumours are positive for AR and growth of 22RV1 is androgen dependent. The recurrent tumour 22RV1 expresses AR, but growth of this tumour becomes androgen independent [38]. As DSMZ could definitely not assure expression of hAR in 22RV1 cells only, receptor expression had first to be characterised. 22RV1 cells proved to express human androgen receptor only. Therefore, conclusion can be made that alteration of gene expression patterns may predominately be related to androgenic/antiandrogenic function of compounds under study. Nevertheless, induction/repression of gene activity due to an androgen independent pathway cannot totally be excluded. However, interferences with oestrogen or progestin signalling pathways are not possible in our system, because of lack of receptor expression. All other commercially available prostate cancer cell lines do express at least one additional steroid receptor. Therefore, they are not suited for selective androgen-related bioresponse analysis. Since one of the primary goals of our study was to define a panel of ARGs that could be used as a readout for androgen signaling status, setting, dosage and time of androgen treatment of 22RV1 cells selected for real-time analysis had to be established. Expression of all genes under study showed to be androgen dependent, although growth of cells was independent from the presence of androgens in the media.

All primers used, amplified the sequences of interest with excellent accuracy and precision. Intra- and inter-assay variances proved to be as low as mentioned by others [39]. Therefore, even slight differences in gene expression were statistically significant. Non-significant differences in expression patterns indicated by higher bars than lower but significant bars, are caused by higher variability in inter-assay performance of respective genes. An important parameter for us when selecting target genes, is that transcriptional alteration is highly sensitive to the treatment. Moreover, gene expression must be directly regulated by androgens/antiandrogens. For this purpose only genes bearing an ARE in were included in this study. In contrast to GAPDH or β-actin, ubiquitin expression was unaffected by cell treatment. Therefore, we chose ubiquitin for normalisation of target gene expression in the different treatment groups.

Finding that gene expression patterns in 22RV1 cells are altered in a time-dependent matter, suggests that studies on endogenous gene expression could be a useful tool for assaying compounds with potential androgenic/

antiandrogenic effects. After treatment with different compounds, each of them revealed a substance-specific expression pattern. *NKX3.1* proved to be the only gene, which was down-regulated in expression in all treatment groups. These observations contrast results of others [43], who were able to show that *NKX3.1* expression is up-regulated upon androgen treatment of LNCaP cells. Tetramethrin treatment for 6 h showed the weakest effect on gene expression. Using this compound in an androgen-receptor binding assay [40] revealed only weak relative binding activity and may therefore not be able to induce strong gene expression activity. In addition, it could be proven that various genes can respond differently to different putative androgens/antiandrogens. Nevertheless, using this system it remains unclear whether expression patterns found, will culminate in an androgenic/antiandrogenic effect when acting on a complex organism. To predict the influence of these compounds on man or livestock, animal-based in vivo assays still have to be carried out. They indicate complex changes in phenotype of test organisms as a result of various cell types interacting differently with the substances under study. Therefore, determination of androgenic/antiandrogenic effects leading to pathological alterations like cryptorchism, hypospadias, testicular cancer, prostate cancer and breast cancer still have to be analysed using animal experiments. Furthermore foetotoxic effects and impairement of fertility in the next generation can be assayed by animal experiments only.

On the other hand, reporter-gene assays are available to test androgenicity of chemicals or metabolites [20–23]. Concerning reporter-gene systems it has to be mentioned that artificially constructed plasmids are far apart from the more complex androgen dependent endogenous gene expression. Nevertheless, these assays show high sensitivity facilitating the distinction between receptor agonists and antagonists. However, expression of one gene of interest only can be measured in these systems, they are not suitable to achieve substance-specific expression patterns. Complex influences on gene expression like induction of one gene and repression of another one cannot be covered by reporter-gene assays. The third method established are the binding assays [16–19] that allow studies on binding efficiencies only. No statement concerning a functional bioresponse can be made. Distinction between different compounds concerning binding efficiencies can only made in a “yes” or “no” manner. Therefore, these tests are hardly suited for functional analysis of substances. Due to the disadvantages of above-mentioned methods, we decided to choose an approach using an endogenous gene expression system. All this has been done with respect to characterisation and achievement of substance-specific expression pattern. Our test system revealed that changes in gene expression levels show a quantitative time-response-correlation. Results obtained, showed that cell harvest and gene expression analysis after 6 h of treatment is sufficient to get substance-specific expression patterns. In conclusion, applying an endogenous-gene expression system facilitates the characterisation of the different activity spectra of androgenic/antiandrogenic substances.

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