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Research paper

Multiple readout assay for hormonal (androgenic and antiandrogenic) and cytotoxic activity of plant and fungal extracts based on differential prostate cancer cell line behavior

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ABSTRACT

Ethnopharmacological relevance: Prostate cancer is one of the most diagnosed forms of cancer among men in western regions. Many traditional applications or phytotherapeutic concepts propose to inhibit the proliferation of prostate cancer cells. In order to detect influences of plant or fungal extracts and derived fractions on androgen receptor signaling pathways, a differentiating cell proliferation assay was established, which enables the simultaneous detection of hormonal and cytotoxic effects.
Material and methods: The well characterized prostate cancer cell lines LNCaP and PC-3 were used in a multiple readout assay. In all, 186 fractions of 23 traditionally used organisms were screened regarding their effects on proliferation of the two prostate cancer cell lines. The fractions were prepared by accelerated solvent extraction followed by gradient extrography. Extracts of the potential hormonally active plants *Cibotium barometz*, *Heteropterys chrysophylla*, and *Sideroxylon obtusifolium* (= *Bumelia sartorum*) were phytochemically investigated.
Results: Fractions from *Cibotium barometz*, *Cortinarius rubellus*, *Cyrtomium falcatum*, *Heteropterys chrysophylla*, *Nephrolepis exaltata, Salvia miltiorrhiza, Sideroxylon obtusifolium, Trichilia emetica*, and *Trimeria grandifolia* exhibited hormonal influences on prostate cancer cells. Cytotoxic activity towards human cell lines was detected for the first time for fractions from *Aglaia spectabilis (A. gigantea)*, *Nephrolepis exaltata* and *Cortinarius brunneus*.
Conclusions: The differential behavior of the two prostate cancer cell lines allows the discrimination between potential androgenic or antiandrogenic activities and effects on the estrogen or glucocorticoid receptor as well as cytotoxic activities. The combined cell lines assay can help to assess the biological activities of material used in traditional medicine.

1. Introduction

The androgen receptor (AR) and its signaling pathways are known to be involved in the development of a variety of diseases and therefore act as a valuable medicinal targets. One example is represented by the therapy of benign prostate hyperplasia, prostate cancer, and skin disorders (e.g. acne, hirsutism or androgenetic alopecia) with androgen receptor signaling pathway inhibitors. Furthermore, androgenic anabolic compounds are used to cure chronic states of exhaustion caused by AIDS or cancer cachexia due to their appetizing effect. In addition to that, they are applied as male contraceptives, in hormone replacement therapies for elderly males and in case of gender dysmorphia. Based on this knowledge, there has been an increasing interest in the use of phytotherapy to prevent or cure androgen dependent diseases. The development of relevant drugs is thereby mainly based on ethnomedicinal knowledge. In South Africa, for example, the folkloric use of the plant *Bulbine natalensis* (= *Bulbine latifolia*) was reported for the management of male sexual dysfunction. Indeed, an anabolic and androgenic activity of the *Bulbine natalenis*

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stem extract could be demonstrated in male Wistar rats (Yakubu & Afolayan, 2010). Furthermore, extracts of *Serenoa repens* (fruit), *Pygeum africanum* (= *Prunus africana*) (cortex), *Urtica dioica* (radix) (Farag et al. 2013), *Secale cereale* (pollen), *Hypoxis rooperi* (= *Hypoxis hemerocallidea*) (herba), and *Cucurbita pepo* (semen) were studied regarding their potential to cure benign prostate hyperplasia (Oelke et al., 2009). *In vitro* activities could mainly be documented for the inhibition of 5α-reductase and growth factors. In addition, effects on prostate cancer cells were shown for the phytocompounds genistein, lycopin, cucurmin, resveratrol, and epigallocatechin gallate from Green Tea. However, only preventive effects, but no therapeutic effects could be proven so far. Moreover, the ecdysteroid 20-hydroxyecdysone, which is also found in plants, has been suggested as an option for the therapy of muscle atrophy because of its anabolic activity (Tóth et al., 2008). Considering the large number of diseases connected to the androgen receptor and its signaling pathway, this study aimed at the evaluation of the antiandrogenic or androgenic potential of extracts from fungi and medicinal plants. For this purpose, the androgen dependent prostate cancer cell line LNCaP and the androgen independent prostate cancer cell line PC3 were employed. Cell proliferation was evaluated with the help of the colorimetric XTT assay. Screening parameters were chosen to reveal any influence on androgen signaling pathways. Extracts of 23 organisms were prepared and fractionated *via* gradient extrography. In total, 186 fractions were submitted to the cell proliferation assay. Beside plant material with an ethnomedicinal background, basidiomycetes were also included in the study, since secondary fungal metabolites can interfere with androgen receptor function (Zaidman et al., 2008).

2. Materials and methods

2.1. Plant materials

Plant and fungi material was provided by cooperation partners or obtained from botanical gardens, commercial sources or the greenhouse collection of the Leibniz Institute of Plant Biochemistry (Table 1). Plant names have been checked with www.theplantlist.org (accessed at 05/14/2014). The identity of commercially procured material was proved by the providers. Fungal fruiting bodies were collected and determined by Dr. Norbert Arnold, IPB. Reference material is available at the IPB.

2.2. Extraction and fractionation

The dried or lyophilized material of 23 organisms was extracted *via* automated accelerated solvent extraction using Dionex ASE 200®. Unless stated otherwise, extraction was performed in two steps: first using the solvent mixture 2-methoxy-2-methylpropane (MTBE)/ethanol (80/20 v/v) and second by applying pure methanol. Two extraction cycles for each solvent were performed in 22 ml extraction cells. The first solvent system was selected as it was found beneficial to also extract more lipophilic compounds well, e.g. steroids (Seipold et al. 2004; Dumri et al. 2008).

The crude extracts obtained by accelerated solvent extraction or provided by cooperation partners were fractionated *via* gradient extrography. For this purpose, extracts were adsorbed to modified diatomaceous earth (Isolute HMN). Afterwards, the loaded diatomaceous earth was eluted using solvents with increasing polarity [*n*-hexane, ethyl acetate, acetone, methanol, acetone/HCl (0.5 ml 2 N HCl/100 ml acetone), whereby the latter is especially useful for fungal extracts and alkaloids] (Scheme 1). Crude extracts and fractions were tested in the proliferation assay (Scheme 1).

2.3. Cell culture conditions

LNCaP and PC-3 cells were obtained from the DSMZ (Braunschweig, Germany) and were cultured in phenol red free RPMI1640 medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with 2 mM L-alanyl-L-glutamine (Biochrom AG, Berlin, Germany), 16 mM HEPES (CC pro, Neustadt, Germany), 100 µg/ml penicillin and streptomycin (both from PAA Laboratories) and 10% FBS (Invitrogen, Carlsbad, CA, USA). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Cell lines were regularly tested for mycoplasm infection.

2.4. Cell proliferation assay

Stock solutions of fractions were prepared in DMSO. For the cell proliferation assay LNCaP and PC-3 cells from passages 5 to 31 were used. The preliminary treatment of the cells included a serum free preincubation period of 7 d after reaching 70% confluency in 25 cm^2 cell culture flasks. Cells were subsequently plated in 100 µl of medium with 10% charcoal-dextran treated fetal bovine serum (CD-FBS) at 8×10^3 LNCaP cells or 5×10^2 PC-3 cells per well as quadruplicates. After 24 h, 100 µl medium with 10% CD-FBS containing 25 µg/ml (exceptions are indicated) of test fraction alone or in addition to 1 nM testosterone (TES) were added. DMSO (0.5%, final v/v) served as negative control. The androgen testosterone (1 nM) was used as positive control for stimulation of LNCaP cell proliferation (group a). The antiandrogenic compounds finasteride and bicalutamide were used in a concentration of 10 µM as positive control for inhibition of testosterone-stimulated LNCaP cell proliferation (group b); the cytotoxic drugs vincristine sulfate and etoposide (10 µM) for inhibition of LNCaP, testosterone-stimulated LNCaP, and PC-3 cell proliferation (group c). The proliferation was determined 5 d later by XTT (Roche Diagnostics Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The absorbance measurements of XTT tests (abs; λ_{max}=490 nm, $\lambda_{reference}$= 600 nm) were compared to the DMSO control to yield percentage values and these values were referred to the proliferation in the DMSO control by subtracting 100%.

$$\text{proliferation in } \% = \frac{\text{abs(test well)}}{\text{abs(DMSO control)}} \times 100 - 100 \ \%$$

Thus, negative proliferation values indicate inhibition and positive values reveal enhancement of cell proliferation. In order to visualize inhibition under testosterone coadministration, the difference to the testosterone positive control was calculated.

$$\text{proliferation in } \% = \frac{\text{abs(test well with TES)}}{\text{abs(DMSO control)}} \times 100 - \frac{\text{abs(TES 1 nM)}}{\text{abs(DMSO control)}} \times 100$$

Plates were also analyzed visually under the microscope to reveal discrepancies between the cell number and XTT values.

2.5. Statistics

The unpaired Lord-test was used to evaluate statistically significant (α=5%) differences.

2.6. Phytochemical investigations

2.6.1. Cibotium barometz (L.) J.Sm

From a fresh rhizome collected in the mountains of Northern Vietnam in March 2008 the hairs were abscised and the remaining rhizome (601 g) extracted exhaustively with methanol. The solvent was removed and the aqueous crude extract was partitioned successively with *n*-heptane (7.65 g) and ethyl acetate