Cytotoxicity of herbal extracts used for treatment of prostatic disease on head and neck carcinoma cell lines and non-malignant primary mucosal cells

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Abstract. Previously, a growth inhibiting effect of PC-Spes on head and neck carcinoma cell lines had been demonstrated. In order to determine the toxic impact of particular herbs in the mixture, we exposed the head and neck cancer cell lines FADU, HLaC79 and its Paclitaxel-resistant subline HLaC79-Clone1 as well as primary mucosal keratinocytes to increasing concentrations of the herbal mixture Prostaprotect, which has a similar formulation as PC-Spes, as well as its single herbal components Dendranthema morifolium, Ganoderma lucidum, Glycyrrhiza glabra, Isatis indigotica, Panax pseudo-ginseng, Rabdosia rubescens, Scutellaria baicalensis and Pygeum africanum. Growth inhibition was measured using the MTT assay. Expression of P-glycoprotein (P-GP), multidrug resistance protein-1 (MRP-1), multidrug resistance protein-2 (MRP-2), breast cancer resistance protein (BCRP) and androgen receptor (AR) were examined by western blot analysis. Pygeum africanum extract clearly turned out as the main cytotoxic component of the Prostaprotect prescription mixture, and initiated apoptosis in sensitive cell lines. All other extracts had only minor toxic effects. Western blot analysis revealed increased expression of P-GP in HLaC79-Clone1 cells, while HLaC79 and FADU cells were negative. All three cell lines were negative for MRP-1 and BCRP but positive for MRP-2. HLaC79 and its descendant HLaC79-Clone1 both expressed AR, as verified by western blotting and immunofluorescence staining. Primary mucosal keratinocytes were negative for all multidrug resistance markers as well as for AR. Growth inhibition rates of the single herbal extracts were compared with previously published results in prostate carcinoma cell lines. The relationship between expression levels of AR and multi-drug resistance markers in relation to the measured toxicity of herbal extracts in our head and neck cancer cell system is critically discussed.

Introduction

PC-Spes is a herbal mixture containing extracts of the herbs Dendranthema morifolium, Ganoderma lucidum, Glycyrrhiza glabra, Isatis indigotica, Panax pseudo-ginseng, Rabdosia rubescens, Scutellaria baicalensis and Serenoa repens. It has been used for a long time by prostate cancer patients as an alternative and/or subsidiary treatment of prostate cancer. Herbal therapy in the treatment of benign prostatic hyperplasia as well as malignant diseases has increased during the last years, especially in the US (2) and there are a variety of clinical studies about the efficiency of PC-Spes chemotherapy in prostate cancer (3-5). In 2002, PC-Spes was recalled and withdrawn from the US market because certain batches were contaminated with prescription drugs. In the Netherlands, PC-Spes was available till 2010. Previously, a growth inhibiting effect of PC-Spes on head and neck carcinoma cell lines and primary mucosal keratinocytes has been shown. This effect occurred consistently through all cell lines tested, even in Paclitaxel-resistant cells (1). Since 2010 PC-Spes is no longer commercially available on the European market. The succeeding herbal remedy called Prospectan is available solely as tablets making it difficult to use for in vitro experiments.

Prostaprotect is available in Germany only as a personal prescription formula, due to the strict German regulation of nutritional supplements. At present there is still a discrepancy between unique admission requirements in the EU and the single European countries. In contrast to PC-Spes, Serenoa repens was replaced in this formulation by an extract of Pygeum africanum, a popular phytotherapeutic preparation, used in Europe and USA to alleviate the symptoms of prostatic hyperplasia (reviewed in ref. 6). Pygeum africanum is also available as Tadenan™ capsules. It is sold as a dietary supplement, but as well as other supplements, it is available only in some European countries such as France and Italy. A variety of active substances such as β-sitosterol (7), N-docosanol (8), artraric acid or N-butylbenzene-sulfonamide (NBBS) (reviewed in ref. 9) have been isolated from Pygeum bark extracts, most of them are growth inhibiting for prostate carcinoma cells and...
mediate their effects via interaction with the intracellular androgen receptor (AR).

The antineoplastic drug Paclitaxel is a natural occurring diterpenoid, isolated from the pacific yew \textit{(Taxus brevifolia)} and is used as a chemotherapeutic agent for the treatment of head and neck cancer patients either alone or in combination therapy with other cytotoxic agents or radiotherapy. The therapeutic effect of Paclitaxel was tested in several studies and proved to be active in patients with squamous cell carcinoma of the head and neck. Response rates varied from 20 to 40\% (reviewed in ref. 10).

We established a Paclitaxel resistant clonal subline of the larynx carcinoma cell line HLaC79, (HLaC79-Clone1) and tested the growth inhibitory/cytotoxic effects of Prostaprotect, and of single herbal ingredients on proliferation of FADU, HLaC79 and HLaC79-Clone1 cell lines and on primary mucosal keratinocytes.

In carcinomas \textit{in situ} and tumour cell lines, multidrug resistance is often associated with overexpression of ATP-binding cassette transporter proteins (ABC proteins). ABC proteins that confer drug resistance include P-glycoprotein (P-GP) and the multidrug resistance associated proteins 1 and 2 (MRP-1, MRP-2) as well as breast cancer resistance protein (BCRP). The expression rates of these multidrug resistance mediating proteins by western blot were analyzed. Since PC-Spes and \textit{Pygeum africanum}, both are growth inhibiting for prostate carcinoma cells, partially exert their effects via interaction with the AR, we determined expression levels of AR in the cell lines and primary cells used in our study.

Results were compared with previous studies concerning PC-Spes and single components of it. Results are critically discussed with respect to convergent observations made in prostate and head and neck cancer cells.

Materials and methods

Cell lines and cell culture. The head and neck squamous carcinoma cell line HLaC79 was established from a lymph node metastase of a laryngeal squamous cell carcinoma (11). The cell line was grown with RPMI-1640 medium (Seromed, Munich, Germany), supplemented with 10\% fetal calf serum (FCS). HLaC79 cells were cultured in the presence of 10 nM Paclitaxel and a resistant clone was isolated by selective trypsination of single clones. The permanent HLaC79 clonal cell line HLaC79-Clone1 was cultured in RPMI-1640 medium, supplemented with 10\% FCS and 10 nM Paclitaxel. FADU cells were grown in RPMI-1640 medium. Mucosal keratinocytes were prepared from tonsillar tissue according to standard protocols (12). In brief mucosa was cut into small pieces, which were incubated overnight with 0.2\% dispase (Sigma-Aldrich, Steinheim, Germany) in Dulbecco's modified Eagle's medium (DMEM; Seromed). The epithelium was separated with sterile forceps and primary cells used in our study.

Results were compared with previous studies concerning PC-Spes and single components of it. Results are critically discussed with respect to convergent observations made in prostate and head and neck cancer cells.

Cell viability and proliferation assay. Cells were seeded at 5000 cells/well in 96-well plates. They were treated with increasing concentrations of Paclitaxel (10-200 nM) Prostaprotect (2-10 µl/ml) or herbal extracts (0.2-10 µl/ml) in RPMI medium for 24 h. Controls were kept in medium supplemented with 10 µl/ml EtOH for the ethanolic extraction analysis without drugs. Cell proliferation was measured after 48 h by replacing the culture medium with medium containing 1 mg/ml MTT. After 4 h of incubation, MTT-staining solution was replaced by isopropanol and cells were incubated at 37\°C for 45 min. The colour conversion of MTT to a blue formazan dye was measured with an ELISA reader at a wavelength of 570 nm. The amount of formazan dye is in direct proportion to the number of metabolically active cells in the culture. Single extracts growth curves were established in triplicate, the mean growth curves were standardized to the percentage of surviving cells, whereas the control cells were set at 100\%.

FACS analysis with Annexin V antibodies. FACS analysis was performed using the Annexin V-APC kit of BD Pharmingen (BD Biosciences, Heidelberg, Germany) according to the kit manual. In brief, cells treated with 2 µl/ml \textit{Pygeum africancum} extract for 24 h, were harvested and washed twice with cold PBS. Cells were then resuspended in 1X binding buffer (0.1 M Hepes, pH 7.4, 1.4 M NaCl, 25 mM CaCl\textsubscript{2}) at a concentration of 1x10\textsuperscript{6} cells/ml. To 100 µl of this cell suspension 5 µl Annexin V-APC and 5 µl 7-Amino-actinomycin D (7-AAD; included in the kit) were added and incubated for 15 min in the dark.
Then 400 µl of 1X binding buffer was added. Within 1 h FACS analysis was performed at an excitation wavelength of 650 nm.

**Western blot analysis.** For western blot analysis, cells were harvested by scraping off, and dissolved in RIPA (PBS, containing 1% NP40, 0.5% sodium deoxycholate, 0.1 % SDS), supplemented with 10 µg/ml phenylmethylsulfonyl fluoride (PMSF). Alternative crude membrane fractions (13) were used for blotting. Protein content was determined according to the method of Lowry (14). Equal amounts of total protein lysates were loaded on 10% SDS-polyacrylamide gels and run at a constant current of 20 mA. Gels were blotted onto nitrocellulose membranes according to the semidry method of Kyhse-Andersen (15). Blots were blocked for 1 h with TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 8.0), containing 5% non-fat dry milk. For detection of AR and multidrug resistance-mediating proteins the following antibodies were used: P-GP: Calbiochem clone C219, supplied by Merck-Millipore, Darmstadt, Germany; Clone F4 (Sigma-Adrich); MRP-1: Santa Cruz Biotechnology (Heidelberg, Germany); MRP-2: Santa Cruz Biotechnology; BCRP: Alexis, supplied by Enzo Life Sciences (Loerrach, Germany); AR: Cell Signaling, supplied by Merck-Millipore; GAPDH: Chemicon, supplied by Merck-Millipore.

Primary antibodies were incubated overnight at 4˚C, after washing 3 times with TBST, cells were incubated with corresponding secondary antibodies, coupled to horseradish peroxidase for 1 h. After washing once again, detection of bound antibody conjugates was performed with the enhanced chemiluminescence system (ECL, Amersham Biosciences, Freiburg, Germany), according to the manufacturer’s protocol.

**Indirect immunofluorescence.** Cells were grown on chamber-slides. Slides were fixed with 4% formaldehyde in phosphate Buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄•2 H₂O, 2 mM KH₂PO₄, pH 7.4) for 15 min. After washing three times with PBS the fixed cells were incubated with anti-androgen receptor antibody (Cell Signaling, Darmstadt, Germany) for 1 h. After washing three times with PBS, cells were incubated with a secondary goat anti-rabbit antibody coupled to Alexafluor 488 (Invitrogen) for 1 h. After washing once again, cell slides were mounted with anti-fade mounting medium (250 mg DABCO [1,4-diazabicyclo(2,2,2)octan] in 90% glycerol, buffered with PBS).

**Results**

**Expression analysis of drug resistance proteins (P-GP, MRP-1, MRP-2 and BCRP).** Expression of P-GP, MRP-1/2 and BCRP was tested by western blot analysis of whole cell lysates. While P-GP was clearly expressed in the Paclitaxel-resistant HLaC79-clone-1 subline (Fig. 1), HLaC79, mucosal keratinocytes as well as Paclitaxel-resistant HLaC79-clone-1 subline (Fig. 1), HLaC79, mucosal keratinocytes as well as FADU cells did not express P-GP. MRP-2 was detectable in all three cell lines, with HLaC79 and its Paclitaxel resistant Clone at a similar high level. Mucosal keratinocytes were negative for both chemoresistance markers. For MRP-1 and BCRP no signal in any cell lysate or membrane fraction was obtained.

**Cell proliferation and viability assay.** For evaluation of cytotoxicity/growth inhibition we exposed cell lines and primary keratinocytes to increasing concentrations of the diluent ethanol, Paclitaxel, Prostaprotect and herbal extracts for 24 h.

Incubation of cells with EtOH exerted only minor cytotoxic effects (data not shown). In order to exclude possible cytotoxic effects of the diluent, the highest concentration of 10 µl/ml EtOH was generally added. Each substance was measured in three separate experiments in 12 wells. Results were expressed in relation to untreated control cells (set at 100% survival rate).

![Figure 2. Treatment of cell lines/primary keratinocytes with increasing concentrations of Paclitaxel. Cell viability was measured by the colour conversion of MTT to a blue formazan at 570 nm. Shown are the data of a representative experiment in triplicate.](image-url)
Paclitaxel and prostaprotect. The cell lines and primary cells were treated with increasing concentrations of Paclitaxel (0-200 nm). After 48 h of incubation cell viability and cytotoxicity of the used drugs were measured with the MTT assay. Paclitaxel suppressed the growth of HLaC79 cells significantly at the low dose of 10 nmol (Fig. 2, one of at least three independent measurements for each cell type is displayed). Cell viability decreased on average to 13.63% at 200 nmol Paclitaxel (untreated controls set as 100% survival) in HLaC79 cells and to 20.85% in FADU cells. HLaC79-Clone1 cells as well as slowly proliferating primary mucosal keratinocytes in contrast showed only weak growth inhibition up to concentrations of 200 nM Paclitaxel (mean growth inhibition: keratinocytes 46.41%. HLaC79-Clone1 52.54% at 200 nm). In case of highly proliferative HLaC79-Clone1 cells this can be explained by up-regulated expression of P-GP (Fig. 1).

Prostaprotect proved to be strongly toxic on all cell types (Fig. 3). The highest concentration of 10 µl extract/ml culture medium dropped proliferation down to 5.12% in HLaC79 cells and to 14.44% in mucosal keratinocytes. In HLaC79-Clone1 cultures 12.09% cells survived after 10 µl/ml prostaprotect application. In FADU cells this treatment decreased proliferation to 8.52% of control cells.

Single plant extracts. Growth inhibiting properties of single herbal ingredients of Prostaprotect were tested using extract concentrations adapted to those used in the capsules. Growth curves in Fig. 4 were fitted by setting OD570 values of untreated control cells as 100% survival, which allows a direct comparison of individual extract concentrations in one diagram. Growth inhibition rates in percent of control cells at the highest extract concentration of 10 µl/ml for each herb are summarized in Fig. 5.

The most toxic plant extract in the Prostaprotect mixture proved to be Pygeum africanum bark extract, dropping cell survival to 16.40% (HLaC79), 14.31% (HLaC79-Clone1), 10.42 (mucosal keratinocytes) and 42.01% (FADU; Fig. 5) at 10 µl/ml. Primary mucosal keratinocytes proved to be selectively sensitive towards high concentrations of Panax ginseng and Ganoderma lucidum extracts (56.32% cell survival for Ganoderma lucidum and 46.99 % cell survival for Panax ginseng at 10 µl/ml applied extract concentration; Figs. 4 and 5).

We observed a remarkable growth stimulation at lower concentrations (2-6 µl/ml) of Glycyrrhiza glabra extract in the carcinoma cell lines, but not in primary mucosa cells (Fig. 4). It has to be pointed out, however, that the concentration of licorice extract used in these experiments is >10-fold higher than the concentration used in PC-Spes (40 mg/ml vs. 3.2 mg/ml in PC-Spes). Aqueous solutions of herbal extracts revealed no acute cytotoxicity on cell cultures, even at high concentrations (data not shown).

Comparison of our experimental design with previously published Pygeum studies revealed a wide variation of extract concentrations used for in vitro experiments, ranging from 10 µg/ml (16) to 750 µg/ml culture medium (7). According to the given formulation in Prostaprotect, we applied concentrations between 100 and 500 µg/ml Pygeum extract for treatment of cell cultures. To cover the different concentrations used in literature so far, we tested Pygeum africanum extract at lower concentrations from 10-50 µg/ml culture medium. Results are displayed in Fig. 6. At low concentrations up to 1 µl/ml (50 µg/ml) Pygeum africanum extract exerted only a weak growth inhibition throughout carcinoma cell lines and primary mucosal keratinocytes.

Expression of AR. To reveal an eventual association between AR expression and toxicity of Pygeum africanum extract AR expression was analyzed by western blotting and immunofluorescence staining. HLaC79 and HLaC79-Clone1 both showed positive reaction with AR-antibodies. Expression of AR appeared weak in comparison to cell lysates of the prostatic carcinoma cell line LNCaP, used as a positive control. FADU cells and primary keratinocytes did not express AR (Fig. 7).
In order to exclude clonal or aberrant expression of AR in our cell lines we performed immunofluorescence staining. Antibody staining showed a weak but specific nuclear staining throughout the population of HLAC79 and HLAC79-Clone1 cells (HLAC79 Fig. 8). FADU cells and mucosal keratinocytes were negative for AR staining.
Apoptosis - FACS analysis with Annexin V antibodies. FACS analysis with the Annexin V-APC kit was carried out for *Pygeum africanum*, the herbal extract acting most toxic in our cell lines and primary cells. *Pygeum africanum* extract significantly increased apoptotic cell fractions after 24 h incubation in both the Paclitaxel-sensitive cell line HLaC79 and the Paclitaxel-resistant cell line HLaC79-Clone1 (Fig. 9: HLaC79-Clone1 14.7% apoptotic fraction; HLaC79 56.4%). HLaC79 and HLaC79-Clone1 differed in sensitivity, which might be caused by the increased detoxification capacity of HLaC79-Clone1 cells. In FADU cells, however, a low concentration of *Pygeum* extract was not able to significantly trigger apoptosis (1.2%; Fig. 9).

Discussion

In advanced laryngeal and hypopharyngeal cancer the chemotherapeutic agent Paclitaxel is commonly used for chemotherapy in order to preserve laryngeal and/or pharyngeal structures. Although Paclitaxel generally seems to be a powerful agent, it failed to reach a local-regional tumour control in 12% of patients according to a previously published study (10). Chemotherapeutic failure may be related either to inherited resistance against the drug or the acquisition of resistance during the therapy. Drug resistance is mostly a multifactorial procedure, in the case of Paclitaxel several mechanisms have been described. One mechanism is the overexpression of multidrug resistance proteins, such as P-glycoprotein (P-GP) (coded by the multidrug resistance gene 1, MDR-1, P-GP), multidrug resistance-associated proteins (such as MRP1 and MRP2) or breast cancer resistance protein (BCRP). P-GP overexpression in Paclitaxel-resistant HLaC79-Clone1 cells was confirmed.

Considering single components combined in the Prostaprotect prescription, we observed a growth stimulating effect of licorice extract in head and neck cancer cell lines. In contrast, Hsieh *et al* (17) observed a clear anti-mitogenic effect of Glycyrrhiza extract on prostate carcinoma cell lines. The Glycyrrhiza extract used in our study was over 10-fold higher concentrated than those used by Hsieh *et al* (17). Kimura *et al* (18) described a growth stimulating effect of Glycyrrhizin and some analogues on primary hepatocytes acting via binding to EGF receptors. Molarities of the single substances used in the above mentioned study can't be related to our extracts, but tyrosin phosphorylation of EGF receptors, which are overexpressed in 90% of head and neck carcinomas (19) might also occur in head and neck cancer cell lines.

In the Prostaprotect mixture *Pygeum africanum* turned out to be the major toxic component. *Pygeum africanum*, also available as Tadenan capsules is sold as a dietary supplement, used to treat prostatic hyperplasia, has been shown to hold a variety of active substances such as β-sitosterol (7), N-docosanol (8), artraric acid or N-butylbenzene-sulfonamide (NBBS) (reviewed in ref. 9). All these substances have been isolated from *Pygeum* bark extracts, most of them are growth inhibiting for prostate carcinoma cells and mediate their effects via interaction with the intracellular androgen receptor (AR). Shenouda *et al* (7) showed a growth inhibiting effect of *Pygeum* extract on AR-dependent LNCaP as well as AR-independent growing PC3 prostate carcinoma cell lines. However, they did not observe any toxic effect on AR-negative CaCO2 colon cancer cells at very high concentrations and concluded a clear action of *Pygeum* extract via the AR.

The role of AR in the development of laryngeal cancer is still controversial. A number of publications are available concerning AR expression in head and neck carcinoma tissue.
expression rates ranging between 0% (20) and 68.3% (21). Even in normal adjacent tissue no common expression rates for AR are available. While Chen et al (21) observed 0% AR expression in normal mucosa, Nehse et al (22) report even higher AR expression in mucosa than in tumour tissue. These controversial results are at least partially caused by the different detection methods used, such as in situ hybridization, or RT-PCR for measuring mRNA transcription, immunohistochemistry and receptor assays for determination of protein expression or activity.

Figure 9. FACS analysis of cultured cells with the Annexin V-APC kit after 24 h of incubation with *Pygeum africanum* extract (2 µl/ml). Cells (10,000) were measured in each single experiment. Q4 shows the percentage of the apoptotic cell fraction.
In the present study AR expression was examined on protein level, using western blot detection and immunofluorescence staining and revealed a weak AR expression signal in HLaC79 and HLaC79-Clone 1 cells. Nevertheless we observed strong toxicity of Pygeum extract on all cell lines, AR-positive or -negative, when used in concentrations adapted to Prostaprotect concentrations. Using lower concentrations of Pygeum extract gained a closer look on cellular changes. All three cell lines survived quantitatively. There was no striking difference between AR-positive and AR-negative cell lines in the MTT assay caused by treatment with Pygeum extract, but apoptosis was more pronounced in AR-positive HLaC79 and HLaC79-Clone 1 cells. On the other hand there is a tremendous difference in Pygeum sensitivity between vulnerable HLaC79 cells and p-GP expressing HLaC79-Clone 1 cells. Furthermore, we observed that Pygeum extract at low doses massively triggered apoptosis in primary keratinocytes, although these cells were clearly AR-negative.

The discrepancy to previous studies is probably based on two major problems: first the diversity of extracts used for experiments is a tremendous black box. There is no standard formulation available, except Tadenan capsules, which are no longer available in most European countries presumably because of art protection constraints (Phytolab Inc., personal communication). Besides Tadenan is not useful for in vitro investigations because peanut oil is the major solvent in the capsules. A variety of undefined Pygeum capsules, powders and tablets circulating at the European market are sold via internet shops. Extracts and concentrations used for investigations are not comparable. The second problem is the lack of a holistic consideration in studies. Most studies have concentrated on cause and effect of drugs applied to cells with one certain aspect focused on, for example the role of the AR. Considering the system of a cell in its entirety, however, includes also a view to the capacity of drug detoxification, growth rates, genetic constellation etc. How tightly the diverse cellular mechanisms are linked has been shown for example by Fedoruk et al (23) who demonstrated, that P-GP increases the efflux of dihydrotestosterone (DHT) from cells and is able to reduce androgen responsive gene activity in prostate cancer cells. This cross-functional features are especially important, when herbal mixtures such as PC-Spes are used for studies, with components influencing different cellular functions such as AR expression (17) or P-GP activity and/or expression (reviewed in ref. 24).

All other components used for formulation of the Prostaprotect mixture exerted only minor cytoxicity on cell lines and primary cells. Solely the extract of Panax ginseng inhibited the growth of mucosal keratinocytes quantitatively. This is in contrast to the study of Hsieh et al (17), who described strong toxic effects of Glycyrrhiza, Isatis, Scutellaria, Dendranthema, Rabdosia, Ganoderma and Panax on prostate carcinoma cell lines even at the concentration of 5 μl/ml medium. One reason for the discrepancy between the studies might be the different cellular systems but again, the problem of diversity of extracts used for treatment of cells exists.

In summary, we demonstrated that individual herbs such as Pygeum africanum extract used for treatment of prostatic diseases might also achieve growth inhibition in head and neck cancer cells, even if these cells are resistant to Paclitaxel. The growth inhibiting effect seems to be affected both by detoxification capacity of cells, as well as the expression of AR. The role of the AR in development and course of head and neck cancer remains to be revealed. Furthermore, it should be reconsidered as to which combinations of natural compounds make sense for practical use. Nevertheless, it seems possible, that combinations of purified herbal compounds may be used in combination with conventional anticancer therapy, to achieve synergistic activities.

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References


