Carcinogenesis vol.26 no.8 pp.1360–1367, 2005
doi:10.1093/carcin/bgi092
Advance Access publication April 21, 2005

Tectorigenin and other phytochemicals extracted from leopard lily *Belamcanda chinensis* affect new and established targets for therapies in prostate cancer

Paul Thelen*, Jens-Gerd Scharf[1], Peter Burfeind[2], Bernhard Hemmerlein[3], Wolfgang Wuttke[4], Barbara Spengler[5], Volker Christoffel[5], Rolf-Hermann Ringert and Dana Seidlová-Wuttke[4]

Department of Urology, [1]Department of Gastroenterology and Endocrinology, [2]Institute of Human Genetics, [3]Department of Pathology, [4]Division of Clinical and Experimental Endocrinology, Georg-August-University, Goettingen, Germany and [5]Bionorica AG, Department of Phytochemistry, Neumarkt, Germany

*To whom correspondence should be addressed. Tel: +49 551 398651; Fax: +49 551 396165;
Email: pthelen@gwdg.de

Isoflavones have been shown to exert antiproliferative effects on cancer cells by steroid receptor signaling. In this study, we demonstrate the potential of plant constituents extracted from *Belamcanda chinensis* as anticancer drugs, which regulate the aberrant expression of genes relevant in proliferation, invasion, immortalization and apoptosis. LNCaP cells were treated with *B.chinensis* extract, tectorigenin or other isoflavones and mRNA expression was quantified by using real time RT–PCR. In addition, ELISA, TRAP assays and western blots were used to measure protein expression or activity. Male nude mice ($n = 18$) were injected subcutaneously with LNCaP cells and were fed with extracts from *B.chinensis,* and tumor development was monitored versus a control animal group ($n = 18$). Tectorigenin and several other phytochemicals downregulated PDEF, PSA and IGF-1 receptor mRNA expression *in vitro*. Furthermore, PSA secretion and IGF-1 receptor protein expression were diminished, and hTERT mRNA expression and telomerase activity decreased after tectorigenin treatments. However, TIMP-3 mRNA was upregulated on tectorigenin treatment. Growth of subcutaneous tumors in nude mice was delayed and diminished in animals fed with extracts from *B.chinensis*. The downregulation of PDEF, PSA, hTERT and IGF-1 receptor gene expression by tectorigenin demonstrates the antiproliferative potential of these agents. The upregulation of *TIMP-3* gene expression indicates a pro-apoptotic function of the drug and a reduction of the invasiveness of tumors. The animal experiments demonstrate that *B.chinensis* markedly inhibited the development of tumors *in vivo*. Thus, these compounds may be useful for the prevention or treatment of human prostate cancer.

Introduction

In Western societies, prostate cancer is the most common malignancy in men. In the US $>230\,000$ and in Germany $>40\,000$ new cases per year will be diagnosed. Prostate cancer is characterized by strong dietary influences and a long disease latency period. This long latency period affords opportunities for intervention with therapies that are designed to delay disease initiation or progression (1). Diets are regarded as important in the transformation of latent into more aggressive prostate cancer considering that the frequency of latent prostate cancer is evenly distributed among populations (2). Progression from latent stages into clinically significant prostate cancer is a process that generally requires several years. During this period of time dietary phytochemicals, such as genistein from soy may have chemopreventive effects, which could slow or obviate hormone-dependent cancer development. This would explain the epidemiological evidence that populations consuming diets rich in soy have lower incidence of prostate cancer (3).

These observations initiated several studies to evaluate the chemopreventive potential of phytochemicals or phytoestrogens. Phytoestrogens are polyphenolic non-steroidal plant compounds with estrogen-like biological activity, which are currently under intensive investigations for their role in human health and the reasons for the geographical differences in prostate cancer incidence rates (4). Phytoestrogens can act as selective estrogen receptor modulators (SERMs) and have been evaluated for potential androgen-blocking activity (5). One indicator of androgen-blocking is the inhibition of androgen-regulated proteins such as prostate-specific antigen (PSA), which is used as a surrogate marker of disease progression in clinical studies. PSA secretion and other actions of androgens are mediated by the androgen receptor. More recently, Chen *et al*. (6) demonstrated that the androgen receptor is the crucial factor in the process of prostate cancer cells to become hormone-refractory and therapy resistant. The findings of their study indicate that androgens are still required for growth in hormone-refractory tumors and androgen receptor overexpression alone is sufficient to detect traces of androgen which remain under antiandrogen therapy. Surprisingly, their experiments showed that androgen receptor antagonists such as bicalutamide, which should overcome the excess of androgen receptor expression, acted as an androgen receptor agonist. The conclusion from these findings is that new strategies have to be developed, which interfere with androgen receptor activation, prevent its nuclear translocation, and adjust an aberrant composition of androgen receptor co-activators and co-repressors in prostate cancer.

One of these androgen receptor co-activators, namely the prostate derived Ets transcription factor (PDEF) could serve as a future therapeutic target. Normally, PDEF is controlled by the tumor modulator NKX 3.1. NKX 3.1 has been demonstrated to interact with PDEF and to suppress the ability of

Abbreviations: ADAM, adamalysin metalloproteinase; DHT, dihydrotestosterone; MMP, matrix metalloproteinases; PDEF, prostate derived Ets transcription factor; PSA, prostate-specific antigen; SERM, selective estrogen receptor modulator; TIMP-3, tissue inhibitor of matrix metalloproteinases-3.

PDEF to transactivate the PSA promoter (7). In humans the *NKX 3.1* gene is localized on chromosome 8p21, a chromosomal region frequently deleted in prostate cancer (8). Furthermore, *PDEF* gene expression is highly restricted in normal human tissues and shows a higher rate of tumor-associated expression as compared with Her-2/neu, CA-125, Bcl-2, survivin and telomerase (9). From this study, the authors conclude that vaccines and/or drugs targeted to PDEF are likely to show a low potential for autoimmunity and/or toxicity against normal human tissues. Another established target of therapeutic strategies is the IGF-1 receptor, which is upregulated in prostate cancer and commonly persists in metastatic disease (10). Similarly, an established goal of anticancer strategies is the impairment of functional telomerase activity, which is modulated through estrogen receptor signaling in human prostate cancer (11). Furthermore, therapeutic strategies include the inhibition of matrix metalloproteinases (MMP) and the induction of apoptosis. Both, MMP inhibition and apoptosis are affected by the tissue inhibitor of matrix metalloproteinases-3 (TIMP-3) acting as a specific inhibitor of several adamalysin metalloproteinases (ADAMs) and MMPs and as an inducer of apoptosis (12,13). High levels of TIMP-3 mRNAs in human breast tumors are associated with successful tamoxifen treatment of breast cancer patients (14). Finally, the novel prostate-specific gene $DD3^{PCA3}$ overexpressed in >95% of prostate cancers is considered for interventions directed specifically towards malignantly transformed cells (15).

In the present study, we demonstrate that established as well as potential targets for strategies against prostate cancer are addressed by phytochemicals. The phytochemicals used in this study were extracted from the rhizome of the leopard lily *Belamcanda chinensis (L.)* DC. Our *in vitro* experiments indicate that these phytochemicals, mostly isoflavones such as tectorigenin, adjust aberrant gene expression profiles in prostate cancer cells. Furthermore, our data obtained from animal studies show that the *B.chinensis* extract has beneficial effects on tumor growth *in vivo*.

Materials and methods

Cell culture

B.chinensis extract and compounds isolated from the extract were provided by Bionorica Arzneimittel AG, Department of Phytochemistry, Neumarkt, Germany. All compounds were dissolved in DMSO. Unless otherwise stated, the concentration of the *B.chinensis* compounds was 100 μM for pure substances. In cases of mixtures (fractions 8 and 9), the concentration was ~100 μM. In treatments lasting 24 h the total *B.chinensis* extract was used at a concentration of 400 μg/ml, whereas dihydrotestosterone (Sigma, Taufkirchen, Germany) treatments were performed at a concentration of 10 nM. The DMSO content in all experiments including untreated controls was adjusted to 0.1%. LNCaP cells between passages 20 and 40 were maintained in phenol red-free DMEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (PAA, Coelbe, Germany), 2% glutamine, 1% sodium-pyruvate and 1% penicillin-streptomycin in culture flasks. Cells were harvested by trypsinization and washed with phosphate buffered saline (PBS) (BioWhittaker, Verviers, Belgium) prior to RNA and protein extraction. Proliferation of cultured cells under hormone treatments was quantified using the AlamarBlue assay (Biosource, Nivelles, Belgium).

Animal experiments

Six-week-old male athymic nude BALB/c-nu mice were purchased from Charles River Laboratory, Sulzfeld, Germany. For acclimatization, mice were kept for 2 weeks in standard cages with air filter hoods and fed with soy-free food pellets. Then, for one group of animals ($n = 18$) the food was changed to soy-free pellets containing *B.chinensis* extract (6.7 mg/g), whereas the control animal group ($n = 18$) was kept on the soy-free diet. Subsequently, all animals received a subcutaneous inoculation dorsal of the forelegs of 10^6 exponentially growing LNCaP cells resuspended in 100 μl PBS mixed with 100 μl Matrigel® (Becton Dickinson, Heidelberg, Germany) through a 26-gauge needle. The weight of the mice and the food intake were determined twice a week and subcutaneous tumors were measured once a week with calipers. Tumor volumes were calculated by the formula (large diameter) × (smaller diameter)^2 × 0.5, as described previously (16). After the experiments, animals were killed by using a CO_2 overdose. All experiments were performed according to protocols approved by the local animal protection committee with the reference number 509.425002/01–11.03.

Expression analysis

Total cellular RNA from pelleted LNCaP cells was extracted with the RNeasy Mini Kit (QIAGEN, Hilden, Germany). RNA integrity and quantity were assessed on an Agilent Bioanalyzer 2100 with a RNA 6000 Nano LabChip-Kit (Agilent Technologies, Waldbronn, Germany). Reverse transcription of 500 ng total cellular RNA with random hexamer primers was performed with an Omniscript RT Kit (QIAGEN). Gene expression at the mRNA level was quantitated by using an iCycler iQ real time detection system (BIORAD, Munich, Germany) with the HotStar Taq DNA Polymerase Kit (QIAGEN). The 20 μl reaction from the kit was supplemented with 2 μl cDNA, 0.6 μM gene-specific primers and 0.2 μM dual-labeled fluorescent probes. Primers and probes (Operon, QIAGEN) were designed using the primer3 on-line primer design program (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Secondary DNA structure, which might impair optimal PCR conditions, was ruled out using the Mfold web server program for nucleic acid folding and hybridization prediction (http://www.bioinfo.rpi.edu/applications/mfold/old/dna/). An optimal choice of non-dimer forming primers was made using the Operon oligo tool kit (http://www.operon.com). Primers for PBGD, androgen receptor, PDEF, PSA, hTERT were used as described in previous experiments (17) and PCR primers for DD3 were used as described by de Kok *et al*. (18).

The TIMP-3 forward primer was 5′-GAGGCTTCACCAAGATGCCC-3′, the TIMP-3 reverse primer was 5′-TGCACAGCCCCGTGTACA-3′ and the TIMP-3 probe was 5′FAM-CCAGTACCTGCTGACAGGTCGCGTC-TAMRA3′. The IGF-1 receptor forward primer was 5′-CCG AAG GTC TGT GAG GAA GA -3′, the IGF-1 receptor reverse primer was 5′-AAT GGC GGA TCT TCA CGT AG -3′ and the IGF-1 receptor probe was 5′-FAM-TGC TCA GAT GCT CCA AGG ATG CA-TAMRA-3′.

Standard curves for quantitative PCR were generated with the same reaction set-up using gene-specific standard cRNA (0.001–1000 a mol) instead of total cellular RNA. *In vitro* transcription of gene-specific standard cRNA was performed with an omniscript RT kit (QIAGEN). In all experiments PBGD served as an internal control. Fluorescence signals were monitored on the iCycler and terminated when all reactions reached an amplification plateau while template-free controls remained at basal levels. Data analysis was done by using the iCycler iQ real time detection system software (BIORAD). To verify specific PCR products 5 μl from the reactions were run on 2% agarose gels and were analyzed with the E.A.S.Y. Win 32 software (HEROLAB, Wiesloch, Germany).

PSA secretion from LNCaP in conditioned media and in serum from mice was measured with the Elecsys® System 2010 (Roche Diagnostics, Mannheim, Germany). Telomerase activity in extracts from LNCaP cells was measured with the TeloTAGGG Telomerase PCR ELISA PLUS (Roche Diagnostics) and calculated as relative telomerase activities according to the instructions provided with the kit. The telomerase-mediated 6 nt reaction products from this assay were separated by polyacrylamide gel electrophoresis on a 12% non-denaturing gel stained with SYBR gold (Molecular Probes Europe BV, Leiden, The Netherlands).

For western blot analysis LNCaP cell pellets were homogenized in lysis buffer (pH 7.4) containing 150 mM NaCl, 50 mM Tris–HCl (pH 7.4), 1 % NP-40, 0.25% sodium deoxycholate, 10 mM PMSF, 4 mM EDTA and 1% CHAPS. Immunoblotting was essentially performed as described previously (19). In brief, nitrocellulose membranes were blocked 2 h at room temperature in PBS blocking buffer containing 5% milk powder and 0.1% Tween-20 followed by an overnight incubation at 4°C with the IGF-1 receptor ß antiserum (Santa Cruz, CA) diluted 1:400 in blocking buffer. The membranes were then washed four times with blocking buffer and incubated at room temperature for 2 h with horseradish peroxidase–conjugated monoclonal mouse anti-rabbit immunoglobulins diluted 1:2000 in blocking buffer. Membranes were rinsed twice in PBS with 0.1% Tween-20 and four times with 0.9% sodium chloride containing 0.05% SDS and 0.5% Triton X-100. Enhanced chemiluminescence detection was performed according to the instructions of the manufacturer. Luminescent membranes were exposed to X-ray films (HyperfilmTMP, Amersham Biosciences, Freiburg, Germany) between 10 and 30 min. No background corrections or subtractions were performed on these data.

Statistical analysis

Data are expressed as means + standard deviation. The statistical significance of differences was determined by using Mann–Whitney *U*-tests with $P < 0.01$ considered statistically significant.

Results

Cultured LNCaP cells were treated with phytochemicals extracted from *B.chinensis* to evaluate their effects on prostate cancer. The compounds extracted from *B.chinensis* were composed of the isoflavones tectorigenin and irigenin, the glycosides of tectorigenin and irigenin, i.e. tectoridin and iridin, respectively, the flavonoid iristectorigenin, the orthomethoxy-substituted catechol apocynine and the diglycoside thereof named tectoruside. *B.chinensis* extracts also yielded two fractions which defied further separation, one fraction comprising the flavone hispidulin and the flavonoid 7-methyl-aromadendrin (F8) as well as another fraction comprising trihydroxyisoflavone, tetrahydroxymethoxiisoflavone and trihydroxydimethoxyflavone (F9). When cultured LNCaP cells were treated with particular compounds most phytochemicals had only minor effects on androgen receptor mRNA expression. Tectorigenin moderately affected the expression of the androgen receptor, whereas fractions 8 and 9 could diminish its expression considerably (Figure 1A). However, the mRNA expression of the co-activator PDEF of the androgen receptor was markedly reduced by tectorigenin, and fractions 8 and 9 (Figure 1B). The mRNA expression of PSA reflected the impact the various phytochemicals have on PDEF expression and tectorigenin followed by fraction 9 reduced PSA expression to the highest extent (Figure 1C). Furthermore, when we measured the PSA secretion in conditioned media tectoridin and iridin representing the glycosides of tectorigenin and irigenin also reduced PSA secretion considerably (Figure 1D). In addition, $DD3^{PCA3}$ expression was also reduced by treatments with these compounds. With the exception of iridin and tectoruside, all phytochemicals could significantly downregulate the expression of this specific marker for prostate cancer (Figure 1E). We also found that $DD3^{PCA3}$ expression, like PSA, can be increased by testosterone or dihydrotestosterone treatments (data not shown). Furthermore, we analyzed the effects of the *B.chinensis* phytochemicals on the MMP inhibitor and pro-apoptotic factor TIMP-3. In contrast to the genes studied so far, expression of TIMP-3 is upregulated by treatments with these compounds. The strongest increase of TIMP-3 mRNA expression in LNCaP cells was evoked by tectorigenin, irigenin and iristectorigenin (Figure 1F).

The phytochemicals also have a strong influence on the expression of the IGF-1 receptor. Again, a minor effect was observed for iridin and tectoruside, whereas fractions 8 and 9 followed by tectorigenin have the strongest impact on IGF-1 receptor expression (Figure 2A). In addition, we also investigated the protein expression of the IGF-1 receptor by western blot analysis. As compared with untreated LNCaP cells or those treated with solvent DMSO alone, cells treated with 400 μg/ml of the total *B.chinensis* extract revealed a complete loss of IGF-1 receptor expression. LNCaP cells treated with the isoflavone tectorigenin showed a distinct reduction of IGF-1 receptor expression (Figure 2B). Next, we measured the androgen-dependent hTERT expression and telomerase activity in LNCaP cells treated with *B.chinensis* phytochemicals. The mRNA expression of hTERT, the catalytic subunit of the telomerase, is strongly reduced by tectorigenin and fraction 9 followed by fraction 8 and iristectorigenin (Figure 3A). To test the effect of tectorigenin on telomerase activity in the absence and in the presence of dihydrotestosterone (DHT), extracts from LNCaP cells were subjected to a TRAP assay. With increasing concentrations of tectorigenin the telomerase activity was reduced. DHT increased the telomerase activity, but with increasing concentrations of tectorigenin the telomerase activity also decreased (Figure 3B).

Next, we queried whether changes in cell proliferation occur concomitantly with these expressional changes measured in LNCaP cells. With increasing concentrations of the *B.chinensis* extract and with 100 μM tectorigenin, we observed a significant decrease of LNCaP cell proliferation (Figure 4).

Since the *B.chinensis* extracts showed these beneficial effects on the expression of genes relevant in proliferation and apoptosis, further investigations were performed to test its relevance in an *in vivo* model. For this purpose nude mice were inoculated subcutaneously with LNCaP cells. In a previous experiment, the acceptance and tolerance of *B.chinensis*-containing food versus control food had been verified in two groups of six animals (data not shown). In the present experiment, one group of animals ($n = 18$) was fed with food pellets containing *B.chinensis* extract (6.7 mg/g). The second animal group ($n = 18$) was fed with soy-free control food. Under *ad libidum* food intake both groups had similar body weights, not taking into account the particular tumor weight. The average daily intake of *B.chinensis* extract was 37 mg per animal. After 6 weeks four animals from the control group had developed measurable subcutaneous tumors (Figure 5A), whereas only one animal fed with *B.chinensis* extract showed a small initial tumor formation at this time. The animal shown in Figure 5B is one out of four animals which were tumor-free in week 6, but these mice developed small tumors in the following weeks. After 13 weeks, the tumor of the same animal shown in Figure 5A had increased considerably in volume (Figure 5C), and a total of 11 animals in the control group had developed such tumors. The mouse from the *B.chinensis* group shown in Figure 5B had also developed a smaller tumor after 13 weeks (Figure 5D), whereas 13 animals from this group remained tumor-free until the end of the experiment. The mean tumor volumes after 8 and 13 weeks for the *B.chinensis* group and the control group are shown in Figure 6. We also measured serum PSA in all animals at the end of the experiment. Since only the subcutaneous tumors proportional to their size secreted PSA, the PSA concentrations were normalized for tumor volume. In mice treated with *B.chinensis* extract, the mean serum PSA level (ng PSA/ml per cm^3 tumor volume) was reduced by 35% as compared with the mean of the control animals (data not shown). However, due to high standard deviations this difference could not be considered significant ($P = 0.44$).

Discussion

In this study, we showed how tectorigenin and several other compounds extracted from *B.chinensis* can affect the expression of genes which are deregulated in prostate cancer. The idea of exploring the potential of *B.chinensis*-derived compounds as an anticancer drug is rather new. However, dried rhizomes of *B.chinensis* have been used in traditional Chinese folk medicine for the treatment of cough and pharyngitis (20). Only recently it has been shown that tectorigenin induces

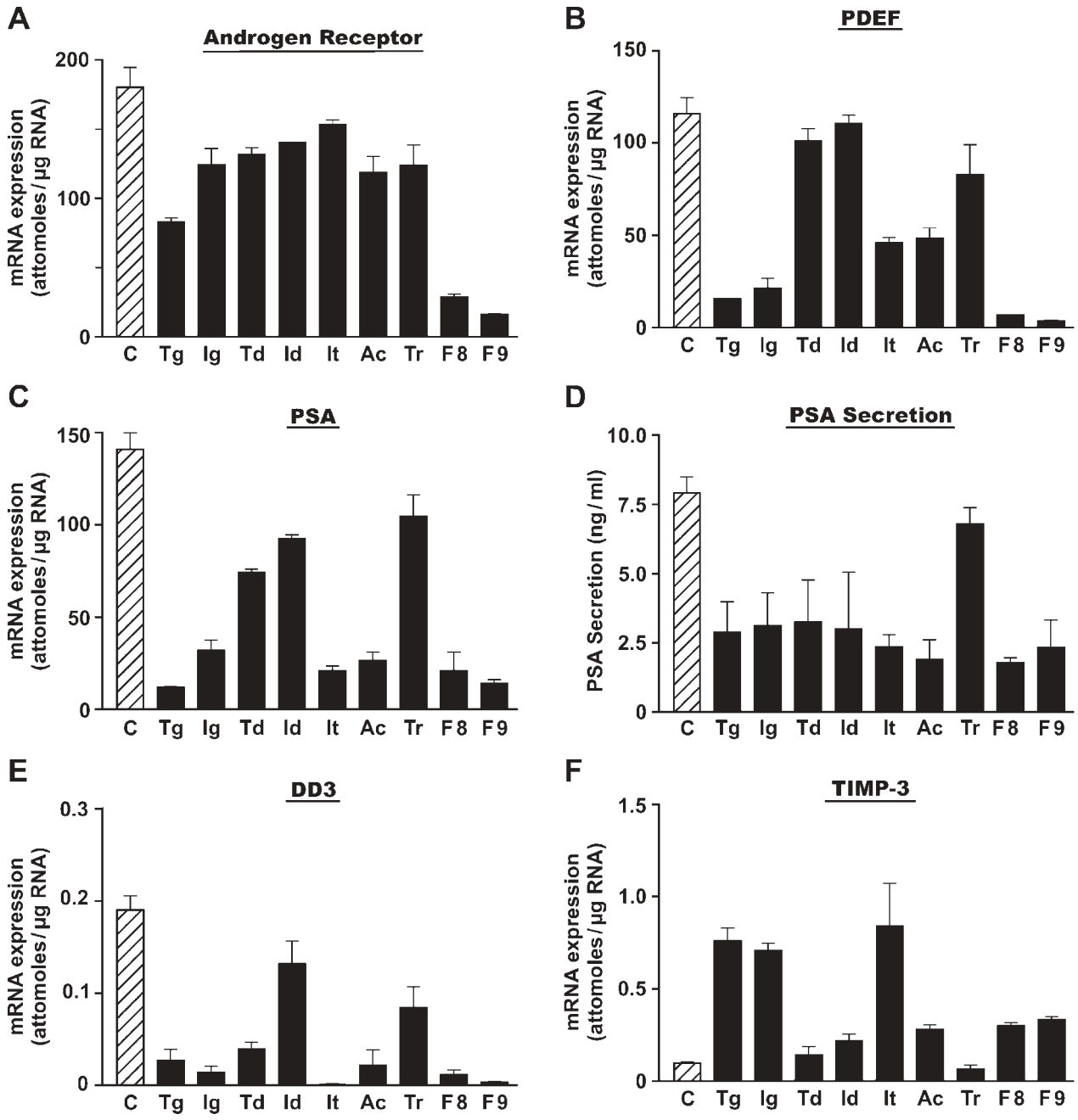

Fig. 1. Expression of the androgen receptor, PDEF, PSA, *DD3* and TIMP-3 in LNCaP cells treated for 24 h with 100 μM tectorigenin (Tg), irigenin (Ig), tectoridin (Td), iridin (Id), iristectorigenin (It), apocynin (Ac), tectoruside (Tr), *B.chinensis* extract fraction 8 (F8) and fraction 9 (F9) versus solvent DMSO (C). (**A–C** and **E–F**) mRNA expression is shown as means of three independent experiments with error bars for standard deviation. (**D**) PSA secretion from LNCaP cells. PSA secretion in conditioned media was measured as described in the Materials and methods section. The effects of treatments are shown as means of three independent experiments with error bars for standard deviation.

beneficial effects in lung carcinoma and leukemia (21,22). The effects we observed here are most probably the consequence of signaling through estrogen receptors. In estrogen receptor binding assays, we and others (23,24) found that tectorigenin exhibited strong binding to the estrogen receptor α and to the estrogen receptor β. Thus, such compounds reveal characteristics of estrogen receptor modulators (SERMs) often featured by phytoestrogens (25). A recent study by Morrissey *et al*. (26) introduced the *B.chinensis* extract as well as the isolated compounds tectorigenin and irigenin for application in prostate cancer. Their *in vitro* experiments demonstrated that tectorigenin and irigenin inhibited the proliferation of LNCaP, RWPE-1 and PC-3 cells, causing G_1 arrest and the induction of p21 (WAF1) or $p27^{kip1}$ protein expression. These data support our findings on the effects of these phytoestrogens on cell proliferation. However, there are possible discrepancies in our findings on the antiandrogenic activity of these compounds. Morrissey *et al*. (26) did not observe a diminished PSA secretion on tectorigenin and irigenin treatments when PSA secretions had been stimulated by DHT. Although we measured the tectorigenin and irigenin effect on the basal, but not DHT-stimulated, expression and secretion of PSA, we observed a more pronounced decrease of PSA as compared with the DHT-free controls in their study. One cause for these

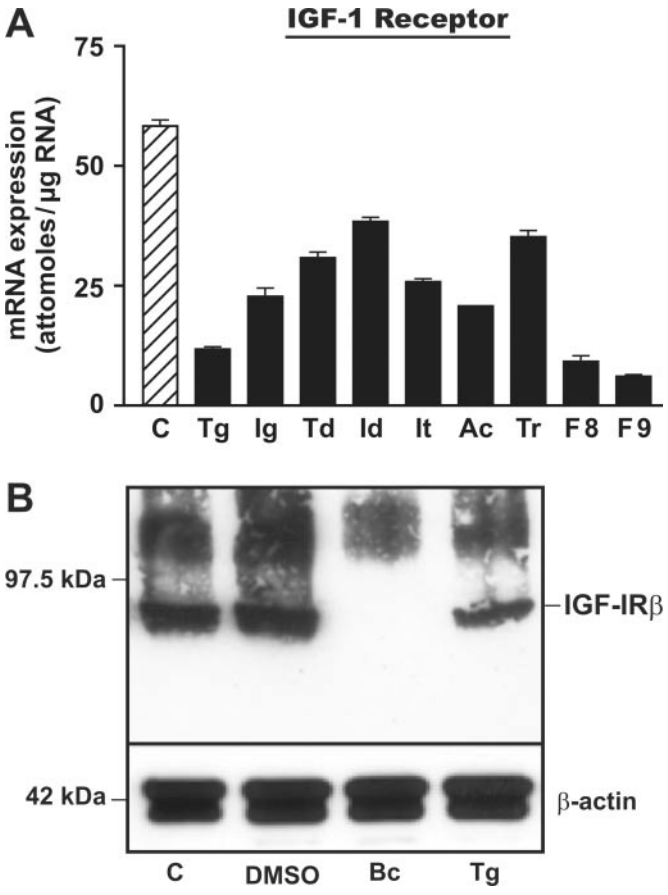

Fig. 2. IGF-1 receptor expression of LNCaP cells treated with various phytochemicals. (**A**) mRNA expression of IGF-1 receptor in the same experiments as described in Figure 1. (**B**) Western blot analysis of IGF-1 receptor β in LNCaP cells treated with 400 μg/ml *B.chinensis* extract (Bc) or 100 μM tectorigenin (Tg) as compared with untreated cells (C) or cells treated with solvent alone (DMSO).

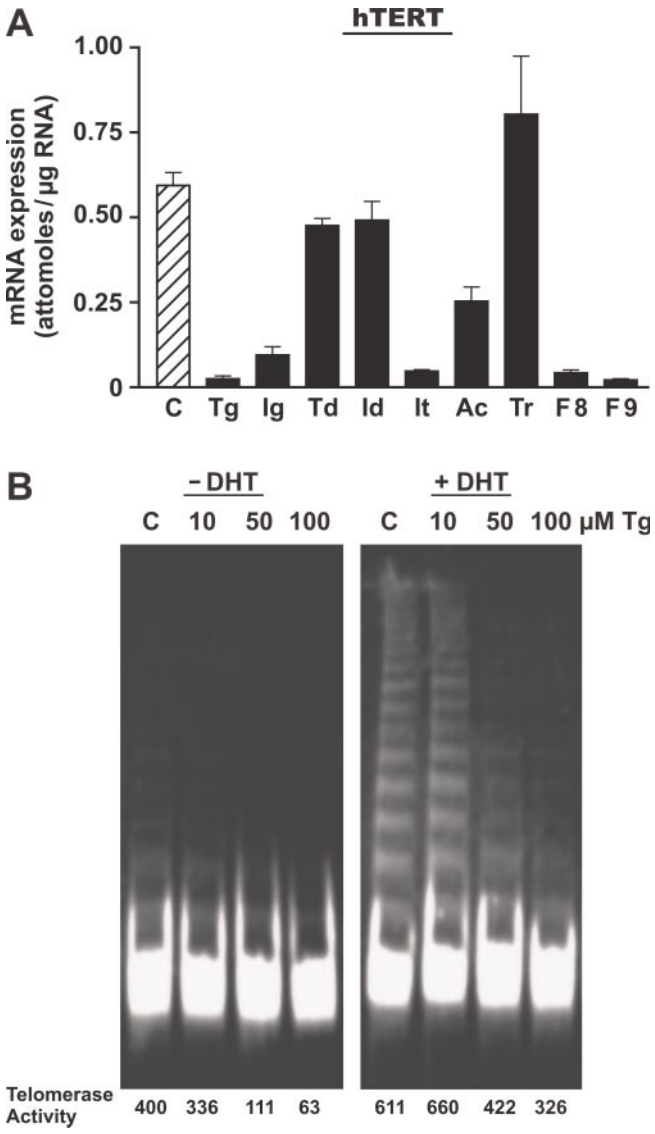

Fig. 3. Telomerase expression of LNCaP cells treated with various phytochemicals. (**A**) mRNA expression of the catalytic telomerase subunit hTERT in the same experiments as described in Figure 1. (**B**) Telomerase activities of LNCaP cells treated with various concentrations of tectorigenin and 10^{-8} M DHT in combinations as indicated. The polyacrylamide gel shows the typical telomerase mediated 6 nt ladders from TRAP assays of LNCaP cell extracts. Experimental protocols were performed and the values for the relative telomerase activities were calculated according to the Materials and methods section.

discrepancies could be the slight difference in cell culture conditions. Another explanation could be the use of different passage numbers of LNCaP cells. In our experiments we only used LNCaP cells between passage numbers 20 and 40. With increasing passage numbers, LNCaP cells acquire a higher basal level of PSA secretion, but become less susceptible to hormone stimuli (16). In such cells, with passages beyond 80, we also observed a less pronounced decrease of the PSA level upon phytoestrogen treatments (data not shown).

Due to recent findings, therapies directed to the proliferative events activated through the androgen receptor are of core interest (6,27,28). Obviously there is a close coordination between PDEF and PSA expression, since PSA expression exhibits the same pattern as compared with PDEF expression in our experiments. The downregulation of PDEF expression affected the downstream expression of PSA and most probably $DD3^{PCA3}$, as well as the IGF-1 receptor, albeit the expression of the androgen receptor itself not being markedly reduced by treatments with most *B.chinensis* compounds. Recently, PDEF has been in demand as a therapeutic target for several cancers, since it shows a higher rate of tumor-associated expression as compared with other cancer-associated molecules. This finding also indicates that drugs targeting the PDEF are likely to show a minor potential for toxicity against normal tissue (9). PDEF could serve as a valuable target, especially in prostate cancer, because its suppressor *NKX 3.1* is frequently deleted in this disease. LNCaP cells do express *NKX 3.1* and an increased expression of *NKX 3.1* could affect the action of PDEF at the protein level (7). However, in our experiments, we did not observe an upregulation of *NKX 3.1* due to phytoestrogen treatments (data not shown).

$DD3^{PCA3}$ exists only as an mRNA product which is not translated; however, it offers immense potential as a prostate-specific marker gene. Although a protein product of $DD3^{PCA3}$ could not be detected, therapeutic implementations for this transcript were considered (15). In the present study, we have shown that $DD3^{PCA3}$ gene expression is hormone-dependent and that $DD3^{PCA3}$ reacts similarly to PSA after LNCaP cells are treated with *B.chinensis* compounds or androgens.

The IGF-1 receptor is an established target for various therapeutic approaches. In experimental models, the growth of

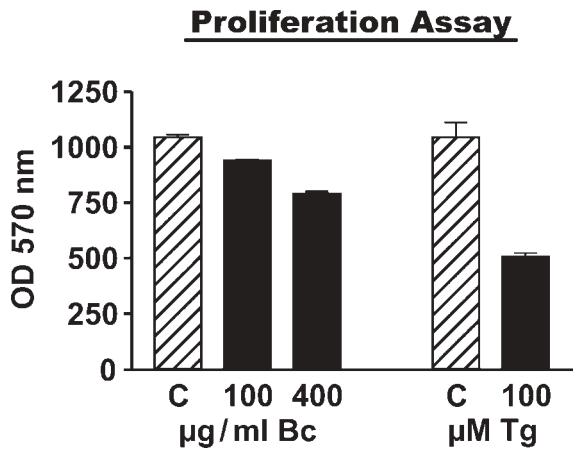

Fig. 4. Proliferation assay in LNCaP cells treated with 100 or 400 μg/ml *B.chinensis* extract (Bc) or 100 μM tectorigenin (Tg) as compared with control cells (C) treated only with solvent DMSO.

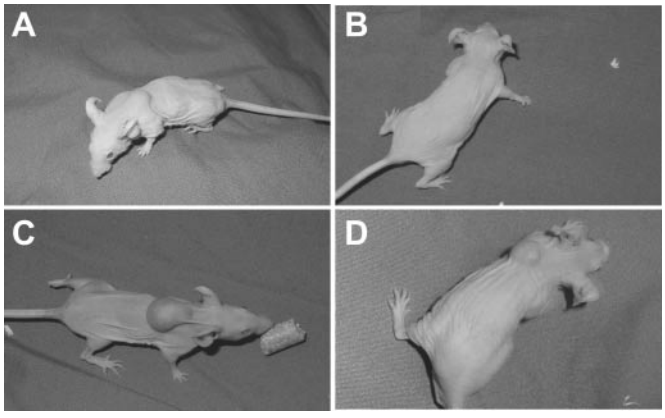

Fig. 5. Images of male nude mice inoculated subcutaneously with LNCaP cells. (**A**) and (**C**) show tumor-bearing mice fed with soy-free control food and (**B**) and (**D**) show mice with no tumor or a small tumor from the group fed with food pellets containing *B.chinensis* extract. (A) and (B) show photographs taken 6 weeks after and (C) and (D) show photographs taken 13 weeks after tumor cell inoculation.

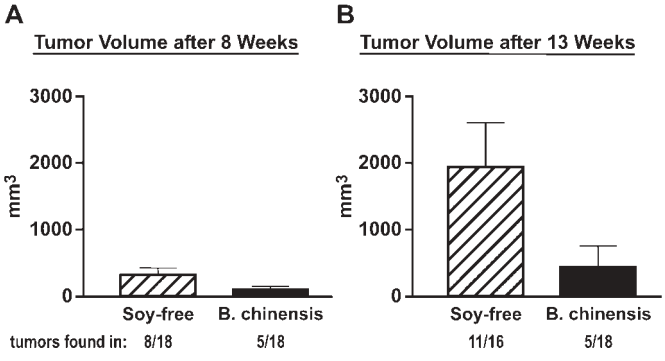

Fig. 6. Mean tumor volumes of nude mice inoculated subcutaneously with LNCaP cells after 8 weeks (**A**) and after 13 weeks (**B**). Columns represent mean tumor volumes with standard deviation in animals either treated with control food (soy-free) or with food pellets containing *B.chinensis* extract (B.chinensis).

many cancers can be inhibited by pharmacological strategies that reduce IGF-1 receptor signaling (29). Tectorigenin and other isoflavones from *B.chinensis* could make further attempts which, presumably, are free of disadvantageous side effects. The expression of the IGF-1 receptor is closely linked to androgen receptor signaling. We and others could show that downregulation of the androgen receptor inhibited IGF-1 receptor expression, which in turn caused an upregulation of IGFBP-3 and a downregulation of MMP-2 expression (30,31).

Similar to the IGF-1 receptor, an aberrant telomerase activity and the inhibition of the invasive potential of tumor cells mediated by MMP are subjects of several therapeutic strategies. In both cases, tectorigenin as well as other isoflavones from *B.chinensis* add an interesting option to such approaches. TIMP-3 has several unique properties setting it apart from other tissue inhibitors of MMP, e.g. its ability to inhibit ADAMs and to induce apoptosis. MMPs or ADAMs are not generally upregulated in cancer and some of their activities serve to inhibit tumor growth (reviewed in ref. 32). Thus, a boost of natural MMP inhibitors such as TIMP-3 are required in order to selectively level out an unbalanced MMP to TIMP ratio in cancers, which synthetic MMP inhibitors usually do not provide. Indeed, there is a loss of TIMP-3 expression in prostate cancer. Recently, we observed that TIMP-3 expression is reduced in central tumor and capsule invasive areas as compared with tumor-free tissue in samples generated from radical prostatectomies by laser microdissection (data not shown). Furthermore, the effect of *B.chinensis* extracts on TIMP-3 expression is also interesting from an experimental point of view, since this MMP inhibitor is upregulated and not downregulated as compared with the other genes analyzed in the present experiments. It is reassuring when after treatments, in addition to unaltered housekeeping gene expression, both directions of expressional changes occur. Thus, the rectification of an aberrant gene expression profile rather than a general loss of gene expression can be assumed.

The beneficial effects that were observed in the present *in vitro* studies could also be demonstrated in our *in vivo* model of subcutaneously implanted prostate tumors into nude mice. However, due to the limited availability of isolated pure tectorigenin in adequate amounts for such extensive animal experiments we decided to use a total extract from *B.chinensis*. This extract consisting of all compounds tested in our *in vitro* experiments contained 5% of tectorigenin. Although the mixtures of different compounds in fractions 8 and 9 and the entire *B.chinensis* extract yielded beneficial results *in vitro*, a therapeutic application would require pure substances in defined dosages. Therefore, further *in vivo* studies of pure isoflavones, e.g. tectorigenin, are warranted.

The outcome of *B.chinensis* treatments in nude mice inoculated with subcutaneous LNCaP tumors was most evident in tumor size and growth rate. The *B.chinensis* extract caused a significant reduction of tumor volume and a delayed onset of tumor growth in treated mice. Moreover, only 5 out of 18 animals from the treated group developed small tumors, whereas 11 out of 16 surviving mice from the control group showed severe tumor formation. An expressional analysis of tumors was hampered by a pronounced heterogeneity in tumor volume, in RNA yield and quality, in necrosis and in the ratio of human- to mouse-derived tissue. However, the mean serum PSA levels normalized for tumor volume in mice treated with *B.chinensis* extract were diminished by 35%, albeit not significantly.

In the present study, we compared the effects of a total extract from *B.chinensis* and various isolated compounds deriving from this mixture. For optimal comparison, we decided to use one single, sufficiently high concentration of

these compounds in order to elicit maximum responses in short time treatments (24 h) of the cells. Thus, in the majority of our studies we used only one concentration (100 μM) for the *in vitro* experiments. This concentration is rather high, but not unusual for *in vitro* experiments using isoflavones including tectorigenin (21,22,26). In animal experiments, we also chose one single concentration of the drug in order to warrant a sufficient systemic concentration of the active compounds. Moreover, in initial animal experiments using this drug we tested the acceptance, tolerance and absence of side effects. Both diets, i.e. the control food and the pellets containing the *B.chinensis* extract were always accepted equally and the animals exhibited equal tumor-free body weights in these experiments (data not shown). The use of high concentrations of isoflavones is also not uncommon in animal feeding experiments. Just recently, Liu *et al*. (33) used 0.5 mg/g of pure isoflavones (genistein and daidzein) in a transgenic erbB-2/*neu* mammary cancer mouse model. On the one hand, they could demonstrate that in tamoxifen-treated mice fed with the high-dose isoflavone-enriched diet, the majority showed no tumor formation and tumor latency was significantly prolonged. On the other hand, in tamoxifen-treated mice fed with the low-dose isoflavone-enriched diet, a much higher rate of mammary tumor development and a shorter tumor latency were observed. These *in vivo* results were also supported by *in vitro* data from human and mouse mammary tumor cell lines. In the *B.chinensis* extract which was used in the present study, the most potent isoflavone is tectorigenin as opposed to less potent isoflavone-glycosides, but tectorigenin only accounts for a fraction of ~5% in *B.chinensis* extracts. In summary, further studies are necessary to investigate candidate drugs derived from *B.chinensis* for optimal concentrations and exposure times.

Taken together, we investigated the effects of phytoestrogens isolated from *B.chinensis* on prostate cancer. The isoflavone tectorigenin significantly rectifies the aberrant expression of several essential gene products involved in prostate cancer. Therefore, each gene product could serve as a valuable target for therapeutic approaches. The combination of these beneficial effects and the absence of undesired side effects indicate that tectorigenin and other isoflavonoids from *B.chinensis* are appropriate tools for future applications in prostate cancer management.

Acknowledgement

This work was supported by the European Union (E)UROESTROGEN(E)S-QLK6-CT-2000–006565.

Conflict of Interest Statement: None declared.

References

1. Nelson,P.S. and Montgomery,B. (2003) Unconventional therapy for prostate cancer: good, bad or questionable? *Nat. Rev. Cancer*, **3**, 845–858.
2. Adlercreutz,H. and Mazur,W. (1997) Phyto-oestrogens and Western diseases. *Ann. Med.*, **29**, 95–120.
3. Denis,L., Morton,M.S. and Griffiths,K. (1999) Diet and its preventive role in prostatic disease. *Eur. Urol.*, **35**, 377–387.
4. Cos,P., De Bruyne,T., Apers,S., Vanden Berghe,D., Pieters,L. and Vlietinck,A.J. (2003) Phytoestrogens: recent developments. *Planta Med.*, **69**, 589–599.
5. Steiner,M.S. and Raghow,S. (2003) Antiestrogens and selective estrogen receptor modulators reduce prostate cancer risk. *World J. Urol.*, **21**, 31–36.
6. Chen,C.D., Welsbie,D.S., Tran,C., Baek,S.H., Chen,R., Vessella,R., Rosenfeld,M.G. and Sawyers,C.L. (2004) Molecular determinants of resistance to antiandrogen therapy. *Nat. Med.*, **10**, 33–39.
7. Chen,H., Nandi,A.K., Li,X. and Bieberich,C.J. (2002) NKX-3.1 interacts with prostate-derived Ets factor and regulates the activity of the PSA promoter. *Cancer Res.*, **62**, 338–340.
8. Abate-Shen,C. (2002) Deregulated homeobox gene expression in cancer: cause or consequence? *Nat. Rev. Cancer*, **2**, 777–785.
9. Ghadersohi,A., Odunsi,K., Lele,S., Collins,Y., Greco,W.R., Winston,J., Liang,P. and Sood,A.K. (2004) Prostate derived Ets transcription factor shows better tumor-association than other cancer-associated molecules. *Oncol. Rep.*, **11**, 453–458.
10. Hellawell,G.O., Turner,G.D., Davies,D.R., Poulsom,R., Brewster,S.F. and Macaulay,V.M. (2002) Expression of the type 1 insulin-like growth factor receptor is up-regulated in primary prostate cancer and commonly persists in metastatic disease. *Cancer Res.*, **62**, 2942–2950.
11. Nanni,S., Narducci,M., Della Pietra,L., Moretti,F., Grasselli,A., De Carli,P., Sacchi,A., Pontecorvi,A. and Farsetti,A. (2002) Signaling through estrogen receptors modulates telomerase activity in human prostate cancer. *J. Clin. Invest.*, **110**, 219–227.
12. Murphy,G., Knauper,V., Lee,M.H., Amour,A., Worley,J.R., Hutton,M., Atkinson,S., Rapti,M. and Williamson,R. (2003) Role of TIMPs (tissue inhibitors of metalloproteinases) in pericellular proteolysis: the specificity is in the detail. *Biochem. Soc. Symp.*, **70**, 65–80.
13. Bond,M., Murphy,G., Bennett,M.R., Newby,A.C. and Baker,A.H. (2002) Tissue inhibitor of metalloproteinase-3 induces a Fas-associated death domain-dependent type II apoptotic pathway. *J. Biol. Chem.*, **277**, 13787–13795.
14. Span,P.N., Lindberg,R.L., Manders,P., Tjan-Heijnen,V.C., Heuvel,J.J., Beex,L.V. and Sweep,C.G. (2004) Tissue inhibitors of metalloproteinase expression in human breast cancer: TIMP-3 is associated with adjuvant endocrine therapy success. *J. Pathol.*, **202**, 395–402.
15. Schalken,J.A., Hessels,D. and Verhaegh,G. (2003) New targets for therapy in prostate cancer: *differential display code 3* (*DD3^{PCA3}*), a highly prostate cancer-specific gene. *Urology*, **62**, 34–43.
16. Igawa,T., Lin,F.F., Lee,M.S., Karan,D., Batra,S.K. and Lin,M.F. (2002) Establishment and characterization of androgen-independent human prostate cancer LNCaP cell model. *Prostate*, **50**, 222–235.
17. Thelen,P., Wuttke,W., Jarry,H., Grzmil,M. and Ringert,R.H. (2004) Inhibition of telomerase activity and secretion of prostate specific antigen by silibinin in prostate cancer cells. *J. Urol.*, **171**, 1934–1938.
18. de Kok,J.B., Verhaegh,G.W., Roelofs,R.W., Hessels,D., Kiemeney,L.A., Aalders,T.W., Swinkels,D.W. and Schalken,J.A. (2002) *DD3^{PCA3}*, a very sensitive and specific marker to detect prostate tumors. *Cancer Res.*, **62**, 2695–2698.
19. Scharf,J.G., Dombrowski,F., Novosyadlyy,R., Eisenbach,C., Demori,I., Kubler,B. and Braulke,T. (2004) Insulin-like growth factor binding protein-1 is highly induced during acute carbon tetrachloride liver injury and potentiates the IGF-I- stimulated activation of rat hepatic stellate cells. *Endocrinology*, **145**, 3463–3472.
20. Ito,H., Onoue,S. and Yoshida,T. (2001) Isoflavonoids from *Belamcanda chinensis*. *Chem. Pharm. Bull.* (Tokyo), **49**, 1229–1231.
21. Jung,S.H., Lee,Y.S., Lee,S., Lim,S.S., Kim,Y.S., Ohuchi,K. and Shin,K.H. (2003) Anti-angiogenic and anti-tumor activities of isoflavonoids from the rhizomes of *Belamcanda chinensis*. *Planta Med.*, **69**, 617–622.
22. Lee,K.T., Sohn,I.C., Kim,Y.K., Choi,J.H., Choi,J.W., Park,H.J., Itoh,Y. and Miyamoto,K. (2001) Tectorigenin, an isoflavone of *Pueraria thunbergiana* Benth., induces differentiation and apoptosis in human promyelocytic leukemia HL-60 cells. *Biol. Pharm. Bull.*, **24**, 1117–1121.
23. Morito,K., Aomori,T., Hirose,T., Kinjo,J., Hasegawa,J., Ogawa,S., Inoue,S., Muramatsu,M. and Masamune,Y. (2002) Interaction of phytoestrogens with estrogen receptors alpha and beta (II). *Biol. Pharm. Bull.*, **25**, 48–52.
24. Seidlova-Wuttke,D., Hesse,O., Jarry,H., Rimoldi,G., Thelen,P., Christoffel,V. and Wuttke,W. (2004) *Belamcanda chinensis* and the thereof purified tectorigenin have selective estrogen receptor modulator activities. *Phytomedicine*, **11**, 392–403.
25. Griffiths,K. (2000) Estrogens and prostatic disease. International Prostate Health Council Study Group. *Prostate*, **45**, 87–100.
26. Morrissey,C., Bektic,J., Spengler,B., Galvin,D., Christoffel,V., Klocker,H., Fitzpatrick,J.M. and Watson,R.W. (2004) Phytoestrogenes derived from Belamcanda chinensis have an antiproliferative effect on prostate cancer cells in vitro. *J. Urol.*, **172**, 2426–2433.
27. Taplin,M.E. and Balk,S.P. (2004) Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence. *J. Cell. Biochem.*, **91**, 483–490.

28. Gao,S., Liu,G.Z. and Wang,Z. (2004) Modulation of androgen receptor-dependent transcription by resveratrol and genistein in prostate cancer cells. *Prostate*, **59**, 214–215.
29. Pollak,M.N., Schernhammer,E.S. and Hankinson,S.E. (2004) Insulin-like growth factors and neoplasia. *Nat. Rev. Cancer*, **4**, 505–518.
30. Grzmil,M., Hemmerlein,B., Thelen,P., Schweyer,S. and Burfeind,P. (2004) Blockade of the type I IGF receptor expression in human prostate cancer cells inhibits proliferation and invasion, up-regulates IGF binding protein-3, and suppresses MMP-2 expression. *J. Pathol.*, **202**, 50–59.
31. Yeh,S., Hu,Y.C., Wang,P.H., Xie,C., Xu,Q., Tsai,M.Y., Dong,Z., Wang,R.S., Lee,T.H. and Chang,C. (2003) Abnormal mammary gland development and growth retardation in female mice and MCF7 breast cancer cells lacking androgen receptor. *J. Exp. Med.*, **198**, 1899–1908.
32. Egeblad,M. and Werb,Z. (2002) New functions for the matrix metalloproteinases in cancer progression. *Nat. Rev. Cancer*, **2**, 161–174.
33. Liu,B., Edgerton,S., Yang,X., Kim,A., Ordonez-Ercan,D., Mason,T., Alvarez,K., McKimmey,C., Liu,N. and Thor,A. (2005) Low-dose dietary phytoestrogen abrogates tamoxifen-associated mammary tumor prevention. *Cancer Res.*, **65**, 879–886.

Received November 9, 2004; revised March 7, 2005; accepted April 10, 2005