Phytoestrogens from *Belamcanda chinensis* regulate the expression of steroid receptors and related cofactors in LNCaP prostate cancer cells

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OBJECTIVE

To investigate the changes in expression underlying the marked reduction of tumour growth *in vivo*, by analysing the effect of *Belamcanda chinensis* extract (BCE) on LNCaP cells *in vitro*, as phytoestrogens are chemopreventive in prostate cancer, and in previous studies we examined the effects of the isoflavone tectorigenin isolated from *B. chinensis* on LNCaP prostate cancer cells, and a BCE consisting of 13 phytoestrogenic compounds on tumour-bearing nude mice.

MATERIALS AND METHODS

LNCaP cells were treated with 100, 400 or 1400 μg/mL BCE; proliferation was assessed with an Alamar Blue assay. We used real-time reverse transcription-polymerase chain reaction to quantify mRNA expression of the androgen receptor (AR), the AR coactivator prostate derived Ets transcription factor (PDEF), NKX3.1, prostate specific antigen (PSA) and oestrogen receptor-β (ER-β) compared with the expression of the housekeeping gene porphobilinogen deaminase (PBGD). PSA secretion from LNCaP cells was measured and protein expression of the AR investigated by Western blot analysis.

RESULTS

Concomitant with a marked decrease of tumour cell proliferation BCE down-regulated the expression of the AR, PDEF, NKX3.1 and PSA. In the same experiments, the expression of PBGD was unaltered, whereas ER-β expression increased. Furthermore, AR protein and PSA secretion were markedly diminished after treatments with the BCE.

CONCLUSION

BCE, comprising 13 different phytoestrogens, decreases the expression of the AR and its co-activator PDEF concomitant with diminished cell proliferation and PSA secretion. NKX3.1 expression was also reduced by BCE. We hypothesise that the positive effects of BCE are initiated by up-regulation of the ER-β, a putative tumour-suppressor gene.

KEYWORDS

prostate cancer, LNCaP, phytoestrogens, androgen receptor, oestrogen, proliferation

INTRODUCTION

In Western societies prostate cancer is the most common malignancy in men; in the USA each year >230 000, and in Germany >40 000, new cases are currently diagnosed. Prostate cancer is characterized by strong dietary influences and a long disease latency period. This long latency affords opportunities for intervention with therapies that are designed to delay disease initiation or progression [1].

Diets are regarded as important in the transformation from 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, dietary phytochemicals might have chemopreventive effects that could slow or obviate the development of hormone-dependent cancer. This is supported by epidemiological evidence that populations consuming diets rich in soya have lower incidence of prostate cancer [3].

Several studies have evaluated the chemopreventive potential of phytochemicals or phytoestrogens. Phytoestrogens are polyphenolic nonsteroidal plant compounds with oestrogen-like biological activity that are currently under intensive investigation for their role in human health, and the reasons for the geographical differences in prostate cancer incidence rates [4].

Phytoestrogens can act as selective oestrogen receptor (ER) modulators and they have been evaluated for potential androgen-blocking activity [5]. One indicator of androgen-blocking is the inhibition of androgen-regulated proteins such as 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 (AR). Chen *et al.* [6] showed that the AR is the crucial factor in the process by which prostate cancer cells become hormone-refractory and therapy-resistant. They showed that cancer progression in hormone-refractory tumours is controlled by traces of androgen that remain even under antiandrogen therapy, and that can be detected by an overexpressed AR. Moreover, their experiments showed that AR antagonists under these conditions acted as AR agonists. This antiandrogen withdrawal syndrome is shown by a PSA decrease when

bicalutamide or flutamide therapy is stopped in patients under combined androgen blockade [7]. Moreover, the androgen withdrawal syndrome is associated with the highest level of AR up-regulation. Considering this, new strategies in the therapy of hormone-refractory prostate cancer must interfere with excessive AR up-regulation and/or activation, and prevent its nuclear translocation. Furthermore, new therapies should remedy the aberrant composition of AR co-activators and co-repressors in prostate cancer.

One of these AR co-activators, the prostate-derived Ets transcription factor (PDEF) could serve as a future therapeutic target. PDEF gene expression is highly restricted in normal human tissues and shows a higher rate of tumour-associated expression [8]. Thus, therapies targeting PDEF are likely to show a low potential for toxicity against normal human tissues. NKX 3.1 was shown to interact with PDEF and to suppress the ability of PDEF to transactivate the PSA promoter [9]. In humans the NKX 3.1 gene is on chromosome 8p21, a chromosomal region that is frequently deleted in prostate cancer [10].

Anti-tumour activities of phytochemicals from the rhizome of the leopard lily *Belamcanda chinensis* were reported previously [11]. Morrissey *et al.* [12] reported antiproliferative effects on prostate cancer cells caused by a total extract of *B. chinensis* and the two purified compounds tectorigenin and irigenin. Recently we reported that established and potential therapeutic targets against prostate cancer are affected by different phytochemicals from *B. chinensis*. Several of the investigated phytochemicals, mostly isoflavones such as tectorigenin, could adjust aberrant gene expression profiles in prostate cancer cells, but with varying efficacy. Furthermore, data obtained from animal studies also showed that the total extract of *B. chinensis* is well tolerated and has beneficial effects on prostate cancer growth *in vivo* [13].

Therefore, we investigated the effects of the *B chinensis* extract (BCE) in *in vitro* studies; the aims were: (i) to test the efficacy of the easily accessible crude extract compared with the individual compounds; (ii) to exclude unwanted side-effects arising from this combination of phytochemicals; and (iii) to elucidate synergistic effects in this combination of compounds compared with the individual purified phytochemicals. As the AR axis is most relevant in hormone-refractory prostate cancer we focused on alterations in the expression of the AR, its co-activator PDEF, the ER-β, the androgen-dependent transcription factor NKX3.1, and PSA.

MATERIALS AND METHODS

BCE was provided by Bionorica Arzneimittel GmbH, Neumarkt, Germany, and used at concentrations of 100, 400 and 1400 μg/mL. The dimethyl sulphoxide content in all experiments, including untreated controls, was adjusted to 0.1%. PC-3 or LNCaP cells at passage 20–40 were maintained in phenol red-free Dulbecco's modified Eagle medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (PAA, Coelbe, Germany), 2% glutamine, 1% sodium pyruvate and 1% penicillin-streptomycin in culture flasks. After 24 h of treatment the cells were harvested by trypsinization and washed with PBS before RNA and protein extraction. The viability of cultured cells after hormone treatment was assessed using the Alamar Blue assay (Biosource, Nivelles, Belgium).

To analyse expression, 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 Bioanalyser 2100 with a RNA 6000 Nano LabChip-Kit (Agilent Technologies, Waldbronn, Germany). Reverse transcription (RT) of 500 ng total cellular RNA with random hexamer primers was done using an Omniscript RT Kit (Qiagen). Gene expression was quantified using an iCycler iQ real-time detection system (Biorad, Munich, Germany) with the QuantiTect™ SYBR®Green Kit (Qiagen). The 20 μL reaction from the kit was supplemented with 2 μL cDNA and 0.6 μM gene-specific primers. Primers (IBA, Göttingen, Germany) were designed using the primer3 on-line primer design program (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and the Operon oligo toolkit (http://www.operon.com). Secondary DNA of PCR products were considered using the Mfold web server program for nucleic acid folding and hybridization prediction (http://www.bioinfo.rpi.edu/applications/mfold/old/dna/). Primer sequences were published previously [13] except NKX3.1: forward primer: 5′-CCG AGA CGC TGG CAG AGA CC-3′ and NKX3.1 reverse primer: 5′-GCT TAG GGG TTT GGG GAA G-3′. Real-time PCR data were analysed using the iCycler iQ real-time detection system software (Biorad). PSA secretion from LNCaP in conditioned media was measured with the Elecsys® System 2010 (Roche Diagnostics, Mannheim, Germany).

For western blot analysis, whole-cell lysates from LNCaP cells were prepared using lysis buffer containing 50 mM Tris-HCl pH 7.4, 150 mM sodium chloride, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL pepstatin A, 1 μg/mL phenylmethylsulphonyl fluoride, 1 mM sodium fluoride and 1 mM sodium orthovanadate. In all, 50 μg of total cell lysates were boiled and denatured in sample buffer containing SDS and dithiothreitol (Invitrogen, Karlsruhe, Germany) followed by gel electrophoresis using the NuPage 4–12% Bis-Tris pre-cast gel in MES buffer (Invitrogen). The proteins were electrotransferred to a PVDF membrane (Macherey & Nagel, Düren, Germany). The membrane was blocked in 2% (for detecting the AR) or 5% (for detecting α-tubulin) dry milk in PBS for 1 h at room temperature and then incubated with primary antibodies at 4 °C overnight. The following concentrations for primary antibodies were used: 1 : 2500 mouse monoclonal anti-α-tubulin (Sigma, Taufkirchen, Germany) and 1 : 2000 rabbit polyclonal anti-AR (Ab-2, NeoMarkers, Montreal, Canada). After washing the membrane three times with 1% dry milk in PBS, the proteins were visualized by using p-nitrotetrazolium blue/5-bromo-4-chloro-3-indolyl phosphate, or by enhanced chemiluminescence according to the manufacturer's instructions (ECL Plus, Amersham, UK).

The data are expressed as the mean (SD), with the statistical significance of differences determined using the Mann–Whitney *U*-test, with $P < 0.05$ considered to indicate statistical significance.

RESULTS

To examine the beneficial effects of BCE on androgen signalling in prostate cancer cells, we assessed tumour cell viability in response to these compounds and changes in expression of the AR and related genes in LNCaP cells.

FIG. 1. Proliferation assay in LNCaP cells treated with solvent (dimethyl sulphoxide, DMSO) alone, or 100, 400 or 1400 μg/mL of BCE.

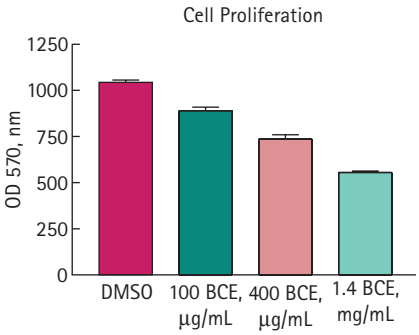


FIG. 2. Expression of the AR in LNCaP cells treated with various concentrations of BCE: (A) mRNA expression as measured by real-time RT-PCR; (B) Western blot analyses.

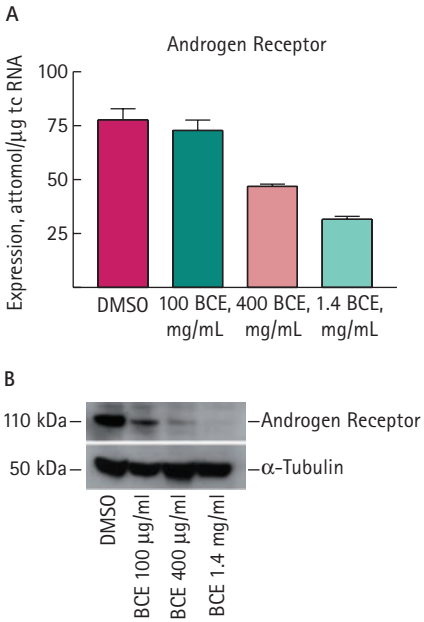


FIG. 3. mRNA expression of the AR coactivator PDEF in LNCaP cells treated with various concentrations of BCE, measured by real-time RT-PCR.

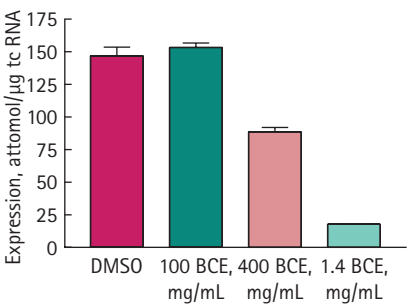


FIG. 4. mRNA expression of NKX3.1 in LNCaP cells treated with various concentrations of BCE, measured by real-time RT-PCR.

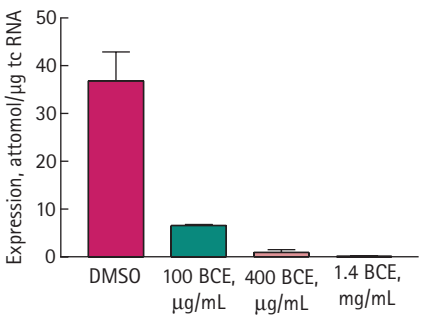
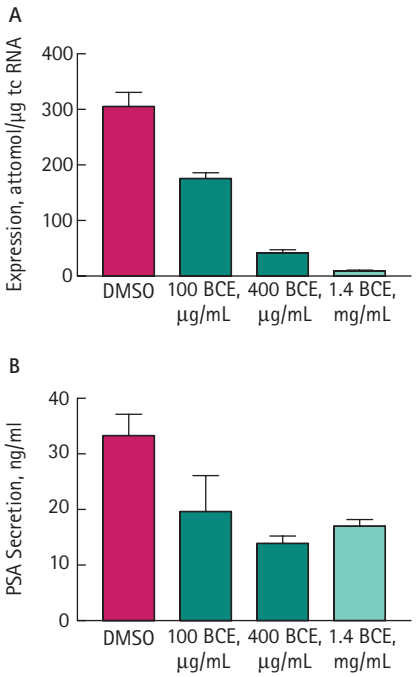


FIG. 5. PSA expression in LNCaP cells treated with various concentrations of BCE: (A) mRNA expression by real-time RT-PCR; (B) analyses of PSA secreted into conditioned cell culture medium.



To evaluate the effect of BCE on the viability of prostate cancer cells we used the Alamar Blue assay on LNCaP cells. In a dosage-dependent manner tumour cell viability was decreased significantly by BCE (Fig. 1). By contrast, androgen-independent PC-3 cells showed no marked reduction of cell viability after BCE treatment. In these cells the maximum decrease of cell viability was 20% at 1400 μg/mL of BCE, showing the marked effect of BCE on tumour cell survival in androgen-sensitive prostate cancer, where it might be accompanied by changes in the expression of genes involved in cell proliferation. Therefore, we investigated the expression of genes highly relevant in prostate cancer cell proliferation.

When androgen-responsive LNCaP cells were treated with increasing doses of BCE the AR mRNA expression decreased accordingly (Fig. 2A). Western blot analyses showed that this reduction of AR expression in response to increasing concentrations of BCE proceeded to the protein level (Fig. 2B). By contrast with this, the expression of the reference genes porphobilinogen deaminase (data not shown) and α-tubulin remain unaffected.

Both the androgen-dependent transcription factors PDEF and NKX3.1 were down-regulated by BCE phytochemicals. The mRNA expression of the AR co-activator PDEF was only reduced by medium and higher doses of the extract (Fig. 3), whereas NKX3.1 expression showed a strong concentration dependence and its expression was completely abolished by higher doses of BCE (Fig. 4). There was also a decrease of PDEF expression depending on BCE dose in PC-3 cells (data not shown).

As the AR and its co-activators enhance PSA expression, we investigated PSA expression in LNCaP cells. PSA mRNA expression was markedly reduced by BCE treatments (Fig. 5A) and almost eliminated by the highest dose of 1.4 mg/mL. Also, PSA secretion from LNCaP cells was reduced during the 24 h treatment with BCE (Fig. 5B). The greatest reduction in PSA secretion was at 400 μg/mL of BCE, whereas, 1400 μg/mL allowed more PSA release, possibly due to unspecific leakage from cells, the viability of which was affected by higher BCE concentrations (Fig. 1).

As many of the compounds in the BCE qualify as specific ligands for ERs we also tested its influence on the expression of the ER-β, the only ER expressed in LNCaP cells. As shown in Fig. 6, doses of >100 μg/mL markedly upregulated the mRNA expression of ER-β. Although PC-3 cells express ER-β we detected no changes in expression for this gene in response to BCE treatments (data not shown).

DISCUSSION

We investigated the beneficial effects the BCE had on prostate cancer cells; the BCE comprises 13 compounds with varying

individual effects on prostate cancer growth [13]. To supplement the previous study, in which we showed the benefit of BCE in a s.c. murine model of human prostate cancer, we tested the crude extract *in vitro* for two reasons: first, the crude BCE is more readily available than the isolated constituents; and second, we hypothesized that synergistic effects from other compounds might add to the action of tectorigenin, a main constituent of the crude extract. Consequently, we examined the changes in expression induced by the BCE in LNCaP and PC-3 prostate cancer cells *in vitro*.

In a concentration-dependent manner BCE reduced tumour cell proliferation, as indicated by diminished cell viability with increasing concentrations of BCE. The regulation of survival and proliferation of prostate cancer cells strongly depends on the AR. Treatments with the BCE resulted in a marked down-regulation of AR expression and consequently down-regulation of PSA expression and secretion. The expression of the AR target gene PSA remains persistently high in hormone-refractory prostate cancer, and the outstanding characteristic of androgen-independent prostate cancer is AR overexpression [6,14]. Thus, these main features of prostate carcinogenesis and androgen independence can be reset by phytoestrogenic treatments of cancer cells. Both tumour cell proliferation and AR expression can be affected more by the present BCE than the pure isoflavone tectorigenin alone [13]. These findings suggest a potential synergistic effect from the combination of phytochemicals in BCE.

In addition to down-regulated AR expression, BCE caused lower expression of the AR-related transcription factors PDEF and NKX3.1. Whereas reduced expression of androgen co-activator PDEF supports the argument in favour of resetting overactive androgen signalling by these phytoestrogens, the rationale behind the down-regulation of NKX3.1 remains elusive. It is also confusing that the expression of NKX3.1, a putative tumour-suppressor gene antagonistic to PDEF and frequently deleted in prostate cancer, is up-regulated by androgens [8,10,15]. However, the present findings of a down-regulation of NKX3.1 expression by phytoestrogens supports previous results by Xing *et al.* [16], who reported such a result from the phytoestrogen quercetin on LNCaP cells. NKX3.1 was recently characterized as maintaining the integrity of the prostatic epithelium by regulating the expression of genes that provide protection against oxidative damage [17]. Further research is warranted to clarify the function of NKX3.1 in prostate cancers not affected by NKX3.1 deletion, such as those represented by LNCaP cells.

As ER-β is the preferred receptor of phytoestrogens [18] it might be crucial in the present effects of BCE. ER-β is the sole ER expressed in LNCaP cells, and is regarded as a tumour-suppressor gene regulating proliferation and apoptosis in prostate carcinoma [19,20]. Bektic *et al.* [21] showed that the down-regulation of AR expression by the phytoestrogen genistein was abolished by ICI 164 384-induced inhibition of ER-β. In the present study BCE extract up-regulated the expression of ER-β, and increased regulatory signalling by this receptor might initiate the resetting of aberrant expression for crucial genes in prostate cancer as reported here. However, further experiments are needed to investigate the underlying correlation between steroid receptor signalling. Notably, BCE did not change the expression of ER-β in PC-3 cells. Furthermore, findings from experiments with ER-β-negative PC-3 suggest that the beneficial effects on androgen-sensitive LNCaP cells is by down-regulation of the AR. Cells devoid of this important feature of androgen-independent prostate cancer respond to a much lesser extent to BCE treatment. Further experiments are warranted to investigate the long-term effect of this compound in androgen-independent cells, which have a highly active PI3K/Akt signalling pathway.

In summary, the phytoestrogenic BCE has beneficial effects on prostate cancer and rectifies the expression of key elements in hormone-refractory prostate cancer, affecting tumour cell viability and proliferation. Being synthesized by secondary plant metabolism, they are readily available as crude plant extracts at low cost, yet might be very effective in prostate cancer prevention and treatment.

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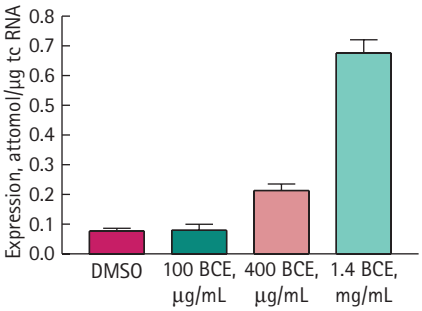
FIG. 6. mRNA expression of ER-β in LNCaP cells treated with various concentrations of BCE, measured by real-time RT-PCR.



CONFLICT OF INTEREST

None declared.

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Abbreviations: **BCE**, *Belamcanda chinensis* extract; **RT**, reverse transcription; **AR**, androgen receptor; **ER**, oestrogen receptor; **PDEF**, prostate-derived Ets transcription factor.