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Characterizing components of the Saw Palmetto Berry Extract (SPBE) on prostate cancer cell growth and traction

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ABSTRACT

Saw Palmetto Berry Extract (SPBE) is applied for prostate health and treatment of urinary tract infections, nonbacterial prostitis and Benign Prostatic Hyperplasia (BPH) in man. An assumption is that SPBE affects tumor cell progression and migration in breast and prostate tissue. In this work, DU-145 cells were used to demonstrate that SPBE and its sterol components, β-sitosterol and stigmasterol, inhibit prostate cancer growth by increasing p53 protein expression and also inhibit carcinoma development by decreasing p21 and p27 protein expression. In the presence of cholesterol, these features are not only reversed but increased significantly. The results show for the first time the potential of SPBE, β-sitosterol and stigmasterol as potential anti-tumor agents.

Since the protein p53 is also regarded as nuclear matrix protein facilitating actin cytoskeletal binding, 2D tractions were measured. The cell adhesion strength in the presence of SPBE, β-sitosterol and cholesterol and the observation was that the increase in p53 expression triggered an increase in the intracellular force generation. The results suggest a dual function of p53 in cells.

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Prostate cancer is the most common male cancer in Western societies, but is significantly less common in Asia. One of the primary differences between these societies is diet, specifically the amount of vegetables compared to the amount of animal lipids consumed. Western diets are high in cholesterol but low in lipids derived from plants, while the opposite is true of the typical Asian diet. The significance of this dietetic difference and the relationship to prostate (and breast) cancer has been studied extensively [1–7].

Lipids derived from plants (*phytosterols*) inhibit the growth and migration of prostate cancer cells and slow down the growth of prostate tumors in mice [8]. Conversely, mice fed a diet supplemented with cholesterol had larger tumors and twice the amount of metastasis in lung and lymph nodes than those receiving the plant lipid diet [8,9]. To date, have been no reports available in the literature of reliable studies investigating the effect of phytosterols on growth and metastasis formation of human prostate cancer [1–3].

Saw Palmetto Berry Extract (SPBE) is a mixture of fatty acids, alcohols and sterols [10–12]. The major constituents of SPBE are free fatty acids, which make up about 90% of the extract and a moderate amount of phytosterols, which include β-sitosterol, stigmasterol and cholesterol.

In this work, human prostate cancer cells DU-145 were exposed to these sterol components (i) to determine their effectiveness on cell proliferation; (ii) to establish their mechanism of action by Western blot analysis, clarifying key cell-cycle-regulating proteins, p53, p27 and p21; and also (iii) to measure their effect on the 2D traction generation on polyacrylamide gel matrices coated with collagen.

Materials and methods

Preparation of SPBE. The stock solution of SPBE was prepared by incubating the Saw Palmetto Berry Powder (Madis Botanical, Inc., New Jersey) in ethanol (20% solution, wt/vol) with constant agitation overnight at room temperature. The whole extract was filtered through a 3-μm filter and finally centrifuged at 2000g to remove herbal debris. The ethanolic extract of Saw Palmetto was concentrated by passing a stream of nitrogen over it and stored at room temperature in the dark [12].

(Phyto)sterol preparation. A 10-ml sterol stock solution of 1 mM concentration was prepared by dissolving 4.15 mg β-sitosterol, 4.13 mg stigmasterol and 3.87 mg cholesterol obtained from Sigma–Aldrich Chemie GmbH, Munich in 98 vol % ethanol. Thereafter,

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the solutions were sonicated for 10 s for complete dissolution and stored in a sealed tube at 4 °C. Aliquots of these solutions were used without further treatment.

Cell culture. The human prostate cancer cell line, DU-145 was obtained from ATCC (Rockville, MD, USA). The cells were grown in media as recommended by the supplier, in DMEM supplemented with 10% serum and 1% penicillin/streptomycin. All cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Viability assay—Calcein-AM. Cells were seeded at a density of 10,000 cells/well in 96-well plates and allowed to attach overnight. To each well, 199 μl of media supplemented with 1 μl SPBE, β-sitosterol, stigmasterol and cholesterol was added. After 24 h, the medium was replaced by 200 μl Calcein-AM/PBS and incubated for 30 min. The amount of living cells was determined fluorescently.

Western blotting. Cells were seeded at a density of 100,000 cells/well in 6-well plates and allowed to attach overnight. To each well, 1990 μl of media supplemented with 10 μl SPBE, β-sitosterol, stigmasterol and cholesterol was added. After 24 h, the cells were washed in PBS and lyzed with RIPA-buffer (Boston Bioproducts, Inc., Worcester, MA) for 5 min on ice. The lysates were centrifuged for 10 min. Aliquots of 20 μl from each sample were used to quantify the protein amount using a Bradford assay. Cell lysates were denatured in Lämmli sample buffer (cf. *Nature, 1970, 227:680*) and 10–20 μg of protein were analyzed by 10% SDS–PAGE. Proteins were transferred electrophoretically onto sheets of Immobilon-P membrane and then visualized by ECL (Boston Bioproducts, Inc., Worcester, MA). Transferred proteins were made visible with Coomassie blue and identified with monoclonal antibodies each against proteins p21, p27 and p53 (BD Biosciences, San Jose, CA). Their intensity was determined densitometrically using the program ImageJ, available from http://rsb.info.nih.gov/ij/download.html.

2D-Traction microscopy. Gels of 6.1% acrylamide and 0.24% bis-acrylamide were prepared on rectangular 75 × 25 mm non-electrostatic silane-coated glass slides according to the procedure described by Pelham and Wang [13]. The Young's modulus of the gels was measured by a magnetically driven plate rheometer and found to be about 12.8 kPa. Red fluorescent, 1 μm carboxylated beads (Molecular Probes, Carlsbad, CA) were suspended in the gels and centrifuged at 300*g* toward the gel surface during polymerization at 4 °C. These beads served as marker for gel deformations. The surface of the gel was activated with Sulfo-SANPAH (Pierce Biotechnology, Rockford, IL) and coated with 50 μg/ml bovine collagen G (Biochrom, Berlin). About 10,000 cells were added to the gel contained within a silicone ring (flexi-perm, *In Vitro*, Göttingen) attached to the glass slide. Cell tractions were computed from an unconstrained deconvolution of the gel surface displacement field measured before and after cell detachment with 80 μM cytochalasin D and trypsin/EDTA (0.25/0.02%) in PBS [14]. During the measurements, the cells were maintained at 37 °C in a humidified atmosphere. Gel deformations were estimated using a Fourier-based difference-with-interpolation image analysis.

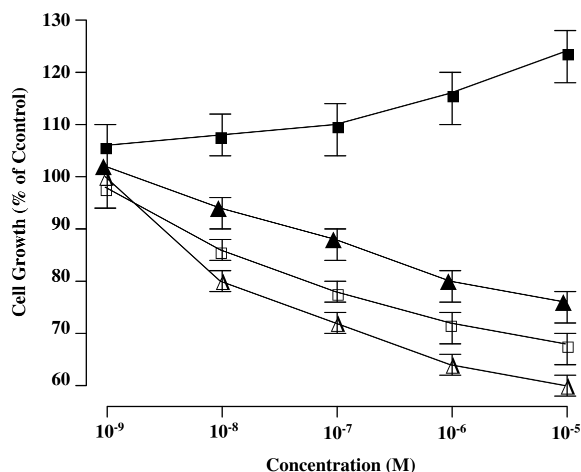

Fig. 1. About 10,000 DU-145 cells were seeded per well in 96-well plates in 200 μl medium (=DMEM supplemented with 10% FCS and 1% penicillin/streptomycin) left overnight and then washed with PBS. Thereafter, the cells were treated for 24 h with SPBE, β-sitosterol, stigmasterol or cholesterol dissolved in 1 μl ethanol added to 199 μl medium before counting. The plot shows the cell growth in the presence of SPBE (Δ), β-sitosterol (□), stigmasterol (▲), and cholesterol (■) at various concentrations. DU-145 cells treated with only 1 μl ethanol were used as control and set at ~100%. The number of experiments were $n = 3$.

Results and discussion

Cell proliferation and viability assay

About 10,000 DU-145 cells were seeded per well in 96-well plates. The cells were left overnight to adhere, briefly washed with PBS and then treated with various substances for 24 h. Each well contained 199 μl of medium supplemented with 1 μl pure ethanol (control) or 1 μl ethanol containing SPBE, β-sitosterol, stigmasterol or cholesterol. After 24 h, the medium was replaced by 200 μl Calcein-AM/PBS and incubated for 30 min. The amount of living cells was then determined fluorescently. Fig. 1 shows a plot of DU-145 cell growth, i.e. the percentage change to control cells (=100%) in

Protein	Control	SPBE	Beta-Sitosterol	Stigmasterol	Cholesterol
p53					
	7 +/-3	29 +/-5	61 +/-8	22 +/-3	31 +/-6
p27					
	43 +/-5	19 +/-2	24 +/-4	32 +/-4	60 +/-5
p21					
	22 +/-4	12 +/-3	12 +/-2	11 +/-2	20 +/-4
Actin					
	57 +/-2	61 +/-5	67 +/-8	58 +/-7	61 +/-4

Fig. 2. The effect of 5 μM SPBE, β-sitosterol, stigmasterol and cholesterol dissolved in 10 μl ethanol or (10 μl ethanol only as control) on protein, p53, p27 and p21 expression of DU-145 cells after 24 h of adhesion in 1990 μl medium (=DMEM supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin). Note, that cells were seeded, left overnight, washed in PBS and then treated for 24 h. Western blot analysis was performed with monoclonal antibodies against p53, p27, p21 and β-actin. Actin was used as loading control. Number of experiments were $n = 3$. The Western blots were analyzed, and the numbers were determined by ImageJ.

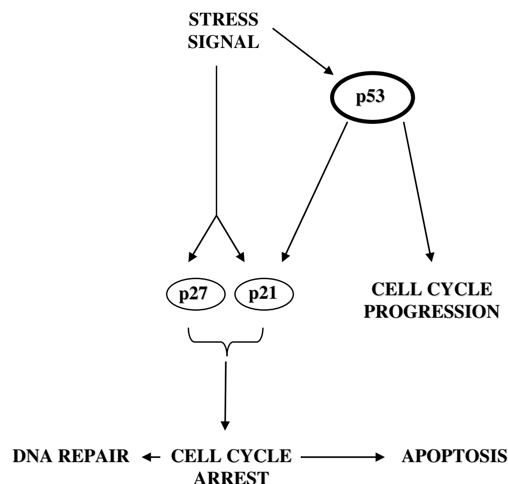

Fig. 3. A schematic representation of a putative molecular mechanism of action in the cell [22].

the presence of different substances and at various concentrations. With increasing concentration, we observed a reduction in cell growth compared to control cells in the following order of substance effectiveness: SPBE > β-sitosterol > stigmasterol, and an increase in cell growth with rising cholesterol concentration.

Western blot analysis

Analyzing the treated DU-145 cells by Western blot analysis, we observed that the expression of the *cell-cycle-regulated repressor protein*, p53 [15] (i.e. guardian of the genome [16]) was notably increased by all substances compared to control (Fig. 2). This has implications on whether cells enter cell-cycle progression or cell-cycle arrest *via* the protein, p21 (Fig. 3). Assuming that the increase of the p53 level is due to treatment with phytosterols, this would affect p21 activity, i.e. cell-cycle arrest, which would allow either DNA repair to proceed before mitosis or, if the damage is severe, could result in apoptosis. Analyzing the expression level of p21 and p27, i.e. two *cyclin-dependent kinase inhibitor proteins*, in DU-145 cells show that SPBE, β-sitosterol and stigmasterol all decrease the expression of these inhibitors compared to control (Fig. 2). Cholesterol, however, showed an increasing effect on p27 expression and similar p21 expression compared to control, with expression levels higher than for SPBE, β-sitosterol and stigmasterol-treated DU-145 cells. Comparing results from viability assays in Fig. 1 and Western blot analysis in Fig. 2 show similar trends in that SPBE has a stronger influence on cell growth and p27 expression compared to control than β-sitosterol and stigmasterol. p21 expression was similar for SPBE, β-sitosterol and stigmasterol-treated cells but lower than the control cells. Therefore, since p21 and p27 expression (*cyclin-dependent kinase inhibitor proteins*) are down-regulated by SPBE, β-sitosterol and stigmasterol compared to control, more cells are arrested and more cells must be apoptotic as observed in cell growth studies (Fig. 1). Cholesterol-treated cells showed an increase in cell growth, which might be explained by the higher p53 and p27, but normal p21 expression compared to control and a pathway that follows cell-cycle arrest and DNA repair rather than cell-cycle progression. To verify this interpretation, further molecular studies need to be done.

2D-Traction microscopy

The motivation for these measurements came from observations that DU-145 cells when treated with phytosterols showed 3- to 9-fold higher p53 expression than control cells (Fig. 2) and from recent reports that p53 also dynamically interacts with the 3-dimensional filamentous protein network referred to as nuclear matrix that is mediated by F-actin [17,18]. If the actin cytoskeleton connects with focal adhesions and the nuclear matrix in cells, then p53 should have a mechanical function. To test this hypothesis, we seeded about 10,000 DU-145 cells on collagen-coated polyacrylamide gels in the presence of phytosterols. The mean elastic strain energy stored in the polyacrylamide gel due to the cell tractions was then calculated according to Butler et al. [14] as the product of local tractions and deformations, integrated over the spreading area of the cells. The contractility of DU-145 cells was substantial (Fig. 4A and B), with strain energies in the pico-Joule range, similar to those reported for other invasive tumor cells [19] and smooth muscle cells [20]. We conclude that the treatment of phytosterols on DU-145 cells leads to higher p53 expression and through its binding to F-actin to higher contractile (adhesive) forces [19,21]. This assumption is supported by the observations that, after phytosterol treatment, cell migratory events on 2D substrates are suppressed which is probably due to increased cell adhesive forces [8].

Further work needs to be done with other prostate or breast cancer cell lines known to respond to phytosterols to verify the molecular and mechanical mechanism of p53 in greater detail. Elucidating the relation of reduced cell growth, increased p53 expression and 2D tractions after phytosterol treatment specifically might be helpful in understanding tumor cell tissue invasion.

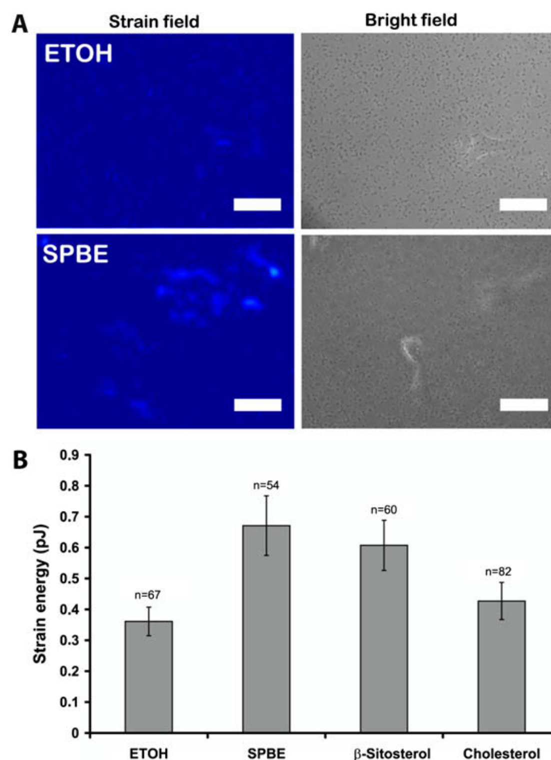

Fig. 4. (A) Strain field and bright field images of DU-145 cells treated with ETOH and SPBE. The bars equal 50 μm. (B) Plot of the mean elastic strain energy in pico-Joule stored in the extracellular matrix due to cell tractions. The cells were treated with 10 μl ethanol (control) or 5 μM SPBE, beta-sitosterol or cholesterol dissolved in 10 μl ethanol and 1990 μl medium (=DMEM supplemented with 10% FCS and 1% penicillin/streptomycin) for 24 h before measurements. Note, that '*n*' indicates the number of cells measured, and ethanol-treated (10 μl) control (ETOH) and untreated (10 μl PBS, data not shown) cells displayed similar tractions.

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