The Effect of a Novel Botanical Agent TBS-101 on Invasive Prostate Cancer in Animal Models

SUSAN EVANS[1], NISHTMAN DIZEYI[1], PER-ANDERS ABRAHAMSSON[1] and JENNY PERSSON[2]

[1]*Department of Clinical Sciences,* [2]*Division of Experimental Cancer Research, Department of Laboratory Medicine, Clinical Research Center, Lund University, University Hospital, 205 02, Malmö, Sweden*

Abstract. *Background: Traditional Botanical Supplement-101 (TBS-101) is a newly developed proprietary botanical agent containing seven standardized botanical extracts, including: Panax ginseng, cranberry, green tea, grape skin, grape seed, Ganoderma lucidum and chamomile. Each of the components has been consumed either in the regular diet or as natural supplement. When used as a single agent, each of these seven botanicals has been implicated in chemoprevention and therapy in various types of cancer. The anticancer effect of TBS-101, with the specific combination of these anti-cancer botanicals for the treatment of prostate cancer (PCa), has not been tested. Materials and Methods: The IC_{50} and the effect of TBS-101 on the proliferation and apoptosis of PC-3 cells were determined. Tumor xenograft mice were generated by subcutaneously implanting PC-3 cells into mice and tumors were allowed to grow to different sizes before starting the treatment. The effects of TBS-101 on tumor growth were assessed by measuring tumor size and by histological, pathological and immunohistochemical analyses. A basic toxicity study was performed to test the tolerance of the mice to high doses of TBS-101. Results: Treatment of the PC-3 cells with TBS-101 resulted in a dose-dependent inhibition of cell growth, with an IC_{50} of 1.4 μg/ml. A concomitant induction of apoptosis in PC-3 cells treated with TBS-101 was also observed. Upon the treatment with TBS-101, all three groups of mice bearing moderate or large tumors showed significant inhibition of tumor growth and invasion. In contrast, control mice treated with vehicle alone had significant tumor growth and lymph node metastasis. In the basic toxicity studies, high doses of TBS-101 exerted no toxicity in healthy or tumor-bearing mice.*

Correspondence to: Jenny Persson, Ph.D., Division of Experimental Cancer Research, Department of Laboratory Medicine, CRC, Lund University, University Hospital, 205 02, Malmö, Sweden. Tel: +46 40338391, Fax: +46 40330352, e-mail: jenny_l.persson@med.lu.se

Conclusion: The natural botanical agent TBS-101 has a good safety profile and significant anticancer activities in hormone-refractory PC-3 cells and large aggressive PC-3 tumors in a xenograft mouse model and has great potential for the treatment of aggressive prostate cancer.

Prostate cancer (PCa) is one of the most prevalent carcinomas and a leading cause of cancer related-death worldwide. The incidence of prostate cancer has significantly increased worldwide (1, 2). Despite recent advances in early diagnosis and treatment, PCa remains the second most lethal cancer in men in the Western world (1, 2). Initially, the majority of prostate carcinomas are responsive to androgen ablation therapy, but most of the tumors will eventually progress to the androgen refractory state (3). Once prostate cancer becomes hormone refractory, the cancer cells may rapidly gain the ability to invade and to metastasize to the lymph nodes and distant organs (4). Tumor metastases are the major cause of mortality in cancer patients. Approximately one-third of treated patients will relapse and no curative treatment currently exists for metastatic disease (2, 3, 5, 6). The progression from hormone-dependent to hormone-refractory and metastatic prostate cancer is poorly understood. Given the high prevalence of the disease, the aging of the population and the lack of effective treatment for cancer metastasis, there is an urgent need to discover and develop novel therapeutic approaches.

Primary PCa is treated with hormonal therapy, which is aimed at suppressing androgen production and blocking the androgen receptor (AR)-mediated proliferation and survival pathways. In advanced PCa, androgen deprivation therapy (ADT) is the mainstay of treatment. Surgical removal of the tumor and the use of hormone agonists or AR antagonists, such as flutamide, are the major types of ADT (7). The current treatment of aggressive cancer benefits from multiple drugs used in combination (8). The combination of multiple drugs that target different pathways may improve the efficacy of the treatment. Docetaxel that targets the mitotic spindle and cell proliferation cycle is being used in combination with avastin, which targets angiogenic pathways for the treatment

of metastatic prostate cancer in clinical trials (8). As a single agent, docetaxel has modest activity, but in combination with avastin, the response rate has been greatly improved. However, the side-effects induced by docetaxel and avastin are very severe, as the individual drug's toxicities are combined to cause multiple adverse effects, in particular myelosuppression and heart failure (9). In addition, the cost of these drugs is enormous. The development of alternative treatments with minimal adverse effect and lower cost would have great clinical and economical benefits.

Overwhelming evidence suggests that some naturally occurring botanicals, such as, ginseng, cranberry, grape, *Ganoderma*, green tea and chamomile may have significant effects against cancer. Many of these botanicals are consumed in our daily diet. Many studies have shown that these natural agents can induce apoptosis and/or cell cycle arrest in a panel of human prostate, breast, lung and colon cancer cell lines and animal models (10-18). Traditional Botanical Supplement-101 (TBS-101) is a proprietary botanical agent consisting of mixed standardized botanical extracts. Each botanical component has been studied and shown to contain natural compounds with proven anticancer (10-16) and anti-inflammatory activity. For example, quercetin, resveratrol, epigallocatechin gallate (EGCG), kaempferol, anthocyanidins, ginsenosides, apigenin and beta-D-glucans are natural compounds found in abundance in these botanicals and which exhibit anticancer and chemopreventive effects (17-26). The anticancer activities of several individual compounds have been tested in many clinical studies and epidemiological studies with therapeutic and chemopreventive indications (10-15, 27). All of these botanicals have a long history of being safely consumed by humans and some have been tested in human clinical trials with minimum adverse effects (28-32), suggesting that these botanicals are safe as potential anticancer therapeutics.

However, the anticancer activity of each individual anticancer compound in TBS-101 is relatively low in comparison to many major pharmaceutical drugs currently being used in cancer treatment (33-37). Since some of these compounds target cell proliferation and apoptosis pathways, whereas others target angiogenesis or inflammatory signals, TBS-101, which contains a combination of these active compounds, may achieve a better effect and higher bioavailability than a single compound for the treatment of PCa. In the present study, the effect of TBS-101 on cell growth, apoptosis, tumor growth and invasion was assessed in hormone-independent PC-3 cells and PC-3 xenograft mice.

Materials and Methods

Materials. TBS-101 is a proprietary blend of *Panax ginseng* (radix), cranberry *(Vaccinium macrocarpon,* fruit), green tea *(Camellia sinensis,* leaf), grape *(Vitis vinifera,* skin and seed), mushroom *(Ganoderma lucidum)* and chamomile *(Matricaria recutita,* flowers) in the form of a mixture of botanical extracts. The preparation was supplied by Titan Biosciences (Mountain View, CA, USA) and dissolved in 100% DMSO diluted to 5% in PBS buffer.

Cell culture and growth inhibition assay. Hormone-independent and metastatic PC-3 prostate cancer cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 supplemented with 10% fetal bovine serum. A total of 1×10^6 cells at a density of 0.3×10^6/ml were seeded in 12-well plates. The cells were treated with TBS-101 at 1 μg/ml, 3 μg/ml, 5 μg/ml or 10 μg/ml for 24 h. The control cells were treated with 5% DMSO in PBS as vehicle alone for 24 h.

Apoptosis assay. A total of 1×10^6 PC-3 cells were seeded in 12-well plates at a density of 0.3×10^6/ml. The cells were treated with TBS-101 at 1 μg/ml, 3 μg/ml, 5 μg/ml or 10 μg/ml for 24 h. The control cells were treated with vehicle for alone 24 h. The cells were labeled with trypan blue and the apoptotic cells were quantified. Microscopic images of cells under each TBS-101 treatment were obtained with a digital holographic microscope.

Xenograft mouse model of tumor growth and metastasis. Athymic Naval Medical Research Institute (NMRI) nude mice, 6-8 weeks old (Taconic; Bomholt, Denmark) were used. The PCa xenograft mouse model was created by implanting hormone-independent PC-3 tumor cells subcutaneously into the right flank of the athymic nude mice as previously described (38). PC-3 cells at 1×10^6/per mouse were used. The mice were divided into three groups (6 mice per group). In the first group, the tumors were allowed to grow to 170-400 mm^3 in size, in the second group to 550-650 mm^3 and in the third group to 700-800 mm^3 before starting the treatment. The tumor diameters were measured twice weekly using calipers. The tumor volumes were calculated using the equation (b)^{2}xa/2 where a and b represent the length and width of the tumor, respectively.

TBS-101 was administered orally once every other day. Tumor growth was assessed periodically. The body weights of the mice on the first and last day of the treatment were recorded and compared. The mice were sacrificed after treatment by euthanasia *via* isofluorene inhalation. The lymph nodes, liver, lung, spleen and femurs were removed from each mouse. Half of the tumor tissues were used for histological and immunohisto-chemical analysis. For the histological analysis, the tissues were fixed in 4% paraformaldehyde and embedded in paraffin. The sections were stained with hematoxylin-eosin (H&E) and analyzed under an optical microscope. The other half of the tissues were used for protein analysis and were snap-frozen in liquid nitrogen. Tumor invasion and metastasis were examined in various tissues from the sacrificed mice.

For comparison, mice with tumors of approximately 300-400 mm^3 in size were treated with the cytotoxic drug etoposide (Sigma, St. Louis, MO, USA) at a dose of 20 mg/kg *via i.v.* injection once every other day. The tumor size was measured periodically. Three mice were treated with etoposide or the vehicle alone as control. The mice were sacrificed and the tissues were collected as described above.

Basic toxicity study. For the assessment of toxicity of TBS-101, healthy mice were treated with TBS-101 by oral gavage at a dose of 200 mg/kg once every other day for 13 days. The body weight of each mouse was measured periodically from Day 1 to Day 13 of the treatment. Cage-side observations including mortality, moribundity, food consumption and activities were performed once every other day.

Blood samples were taken on Day 1 and Day 13 for the measurement of blood cell counts. To assess the toxicity of TBS-101 on the mice with tumors, mice bearing subcutaneous PC-3 tumors were treated with TBS-101 at the highest dose that the mouse was able to tolerate: 1,000 mg/kg once every other day for 19 days. The body weight was measured and cage-side observations were performed once every other day. The toxicity of TBS-101 was also assessed in tumor-bearing mice treated with 80 mg/kg of TBS-101 daily.

Immunohistochemistry and quantification of angiogenesis. Immunohistochemistry on the tumor tissues was performed as previously described (39) by using antibodies to CD31, a protein expressed in tumor vasculature and important for tumor invasion, (Dako, Golstrup, Denmark). Anti-rabbit peroxidase-conjugated secondary antibodies were applied. Diaminobenzidine colorimetric reagent solution (Dako) was used. The slides were counterstained with hematoxylin (Sigma). The specimens were viewed with an Olympus BX51 microscope at a magnification of ×20 or ×40. For the analysis of tumor angiogenesis, the tumor sections stained with antibody against CD31 were examined and quantified. Regions of high vascular density within the tumors were examined. The number of CD31-positive pixels per microscopic field was recorded. At least two sections per tumor and three views per section were determined. The slides were scanned using a MIRAX scanner and the microphotographs were taken using the MIRAX Viewer program at ×20 magnification.

Results

Proliferation of prostate cancer cells. As shown in Figure 1A, treatment of the PC-3 cells with increasing concentrations of TBS-101 induced dose-dependant inhibition of cell growth. Treatment with 1 μg/ml of TBS-101 readily resulted in a significant reduction in cell numbers to approximately 45% of that in the control and with 10 μg/ml almost completely eliminated viable PC-3 cells after 24 h. The IC_{50} for growth inhibition was calculated to be 1.4 μg/ml based on the dose-response curve. The dose-dependant inhibition of proliferation was also measured by BrdU labeling and a similar result was obtained (data not shown).

Apoptosis of prostate cancer cells. The rate of apoptosis was 20% in the PC-3 cells treated with 1 μg/ml of TBS-101, increased to approximately 60% with 3 μg/ml of TBS-101, more than 80% with 5 μg/ml of TBS-101 and finally almost 100% in the cells treated with 10 μg/ml of TBS-101 (Figure 1B). The concentration required for 50% apoptosis was calculated to be 2.4 μg/ml based on the dose-response curve. The result clearly showed that TBS-101 significantly induced a dose-dependent increase in apoptosis in the PC-3 cells.

Safety in basic toxicity study. As shown in Figure 2A, there was no significant change in the body weight of the healthy mice throughout the 13-day treatment period with a high dose of TBS-101. The cage-side observations showed no abnormality of food consumption and daily activities, nor
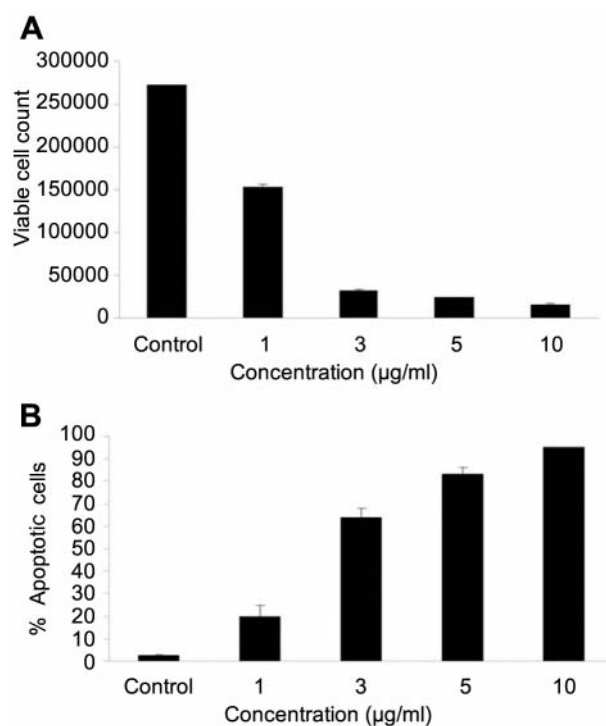

Figure 1. *Effect of TBS-101 on proliferation and apoptosis in PC-3 cells. A, The number of viable cells after 24 h treatment. B, The proportion of apoptotic cells after 24 h treatment. Values are the mean±standard deviation (SD) of three parallel experiments.*

any observable toxic effect for the 13 days. No abnormality in the histology of the liver and kidneys was detected (data not shown). These basic toxicity tests showed that TBS-101 was safe at a high dose in healthy mice. As shown in Figure 2B, the body weights of the tumor-bearing mice were steady throughout the study period. The cage-side observations showed no abnormality of food consumption and daily activities, or any observable toxic effect.

The dose of 80 mg/kg which generated significant antitumor activity in the xenograft mouse model was also tested. This dose is similar to the proposed prophylactic dose for human consumption, thus the daily treatment of tumor-bearing mice with this dose may suggest the safety of TBS-101 for human treatment. The body weights of the tumor-bearing mice treated with TBS-101 remained constant throughout the period of treatment (data not shown). The cage-side observations showed no abnormality of food consumption and/or daily activities, or any other observable toxic effect.

Tumor growth in xenograft animal model. When the tumors had grown to 170-400 mm³ in size, the mice, in group one, were treated orally with TBS-101 at 80 mg/kg or the vehicle alone as control for 29 days. The tumors in the control group
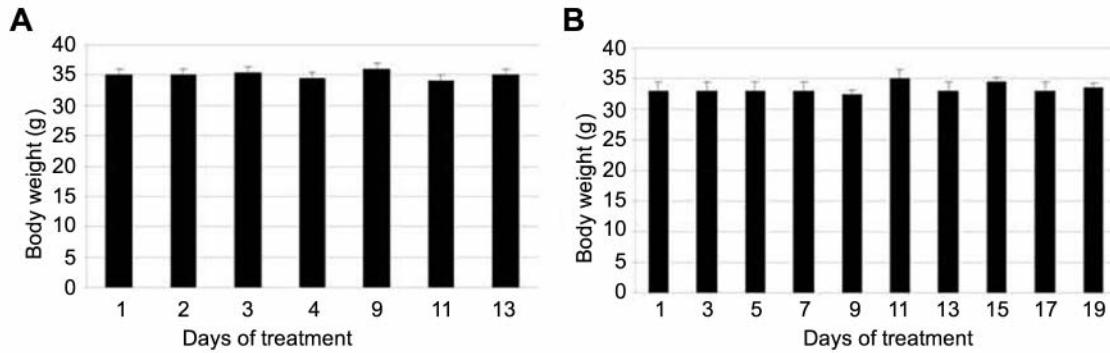
Figure 2. *Tolerance of healthy and tumor-bearing mice to TBS-101 treatment at various doses. A, Healthy mice were treated with 200 mg/kg of TBS-101 once every other day for 13 days and weighed periodically from Day 1 to Day 13. B, Tumor-bearing mice were treated with 1,000 mg/kg of TBS-101 once every other day for 19 days and weighed every other day. Values are the mean±standard deviation (SD).*
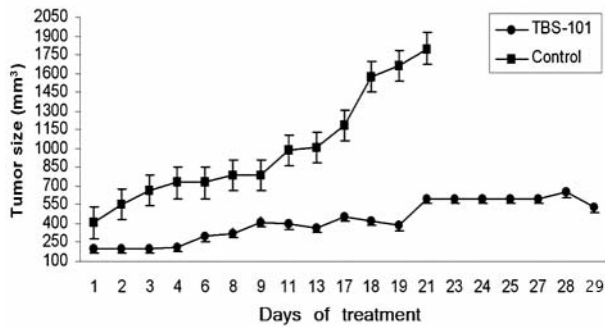

Figure 3. *Effect of TBS-101 on tumor growth in xenograft mice bearing PC-3 tumors of regular size. Mice bearing 170-400 mm³ tumors were treated with 80 mg/kg of TBS-101 or solvent alone (control) orally once every other day for 29 days and the tumor size was measured periodically. On Day 21, the control mice had to be sacrificed due to the tumor size exceeding the allowable tumor burden limit set by the animal facility. Values are the mean±standard deviation (SD).*

Figure 4. *Effect of TBS-101 on tumor growth in the xenograft animal model with large-sized tumors. When implanted PC-3 tumors had grown to 550-650 mm³ in size, the mice were treated with 80 mg/kg of TBS-101 or solvent alone (control) orally for 13 days and the tumor size was measured periodically. Values are the mean±standard deviation (SD).*

grew exponentially to a large size and after 21 days the animals had to be sacrificed for humanitarian reasons, while tumor growth in the mice treated with TBS-101 was significantly lower (Figure 3). This suggested a significant inhibitory effect of TBS-101 on tumor growth in xenograft mice *in vivo*.

Tumor growth and invasion in xenograft mice with large and invasive tumors. We previously found that PC-3 tumors grown to 400 mm³ in size could become metastatic and invade into the lymph nodes, liver or lung (unpublished observations). Therefore, some tumor transplants were allowed to grow to approximately 550-650 mm³ in size before starting to treat the mice with TBS-101 or the solvent alone as control for 13 days. While the large tumors in the control group continued to grow rapidly, the tumors in the TBS-101 treated mice did not grow, but showed a slight
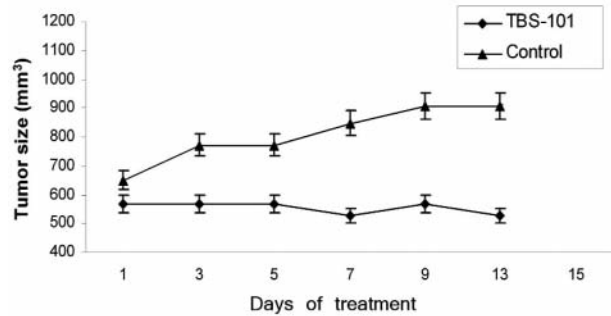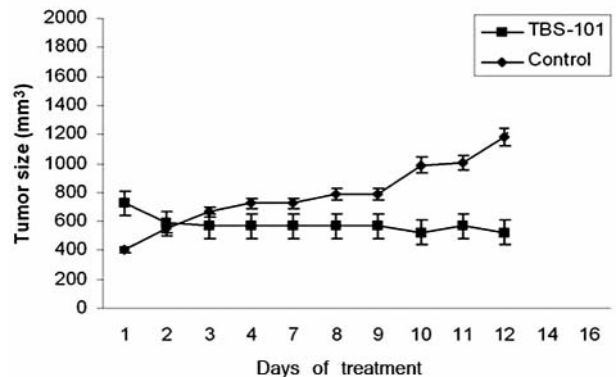

Figure 5. *Effect of TBS-101 on tumor growth in the xenograft animal model with very large sized tumors. When the PC-3 implanted tumors had grown to 700-800 mm³, the mice were treated with 80 mg/kg of TBS-101 or solvent alone (control) orally for 12 days and the tumor size was measured periodically. Values are the mean±standard deviation (SD).*

decrease in size at the end of the treatment (Figure 4). Histological and immunohistochemical analysis did not detect any infiltration of tumor cells into secondary sites in any of these mice at the end of the study. In contrast, the tumors in the control mice were found to have metastasized into the lymph nodes (data not shown).

When the effect of TBS-101 on very large and invasive tumors (700-800 mm^3) was tested, the tumor growth in the TBS-101 treated mice was inhibited and the tumors shrank slightly during the treatment, while the tumors in the control group grew exponentially and became metastasized (Figure 5).

Side-by-side comparison of the histology sections containing the whole tumors from the control and TBS-101 treated mice clearly revealed differences in tumor size and morphology between the two groups (Figure 6). The tumors from the mice treated with TBS-101 appeared to be much smaller than those from the control mice. Interestingly, there were large empty areas in the tumors treated with TBS-101, suggesting ongoing tumor reduction may be associated with the clearance of dead tumor cells. These results further confirmed that TBS-101 is capable of inhibiting growth and inducing apoptosis in large and invasive tumors.

Effect of TBS-101 on vascularization of tumors in xenograft mice. The extent of tumor vascularization was determined by quantifying the CD31-positive vessels in the tumors, including, the edge and the center areas. There was a statistically significant decrease in CD31 expression and the number of CD31-expressing vessels in the tumors treated with TBS-101 as compared with that of the control tumors (Figure 7).

Effect of etoposide on tumor growth in xenograft mice. As shown in Figure 8, the tumors in the control group grew exponentially to a large size (approximately 300 mm^3) after 18 days and the mice had to be sacrificed for humanitarian reasons. In contrast, the tumors in the etoposide treatment group grew slowly and the size increased to approximately 650 mm^3 by Day 34.

Discussion

Each of the botanical compounds in TBS-101 has been shown to have anticancer and/or chemopreventive effects on PCa in animal models or cancer patients (11, 12, 40, 41). However, no individual compound has been shown to have a significant inhibitory effect on aggressive hormone-refractory PCa. In the present study, TBS-101 had a potent inhibitory activity on the growth and invasion of hormone-refractory and aggressive human prostate tumors in a xenograft mouse model. The significant anticancer activity of TBS-101 may have been due to the multiple active compounds acting synergistically or additively to target the multiple cellular pathways that control tumor growth,

invasion and metastasis. Thus, this work is among the first to demonstrate that specific combinations of multiple active natural anticancer compounds may be effective for the treatment of PCa.

The PC-3 cell line was used because it has several properties that represent hormone-independent and invasive PCa. PC-3 cells lack functional AR signaling, grow rapidly in culture medium and can form large and very aggressive tumors when implanted into nude mice. Even the widely used cancer drugs such as Avastin or docetaxal alone or in combination had low inhibitory effect on PC-3 cells (42). In the present study, treatment of the PC-3 cells with TBS-101 resulted in a dose-dependent inhibition of cell proliferation and dose-dependent increase in apoptosis, with the IC$_{50}$ at 1.4 μg/ml and 2.4 μg/ml, respectively. This suggested that the effect of TBS-101 on PC-3 cells was strong.

To mimic the gradual dissemination of PCa cells *in vivo*, the PC-3 xenograft mouse model was used. No lymph node metastasis was observed in the mice treated with TBS-101. However, all the mice treated with vehicle alone had metastasis to the lymph nodes. Despite the large size and the aggressive and metastatic properties of PC-3 tumors, the TBS-101 treatment was very effective with a pronounced effect on tumor growth and invasion suggesting that TBS-101 may target multiple cellular pathways that are associated with growth, angiogenesis and metastasis. More surprisingly, the effect of TBS-101 on the PC-3 xenograft tumors in mice appeared to be similar or better compared to a commonly used chemotherapy drug etoposide, typically used to treat invasive tumors.

One striking observation in this study was that the animals were able to tolerate TBS-101 well even at the highest concentration of 1,000 mg/kg, which mimicked its effect on PCa patients with no observable adverse effects, such as weight loss or other symptoms. A dose of 80 mg/kg showed a significant effect on large and invasive tumors. The potent anticancer activities achieved at low dose coupled with complete safety at high dose suggest that TBS-101 may have a large therapeutic window and may be an effective and safe treatment for PCa.

In the present study, we are among the first who have tested a combination of several active compounds for the purposes of targeting multiple cellular pathways. TBS-101 is a natural botanical agent that contains many active anticancer and anti-inflammatory compounds including: quercetin, resveratrol, EGCG, kaempferol, anthocyanidins, ginsenosides, apigenin and beta-D-glucans. The molecular and cellular mechanisms by which TBS-101 inhibits the growth of invasive and metastatic PCa remain to be investigated. Even so, the mechanisms underlying the actions of some of the individual active compounds in TBS-101 have been extensively studied. For example, green tea contains the polyphenol EGCG that has been shown to induce cell cycle
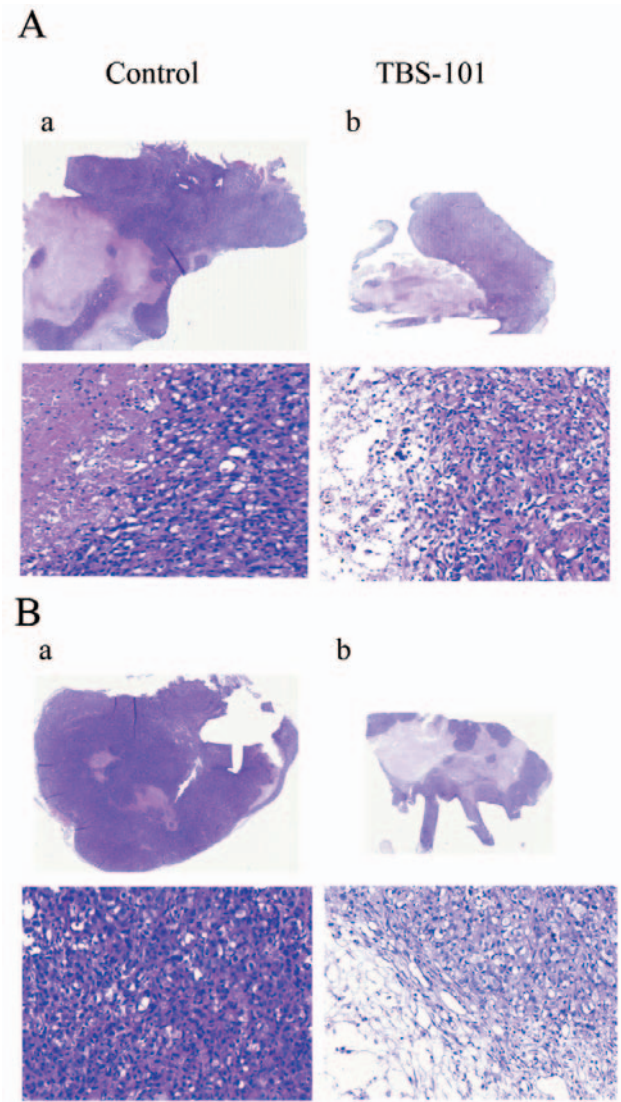
Figure 6. *Histology section of whole tumors from control and TBS-101 treated mice. Appearance and histology of two pairs (A and B) of H&E stained tumors (group 2 mice). Upper panel: section of the whole tumor (×20 magnification). Lower panel: selected tumor areas of the above tumors at higher magnification (×40). Panel a: control, vehicle alone; Panel b: from a mouse treated with TBS-101.*

Figure 7. *Effect of TBS-101 on the vascularization in tumors. A, Tumors from group 2 mice (×40 magnification) were stained with antibody against CD31. B, The number of CD31-positive pixels per microscopic field of regions of high vascular density at the center or the edge of the tumors. At least two sections per tumor and three views per section were determined. *Two-sided t-test p-value.*

arrest and apoptosis in human prostate cancer cells (40, 41). In addition, EGCG has also been shown to inhibit the expression of matrix metalloproteinases as well as to bind to Bcl-2 and vimentin with high affinity (43). Resveratrol, kaempferol, and the bioflavonoid quercetin in grape skin have been shown to inhibit growth and induce apoptosis in prostate cancer cells while exerting no quantifiable effect on normal prostate epithelial cells (17). These compounds, in general, affect the Bax and Akt apoptotic pathways (44, 45). Anthocyanidins from grape seed and cranberry have been
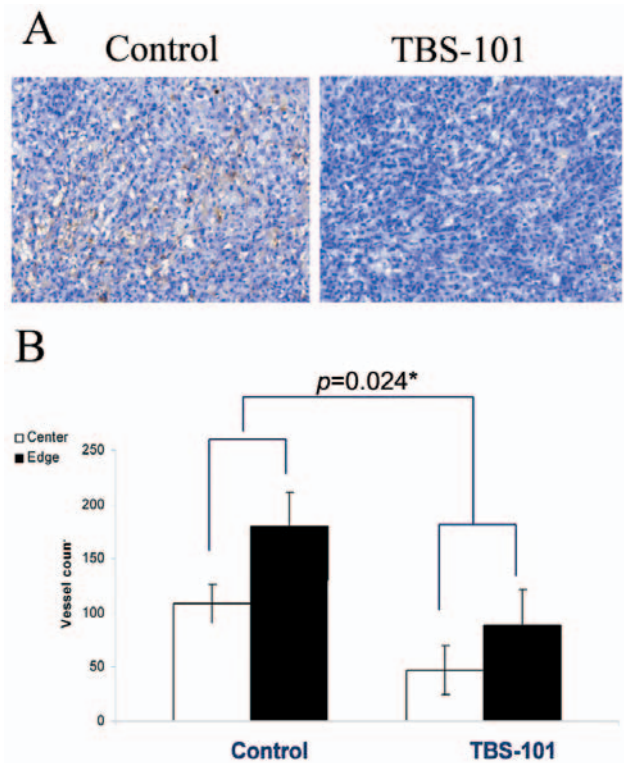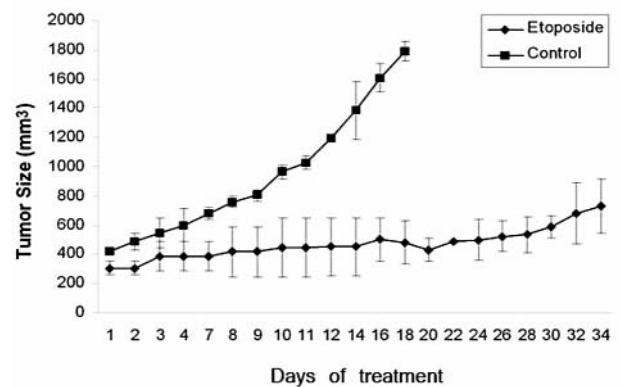

Figure 8. *Effect of etoposide on tumor growth in the xenograft animal model. When the PC-3 implanted tumors had grown to 300-400 mm³ in size, the mice were treated with 20 mg/kg of etoposide or solvent alone (control) i.v. once every other day for 34 days. Tumor size was measured periodically. Values are the mean±standard deviation (SD).*

shown to induce apoptosis through the nuclear factor (NF)-κB signaling pathway in PC-3 cells and animal model studies (22). Ginsenosides in *Panax ginseng* have been studied extensively and found to inhibit proliferation of prostate cancer PC-3 and LNCaP cells, induce cell detachment and modulate the activities of Erk1 and Erk 2 of MAP kinases actions (46, 47). Apigenin is a plant flavone in chamomile that has been tested and found to inhibit cell proliferation and induce apoptosis by inhibiting insulin growth factor (IGF) and its receptor signaling and the PI3K/Akt pathway (48). Lastly, beta-D-glucans are one of the main polysaccharides in *Ganoderma* and has been shown to possess antitumor effects through immunomodulation and anti-angiogenesis (26). The potent effect of TBS-101 on tumor growth and invasion may indicate strong synergistic or additive effects resulting from the optimal combination of these multiple active compounds.

As cancer is a multi-step, multi-pathway and multi-focal process which involves a series of epigenetic and genetic alterations and alterations in cellular pathways, multi-target or combination therapies may provide higher efficacy and a better treatment outcome than single target or single drug therapies, especially for late stage and aggressive carcinomas. The development and validation of effective natural botanical products for cancer chemoprevention and therapy will have a significant impact both medically and economically. Prostate cancer is a good target for developing cancer prevention and treatment as it has a high prevalence and disease-related mortality. In addition, research on PCa mechanism and treatment is far behind as compared to what has been achieved in the field of breast cancer. The development of TBS-101 as a potentially effective, safe and undesirable side-effects-free anticancer treatment is of great interest. More complete evaluation of the effects of TBS-101 and the underlying mechanisms will be performed in the hope of improving the treatment of PCa.

Acknowledgements

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