

## Grape seed proanthocyanidins induce apoptosis and inhibit metastasis of highly metastatic breast carcinoma cells

Sudheer K.Mantena<sup>1</sup>, Manjeshwar S.Baliga<sup>1</sup> and Santosh K.Katiyar<sup>1,2,3,4,\*</sup>

<sup>1</sup>Department of Dermatology, <sup>2</sup>Department of Environmental Health Sciences, <sup>3</sup>Clinical Nutrition Research Center and <sup>4</sup>Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL 35294, USA

\*To whom correspondence should be addressed. Tel: +1 205 975 2608; Fax: +1 205 934 5745; Email: skatiyar@uab.edu

**The strategies available for the treatment of metastatic breast cancer are limited. Dietary botanicals may have a better protective effect on this disease. We therefore investigated the effects of grape seed proanthocyanidins (GSPs) on a highly metastatic mouse mammary carcinoma cell line. *In vitro* treatment of breast cancer cells, 4T1, MCF-7 and MDA-MB-468, with GSPs resulted in significant inhibition of cellular proliferation and viability, and induction of apoptosis in 4T1 cells in a time- and dose-dependent manner. Further analysis indicated an alteration in the ratio of Bax/Bcl-2 proteins in favor of apoptosis, and the knockdown of Bax using Bax siRNA transfection of 4T1 cells resulted in blocking of GSPs-induced apoptosis. Induction of apoptosis was associated with the release of cytochrome *c*, increased expression of Apaf-1 and activation of caspase 3 and poly (ADP-ribose) polymerase. Treatment with the pan-caspase inhibitor (Z-VAD-FMK) resulted in partial but significant inhibition of apoptosis in 4T1 cells suggesting the involvement of both caspase activation-dependent and activation-independent pathways in the apoptosis of 4T1 cells induced by GSPs. The effects of dietary GSPs were then examined using an *in vivo* model in which 4T1 cells were implanted subcutaneously in Balb/c mice. Dietary GSPs (0.2 and 0.5%, w/w) significantly inhibited the growth of the implanted 4T1 tumor cells and increased the ratio of Bax:Bcl-2 proteins, cytochrome *c* release, induction of Apaf-1 and activation of caspase 3 in the tumor microenvironment. Notably, the metastasis of tumor cells to the lungs was inhibited significantly and the survival of the mice enhanced. These data suggest that GSPs possess chemotherapeutic efficacy against breast cancer including inhibition of metastasis.**

### Introduction

Epidemiologic studies indicate that dietary habits play crucial roles in both the induction as well as the prevention of cancer, including breast cancer (1,2). It has been recognized that incidence of breast cancer in Asian women is lower than

the incidence in women in Western countries and that migration of Asian women to the USA increases the risk of and mortality associated with breast cancer (3,4). The Asian diets are typically richer in natural plant products, like fruits and vegetables, than Western diets. It has been reported that 12% of the post-menopausal women without a history of breast cancer and 23% of post-menopausal women with a history of breast cancer used plant products as a complementary and alternative medicines for the prevention of breast cancer risk (5). Therefore, efforts are needed to develop newer and effective botanicals, in particular, dietary supplements which can reduce the risk of breast cancer incidence and its metastasis.

Grapes (*Vitis vinifera*), which are one of the most widely consumed fruits in the world have enormous health benefits. Grapes are rich in proanthocyanidins with ~60–70% of the proanthocyanidins being contained in the seeds. The grape seed proanthocyanidins (GSPs) are composed mainly of dimers, trimers, tetramers and oligomers of monomeric catechins (6,7). GSPs have been shown to have cytotoxic effects on tumor cells without having adverse effects on normal cells (8). We showed that supplementation of the diet with GSPs prevents photocarcinogenesis in animals (9), and prevents the expression and activation of metastasis-specific matrix metalloproteinases in human prostate cancer cells (10). Recently, Kim *et al.* (11) have shown that dietary GSPs inhibit chemical carcinogen-induced mammary tumors in rats, however, its chemopreventive mechanism is quite unknown. Although several modalities are available for the treatment of breast cancer, including surgery, hormone therapy and radiation therapy, these treatment modalities are not effective once metastasis has occurred. Moreover, the therapies available currently for the treatment of breast cancer can be compromised by the development of drug resistance or toxicity (12).

Mouse mammary carcinoma 4T1 cells have been employed to examine the therapeutic efficacy and molecular mechanism of chemotherapeutic agents which are relevant to humans (13–15). The 4T1 cells are poorly immunogenic and exhibit characteristics that resemble to those of stage IV breast cancer in humans (16–19). They are highly invasive and metastasize as early as 2 weeks after implantation from the primary tumor site to distant organs (16–18). Therefore, we examined the chemoprotective efficacy of GSPs in 4T1 cells in both *in vitro* and *in vivo* model systems. Here, we report that (i) *in vitro* treatment of 4T1, MCF-7 and MDA-MB-468 breast cancer cells with GSPs inhibits their proliferation and viability, (ii) *in vitro* treatment of 4T1 cells with GSPs results in induction of apoptosis which is associated with enhanced expression of the pro-apoptotic protein Bax and activation of caspase 3 and cleavage of poly (ADP-ribose) polymerase (PARP), and (iii) administration of dietary GSPs to immunocompetent Balb/c mice inhibits subcutaneous tumor growth of 4T1 cells, inhibits their metastasis to lungs and increases the survival of the animals.

**Abbreviations:** Apaf-1, apoptotic protease-activating factor-1; GSPs, grape seed proanthocyanidins; PARP, poly (ADP-ribose) polymerase.

## Materials and methods

### Chemicals and antibodies

The GSPs used in this study was obtained from Kikkoman Corporation, Japan. Proanthocyanidins constitute a major component of GSPs (90%) with some catechin monomers, as described previously (9). The Annexin V-conjugated Alexafluor488 Apoptosis Detection Kit was purchased from Molecular Probes (Eugene, OR). The primary antibodies to Bax and cleaved caspase 3 were purchased from Cell Signaling Technology (Beverly, MA). The antibodies for Bcl-2, Apaf-1 and cytochrome *c*, and Bax siRNA transfection kit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and PARP from Upstate Cell Signaling Solutions (Lake Placid, NY). The respective secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA). For assay of caspase 3 activity, ApoTarget Kit was procured from Biosource International, (Camarillo, CA). The ECL Detection Kit was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) and all other chemicals employed in this study were of analytical grade and purchased from Sigma Chemical (St Louis, MO).

### Cell line and cell culture

The 4T1 mouse mammary carcinoma cell line, and human breast cancer cell lines, MDA-MB-468 and MCF-7, were obtained from the American Type Culture Collection (Rockville, MA). The cells were cultured in monolayers in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT), 100 µg/ml penicillin and 100 µg/ml streptomycin from Invitrogen (Carlsbad, CA), and maintained in an incubator with a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

### Clonogenic assay for cellular proliferation

The anti-proliferative effect of GSPs on 4T1 cells was determined using a colony formation assay, as described previously (20). Briefly,  $\sim 1 \times 10^3$  cells were plated into each well of 6-well plates in triplicates and incubated for 24 h prior to the addition of GSPs (5–80 µg/ml), then incubated for 15 days. This 15-day time period was chosen as during this time it was expected that each cell would have the chance to proliferate and form a colony. On Day 15, the colonies were washed with phosphate-buffered saline (PBS), fixed with 70% ethanol and stained with 0.02% aqueous trypan blue solution. The colonies that had  $\geq 50$  cells/colony were counted. The colony forming potential of 4T1 cells after treatment with GSPs was then expressed as the percentage of the colonies formed by the control cells (not treated with GSPs).

### MTT assay

The effect of GSPs on cell viability was determined using the MTT assay as described previously (20,21). Briefly,  $\sim 5 \times 10^3$  4T1, MDA-MB-468 or MCF-7 cells were plated per well in 96-well culture plates and incubated overnight. GSPs were then added to attain final concentration ranging from 10 to 80 µg/ml and the cells incubated for a further 24, 48 or 72 h. At the end of the stipulated period, MTT (50 µl of 5 mg/ml) was added into each well and the resulting formazan was then dissolved in 150 µl of dimethyl sulfoxide and the absorbance recorded at 540 nm using a Bio-Rad 3350 microplate reader.

### Quantification of apoptosis by flow cytometry

Apoptotic cell death of 4T1 cells on treatment with GSPs was measured as described previously using flow cytometric analysis of cells stained with the Annexin V-Alexafluor488 (Alexa488) Apoptosis Detection Kit according to the manufacturer's instructions (21). Briefly, after overnight serum starvation, 4T1 cells were treated with GSPs (0, 20, 40, 60 and 80 µg/ml) for 24 or 48 h. The cells were then harvested, washed with cold PBS and stained with Alexa488 and propidium iodide (PI) in binding buffer at room temperature in the dark. The stained cells were analyzed by fluorescence-activated cell sorting using a FACSCalibur instrument (BD Biosciences, San Jose, CA) equipped with CellQuest 3.3 Software. In experiments in which the pan-caspases inhibitor (Z-VAD-FMK) was used, the inhibitor was added 2 h before addition of the GSPs.

### Preparation of cell and tumor lysates and western blot analysis

The 4T1 cells were grown in complete DMEM medium and incubated in the absence or presence of GSPs (20, 40, 60 and 80 µg/ml) for 24 or 48 h. Thereafter, the cells were harvested and lysates prepared, as described previously (21). The concentration of protein in the lysates was determined using the DC Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Similarly, the lysates of tumors harvested at the end of the experiments as well as age-matched skin samples were prepared using a homogenizer in ice-cold lysis buffer with protease inhibitors as described above. For western blot analysis, an appropriate amount of lysate (25–50 µg protein) was subjected to SDS-PAGE using a 12% gel. Thereafter,

separated proteins were transferred electrophoretically to nitrocellulose membranes (21). Non-specific sites on the membrane were blocked by incubation with blocking buffer (5% non-fat dry milk in 1% Tween-20 in 20 mM Tris-buffered saline, pH 7.5) for 1 h. The membranes were then probed overnight at 4°C with the primary antibody. After washing, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h, washed and the expression of the protein determined using the ECL detection system and autoradiography with HXR-film (Oneonta, AL). To ensure equal protein loading, the membranes were stripped and reprobed with anti-β-actin antibodies using the protocol detailed above. The relative intensity of each protein band in a blot was measured using a computerized software program OPTIMAS 6.2 and normalized with the β-actin bands to compare the expression of proteins in different treatment groups. The experiments were repeated three times.

### Bax siRNA transfection of 4T1 cells

Mouse specific Bax siRNA (Cat no. sc-29213) and control siRNA (Cat. no. sc-37007) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). One day before transfection, cells were plated in 10% FBS containing DMEM and without antibiotics. Cells were transfected with 100 nM of siRNA targeted against Bax using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Briefly, Bax siRNA (100 nM) and Lipofectamine 2000 (6 µl) were diluted separately in 250 µl each of Opti-MEM I reduced serum medium without serum and incubated for 5 min. After incubation, the diluted siRNA was added in diluted Lipofectamine 2000, mixed gently and incubated for 20 min at room temperature to allow the formation of siRNA and Lipofectamine 2000 complex. The complex was then added to each well of 6-well culture plate containing cells and medium (Opti-MEM, 1.5 ml), mixed gently and incubated at 37°C in a CO<sub>2</sub> incubator. After 5 h, 2 ml of fresh 10% FBS containing DMEM medium was added to the cells. At 24 h post-transfection, fresh medium was added to the cells and at 48 h post-transfection cells were treated with 20 and 40 µg/ml of GSPs for an additional 24 h and analyzed for apoptosis using FACS. Knock-down of Bax after transfection was confirmed by western blotting of whole cell lysates prepared from 48 h post-transfected cells.

### Caspase 3 activity assay

The ApoTarget Kit (BioSource International) was employed to determine the activity of caspase 3 following the manufacturer's protocol. Briefly, the cells were treated with GSPs (40 and 60 µg/ml) with or without the pan-caspases inhibitor Z-VAD-FMK. The Z-VAD-FMK (80 µmol/l) was added 2 h prior to the addition of the GSPs. After 48 h, the cells were harvested and cell lysates were prepared as described above. Samples of the cell lysates (100 µg protein per sample) were mixed with reaction buffer and 200 µmol/l substrate (DEVD-pNA for caspase 3) and incubated for 3 h at 37°C. The absorbance was then measured at 405 nm and the sample readings calculated by subtracting the absorbance of blank samples.

### Animals and animal diet

Female Balb/c mice of 6–7 weeks of age were purchased from Charles River Laboratories (Wilmington, MA) and housed in the animal research facility of the University of Alabama at Birmingham, AL. Mice were kept in groups of five per cage and fed with AIN76A control diet and water *ad libitum*. The animals were acclimatized for 1 week before use and maintained throughout at standard conditions as follows: 24 ± 2°C temperature, 50 ± 10% relative humidity and 12 h light/dark cycle. To determine the effect of GSPs, GSPs were given with the control diet (0.2 and 0.5% w/w). The supplemented diet was started at least 2 weeks before tumor cell inoculation and continued until the end of the experiment.

Experimental diets containing GSPs (0.2 and 0.5%, w/w) were prepared separately in the AIN76A powdered control diet, as described previously (9). Briefly, the GSPs and AIN76A powdered control diet were mixed for at least 4 h in a rotating pan to obtain a uniform mixture. Water was then added (approximately one-tenth of the diet) and the ingredients further mixed by hand and spread uniformly in shallow pans such that a compact texture was achieved. The diet was then cut into small pieces and air dried at room temperature. The experimental diet was prepared once a month and stored at 4°C.

### In vivo tumor experiment

The 4T1 cells were inoculated subcutaneously in the pre-shaved skin of the back of the mouse. The mice were inoculated with either  $1 \times 10^6$  or  $1 \times 10^4$  viable cells with the higher dose being considered as placing the mice at high risk of tumor growth and metastasis and the lower dose being considered as placing the mice at low risk of tumor growth and metastasis. Each treatment group had 10 animals. The growth of tumor was monitored throughout the experiment with the tumor size being measured regularly twice or thrice weekly using Vernier calipers and represented in terms of tumor volume. At the termination of the experiment, the animals were sacrificed. At the

time of sacrifice, the tumors and lungs were excised from the animals. The lungs were fixed in Bouin's solution for 24 h. The number and size of metastatic tumor nodules on lungs were recorded using a dissection microscope. The median survival time of the mice also was recorded for each treatment group.

#### Statistical analysis

The statistical significance of differences between the experimental groups (not treated and treated with GSPs) was analyzed using the Student's *t*-test. The *in vivo* tumor growth data were statistically analyzed using ANOVA. A *P*-value <0.05 was considered statistically significant.

## Results

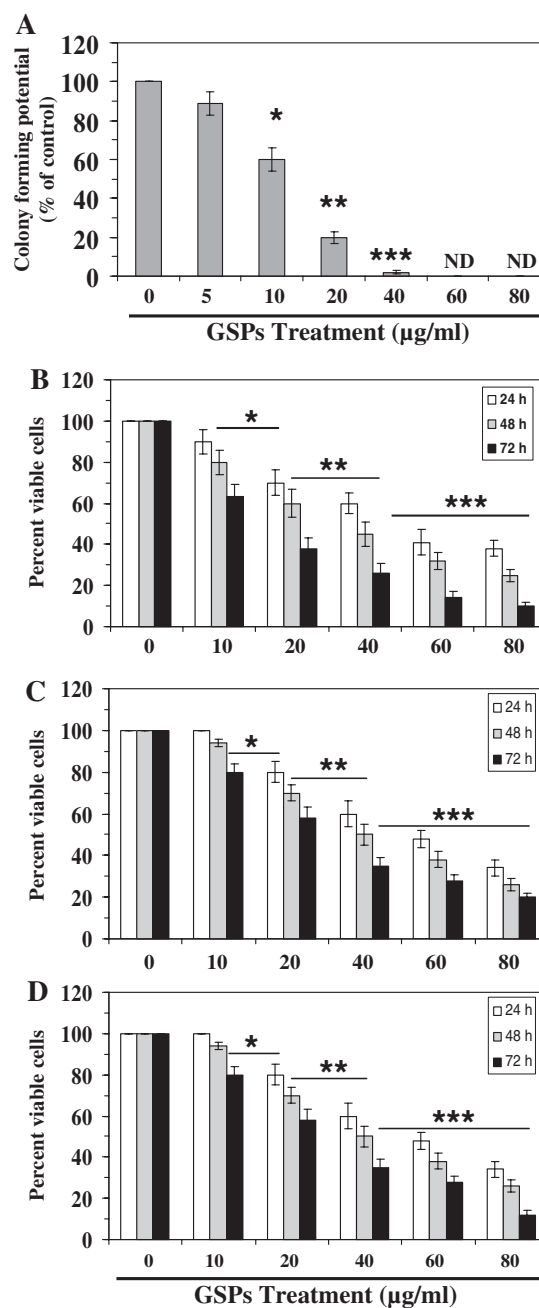
### GSPs inhibit cellular proliferation and diminish the viability of breast cancer cells: 4T1, MCF-7 and MDA-MB-468 cells

The anti-proliferative and cytotoxic effects of GSPs on 4T1 cells were first determined *in vitro* by using anchorage-dependent colony formation and MTT assays. Treatment of 4T1 cells with GSPs at all concentrations tested (5–80 µg/ml) for 15 days resulted in inhibition (10–100%, *P* < 0.05–0.001) of the colony forming potential (Figure 1, Panel A). The ability of the cells to form colonies was reduced significantly at a concentration of GSPs ≥10 µg/ml, and was dose-dependent with doses of ≥40 µg/ml of GSPs almost completely inhibiting the proliferation potential of cells in these groups (Figure 1, Panel A).

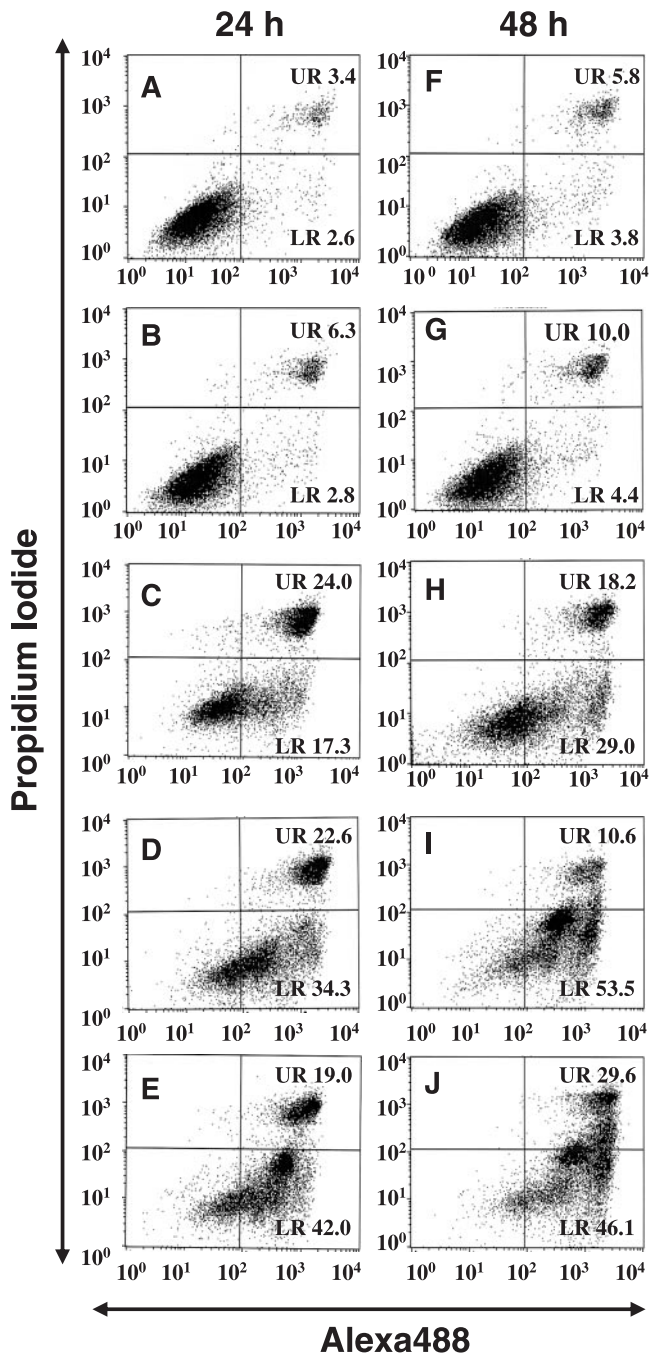
The effect of GSPs on the cell viability of 4T1 cells was further determined and confirmed using the MTT assay. As shown in Figure 1 (Panel B), treatment of 4T1 cells with GSPs (10–80 µg/ml) resulted in a significant reduction in cell viability in a concentration-dependent manner. This effect was also time-dependent, with a 10–62% reduction after 24 h, a 20–75% (*P* < 0.05–0.001) reduction after 48 h and 37–90% (*P* < 0.05–0.001) reduction after 72 h. These results indicate the cytotoxic activity of GSPs against highly metastatic breast cancer cells. Further, we examined whether GSPs have similar anti-carcinogenic effects in human breast cancer cells. For this purpose, the MDA-MB-468 (estrogen receptor-negative) and MCF-7 (estrogen receptor-positive) human breast carcinoma cells were treated with GSPs with identical doses and for different time points as was done with 4T1 cells. As shown in Figure 1, treatment of MCF-7 (Panel C) and MDA-MB-468 (Panel D) tumor cells with GSPs (10–80 µg/ml) resulted in a significant reduction in cell viability in a concentration- and time-dependent (24–72 h) manner. These observations indicated that GSPs possess identical cytotoxic effects against both mouse as well as human breast carcinoma cells. Since our interest was to examine whether GSPs have the ability to prevent metastatic potential of tumor cells, we selected mouse 4T1 cells, which are highly metastatic in nature, for further studies. Our results indicate that *in vitro* concentrations of GSPs >10 µg/ml induce significant reduction in the viability of 4T1 cells; therefore, we used concentrations of 20, 40, 60 and 80 µg/ml of GSPs in the *in vitro* mechanistic studies described below.

### GSPs induce apoptosis in 4T1 cells

A quantitative analysis of the induction of apoptosis of 4T1 cells was then performed using flow cytometry as described previously (20,21). The early apoptotic cells stain with Annexin V-conjugated Alexa Fluor 488 dye, which detects apoptotic cell with externalized phosphatidyl-serine leaflets. These cells are shown in the lower right (LR) quadrant of the histograms (Figure 2), whereas late apoptotic cells are shown



**Fig. 1.** *In vitro* treatment of murine or human breast cancer cells with GSPs inhibits the proliferation potential and cell viability of the cells in a dose- and time-dependent manner. (A), Cellular proliferation assayed using the clonogenic assay in murine 4T1 cells as described in the Materials and methods. A survival of 100% corresponds to the number of colonies obtained with cells that were not treated with GSPs. Treatment of 4T1 cells with ≥40 µg/ml of GSPs concentration almost completely inhibited the colony forming potential of breast cancer cells when recorded after 15 days of treatment. The experiment was repeated three times, and the colony forming potential of the cells at each dose of GSPs is expressed in terms of percent of control and is reported as the mean ± SD. ND = colonies not detectable. Dose- and time-dependent effect of GSPs on the viability of mouse 4T1 (B), and human breast carcinoma MCF-7 (C) and MDA-MB-468 (D) cells were performed. The cell viability was determined using the MTT assay as described in the Materials and methods. Significant reduction in viability of the breast carcinoma cells was observed on treatment with GSPs (10–80 µg/ml concentrations) after 24, 48 and 72 h of treatment. Cell viability after treatment with GSPs is expressed in terms of percent of control cells not treated with GSPs and is presented as the mean ± SD, of eight replicates. \**P* < 0.05 versus control; \*\**P* < 0.01 versus control; \*\*\**P* < 0.001 versus control group.



**Fig. 2.** GSPs induce apoptosis of 4T1 cells in a dose-dependent manner after 24 and 48 h of treatment *in vitro*. 4T1 cells were treated with varying concentrations of GSPs, including 0 µg/ml (A and F), 20 µg/ml (B and G), 40 µg/ml (C and H), 60 µg/ml (D and I) and 80 µg/ml (E and J) for 24 h (left panels) and 48 h (right panels) then harvested for analysis of apoptosis. The Annexin V-Alexa Fluor 488 (Alexa488) Apoptosis Vybrant Assay Kit was used to analyze the number of apoptotic cells by flow cytometry following the manufacturer's protocol, as detailed in Materials and methods and the stained cells analyzed using flow cytometry. The lower right (LR) quadrant of histograms indicates the percentage of early apoptotic cells (Alexa488-stained cells) and the upper right (UR) quadrant indicates the percentage of late apoptotic cells (Alexa488 + propidium iodide-stained cells) after GSPs treatment.

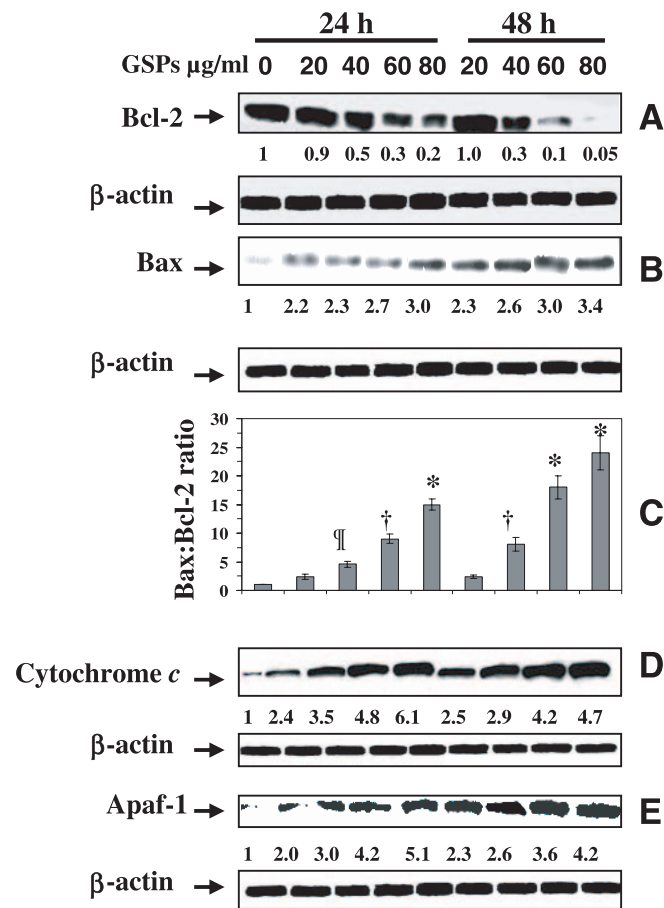
in the upper right (UR) quadrant. We found that treatment of 4T1 cells with GSPs (20, 40, 60 and 80 µg/ml, Panels B–E, respectively) for 24 h resulted in a dose-dependent increase in the percentage of early apoptotic cells (LR), respectively, from

2.8 to 42.0% (Panels B–E) compared to a percentage of 2.6% in control cells that were not treated with GSPs (Panel A). Similarly, treatment with GSPs resulted in a dose-dependent increase in the numbers of late apoptotic cells (UR) 6.3–19.0% (Panels B–E) compared to a percentage of 3.4% in cells not treated with GSPs (Panel A). Thus, the percentage of all apoptotic cells (UR + LR) was significantly higher (61.0%; Panel E) for 4T1 cells treated with 80 µg/ml GSPs for 24 h than for 4T1 cells not treated with GSPs (6%; Figure 2, Panel A). The induction of apoptosis was greater when the 4T1 cells were treated with the same concentrations of GSPs for 48 h (Figure 2, Right Panels; F–J). At this time point, the percentage of early apoptotic cells showed a dose-dependent increase from 4.4 to 46.1% for concentrations of GSPs ranging from 20 to 80 µg/ml (Figure 2, Right Panels; G–J) compared to 3.8% for cells not treated with GSPs (Panel F). Thus at 48 h, the percentage of all apoptotic cells (UR + LR) ranged from 14.4 to 75.7% compared to 9.6% for cells that were not treated with GSPs (Panels F–J). This significant induction of apoptosis of 4T1 cells caused by treatment with GSPs is sufficient to account for the inhibition of cell growth and cell viability in these highly metastatic breast cancer cells.

*GSPs increase the expression of Bax, induce the release of cytochrome c, induce the expression of Apaf-1 while decreasing the expression of Bcl-2 in 4T1 cells*

The anti-apoptotic protein Bcl-2 has been associated with inhibition of apoptosis whereas the increased expression of the pro-apoptotic protein, Bax, has been associated with the induction of apoptosis (22,23). We therefore reasoned if the proteins of the Bcl-2 family are involved in the induction of apoptosis of 4T1 cells by GSPs, there would be reduced expression of Bcl-2 and enhanced expression of Bax in the cells treated with the GSPs. As shown in Figure 3, the treatment of the 4T1 cells with GSPs resulted in a dose-dependent reduction in Bcl-2 expression (Panel A) and increased expression of Bax (Panel B) as determined by western blot analysis of cells at 24 and 48 h after addition of the GSPs. It has been recognized that the ratio of Bax:Bcl-2 proteins is the determining factor in the transmission of the apoptotic signal. We therefore calculated the ratio of these proteins and, as shown in Panel C of Figure 3, found that the ratio of Bax/Bcl-2 exhibited a significant dose-dependent increase ( $P < 0.05$ – $0.001$ ) both at 24 and 48 h after treatment of GSPs. Thus, the induction of apoptosis of 4T1 cells by GSPs is mediated through this apoptotic signal.

It has been recognized that intracellular movement of pro-apoptotic proteins of Bcl-2 family, such as Bax, depolarizes mitochondria and induces the release of cytochrome *c* through openings in the outer membrane formed as a consequence of permeability transition and loss of mitochondrial membrane potential (24). The released cytochrome *c* triggers a post-mitochondrial pathway, forming an 'apoptosome' of Apaf-1, cytochrome *c* and caspase 9 that subsequently cleaves the effector molecule caspase 3 (25). Cleaved caspase 3 is the executioner of apoptosis, cleaving a broad spectrum of cellular targets, including PARP (26,27). We therefore determined the effect of GSPs on these proteins. Western blot analysis indicated that treatment of 4T1 cells with GSPs resulted in a dose-dependent increase in cytochrome *c* release (Figure 3, Panel D) and increased expression of Apaf-1 (Figure 3, Panel E) at 24 and 48 h after treatment with GSPs. These changes were not owing to differences in the amounts of proteins

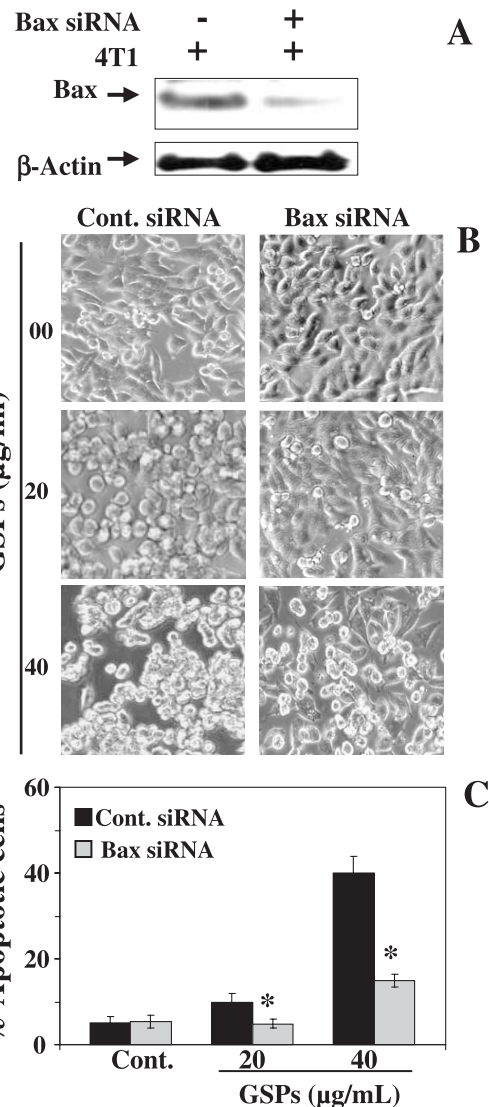


**Fig. 3.** *In vitro* treatment of 4T1 cells with GSPs results in a dose-dependent reduction in the expression of the anti-apoptotic protein Bcl-2 (A) while increasing the expression of the pro-apoptotic protein, Bax (B) after 24 and 48 h of treatment. Treatment of 4T1 cells with GSPs significantly increased the Bax:Bcl-2 protein ratio (C) which is expressed as the mean  $\pm$  SD from three independent experiments.  $^{\ddagger}P < 0.05$ ;  $^{\dagger}P < 0.01$  and  $^*P < 0.001$ . GSPs also induced the release of cytochrome *c* from mitochondria (D) and increased the expression of Apaf-1 (E) in 4T1 cells, as demonstrated by western blot analysis. A representative blot is shown from three independent experiments with identical observations, and equivalent protein loading was confirmed by probing stripped blots for  $\beta$ -actin as shown. The relative intensity of each band after normalization for  $\beta$ -actin is shown under each blot.

loaded on the gels as indicated by the levels of  $\beta$ -actin and held true when the relative intensity of each band was normalized to the level of  $\beta$ -actin in each sample, as indicated by the numbers shown under each blot.

#### GSPs-induced apoptosis is blocked by knockdown of Bax in 4T1 cells

As GSPs-induced Bax may have a role in induction of apoptosis in 4T1 cells, we further checked the role of Bax in GSPs-induced apoptosis by knockdown of Bax from the cells using siRNA transfection. The transfection of Bax siRNA resulted in significant reduction of Bax expression ( $>75\%$ ,  $P < 0.001$ ) in 4T1 cells (Figure 4A). Identical results were also obtained when 4T1 cells were treated with GSPs (data not shown). The treatment of 4T1 cells with GSPs (20 or 40  $\mu\text{g/ml}$ ) for 24 h resulted in marked cell death but this effect was significantly less in GSPs-treated Bax siRNA transfected



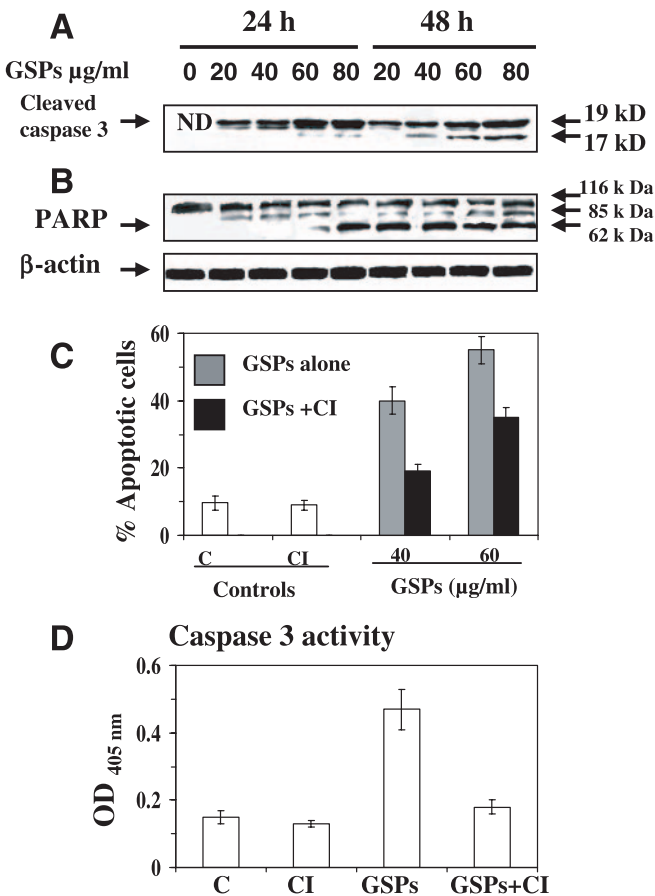
**Fig. 4.** GSPs-induced apoptosis is blocked by knockdown of Bax in 4T1 cells. Cells were transfected with Bax siRNA to knockdown the Bax expression (A). Treatment of 4T1 cells with GSPs (20 or 40  $\mu\text{g/ml}$ ) for 24 h resulted in induction of apoptosis in control siRNA treated cells but GSPs-induced apoptosis was blocked in Bax siRNA transfected cells. Morphological changes indicating the apoptosis in 4T1 cells (Panel B). Representative pictures are shown from three independent experiments (B). The percentage of apoptosis in different treatment groups was determined using FACS as detailed in Materials and methods (C). Data are presented in terms of percent apoptotic cells as a mean  $\pm$  SD from three independent experiments.  $^*P < 0.001$ ; significant blocking of apoptosis versus non-Bax siRNA transfected 4T1 cells.

cells, as was evident from the morphology of the cells under microscope (Figure 4B). The percentage of apoptotic cells under identical conditions in different treatment groups were quantitatively analyzed using FACS. Treatment of 4T1 cells with GSPs at the dose of 20 and 40  $\mu\text{g/ml}$  along with the control siRNA for 24 h resulted in 10 and 40% apoptosis, respectively, whereas GSPs-induced apoptosis in Bax siRNA transfected cells was significantly blocked ( $P < 0.001$ ) (Figure 4C). These data further confirm our observation that GSPs-induced apoptosis in 4T1 cells may be mediated through the overexpression of pro-apoptotic protein Bax, which may have resulted in high Bax:Bcl-2 ratio.

### GSPs induce caspase 3 activation and PARP cleavage in 4T1 cells

We also examined the effect of GSPs on the activation of caspase 3 and PARP proteins. As shown in Figure 5, treatment with GSPs induced the cleavage of caspase 3 (Panel A) and PARP (Panel B) in a dose-dependently manner when observed at 24 and 48 h of treatment. The cleavage of caspase 3 and PARP support the evidence that the induction of apoptosis of 4T1 cells by GSPs is mediated through activation of the caspase 3 pathways and via disruption of mitochondrial function. To further check whether activation of caspases is involved in the apoptosis of 4T1 cells induced by GSPs, we utilized the pan-caspase inhibitor, Z-VAD-FMK. The 4T1 cells were

treated with GSPs (40 and 60  $\mu\text{g/ml}$ ) with and without the pan-caspase inhibitor, Z-VAD-FMK (80  $\mu\text{mol/l}$ ) and the numbers of apoptotic cells were analyzed by flow cytometry after 48 h (Figure 5, Panel C). We found that GSPs-induced apoptosis of 4T1 cells was blocked by 65 and 45% at the doses of 40 and 60  $\mu\text{g/ml}$ , respectively, by the treatment of Z-VAD-FMK. The GSPs were still able to induce apoptosis of some 4T1 cells in the presence of inhibitor. After subtraction of the number of control cells, treated with Z-VAD-FMK but not GSPs, that underwent apoptosis, an estimated 10% of cells were capable of undergoing apoptosis in the presence of the inhibitor when treated with 40  $\mu\text{g/ml}$  of GSPs, and 26% of cells when treated with 60  $\mu\text{g/ml}$  of GSPs. To confirm that the concentration of pan-caspase inhibitor used (80  $\mu\text{mol/l}$ ) was able to completely inhibit the activity of caspases, we treated the cells with GSPs with and without Z-VAD-FMK for 48 h and then determined caspase 3 activity using a colorimetric assay. This experiment indicated that treatment of 4T1 cells with GSPs resulted in increased activation of caspase 3, which was inhibited on treated with pan-caspase inhibitor (Figure 5, Panel D), and confirmed that at a dose of 80  $\mu\text{mol/l}$  which had been selected in pre-trial experiments, Z-VAD-FMK resulted in >90% inhibition of caspase activation. Taken together, these results suggested that the apoptosis of 4T1 cells induced by GSPs is mediated primarily by caspase-dependent pathways, but that caspase-independent pathways also play a role.

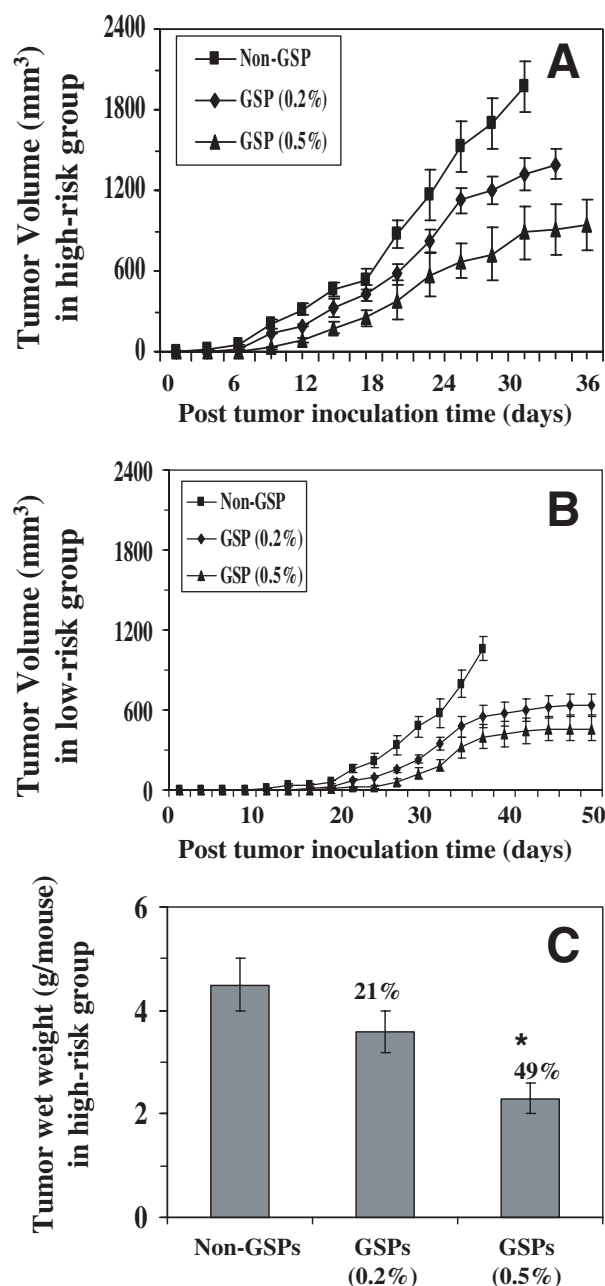


**Fig. 5.** *In vitro* treatment of 4T1 cells with GSPs increases the cleavage of caspase 3 and PARP. 4T1 cells were treated with varying concentrations of GSPs (0, 20, 40, 60 and 80  $\mu\text{g/ml}$ ) for 24 and 48 h, thereafter cells were harvested and samples were prepared to analyze the cleavage of caspase 3 (A) and PARP (B) using western blotting, as detailed in the Materials and methods. Equal loading of samples was confirmed by  $\beta$ -actin and a representative blot is shown from three independent experiments with almost identical observations. (C) The effect of GSPs (40 and 60  $\mu\text{g/ml}$ ) on apoptosis of 4T1 cells was determined after 48 h treatment in the absence or presence of 80  $\mu\text{mol/l}$  of the pan-caspase inhibitor (Z-VAD-FMK). The inhibitor was added to the cells 2 h prior to the addition of GSPs. Percent apoptotic cells in different treatment groups, as shown in the figure, were analyzed by flow cytometry as detailed in Materials and methods. The number of apoptotic cells in the different treatment groups is as the mean  $\pm$  SD from three independent experiments. (D) The activity of caspase 3 in cell lysates from different treatment groups was determined using a colorimetric protease assay (the ApoTarget Kit). Absorbance as a measure of caspase 3 activity is reported as the mean  $\pm$  SD of three independent experiments. C, control (non-GSP); CI, pan-caspase inhibitor.

### Dietary GSPs inhibit *in vivo* growth of 4T1 breast tumor cells in Balb/c mice

In order to examine the applicability of *in vitro* observations in *in vivo* system, we determined the effect of dietary GSPs on the growth of 4T1 cells in immunocompetent Balb/c mice. High-risk ( $1 \times 10^6$  4T1 cells) and low-risk ( $1 \times 10^4$  4T1 cells) tumor models were employed in this study, as described in Materials and methods section. After subcutaneous inoculation of the tumor cells, it was observed that growth of the tumors in terms of tumor volume was inhibited in the animals fed GSPs as compared to the growth of the tumors in the mice that were not fed GSPs (Figure 6). In the high-risk control group in which  $1 \times 10^6$  4T1 tumor cells were inoculated in mice that were not fed GSPs, tumor growth was observed on Day 3. The first recordable tumor growth was delayed by 3 days in mice inoculated with  $1 \times 10^6$  4T1 cells and that were fed a diet supplemented with 0.2 or 0.5% GSPs (Figure 6, Panel A). In the low-risk group in which the mice were inoculated subcutaneously with  $1 \times 10^4$  tumor cells (Panel B), tumor growth first observed on Day 12. In those animals fed 0.2 and 0.5% GSPs tumor growth started on Days 12 and 19, respectively. Thus, dietary 0.5% GSPs resulted in a delay of 7 days in tumor appearance.

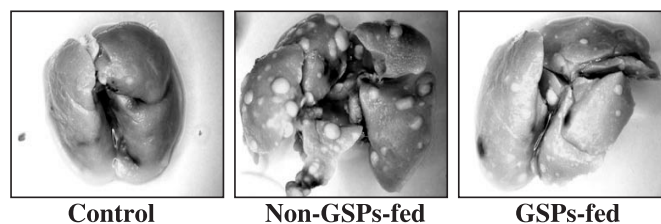
Measurement of tumor volume at regular intervals indicated that dietary GSPs also inhibited tumor development. In the high-risk groups (Figure 6, Panel A), administration of 0.2 and 0.5% GSP resulted in a 30% ( $P < 0.05$ ) and 52% ( $P < 0.01$ ) inhibition in tumor volume, respectively, when analyzed at the termination of the experiment. Similarly, dietary GSPs, 0.2 and 0.5%, significantly inhibited tumor growth in the low-risk group by 42 and 60% ( $P < 0.005$ ), respectively, at Day 36 when the tumor yield was maximal in the low-risk group of mice that were not fed GSPs (Figure 6, Panel B). Finally, the wet weight of the tumors was measured in the high-risk groups at the termination of the experiment, and it was found that the weight of the tumors in the high-risk mice



**Fig. 6.** Dietary GSPs (0.2 and 0.5%, w/w) inhibit the growth of tumors *in vivo* (A and B), and increase the survival period of the tumor-bearing Balb/c mice. Mice were inoculated subcutaneously on the shaved right flank with either  $1 \times 10^6$  (high-risk group, A) or  $1 \times 10^4$  (low-risk group, B) 4T1 cells, as detailed in the Materials and methods. Experiments were performed for 36 days (A) and 50 days (B). Tumor volumes were recorded on a regular basis to determine the chemotherapeutic effect of GSPs on 4T1 tumor growth. (C) At the termination of the experiment in the high-risk group, the tumor mass was dissected out from each mouse and the wet weight recorded. \* $P < 0.01$ ; significant difference versus mice not fed GSPs.

was reduced by 21% in mice fed 0.2% GSPs and was reduced significantly by 49% ( $P < 0.01$ ) in mice fed 0.5% after 36 days when compared to mice not fed GSPs, as shown in Figure 6, Panel C.

The *in vivo* tumor experiments also indicated that dietary administration of GSPs increased the survival time of the tumor-bearing animals. In the high-risk control group that



**Fig. 7.** Dietary GSPs (0.5%, w/w) inhibit metastasis of tumor cells from the primary tumor site to the lungs in the high-risk group of mice. Representative photographs of lungs are shown from each treatment group. Whitish tumor lesions can be seen on the surface of the lungs. The lungs were dissected out at the termination of the experiment.

were not fed GSPs, the first death was observed on Day 27 and all animals had died by Day 32 after inoculation of the tumor cells. Administration of 0.2% GSPs in the diet did not significantly alter the survival time of the high-risk group of mice compared to the control group. A significant chemoprotective effect was observed in the animals fed 0.5% GSPs, however, the average survival period of the mice being increased by 6 days. In the low-risk groups, dietary GSPs resulted in an enhancement in the mean survival period which was of 47.5 days compared with 36 days in the mice that were not fed GSPs. Importantly, there was no significant difference in body weight of the mice and diet consumption/weight in between various treatment groups (data not shown).

#### *Dietary GSPs inhibit in vivo metastasis of the tumor cells from the primary tumor site to the lungs of Balb/c mice*

As 4T1 breast tumor cells have been shown to metastasize within 2 weeks (16–18) from primary tumor site to distant organs, two separate time points were selected to examine the chemotherapeutic effect of GSPs on metastasis, i.e. Days 16 and 30 post-tumor cell inoculation. The mice were subcutaneously inoculated with the 4T1 tumor cells ( $1 \times 10^6$ ) in two separate experiments. After 16 and 30 days mice were sacrificed and lungs were examined. It was observed that metastasis has already been occurred at Day 16 which was supported by the presence of  $4 \pm 1$  metastatic nodules/mouse on the surface of the lungs, whereas the degree of metastasis was significantly higher after 32 days of tumor cells inoculation which was evident by the presence of  $38 \pm 6$  metastatic nodules/mouse on the lungs (Figure 7, Table I). The size of the metastatic lung nodules was also larger after 32 days of tumor cell inoculation compared to 16 days of tumor cells inoculation. Chemoprotective effect of dietary GSPs was simultaneously evaluated and it was observed that dietary GSPs at the level of 0.2 and 0.5% could not delay the metastasis but significantly inhibited the metastasis of tumor cells from the primary site to the lungs in terms of the number and growth of the tumor nodules on the lungs. Administration of 0.2% GSPs in the diet did not prevent significantly the metastasis of tumor cells in terms of number of tumor nodules and their size from the primary tumor site to the lungs after 16 days of the treatment. However, the growth of the metastatic tumor nodules was significantly inhibited (27%,  $P < 0.05$ ) after 32 days of the treatment compared to non-GSPs-fed mice (Table I). The administration of 0.5% GSPs resulted in a significant reduction in the number of metastatic tumor nodules or lesions by 45% ( $P < 0.01$ ) after 16 days and 42% ( $P < 0.01$ ) reduction being observed after 30 days. As compared with the size of the metastatic lesions in the animals that did not receive GSPs,

**Table I.** Effect of dietary GSPs on the metastasis of 4T1 tumor cells from primary tumor site to lungs in Balb/c mice<sup>a</sup>

Treatment groups	After 16 days		After 30 days	
	No. of metastatic lung nodules/mouse	Diameter of metastatic lung nodules (mm)	No. of metastatic lung nodules/mouse	Diameter of metastatic lung nodules (mm)
Control (GSPs 0.0%)	—	—	—	—
GSPs (0.5%) alone	—	—	—	—
4T1 + GSPs (0.0%)	4.0 ± 1.0	1.1 ± 0.2	38 ± 6	3.0 ± 0.5
4T1 + GSPs (0.2%)	3.5 ± 0.5 (13) <sup>b</sup>	0.9 ± 0.2 (18)	31 ± 6 (18)	2.2 ± 0.5 (27) <sup>c</sup>
4T1 + GSPs (0.5%)	2.2 ± 0.5 (45) <sup>d</sup>	0.5 ± 0.2 (55) <sup>d</sup>	22 ± 5 (42) <sup>d</sup>	1.8 ± 0.5 (40) <sup>d</sup>

<sup>a</sup>One million 4T1 cells were inoculated on the shaved right flank of the mouse and considered as a high-risk group. Mice were sacrificed on Days 16 and 30 after tumor cell inoculation and tumor cell metastasis was recorded. For determination of lung metastasis, the lungs were removed and the number and size of the lung nodules were determined using a dissection microscope;  $n = 10$ .

<sup>b</sup>The data in parentheses indicate the percent inhibition on GSPs treatment.

<sup>c</sup>Significant versus non-GSPs-fed and 4T1 cells inoculated group,  $P < 0.05$ .

<sup>d</sup>Significant versus non-GSPs-fed and 4T1 cells inoculated group,  $P < 0.01$ .

the size of the metastatic lung nodules was reduced significantly after treatment with 0.5% of GSPs for 16 and 30 days by 55% ( $P < 0.01$ ) and 40% ( $P < 0.01$ ), respectively (Table I). Representative pictures of the lungs of animals in the different treatment groups are shown in Figure 7 to illustrate the number and size of the metastatic lung nodules.

#### *Dietary GSPs increase the expression of pro-apoptotic protein Bax and the ratio of Bax:Bcl-2 proteins in 4T1 tumors in Balb/c mice*

The effect of dietary GSPs on the expression of pro-apoptotic and anti-apoptotic proteins involved in disruption of mitochondrial pathway of 4T1 tumor cells *in vivo* was analyzed as described for the tumor cells grown *in vitro*. This study was conducted using tumor tissues from the high-risk group of mice which were inoculated subcutaneously with  $1 \times 10^6$  4T1 cells. As shown in Figure 8, western blot analysis revealed that dietary GSPs downregulated the expression of the anti-apoptotic protein Bcl-2 (Panel A) while increasing the expression of the pro-apoptotic protein Bax (Panel B) in the 4T1 tumors in comparison to the expression of these proteins in the tumors grown in mice that were not fed GSPs. The significant increase in the ratio of Bax:Bcl-2 proteins (Panel C;  $P < 0.001$ ) in the tumors grown *in vivo* in mice fed GSPs indicates the susceptibility of 4T1 tumor cells to apoptosis, and this may be the reason that tumor growth was inhibited in the Balb/c mice that were fed GSPs.

#### *Dietary GSPs increase the release of cytochrome c, induce the expression of Apaf-1 and result in the activation of caspase 3 in 4T1 tumors in Balb/c mice*

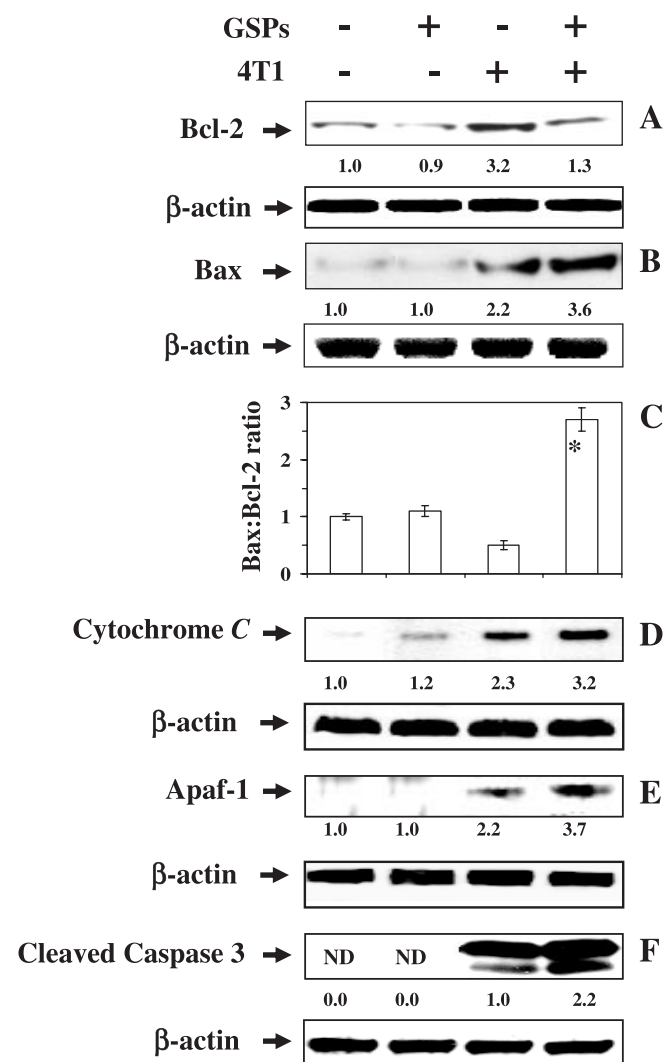
The *in vitro* studies indicated that treatment of 4T1 cells with GSPs induces apoptosis that is associated with disruption of the mitochondrial pathway. Therefore, we examined whether inhibition of tumor growth by dietary GSPs *in vivo* is associated with the induction of apoptosis and disruption of mitochondrial pathway. As shown in Figure 8, western blot analysis indicated that the supplementation of GSPs in the diet resulted in higher levels of cytochrome *c* (Panel D), induction of Apaf-1 (Panel E) and activation (cleavage) of caspase 3 (Panel F) in the tumor samples. As shown in Figure 8 (Panel F), the level of cleaved caspase 3 in the tumors was markedly higher in mice fed GSPs than in tumors from mice not fed GSPs. The basal levels of caspase 3 in the normal age- and sex-matched mouse

skin were not detectable using this technique as the antibodies used only recognize cleaved caspase 3. These observations suggest that dietary GSPs inhibit tumor growth through the induction of apoptosis mediated through caspase 3 activation and the mitochondrial pathway in the 4T1 tumor cells. The administration of GSPs alone did not affect the expression of pro-apoptotic or anti-apoptotic proteins in mouse skin (Figure 8).

## Discussion

The *in vitro* studies presented here indicate that treatment of 4T1 cells with GSPs results in a dose-dependent inhibition of cell viability and proliferation together with induction of apoptosis. It is known that apoptosis plays a crucial role in eliminating the mutated neoplastic and hyperproliferating cells from the system, and therefore may be considered as a protective mechanism against cancer progression. To gain insight into the protective mechanism controlling the apoptosis induced by GSPs, we looked at the levels of several components of the known apoptosis pathways. Apoptosis is governed by a complex network of anti-apoptotic and pro-apoptotic effector molecules. The proteins of the Bcl-2 family play an important role in apoptosis and are considered as a target for anticancer therapy (28,29). Bcl-2 protein functions as a suppressor of apoptosis while Bax is a pro-apoptotic protein of the Bcl-2 family (27,30). Bax normally acts as a sensor of cellular damage and stress. In response to significant damage or stress Bax relocates to the mitochondrial surface and disrupts the normal functioning of the anti-apoptotic Bcl-2 protein (24). Bax can form transmembrane pores across the outer mitochondrial membrane, which leads to loss of membrane potential (24) and efflux of cytochrome *c* and the apoptosis inducing factors. Cytochrome *c* together with Apaf-1 and ATP forms a complex with pro-caspase 9 (apoptosome) and finally leading to the activation of caspase 3 (25). In this scheme of events, anti-apoptotic molecules, such as Bcl-2, act to prevent mitochondrial pore formation. When Bax heterodimerizes with Bcl-2 or Bcl-x1 it blocks the anti-apoptotic effects of Bcl-2 or Bcl-x1 (31,32). Studies have shown that the ratio of Bax to Bcl-2 proteins increases during apoptosis (22,30). In line with these observations, we found that treatment of 4T1 cells with GSPs *in vitro* decreases the expression of Bcl-2 (Figure 3,





**Fig. 8.** Dietary GSPs (0.5%, w/w) downregulate the expression of the anti-apoptotic protein Bcl-2 (A) and upregulate pro-apoptotic protein Bax (B) in 4T1 cells grown as tumors in Balb/c mice from inoculation of  $1 \times 10^6$  viable cells. The ratio of Bax:Bcl-2 protein expression was determined by comparing the relative intensities of protein bands and shown as the mean  $\pm$  SD (C). Administration of GSPs also induces the release of cytochrome *c* from mitochondria (D), induction of Apaf-1 (E) and increases the activation (cleavage) of caspase 3 (F) in the 4T1 tumors. Skin and tumor lysates were prepared for western blot analysis as detailed in the Materials and methods. Western blot analyses were repeated using skin or tumor samples from each individual mouse, thus tumors and skin lysates were prepared separately from 10 animals in each group. A representative blot is shown from 10 independent experiments with identical results. The mean of relative intensities of each band after normalization for the levels of  $\beta$ -actin are shown under each blot. \* $P < 0.001$  versus control tumors from mice that were not treated with GSPs. ND, not detectable.

Panel A) while increasing the expression of Bax (Figure 3, Panel B) indicating that the increased ratio of Bax:Bcl-2 proteins might be responsible for the induction of apoptosis by GSPs in 4T1 cells. The role of increased expression of Bax by GSPs in induction of apoptosis was further confirmed by knockdown of Bax from the 4T1 cells. It was observed that GSPs-induced apoptosis in 4T1 cells was blocked when these cells were transfected with Bax siRNA (Figure 4). These data further confirm the role of Bax or increased ratio of Bax/Bcl-2

in GSPs-induced apoptosis in highly metastatic breast cancer 4T1 cells.

In our *in vitro* system, GSPs caused a dose- and time-dependent increase in levels of cytochrome *c*, the adaptor Apaf-1 and cleaved caspase 3 (Figures 3 and 5). As activated caspase 3 is the key executioner of apoptosis, and activated caspase 3 cleaves intracellular proteins vital to cell survival and growth, such as PARP, it has been used as an important marker of apoptosis (33,34). Our data demonstrate that cleavage of caspase 3 and PARP are prominent after treatment of 4T1 cells with GSPs (Figure 5) and therefore suggest a role for caspase 3 and PARP activation in the apoptosis induced by GSPs in 4T1 cells. The involvement of caspase 3 activation in the apoptosis induced by GSPs in 4T1 cells was further confirmed using the inhibitor of caspases. Thus, the *in vitro* data provide strong evidence that GSPs mediate apoptosis in 4T1 cells via the mitochondrial disruption pathway and activation of caspase 3.

Our *in vivo* study conducted in immunocompetent Balb/c mice indicates clearly that dietary GSPs (0.2 and 0.5%, w/w) significantly inhibit the growth of tumors formed on subcutaneous inoculation of viable 4T1 breast cancer cells (Figure 6, Panels A and B). The survival period of the tumor-bearing mice also was increased by 6–7 days with administration of the dietary GSPs (0.5%, w/w). In contrast to the present breast cancer model, the treatment of GSP in diet at a very low concentration (0.002%, w/w) was able to inhibit azoxymethane-induced colonic pre-neoplastic aberrant crypt foci formation in F344 rats (35). The variation in the effective doses of GSPs may be dependent on the model used.

The highly metastatic nature of 4T1 cells is well documented (16–18). These cells can metastasize from the primary tumor site to distant sites, such as the lymph nodes, lungs, and livers, while the primary tumor is in place (33), and death in recipient animals can occur owing to metastasis rather than the primary tumor (33). We found that dietary GSPs inhibited metastasis of 4T1 cells from the primary tumor site to the lungs as determined by counting the number and size of the metastatic tumor nodules in lungs (Figure 7 and Table I), and this may be the reason that the mice fed GSPs survived longer than the mice not fed GSPs.

We further examined the mechanisms of inhibition of tumor growth associated with the administration of dietary GSPs in the *in vivo* mouse model to determine if they were similar to those observed using the *in vitro* model. We observed that dietary GSPs resulted in decreased expression of the anti-apoptotic protein Bcl-2 while increasing the expression of Bax, cytochrome *c*, Apaf-1 and activation of caspase 3 in the 4T1 tumors as compared to tumors from mice that were not given GSPs in the diet. As cleaved caspase 3 is considered as a key executioner of apoptosis, the present *in vivo* data further confirm that GSPs induce apoptosis in tumor cells through disruption of mitochondrial pathways and activation of caspase 3 which may be responsible for the inhibition of tumor growth and subsequent inhibition of metastasis.

In summary, the *in vitro* and *in vivo* findings of our study suggest that GSPs induce apoptosis, and inhibit tumor growth and metastasis of highly metastatic breast cancer cells through disruption of mitochondrial pathway and increased activation of caspase 3. The outcome of this study holds promise for further *in vitro* and *in vivo* molecular target-oriented studies to examine the chemoprotective efficacy of GSPs. Additionally,

these studies underscore the necessity for further studies on the bioavailability of GSPs in *in vivo* system.

## Acknowledgements

Partial financial support from the Cancer Research and Prevention Foundation is gratefully acknowledged.

*Conflict of Interest Statement:* None declared.

## References

- World Health Organization. (2003) World cancer report. Steward, B.W. and Kleihues, P. (eds) IARC Press, Lyon, pp. 188–193.
- Cancer facts and figures, 2005 on the web of American Cancer Society at [www.acs.org](http://www.acs.org).
- Haenszel, W. and Kurihara, M. (1968) Studies of Japanese migrants I. Mortality from cancer and other disease among Japanese in the United States. *J. Natl Cancer Inst*, **40**, 43–68.
- Ziegler, R.G., Hoover, R.N., Pike, M.C. *et al.* (1993) Migration patterns and breast cancer risk in Asian-American women. *J. Natl Cancer Inst*, **85**, 1819–1827.
- Duda, R.B., Zhong, Y., Navas, V., Li, M.Z., Toy, B.R. and Alavarez, J.G. (1999) American ginseng and breast cancer therapeutic agents synergistically inhibit MCF-7 breast cancer cell growth. *J. Surg. Oncol*, **72**, 230–239.
- Silva, R.C., Rigaud, J., Cheynier, V. and Chemina, A. (1991) Procyanidin dimers and trimers from grape seeds. *Phytochemistry*, **30**, 1259–1264.
- Prieur, C., Rigaud, J., Cheynier, V. and Moutounet, M. (1994) Oligomeric and polymeric procyanidins from grape seeds. *Phytochemistry*, **30**, 781–789.
- Ye, X., Krohn, R.L., Liu, W., Joshi, S.S., Kuszynski, C.A., McGinn, T.R., Bagchi, M., Preuss, H.G., Stohs, S.J. and Bagchi, D. (1999) The cytotoxic effects of a novel IH636 grape seed proanthocyanidin extract on cultured human cancer cells. *Mol. Cell. Biochem*, **196**, 99–108.
- Mittal, A., Elmets, C.A. and Katiyar, S.K. (2003) Dietary feeding of proanthocyanidins from grape seeds prevents photocarcinogenesis in SKH-1 hairless mice: relationship to decreased fat and lipid peroxidation. *Carcinogenesis*, **24**, 1379–1388.
- Vayalil, P.K., Mittal, A. and Katiyar, S.K. (2004) Proanthocyanidins from grape seeds inhibit expression of matrix metalloproteinases in human prostate carcinoma cells, which is associated with the inhibition of activation of MAPK and NF $\kappa$ B. *Carcinogenesis*, **25**, 987–995.
- Kim, H., Hall, P., Smith, M., Kirk, M., Prasain, J.K., Barnes, S. and Grubbs, C. (2004) Chemoprevention by grape seed extract and genistein in carcinogen-induced mammary cancer in rats is diet dependent. *J. Nutr*, **134S**, 3445S–3452S.
- Peto, R., Boreham, J., Clarke, M., Davies, C. and Beral, V. (2000) UK and USA breast cancer deaths down 25% in year 2000 at ages 20–69 years. *Lancet*, **355**, 1822.
- Heppner, G.H., Miller, F.R. and Shekhar, P.M. (2000) Nontransgenic models of breast cancer. *Breast Cancer Res*, **2**, 331–334.
- Wang, H., Mohammad, R.M., Werdell, J. and Shekhar, P.V. (1998) p53 and protein kinase C independent induction of growth arrest and apoptosis by bryostatin 1 in a highly metastatic mammary epithelial cell line: *In vitro* versus *in vivo* activity. *Int. J. Mol. Med*, **1**, 915–923.
- Bove, K., Lincoln, D.W. and Tsan, M.F. (2002) Effect of resveratrol on growth of 4T1 breast cancer cells *in vitro* and *in vivo*. *Biochem. Biophys. Res. Commun*, **291**, 1001–1005.
- Hiraga, T., Ueda, A., Tamura, D., Hata, K., Ikeda, F., Williams, P.J. and Yoneda, T. (2003) Effects of oral UFT combined with or without zoledronic acid on bone metastasis in the 4T1/luc mouse breast cancer. *Int. J. Cancer*, **106**, 973–979.
- Michigami, T., Hiraga, T., Williams, P.J., Niewolna, M., Nishimura, R., Mundy, G.R. and Yoneda, T. (2002) The effect of the bisphosphonate ibandronate on breast cancer metastasis to visceral organs. *Breast Cancer Res. Treat*, **75**, 249–258.
- Yoneda, T., Michigami, T., Yi, B., Williams, P.J., Niewolna, M. and Hiraga, T. (2000) Actions of bisphosphonate on bone metastasis in animal models of breast carcinoma. *Cancer*, **88**, 2979–2988.
- Samoszuk, M. and Corwin, M.A. (2003) Mast cell inhibitor cromolyn increases blood clotting and hypoxia in murine breast cancer. *Int. J. Cancer*, **107**, 159–163.
- Mittal, A., Pate, M.S., Wylie, R.C., Tollefsbol, T.O. and Katiyar, S.K. (2004) EGCG down-regulates telomerase in human breast carcinoma MCF-7 cells, leading to suppression of cell viability and induction of apoptosis. *Int. J. Oncol*, **24**, 703–710.
- Roy, A.M., Baliga, M.S. and Katiyar, S.K. (2005) Epigallocatechin-3-gallate induces apoptosis in estrogen-receptor negative human breast carcinoma cells via modulation in protein expression of p53 and Bax, and caspase 3 activation. *Mol. Cancer Ther*, **4**, 81–90.
- Oltvai, Z.N., Milliman, C.L. and Korsmeyer, S.J. (1993) Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*, **74**, 609–619.
- Zhan, Q., Fan, S., Bae, I., Guillouf, C., Liebermann, D.A., O'Connor, P.M. and Fornace, A.J.Jr (1994) Induction of Bax by genotoxic stress in human cells correlates with normal p53 status and apoptosis. *Oncogene*, **9**, 3743–3751.
- Green, D.R. and Reed, J.C. (1998) Mitochondria and apoptosis. *Science*, **281**, 1309–1312.
- Thornberry, N.A. and Lazebnik, Y. (1998) Caspases: enemies within. *Science*, **281**, 1312–1316.
- Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T.I., Jones, D.P. and Wang, X. (1997) Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. *Science*, **275**, 1129–1132.
- Kluck, R.M., Bossy-Wetzel, E., Green, D.R. and Newmeyer, D.D. (1997) The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science*, **275**, 1132–1136.
- Baell, J.B. and Huang, D.C. (2002) Prospects for targeting the Bcl-2 family of proteins to develop novel cytotoxic drugs. *Biochem. Pharmacol*, **64**, 851–863.
- Goodsell, D.S. (2002) The molecular perspective: Bcl-2 and apoptosis. *Stem Cells*, **20**, 355–356.
- Sedlak, T.W., Oltvai, Z.N., Yang, E., Wang, K., Boise, L.H., Thompson, C.B. and Korsmeyer, S.J. (1995) Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. *Proc. Natl Acad. Sci. USA*, **92**, 7834–7838.
- Salomons, G.S., Brady, H.J., Verwijns-Janssen, M., Van Den Berg, J.D., Hart, A.A., Van Den Berg, H., Behrendt, H., Hahlen, K. and Smets, L.A. (1997) The Bax:Bcl-2 ratio modulates the response to dexamethasone in leukaemic cells and is highly variable in childhood acute leukaemia. *Int. J. Cancer*, **71**, 959–965.
- Wolter, K.G., Hsu, Y.T., Smith, C.L., Nechushtan, A., Xim, X.G. and Youle, R.J. (1997) Movement of Bax from the cytosol to mitochondria during apoptosis. *J. Cell Biol*, **139**, 1281–1292.
- Darmon, A.J., Nicholson, D.W. and Bleackley, R.C. (1995) Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature*, **377**, 446–448.
- Budihardjo, I., Oliver, H., Lutter, M., Luo, X. and Wang, X. (1999) Biochemical pathways of caspase activation during apoptosis. *Ann. Rev. Cell Dev. Biol*, **15**, 269–290.
- Nomoto, H., Iigo, M., Hamada, H., Kojima, S. and Tsuda, H. (2004) Chemoprevention of colorectal cancer by grape seed proanthocyanidin is accompanied by a decrease in proliferation and increase in apoptosis. *Nutr. Cancer*, **49**, 81–88.

Received January 4, 2006; revised February 26, 2006; accepted March 19, 2006