

Growth Inhibitory and Antimetastatic Effect of Green Tea Polyphenols on Metastasis-Specific Mouse Mammary Carcinoma 4T1 Cells *In vitro* and *In vivo* Systems

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ABSTRACT

Purpose: Breast cancer is the second leading cause of cancer-related deaths among females. Dietary habits may have a role in breast cancer risk and prevention as well. Here, we examined the effect of green tea polyphenols (GTP) on growth and metastasis of highly metastatic mouse mammary carcinoma 4T1 cells *in vitro* and *in vivo* systems.

Experimental Design: 4T1 cells were treated with (–)-epigallocatechin-3-gallate (EGCG), and the effect was determined on cellular proliferation, induction of apoptosis, proapoptosis, and antiapoptotic proteins of Bcl-2 family, and caspase 3 and poly(ADP-ribose) polymerase activation following 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, flow cytometry, and Western blot analysis. Anticarcinogenic and antimetastatic effect of GTP in 4T1 cells was assessed in immunocompetent BALB/c mice.

Results: Treatment of 4T1 cells with EGCG resulted in inhibition of cell proliferation, induction of apoptosis in dose- and time-dependent manner. The increase in apoptosis was accompanied with decrease in the protein expression of Bcl-2 concomitantly increase in Bax, cytochrome *c* release, Apaf-1, and cleavage of caspase 3 and PARP proteins. Treatment of EGCG-rich GTP in drinking water to 4T1 cells bearing BALB/c mice resulted in reduction of tumor growth accompanied with increase in Bax/Bcl-2 ratio, reduction in proliferating cell nuclear antigen and activation of caspase 3 in tumors. Metastasis of tumor cells to lungs was inhibited and survival period of animals was increased after green tea treatment.

Conclusion: This study suggests that GTP have the ability to prevent the development of breast cancer and its metastasis; however, further *in vivo* studies are required to identify the molecular targets.

INTRODUCTION

Breast cancer is the second leading cause of cancer related deaths among females in the United States (1). Statistics from the year 2003 indicates that incidence of breast cancer was higher in White women; however, mortality was greater in Black women (2). Studies also show that incidence of breast cancer in Asian women is less in comparison with women in Western countries. Moreover, the migration of young Asian women to the United States dramatically increases their risk and mortality from breast cancer (3, 4). In an effort to explain this phenomenon, epidemiologists have put forth various hypotheses, including differences in diet and environmental exposure to carcinogens (3, 4). Dietary comparisons of the Asian diet with that of a typical Western diet show, among many differences, that Asian population, mainly in China, Japan, Korea, and some parts of India, consume more green tea than Western countries.

Next to water, tea (*Camellia sinensis* L.) is widely consumed as a popular beverage worldwide because of its characteristic aroma, flavor, and health benefits (5, 6). Green tea polyphenols (GTP) mainly constitutes epicatechin derivatives, such as (–)-epicatechingallate, (–)-epigallocatechin-3-gallate (EGCG), (–)-epicatechin, and (–)-epigallocatechin which possess antioxidant and anti-inflammatory properties (5–7). Epidemiologic studies have indicated that consumption of green tea reduces risk of many cancers, including stomach, lung, colon, rectum, liver, breast, and pancreas cancer etc. (7–10). Epidemiologic studies also suggest that incidence of breast cancer in regions where green tea is consumed in large quantities, including China and Japan, is much lower than in Western countries (11). Furthermore, several lines of evidence from experimental studies have shown that GTP induced growth inhibitory effects on cancerous cells but does not adversely affect normal cells (12).

Breast cancer is one of the few cancers that have several active modalities available for its treatment like surgery, hormone therapy, cytotoxic therapy, and radiation therapy (13). However, all these modalities are in vain in advanced stage, where metastasis has already set and the median survival time in most conditions is not more than 2 to 3 years (13). Some studies show growth inhibitory effect of EGCG and GTP (a mixture of polyphenols) in breast cancer cells in animal models, these studies were carried out in nude mice with the aim of deciphering the mode/s of action (14). There are a number of human breast cancer lines that will metastasize in xenograft models, but none of them fully reflect the complexity of tumor progression operating in humans, because these models lack them (14–16). Once the metastasis of breast cancer occurred in the body the chances of survival is very less (17).

4T1 cells are transplantable mouse mammary carcinoma cells and are poorly immunogenic with growth characteristics and resembling exactly to that of stage IV in humans (18–21).

Received 9/27/04; revised 11/19/04; accepted 12/2/04.

Grant support: Cancer Research and Prevention Foundation.

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These cells are highly invasive and primary tumor metastasizes as early as 2 weeks (after inoculation) to lungs, liver, bone, and brain (15, 18–20). 4T1 cells have also been employed to study therapeutic effects as these cells lend itself to deciphering and confirming cellular and molecular events, and interactions among them which is of clinical relevance to humans (22, 23).

In anticarcinogenic effects of chemopreventive agents, induction of apoptosis in tumor cells plays a decisive role, and disruption of mitochondrial function plays a crucial role in apoptotic cell death of tumor cells (24–26). Therefore, for the first time we attempted to determine the chemopreventive effect of EGCG and GTP in highly metastatic breast cancer 4T1 cells in both *in vitro* and *in vivo* model systems. Here, we report that (i) treatment of EGCG to 4T1 cells resulted in induction of apoptosis which is associated with enhanced expression of Bax and activation of caspase 3 and poly(ADP-ribose) polymerase (PARP) cleavage following disruption of mitochondrial pathway, and (ii) oral administration of GTP to immunocompetent BALB/c mice inhibits tumor growth of 4T1 cells, inhibits metastasis to lungs, and increases survival period of the animals.

MATERIALS AND METHODS

Chemicals and Antibodies. Purified EGCG (>98% pure) and GTP were obtained from Mitsui Norin, Co., Ltd. (Shizuoka, Japan). Annexin V–conjugated Alexafluor488 Apoptosis detection kit was purchased from Molecular Probes, Inc. (Eugene, OR). The primary antibodies to Bax, cleaved caspase 3 and all respective secondary antibodies anti-rabbit IgG conjugated with horseradish peroxidase were purchased from Cell Signaling Technology (Beverly, MA). The mouse monoclonal antibodies for Bcl-2, Apaf 1, Cytochrome *c*, and proliferating cell nuclear antigen (PCNA) were procured from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and PARP from Upstate Cell Signaling Solutions (Lake Placid, NY).

Cell Culture Conditions. The 4T1 mouse mammary carcinoma cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in monolayers in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 100 µg/mL penicillin and 100 µg/mL streptomycin from Invitrogen (Carlsbad, CA) and maintained in humidified incubator at 37°C in a 5% CO₂ atmosphere.

Animals. Female BALB/c mice of 6 to 7 weeks old were purchased from Charles River Laboratories (Wilmington, MA) and were housed in our animal research facility. 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: 24 ± 2°C temperature, 50 ± 10% relative humidity, and 12-hour light/12-hour dark cycle. To determine the chemopreventive effect of GTP, GTP was given in drinking water (0.2% and 0.5% w/v), and was started 7 days before tumor cells inoculation and continued till end of the experiment.

***In vivo* Tumor Experiment.** The 4T1 cells were inoculated s.c. with either 1 × 10⁶ or 1 × 10⁴ viable cells in preshaved back of the mouse skin. The treatment groups of 1 × 10⁶ and 1 × 10⁴ cells were termed as high- and low-risk groups, respectively, based on the risk generated by tumor cells. Each treatment group

had 10 animals. The growth of tumor was monitored throughout the experiment and tumor size was measured regularly twice or thrice weekly using Vernier calipers. At the termination of the experiment, animals were sacrificed. At that time tumors and internal organs, such as, livers, spleens and lungs were excised from animals. The lungs were fixed in Bouin's solution for 24 hours. The number and size of metastatic tumor nodules on lungs were observed and counted under dissection microscope and the volumes were measured. The length, width, and weights of spleens were recorded to evaluate the organ toxicity. The median survival time and tumor-free survival of the mice were recorded in different treatment groups. The sick and moribund animals were euthanized and excluded from the study. The median survival time (MST) and the average survival time (AST) of the animals were calculated, as follows:

$$\text{MST} = \text{first death} + \text{last death in the group} / 2$$

$\text{AST} = \text{sum of animal death on different days} / \text{number of animals}$

The percent increase in median life span and percent increase in average life span were also calculated using the following formulae:

$$\text{Percent increase in median life span} = (\text{MST of treated mice} - \text{MST of control}) \times 100 / \text{MST of control}$$

$$\text{Percent increase in average life span} = (\text{AST of treated mice} - \text{AST of control}) \times 100 / \text{AST of control}$$

Cell Viability Assay. The effect of EGCG on the viability of 4T1 cells was determined by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazoliumbromide (MTT) assay as described previously (27–29). Briefly, ~5,000 4T1 cells per well were plated in 96-well plates and treated with or without EGCG (210–100 µg/mL) for 24, 48, and 72 hours. At the end of stipulated time following EGCG treatment, the medium was aspirated and MTT (50 µL of 5 mg/mL stock solution in PBS) was added in to each well and incubated at 37°C for another 2 hours. After centrifugation, the purple colored precipitates of formazan were dissolved in 150 µL of dimethyl sulfoxide. The color absorbance of each aliquot was recorded at 540 nm with a Bio-Rad 3350 microplate reader with a reference at 650 nm serving as blank. Effect of EGCG on cell viability was assessed as percent cell viability in terms of non-EGCG treated control cells. Control cells were considered as 100% viable.

Assay of Apoptotic Cells by Flow Cytometry. Induction of apoptosis in 4T1 cells caused by EGCG was quantitatively determined by flow cytometry using the Annexin V–conjugated Alexafluor 488 Apoptosis Detection Kit following the manufacturer's instructions. Briefly, after treatment of cells with EGCG for 24 and 48 hours, cells were harvested, washed with PBS and incubated with Annexin V Alexafluor 488 (Alexa488) and propidium iodide for cellular staining in binding buffer at room temperature for 10 minutes in the dark, as previously used (28, 29). Stained cells were analyzed by fluorescence activated cell sorting (FACSCalibur, BD Biosciences, San Jose, CA) using CellQuest 3.3 software. The early apoptotic cells stained with Alexa488 give green fluorescence and present in lower right (LR) quadrant of the fluorescence-activated cell sorting histogram, and the late apoptotic cells stained with both Alexa488 and propidium iodide gives red-green fluorescence and present in the upper right (UR) quadrant of the fluorescence-activated cell sorting histogram.

Preparation of Cell and Tumor Lysates and Western Blot Analysis. Western blot analysis was done to determine the expression of different proteins. Cells were treated with EGCG (20, 40, 60, and 80 $\mu\text{g}/\text{mL}$) for 24 and 48 hours. Cells were harvested, washed with cold PBS [10 mmol/L (pH 7.4)], and lysed with ice-cold lysis buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na_3VO_4 , 1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{mL}$ aprotinin, and 10 $\mu\text{g}/\text{mL}$ leupeptin (pH 7.4)] for 30 minutes and centrifuged at $14,000 \times g$ for 20 minutes at 4°C as detailed previously (29). The supernatant was collected and either used immediately or stored at -80°C . Similar to cell lysates, tumor lysates were also prepared. Tumor or skin tissues were collected at the termination of the experiment, minced and homogenized with homogenizer in ice-cold lysis buffer. Supernatants were collected and used to examine the expression of different proteins by Western blot analysis. The nuclear fractions were prepared as described previously (29–31). Protein concentration was determined using DC Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol.

Western blot analysis was done to analyze the expression of various proteins as described previously (29). Briefly, aliquots of equal amounts of protein (25–50 μg) from the cell or tumor lysates were subjected to SDS-PAGE electrophoresis. Thereafter, proteins were electrophoretically transferred to nitrocellulose membranes and nonspecific sites were blocked with blocking buffer [5% nonfat dry milk in 1% Tween 20 in 20 mmol/L TBS (pH 7.5)] by incubating for 1 hour at room temperature. The membranes were then probed overnight with the desired primary antibody at 4°C . After washing, membranes were incubated with the respective horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. After washing, the protein expression was detected by enhanced chemiluminescence detection systems (Amersham Life Science, Inc., Arlington Heights, IL) and autoradiography with HXR- film (Hawkins Film, Oneonta, AL). To verify equal protein loading and transfer, the blots were stripped and re probed for β -actin using an anti-actin rabbit polyclonal antibody and thereafter the same protocol was followed as detailed above. The relative intensity of each protein band in a blot was measured by using computerized software program OPTIMAS 6.2.

Immunohistochemical Detection of Cleaved Caspase 3-Positive Cells. Cleaved caspase 3^+ cells were detected in tumor or untreated skin biopsies as a marker of apoptotic cells, following the immunoperoxidase staining. Biopsies were fixed in 10% buffered formalin for not more than 24 hours and processed for paraffin block formation. After deparaffinization, the sections (5 μm thick) were stained to detect cleaved caspase 3^+ cells. Briefly, after antigen retrieval sections were treated with 3% H_2O_2 for 15 minutes to quench endogenous peroxidase. Sections were incubated with preimmune goat serum (3%) for 30 minutes followed by incubation with monoclonal antibodies for cleaved caspase 3 overnight at 4°C . After washing, sections were incubated with biotinylated rabbit anti-rat IgG (Vector, Burlingame, CA) thereafter with peroxidase labeled streptavidin for 1 hour at room temperature. After washing with PBS buffer, sections were incubated with diaminobenzidine substrate

(Kirkegaard & Perry, Gaithersburg, MD) and counterstained with methyl green (2% in HBBS buffer). The cleaved caspase 3^+ cells in different treatment groups were counted at least 6 to 8 different places in a section using Olympus microscope (Model BX40F4, Tokyo, Japan). The cleaved caspase 3^+ cells were expressed as a percent of total cells.

Statistical Analysis. The results of MTT assay are expressed as means \pm SD in terms of percent of control and statistical analysis was done by Student's *t* test. The statistical significance of difference for tumor weight, metastasis lung nodules and tumor size among different treatment groups were determined by Wilcoxon rank sum test, and statistical significance of survival of animals after green tea treatment was determined by log-rank test. A $P < 0.05$ was considered statistically significant.

RESULTS

(-)-Epigallocatechin-3-Gallate Treatment Inhibits Cell Viability in 4T1 Cells. The cytotoxic effect of EGCG on mammary carcinoma 4T1 cells was determined with varying concentration of EGCG treatment for 24, 48, and 72 hours by MTT assay. As is evident from Fig. 1 (*top*), increasing concentration and treatment time of EGCG to 4T1 cells resulted in increased inhibition of cells viability. Treatment of 10 $\mu\text{g}/\text{mL}$ concentration of EGCG did not produce any significant reduction in cell viability however treatment of higher concentrations of EGCG (20–100 $\mu\text{g}/\text{mL}$) resulted in significant dose- and time-dependent reduction in cell viability of 4T1 cells. Reduction in cell viability by EGCG treatment at the concentration of 20 to 100 $\mu\text{g}/\text{mL}$ after 24 hours ranged from 10% to 53% ($P < 0.05$ to $P < 0.001$), whereas after 48 and 72 hours ranged from 26% to 65% ($P < 0.05$ to $P < 0.001$) and 30% to 75% ($P < 0.05$ to $P < 0.001$) respectively, as shown in Fig. 1 (*top*). Based on significant reduction in cell viability of 4T1 cells after EGCG treatment, 20, 40, 60, and 80 $\mu\text{g}/\text{mL}$ concentrations of EGCG, and treatment time for 24 and 48 hours were selected for further mechanistic studies.

(-)-Epigallocatechin-3-Gallate Treatment Induces Apoptosis in 4T1 Cells. In milieu of the MTT assay results, we extended our study to examine whether breast cancer cells are undergoing apoptosis after EGCG treatment by using flow cytometry. The number of apoptotic cells was determined as late apoptotic cells shown in UR quadrant, and early apoptotic cells as shown in LR quadrant of the fluorescence-activated cell sorting histograms, as described previously (28, 29, 32).

It was observed that treatment of 4T1 cells with 20 to 80 $\mu\text{g}/\text{mL}$ of EGCG for 24 hours increased the number of early apoptotic cells (LR), respectively, from 7% to 32.5% in a dose-dependent manner compared with that of 2.2% in non-EGCG treated control cells. The number of late apoptotic cells (UR) had increased from 3.1% to 8.9% compared with that of 1.8% in non-EGCG-treated cells. The total percentage of apoptotic cells (UR + LR) were increased from 4.0% in non-EGCG-treated 4T1 cells to 41.4% in 80 $\mu\text{g}/\text{mL}$ of EGCG treatment for 24 hours (Fig. 1A–E). As expected, the induction of apoptosis was higher when cells were treated with EGCG for 48 hours (Fig. 1F–J). The number of early apoptotic cells were increased from 2.6% in

non-EGCG-treated cells to 15.3% to 40.9 % by 20 to 80 $\mu\text{g}/\text{mL}$ of EGCG treatment for 48 hours (Fig. 1*F-J*). The total percentage of apoptotic cells (UR + LR) were increased from 5.0% in non-EGCG-treated cells to 21.8% to 49.1% following the treatment of 4T1 cells with EGCG in a dose-dependent manner (20-80 $\mu\text{g}/\text{mL}$) for 48 hours. Thus, significant induction of apoptosis caused by EGCG explained the reduction in cell viability and its anticarcinogenic effect against mouse breast cancer 4T1 cells.

(-)-Epigallocatechin-3-Gallate Treatment Down-Regulates Antiapoptotic Protein Bcl-2 with a Concomitant Up-Regulation in Proapoptotic Protein Bax in 4T1 Cells.

Antiapoptotic protein Bcl-2 has been associated with cell survival and to inhibit programmed cell death, whereas increase in proapoptotic protein Bax results in apoptosis (24). As determined by Western blot analysis, treatment of 4T1 cells with EGCG resulted in dose-dependent reduction of Bcl-2 protein expression after 24 and 48 hours of treatment, as shown by the relative intensity of each band below the blot (Fig. 2*A*). The relative intensity in non-EGCG-treated control sample was considered as 1.0. The protein expression of Bax was correspondingly up-regulated from 1.0 in non-EGCG-treated cells to 5.2-fold with increasing concentrations (20-80 $\mu\text{g}/\text{mL}$) and time (24 and 48 hours) of EGCG treatment (Fig. 2*B*). It has been suggested that the ratio of Bax/Bcl-2 proteins expression plays a determinant role in transducing the signal of apoptosis (24). As shown in Fig. 2*C*, the ratio of Bax/Bcl-2 was significantly increased ($P < 0.01$ and $P < 0.001$) dose- and time-dependently after EGCG treatment, which suggested the susceptibility of 4T1 cells for apoptosis through the involvement of proteins of Bcl-2 family.

(-)-Epigallocatechin-3-Gallate Treatment Induces Mitochondrial Disruption and thus Releases Cytochrome *c*, Induction of Apaf-1, and Cleavage of Caspase 3 and Poly(ADP-ribose) Polymerase in 4T1 Cells. In mitochondrial pathway, the proapoptotic members of the Bcl-2 family, such as Bax, interact with mitochondria and direct the release of cytochrome *c*, whereas Bcl-2 prevents its release (33). After stimulation by the proapoptotic signals, cytochrome *c* is released from mitochondria into the cytosol and binds to Apaf-1, and leads to the activation of caspase 9. The initiator caspases then stimulate the effector caspases, such as caspase 3, which are the

executioners of apoptosis and are responsible for the degradation of other cellular proteins (e.g., cytoskeletal proteins, PARP; ref. 26). Therefore, we determined the effect of EGCG on the expression of proteins associated with the mitochondrial disruption. As shown in Fig. 3, EGCG treatment resulted in a marked increase in cytochrome *c* (*A*), induction of Apaf-1 (*B*), and activation or cleavage of caspase 3 (*C*) and PARP (*D*) in a dose- and time-dependent manner (Fig. 3). The cleaved caspase 3 (19 and 17 kDa) and PARP (116, 85, and 62 kDa) are the

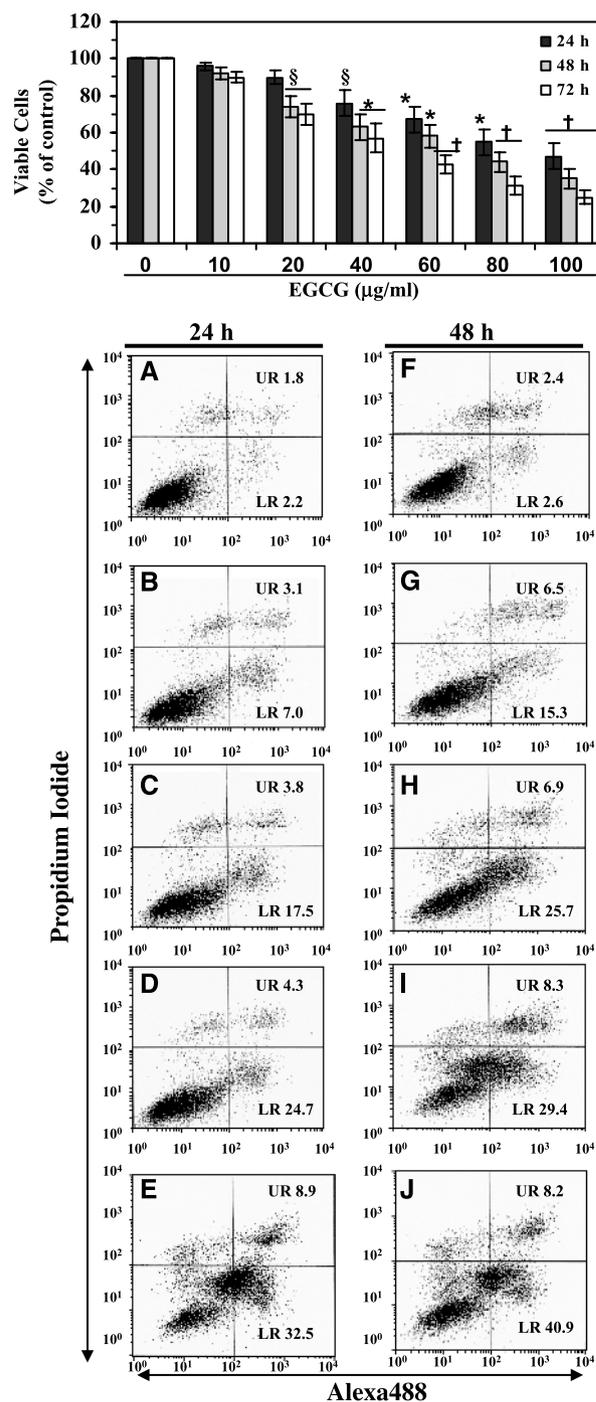


Fig. 1 EGCG inhibits cellular proliferation and induces apoptosis in mouse mammary cancer 4T1 cells. EGCG inhibits proliferation and cell viability of 4T1 cells in a dose- and time-dependent manner (*top*). Inhibitory effect of EGCG on cell viability of 4T1 cells was determined by the MTT assay as described in Materials and Methods. Columns, mean % viable cells of eight replicates; bars, \pm SD. §, $P < 0.05$ versus control (non-EGCG); *, $P < 0.01$ versus control; †, $P < 0.001$ versus control. EGCG induced apoptosis in 4T1 cells was determined by the flow cytometry using Annexin V-Alexa Fluor 488 (Alexa488) Apoptosis Vybrant Assay kit following the manufacturer's protocol (*bottom*). Apoptosis was determined after 24 and 48 hours of EGCG treatment. *A* and *F*, control cells (non-EGCG treatment). Cells in *B*, *C*, *D*, and *E* were treated with EGCG (20, 40, 60, and 80 $\mu\text{g}/\text{mL}$, respectively) for 24 hours, and cells in *G*, *H*, *I*, and *J* were treated with EGCG (20, 40, 60, and 80 $\mu\text{g}/\text{mL}$, respectively) for 48 hours, as detailed in Materials and Methods. Cells undergoing early apoptosis are shown in LR quadrant (Alexa488-stained cells) and late apoptotic cells are shown in UR quadrant of the FACS histogram (Alexa488 + propidium iodide-stained cells).

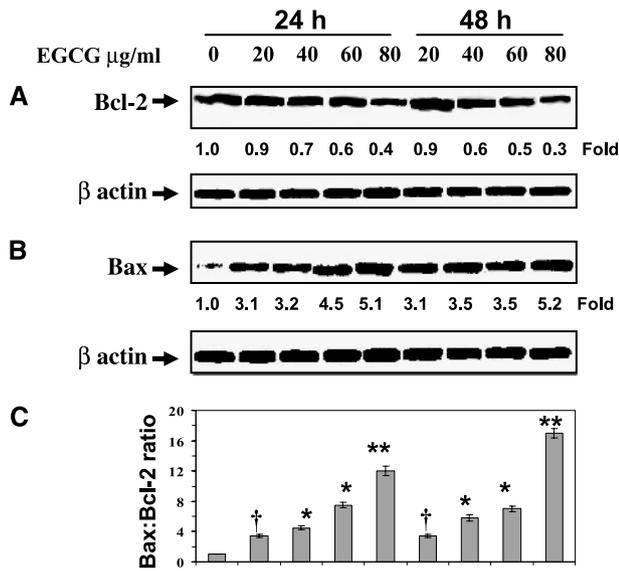


Fig. 2 Treatment of EGCG decreases the expression of antiapoptotic protein Bcl-2 (A), and increases the expression of proapoptotic protein Bax (B) in mouse mammary carcinoma 4T1 cells. 4T1 cells were starved in 0.5% FBS/DMEM overnight and then treated with EGCG (20–80 μ g/mL) in serum containing media for another 24 and 48 hours. Cell lysates were prepared, and the expression of the proteins was determined by the Western blot analysis using the corresponding antibodies, as detailed in Materials and Methods. Representative blot from three independent experiments with identical results. Relative intensity of each band after normalization with the intensity of β -actin in a blot (below each Western blot). The ratio of Bax and Bcl-2 protein expression was determined from three separate experiments by comparing the relative intensities of protein bands. Columns, mean; bars, \pm SD (C). β -Actin was used as an internal control to monitor equal protein loading and transfer of proteins from gel to the membranes after stripping them and reprobing them with the actin antibody. †, $P < 0.05$ versus control (non-EGCG); *, $P < 0.01$ versus control; **, $P < 0.001$ versus control.

characteristic hallmarks of apoptosis which were observed in this system. These observations further support the involvement of disruption of mitochondrial pathway in EGCG-induced apoptosis in mouse breast cancer 4T1 cells.

Green Tea Polyphenol Inhibits *In vivo* Growth of 4T1 Breast Cancer Cells and Metastasis to Lungs in BALB/c Mice. *In vivo* animal experiments are considered as gold standard in chemopreventive studies, as they give clear indication on pharmacologic and therapeutic effect of chemopreventive agents, which can then be extrapolated to humans. Therefore, in order to confirm the applicability of our *in vitro* observations we studied the chemopreventive effect of purified GTP, which was rich in EGCG content (60%). It was observed that the tumor growth caused by 4T1 cells was delayed in GTP fed animals compared with non-GTP fed control animals. In the high-risk group (Fig. 4A), tumor appearance was observed on day 3. Administration of GTP delayed the appearance by 3 days in both 0.2% and 0.5% GTP fed groups. In the low risk group (C), the growth of tumor was observed on day 13 in 0.5% GTP treatment group thus delayed their appearance by 7 days compared with non-GTP-fed animals. Furthermore, GTP administration decreased the rate of tumor growth as evaluated

by measuring tumor volume at regular intervals. In high-risk group (Fig. 4A), administration of 0.2% and 0.5% GTP resulted in 42% ($P < 0.01$) and 60% ($P < 0.005$) inhibition in tumor volume, respectively, when recorded at the termination of the experiment. Similarly, GTP significantly inhibited tumor growth in low-risk group (90%, $P < 0.001$) at day 40 when tumor yield was maximum in non-GTP-fed group (Fig. 4C). It was also observed that feeding of GTP inhibited the toxicity in spleens that may be caused by tumor metastasis. Because of tumor toxicity, the size of the spleens was increased, and GTP treatment prevented this toxic effect which was evident from the reduction

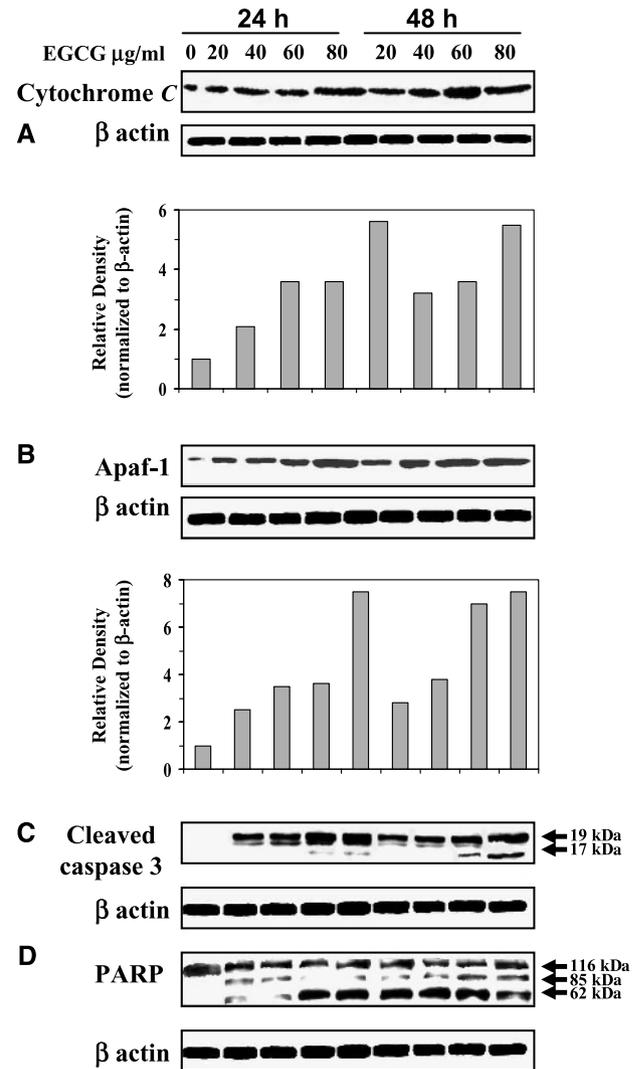


Fig. 3 Treatment of EGCG increases the release of cytochrome *c* (A), expression of apoptotic protease-activating factor-1 (Apaf-1, B), and cleaved caspase 3 (C) in 4T1 cells. Antibody for caspase 3 specifically recognizes the cleaved products of caspase 3 (19 and 17 kDa). Treatment of EGCG also induces the cleavage of PARP (D). Representative blot from three independent experiments with identical results. Cells were cultured as described in Fig. 2, and protein levels were analyzed by Western blot analysis as detailed in Materials and Methods. Relative intensity of bands in each panel of cytochrome *c* and Apaf-1 was determined (below each respective panel) after normalization with β -actin bands.

in spleen's length (30-53%) and width (32-75%) when observed after 16 and 30 days of treatment. The tumor wet weight of animals at the termination of the experiment was taken in high risk group, and it was found that tumor weight in GTP-fed group (0.2% and 0.5%) was reduced by 16% and 42% ($P < 0.01$) after 16 days animal protocol, whereas 24% and 53% was reduced after 30 days of treatment of 0.2% and 0.5% of GTP, respectively, than that of non-GTP-fed group of animals, as shown in Table 1.

Oral administration of GTP also increased survival time of the animals demonstrating its overall chemopreventive effect (Fig. 4B and D). In high-risk control group, the first death of animal was observed on day 27 and all animals died on day 30 post-tumor inoculation (Fig. 4B). The MST was found to be 28.5 days, whereas AST was 28.3 days in high risk group (Fig. 4). Administration of 0.2% GTP did not significantly alter the MST (31 days) and AST (31 days) when compared with control. However, significant chemopreventive effect was observed in 0.5% GTP-fed animals ($P < 0.001$), where the MST and AST increased up to 34.5 days (Fig. 4B). In low-risk group (Fig. 4D), administration of 0.5% GTP resulted in enhancement in MST and AST ($P < 0.001$) when compared with non-GTP-fed animals. MST was found to be 47.5 days whereas the AST was 45.7 days in GTP-fed group compared with 32.5 and 32.6 days, respectively, in non-GTP-fed animals.

As 4T1 tumor cells metastasize relatively early from primary tumor growth, two time points (after the 16th and 30th days post tumor inoculation time) were selected to examine this effect in separate sets of experiment. As shown in Table 1, the administration of 0.2% and 0.5% GTP resulted in reduction of number of metastatic tumor nodules by 25% ($P < 0.05$) and 50% ($P < 0.01$) respectively after 16 days treatment, and 19% and 43% ($P < 0.01$) reduction was observed after 30 days of GTP treatment. Additionally, the size of the metastatic lung nodules was also reduced by 32% ($P < 0.05$) and 42% ($P < 0.01$) after 0.2% and 0.5% after 30 days of GTP treatment compared with non-GTP-treated animals, as shown in Table 1. Total primary tumor wet weight on the mouse skin was found to be reduced by 16% and 42% ($P < 0.01$) after 16 days whereas 24% and 53% ($P < 0.01$) reduction in tumor weight was observed after 30 days of 0.2% and 0.5% GTP treatment, respectively (Table 1).

Green Tea Polyphenol Administration Down-Regulates the Expression of Bcl-2 and Up-Regulates the Expression of Bax Protein in Tumors in BALB/c Mice. Furthermore, we were interested to examine the effect of GTP on the apoptotic proteins involved in mitochondrial disruption pathway in *in vivo* tumor development similar to that observed in *in vitro* system. This study was extended to high-risk groups. As shown in Fig. 5,

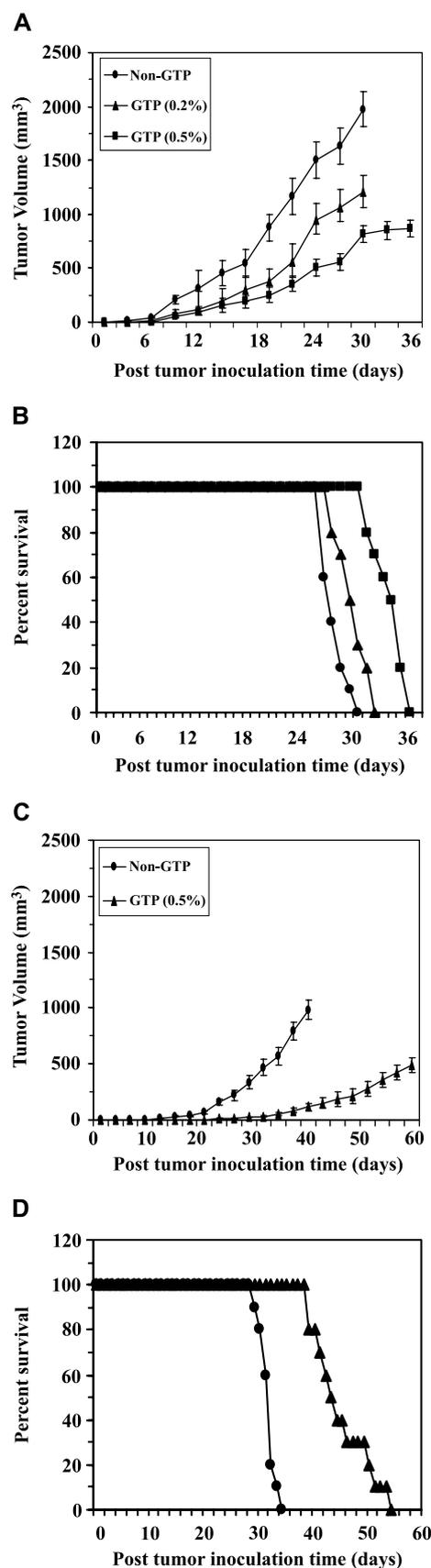


Fig. 4 Administration of GTP (0.2% and 0.5%, w/v) in drinking water inhibits the growth of mouse mammary carcinoma 4T1 cells (A and C), and increases the survival period of the BALB/c mice (B and D). 4T1 tumor cells were inoculated either 1×10^6 (high-risk group, A and B) or 1×10^4 (low-risk group, C and D) to the right flank of each mouse, as detailed in Materials and Methods. Experiments done for 36 days (A and B) and 60 days (C and D). Tumor volumes were recorded on regular basis to determine the chemopreventive effect of GTP on 4T1 tumor cells growth. % Survival of animals was recorded post-tumor cells inoculation.

Table 1 Effect of oral administration of GTP on the tumor development and 4T1 tumor cell metastasis in lungs of BALB/c mice

Treatment groups	After 15 d			After 30 d		
	Tumor wet weight (g)	No. metastatic lung nodules/mouse	Diameter of metastatic lung nodules (mm)	Tumor wet weight (g)	No. metastatic lung nodules/mouse	Diameter of metastatic lung nodules (mm)
Normal (no treatment)	—	—	—	—	—	—
GTP (0.5%) alone	—	—	—	—	—	—
4T1 (GTP 0%)	3.1 ± 0.8	4.0 ± 1.0	1.1 ± 0.2	4.5 ± 0.3	37 ± 6	3.1 ± 0.5
4T1 + GTP (0.2%)	2.6 ± 0.8 (16)	3.0 ± 0.5 (25)*	0.9 ± 0.2 (18)	3.4 ± 0.3 (24)*	30 ± 6 (19)	2.1 ± 0.5 (32)*
4T1 + GTP (0.5%)	1.8 ± 0.7 (42)†	2.0 ± 0.5 (50)†	0.6 ± 0.2 (45)†	2.1 ± 0.3 (53)†	21 ± 5 (43)†	1.8 ± 0.5 (42)†

NOTE. One million mouse breast cancer 4T1 cells were inoculated on right flank of each mouse and considered as a high-risk group. Mice were sacrificed after 15 and 30 days of tumor cell inoculation and observations were recorded at the same time. Each treatment group has 10 mice.

The data in parentheses indicate % inhibition by GTP treatment.

*Significant versus non-GTP-fed animals ($P < 0.05$).

†Significant versus non-GTP-fed animals ($P < 0.01$).

Western blot analysis revealed that oral administration of GTP down-regulated the expression of antiapoptotic protein Bcl-2 (A), whereas increased the expression of proapoptotic protein Bax (B). The increase in the ratio of Bax/Bcl-2 (C) in *in vivo* tumors suggested the susceptibility of tumor cells for apoptosis, and this may be the reason that tumor growth was blocked or inhibited in GTP-treated BALB/c mice.

Green Tea Polyphenol Administration Inhibits the Surrogate Markers of Proliferation and Apoptosis (Caspase 3) in 4T1-Induced Tumors in BALB/c Mice. As treatment of EGCG inhibited the cell proliferation and viability in *in vitro* system, we examined the effect of GTP on the marker of cell proliferating in tumors by assessing the protein expression of proliferation cell nuclear antigen (PCNA). PCNA is a requisite auxiliary protein for DNA polymerase δ -driven DNA synthesis. Western blot analysis revealed that PCNA expression was increased by >5-fold in tumors in comparison with age-matched normal skin of the mice. The administration of GTP inhibited the expression of PCNA in developing tumors as compared with non-GTP-treated animals (Fig. 5D). As determined by densitometric analysis of bands, the expression of PCNA was decreased by about 70% in tumors of those mice that were given GTP in drinking water. Similarly, we examined the expression of activated caspase 3 in tumors because cleaved caspase 3 is considered as a hallmark of apoptosis. As determined by Western blot analysis, the level of cleaved caspase 3 in tumors was markedly increased in GTP-fed animals compared with non-GTP-fed animals (Fig. 5E). The expression of basal level of caspase 3 was not detectable in normal mouse skin because the antibodies that we used only recognize cleaved caspase 3. Furthermore, the induction of apoptosis in *in vivo* tumors was confirmed by immunohistochemical detection of cleaved caspase 3⁺ cells in tumors and skin biopsies from untreated mice. As shown in Fig. 5F, the percent of cleaved caspase 3⁺ cells in GTP-treated tumors were >3-fold in comparison to non-GTP-treated tumors. These observations support the evidence that administration of GTP inhibited tumor growth probably through the induction of apoptosis in 4T1 tumor cells. The administration of GTP alone did not affect the expression of PCNA and activation of caspase 3 in the skin of normal mice. These observations in *in vivo* tumors further support the involvement of mitochondrial pathway in GTP-induced apoptosis in highly metastatic breast cancer 4T1 cells.

DISCUSSION

WHO and current cancer statistics revealed that breast cancer is the most common malignancy affecting women all over the world (1, 34). In normal practice, surgery and radiation therapy are the local treatments to reduce the risk of cancer in the breast, chest wall, and regional lymph nodes, whereas chemotherapy and hormonal therapy are the systemic treatments to reduce recurrences and overall mortality (35, 36). However, patients receiving radiation and chemotherapy experience treatment-induced adverse effects, which are a major hindrance towards successful treatment. Furthermore, the best possible treatment is mostly not effective in advanced stages where metastasis has already occurred. Therefore, there is an imperative need to develop such chemopreventive agents that are nontoxic or less toxic and should be effective at metastasis stages also. In this regard, the dietary botanicals have attracted considerable attention because of their intriguing biological activities at nontoxic levels. A survey assessing the frequency of use of alternative therapies in postmenopausal women indicated that 12% of the postmenopausal women without a history of breast cancer, and 23% of postmenopausal women with a history of breast cancer used complementary and alternative medicines (37). Epidemiologic and laboratory studies have shown that consumption of green tea reduces the incidence of cancers including breast in humans (38). However, the information on the prevention of metastatic spread of breast tumor cells and their mechanism is lacking.

We observed that EGCG treatment resulted in dose- and time-dependent inhibition of cell viability and induction of apoptosis in 4T1 cells (Fig. 1). This information shows that inhibition of cell viability may be in part due to induction of apoptosis in 4T1 cells. Apoptosis plays a crucial role in eliminating the mutated preneoplastic and hyperproliferating cells from the system. Thus, induction of apoptosis in tumor cells may be considered as a protective mechanism against development and progression of cancer. Apoptosis is modulated by antiapoptotic and proapoptotic effectors, which involve a large number of proteins. Therefore, to gain insight in to mechanisms controlling apoptosis, we looked at the effect of EGCG on proapoptotic and antiapoptotic proteins of the Bcl-2 family. The proteins of Bcl-2 family play an important role in induction of apoptosis and are considered as a target for anticancer therapy (39, 40). Bcl-2, an oncoprotein, functions as a suppressor of apoptosis, a fact valued when its down-regulation causes tumor

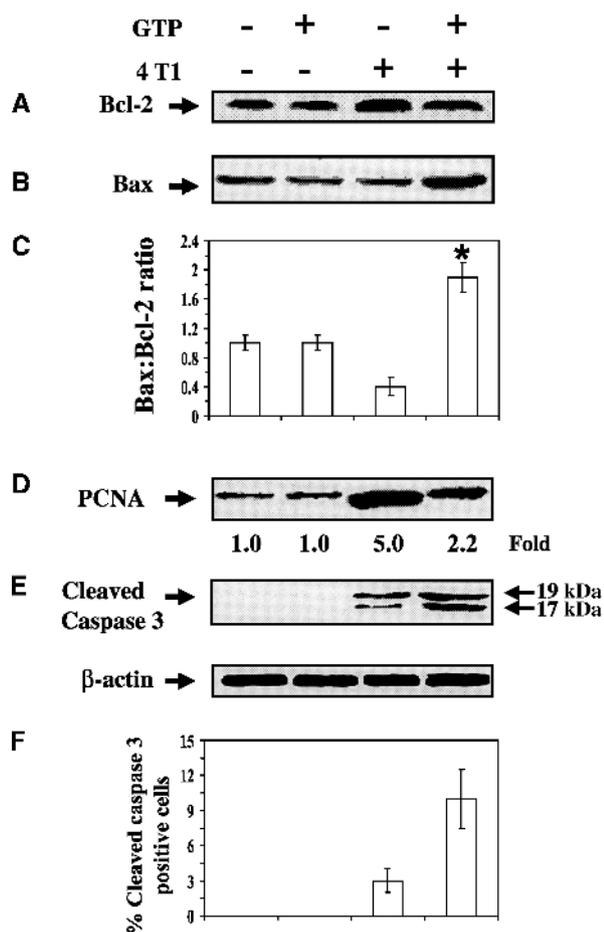


Fig. 5 Administration of GTP in drinking water (0.5%, w/v) to BALB/c mice in high risk group down-regulates the expression of antiapoptotic protein Bcl-2 (A) and up-regulates proapoptotic protein Bax (B) in 4T1 tumors. The ratio of Bax and Bcl-2 proteins expression was determined from three separate experiments by comparing the relative intensities of protein bands. Column, mean; bars, \pm SD (C). Administration of GTP inhibits the expression of PCNA (D) and increased cleaved caspase 3 (E) in 4T1 tumors. Skin lysates from normal mouse (non-GTP treated and/or non-4T1 cells), GTP alone administration in drinking water (0.5%) to mice, and tumor lysates (from 4T1 cells alone and GTP + 4T1 cells groups) were prepared similar to cell lysates and the expressions of proteins were examined by Western blot analysis, as detailed in Materials and Methods. Western blot analysis was repeated thrice by taking skin and tumor lysates from two mice each time; thus, tumors and skin lysates were prepared from six animals in each group. Representative blot from three independent experiments with identical results. *, $P < 0.001$ versus control (non-GTP).

regression (33, 41). Although Bax is a proapoptotic protein and its predominance over Bcl-2 promotes apoptosis (42, 43). Studies have also shown that the ratio of Bax to Bcl-2 proteins increases during apoptosis (24, 41). We found that treatment of EGCG to 4T1 cells resulted in reduction of Bcl-2 protein expression (Fig. 2), whereas increases the expression of Bax (Fig. 2), indicating that the increased ratio of Bax/Bcl-2 proteins (Fig. 2C) may be responsible for the induction of apoptosis in 4T1 cells.

The mitochondrion is a prominent participant in apoptosis and the proapoptotic Bax protein plays an essential role for onset of mitochondrial dysfunction (44). The intracellular movement of Bax induces release of cytochrome *c* through openings in the

outer membrane, formed as a consequence of permeability transition and loss of mitochondrial membrane potential (44). The released cytochrome *c* forms an "apoptosome" of Apaf-1, cytochrome *c*, and caspase-9, which subsequently cleaves the effector caspase 3 (45). In our *in vitro* system, EGCG caused a dose- and time-dependent increase in levels of cytochrome *c*, the adaptor Apaf-1 and activated cleaved caspase 3 (Fig. 3). The activated caspase 3 is the key executioner of cell apoptosis. Activated caspase 3 cleaves intracellular proteins vital to cell survival and growth, such as PARP, and this has been used as an important marker of apoptosis (46). From the present observations it can be inferred that PARP cleavage was very prominent (Fig. 3D) and therefore indicates the involvement of caspase 3 and PARP in induction of apoptosis in 4T1 cells caused by EGCG. Thus, the data obtained in the present study strengths our conviction that EGCG mediates apoptosis via mitochondrial disruption pathway.

Studies have shown that polyphenols from green tea have antitumor and antimetastatic activity in animal xenograft and allograft models, suggesting a possible therapeutic potential (47, 48); however, studies with normal animals which have active immune system are lacking. In view of this fact, we investigated whether GTP can prevent tumor development *in vivo* immunocompetent mouse model following the mitochondrial pathway. For this purpose, we used purified mixture of GTP that has EGCG as a major component. The use of GTP in *in vivo* system seems more practical and relevant because it can be easily available in day-to-day life from the green tea beverage and cost effective in comparison to purified EGCG. Our observation clearly indicates that *in vivo* treatment of GTP in drinking water (0.2% and 0.5%, w/v) to BALB/c mice significantly inhibited tumor growth caused by inoculation of viable 4T1 cells (Fig. 4A and C), and simultaneously increased both the median and average survival time of the mice compared with non-GTP-fed mice (Fig. 4B and D). Additionally, GTP administration was also resulted in reduction of toxicity in internal organs as was observed in spleen and liver.

4T1 cells are documented to be very aggressive and primary tumors that have been established for 2 to 3 weeks in BALB/c mice typically metastasize to the lymph nodes, lungs, and livers, whereas primary tumor is in place (49). It is also reported that death in recipient animals is due to metastasis and not due to the primary tumor (49). We observed that administration of GTP in drinking water to BALB/c mice inhibited metastasis which was determined by counting the number and size of the metastatic tumor nodules in lungs (Table 1), and this may be the reason that animals were survived more than non-GTP-fed animals (Fig. 4B and D).

We further emphasize our examination on the effect of GTP on different surrogate markers of apoptosis in *in vivo* tumors to confirm that the mechanism which was observed in *in vitro* system is also occurring in *in vivo* tumors. We observed that administration of GTP increased the ratio of Bax/Bcl-2 in tumors suggesting the role of these proteins in prevention of tumor development. PCNA, a subunit of DNA polymerase, plays a crucial role in DNA synthesis and serves as a biomarker of proliferation. GTP treatment inhibited cell proliferation in 4T1 cells-induced breast cancer tumors as evident by the inhibition of PCNA expression in tumors (Fig. 5D), suggesting the possible

role of *in vivo* antiproliferating effect of GTP. As the cleaved caspase 3 is considered as the key executioner of apoptosis, GTP treatment increased the activation of caspase 3 in 4T1 tumors in BALB/c mice which is evident from Western blot analysis (Fig. 5E) and cleaved caspase 3⁺ cells (Fig. 5F). These observations suggest that GTP might involved in chemoprevention of breast cancer and their metastatic spread through disruption of mitochondrial pathway, as summarized in Fig. 6. It is often a point of interest to suggest the amount of consumption of green tea on per day basis for the prevention of cancer. Usually a cup of green tea contains about 300 to 350 mg of EGCG. Epidemiologic and experimental studies suggest that consumption of six to seven cup of green tea per day should be sufficient to prevent from the cancer risk in humans. The doses of EGCG and GTP used in this study are in agreement with the suggested consumption of green tea.

In summary, the *in vitro* and *in vivo* findings of our study suggest that EGCG or GTP induces apoptosis and inhibits tumor development and metastasis of highly metastatic mouse breast cancer cells through disruption of mitochondrial

pathway (as summarized in Fig. 6). This observation holds promise for further *in vivo* detailed and molecular target oriented studies to examine the chemopreventive efficacy of green tea against breast cancer in animal model and high-risk women population.

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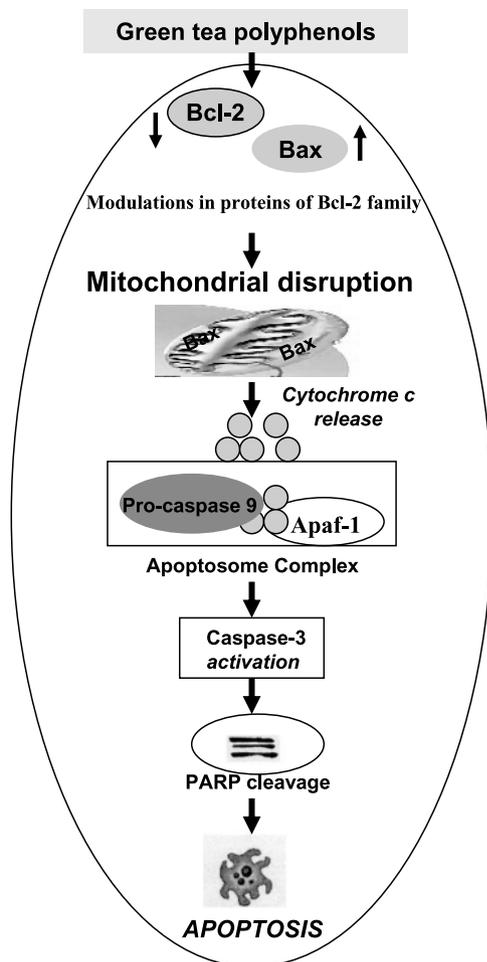


Fig. 6 Schematic diagram depicts the proposed model for EGCG/GTP-induced apoptosis in mouse breast carcinoma 4T1 cells *in vitro* and *in vivo* systems, which may result in prevention of 4T1 tumor growth in BALB/c mice. ↑, up-regulation; ↓, down-regulation of Bax and Bcl-2 proteins.

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