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Full Length Research Paper

Antiproliferative effect of *Lactobacillus helveticus* and gamma radiation on the mammary carcinogenesis

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Immunotherapy play important role in cancer prevention and treatment. Low dose of gamma radiation also probiotics and fermented foods activate immune functions leading to tumor growth delay. This study aimed to investigate the role of milk fermented by *Lactobacillus helveticus* and low dose of gamma irradiation (0.25 Gy) in the protection and treatment of mammary tumor cells. Fifty six female rats were divided into 7 groups (n=8): Group 1: control, Group 2: received fermented milk with *L. helveticus* (FM), group 3: whole body rats exposed to gamma radiation, group 4: rats i.p. injected with 17β-estradiol (E2), group 5: rats received FM for 15 days, then injected with E2 and continued receiving FM, group 6: rats exposed to gamma radiation and injected with E2, group 7: rats received FM for 15 days, exposed to gamma radiation and injected with E2 while receiving FM. The results of this study showed that E2 induced negative effects on immune and antioxidant parameters, showed a reduction in CD4, CD8 count, GSH level, GPx, CAT and SOD activities as well as an increase in the proliferation marker Ki-67. In addition, hyperplasia appeared in the histological examinations of mammary tissue epithelium. Milk fermented by *L. helveticus* and combined treatment of fermented milk and low dose of gamma radiation significantly increased CD4 and CD8 count, ameliorated GSH level, GPx, CAT and SOD activities and reduced ki-67% , showing normal histological mammary gland tissue compared to the control. This study demonstrated the immunoregulatory, antioxidant capacity of milk fermented by *L. helveticus* and low dose of gamma radiation in preventing carcinogenic effect of E2 on breast tissue and restoration tissue of histological texture.

Key words: Estradiol, low dose gamma radiation, CD4, CD8, Ki-67, antioxidant parameters, lipid peroxidation, nitric oxide.

INTRODUCTION

Breast cancer is one of the most common cancers in women and many dietary factors are related with this disease either positively or negatively (Esther et al., 2011). Estrogens have been implicated to be complete carcinogens through a mechanism involving oxidative stress in the kidney, liver and breast tissues (De Le-Blanc et al., 2005).

Numerous research studies have focused on anticancer property as protective adjuncts against a host of diseases. Data from epidemiological and experimental studies have also indicated that the ingestion of certain Lactic acid bacteria strains or its fermented dairy products might alleviate the risk of certain type of cancers and inhibit tumor growth (Liu and Pan, 2010). LAB and other probiotic organisms in fermented milks have been shown to be beneficial to the immune system of the consumer and to increase the resistance to neoplasia and infections. For these and other reasons, there is a steady increase in the consumption of fermented dairy products containing viable microorganisms (Kato, 2000; De Le-Blanc et al., 2005).

Cellular detoxification, DNA reduction, decreasing the probability of neoplastic transformation, tumor growth

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delay, antimetastatic effects and sensitization of tumor could be achieved via exposure to low doses of ionizing radiation (Hosoi, 2006; Redpath and Elmore, 2007; Feinendegen et al., 2008). Such exposures may also enhance immune reactions and attenuate harmful effects of higher doses of radiation (Safwat, 2000; Safwat et al., 2003). These mechanisms may explain that the incidence of leukemia and some solid tumors has been reported to be lower in nuclear workers and in the survivors of the Hiroshima and Nagasaki bombings whose absorbed doses did not exceed 0.25 Gy, compared to the respective control groups (Matanoski et al., 1990; Katayama et al., 2002).

Previous studies have shown that compounds released during milk fermentation by *Lactobacillus helveticus* are implicated in the antitumor effect of fermented milk (De Le-Blanc et al., 2005). There are also studies on the beneficial effects of fermented products in the prevention of different types of cancer such as colon cancer showing an inhibition of tumor growth during cyclical yoghurt feeding. This effect was observed by long-term cyclic yoghurt consumption, which inhibited promotion and progression of the experimental intestinal tumor (Brady et al., 2000; De Le-Blanc et al., 2005).

Thus, this study aimed to evaluate the effects of *L. helveticus* fermented milk consumption on the immune, antioxidant responses and a tumor marker of estradiol dependent breast hyperplasia.

MATERIALS AND METHODS

Preparation of fermented milk with *Lactobacillus helveticus* (FM)

Lactobacillus helveticus (*L. helveticus*) ATCC 15009 were obtained from Microbiological Resources Center (MIRCEN, Cairo, Egypt). Milk used was sterile non-fat milk by autoclaving at 115 °C for 15 min and yeast extract (0.4%) was added to the milk used to grow *L. helveticus* before autoclaving. Sterile milk was inoculated with *L. helveticus* (2% vol/vol) and incubated statically at 37 °C for 17 h. Fermented milk had a concentration of $1x10^9$ cfu/ml at the end of the fermentation period (De Le-Blanc et al., 2005).

Animals and treatments

Female adult Wistar albino rats weighing 100 ±10g were housed in the animal house of National Centre of Radiation Research and Technology (NCRRT) (Cairo, Egypt), in a controlled temperature, humidity and lighting conditions. This facility is accredited by the American Association for ACI rat. Accreditation of Laboratory Animal Care and is operated in accordance with the standards outlined in Guide for the Care and Use of Laboratory Animals (DHHS publication 85-23), and received food and water ad libitum. Animals were divided into seven groups (n=8): group 1 (control): rats were received 1 mL of 0.9% saline by gavages, group 2 (FM): rats received fermented milk with *L. helveticus* 1 mL/kg body weight/day by gavages, group 3 (Radiation): whole body rats were exposed to 0.25 Gy of gamma radiation, group 4 (E2): rats were i.p. injected once with E2 (50 mg/kg body weight) (17β-estradiol of Sigma Chemical Co. (St. Louis, MO, USA), group 5 (E2+ FM): rats were received FM 1 mL/kg body weight/day, for 15 days then injected with E2 (50 mg/kg body weight) and continued receiving FM, group 6 (E2+radiation): rats were exposed once to gamma radiation (0.25 Gy) and injected with E2 (50 mg/kg body weight) and group 7 (E2+radiation+ FM): rats were received FM for 15 days then exposed once to gamma radiation (0.25 Gy) then injected once with E2 (50 mg/kg body weight) and continued receiving FM for 28 days.

Irradiation

Whole-body gamma irradiation was performed at the National Centre for Radiation Research and Technology (NCRRT), using ^{137}Cs Gamma Cell-40 biological irradiator. Animals were exposed to a single dose of (0.25Gy) gamma radiation at a dose rate of 0.006 Gy/s.

Antioxidant assays

Antioxidant parameters were measured in the liver homogenate (10 % in 0.9 % saline). Glutathione (GSH) content was measured calorimetrically according to the method described by (Beutler et al., 1963**).** Glutathione peroxidase activity was measured calorimetrically according to the method described by (Gross et al., 1967**).** Superoxide dismutase activity was measured according to the method described by (Minami and Yoshikawa 1979) and catalase activity was measured calorimetrically according to the method described by (Sinha, 1972).

Determination of CD4, CD8 and Ki-67

FACS calibar flow cytometer in Mansoura Children Hospital was used (Becton Dickinson, sunnyvale, CA, USA) equipped with a compact air cooked low power 15 m watt Argon ion laser beam (488 nm). The average number of evaluated nuclei per specimen 20.000 and the number of nuclei scanned were 120 per second. CD4 and CD8 histogram were obtained by a mathematical analysis according to Dean and Jett, (1974)**.** Determination of cell proliferation marker (Ki-67) in breast tissue was determined using Anti-Ki-67 antigen conjugate (Monoclonal Mouse Anti-Human Ki-67 Antigen, Clone Ki-67) according to methodology described by (Tribukati,1984).

Evaluation of apoptosis and cell cycle analysis

Flow cytometric analysis was performed for cell cycle analysis and evaluation of apoptosis via DNA stained with propidium iodide. As the DNA content is duplicated prior to cell division, mathematical models can estimate the percentage of cells in different phases of the cell cycle (G0/1, S-phase, G2/M). Data analysis was conducted using DNA analysis program MODFIT (verity software house, Inc. Topsham, ME 04086 USA, version: 2.0.

Histopathological study

Sections of mammary glands were stained with hematoxylin & eosin (H&E) and examined by light microscope (Banchroft et al., 1996).

Statistical analysis

The values representing mean ± standard deviation (SD), values were considered statistically significant if the P value was less than or equal 0.05. Comparisons among the different groups were carried out by ANOVA tests using SPSS (statistical package for social sciences, 1999; ver.10.0).

RESULTS

To evaluate the effect of FM and gamma radiation on antioxidant state glutathione (GSH) level and glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) activities were assayed. The results showed that E2 injection significantly decreased GSH and CAT activity with significant increase in GPx and SOD activities compared to the control. Oral intake of FM with low dose of gamma irradiation significantly ameliorated GPx, CAT and SOD activities, while a marked ameliorative effect in antioxidant parameters was the result of combined treatment of FM and gamma radiation in E2 treated rats compared to E2 group (Table 1).

To determine the effect of FM and gamma radiation on oxidative stress parameters in E2 treated group, lipid peroxidation and nitric oxide levels were measured. Estradiol significantly increased lipid peroxidation (MDA) and nitric oxide (NO) levels which were ameliorated by FM intake or combined treatment of FM and gamma radiation when compared to E2 treated group (Table 2).

Estradiol treatment significantly decreased CD4 and CD8 percentage count which was ameliorated by pretreatment with FM compared to E2 group. Low dose of gamma radiation induced CD4 and CD8 count percentage compared to control (Figure1).

To study the effect of FM and gamma irradiation on cell cycle of mammary gland tissue induced hyperplasia by E2, mammary tissue cells were analyzed by flow cytometr and the results shows that, E2 markedly increase cell count percentage of most phases compared to that of control. Pretreatment with FM also gamma irradiation and combined treatment of FM and gamma irradiation ameliorated G0, S and M phases (Table 3).

Estradiol induced proliferation marker ki-67 percentage and apoptosis, FM significantly reduced Ki-67 marker with an increase in apoptotic cell and caused reduction in growth index compared to E2 group, while combined treatment with low dose of gamma radiation markedly caused reduction in ki-67 marker with same growth index as that of FM alone, (Figure 2).

Histopathological study by light microscope showed, female rat healthy mammary gland distinguished with acini and lactiferous ducts as a control, FM treated group also gamma irradiated rats showed no observed alteration with normal histological structure of the acini and lactiferous ducts. Estradiol treatment caused dysplatic and anaplastic alterations associated with necrosis, also there was hyperplasia in the lining epithelium of the lactiferous duct. Rats supplemented with FM before and after E2 injection with radiation exposure showed active acini and duct system and well differentiated lining epithelium, (Figure 3).

DISCUSSION

Spontaneous tumors frequently express antigens that can be recognized by the immune system. Specific CD4 with CD8 cells can reject and deal with tumor formation in the host body (Klein et al., 2003). Numerous research studies have focused on probiotics anticancer property exerting resistance to neoplasia and infections (Kato, 2000). Data from epidemiological and experimental studies have also indicated that the ingestion of certain LAB strains or its fermented dairy products might alleviate the risk of certain type of cancers and inhibit the growth of tumors (Liu and Pan 2010).

Absorption of low doses of ionizing radiation may stimulate cellular detoxification and repair mechanisms leading to reduction of the DNA damage even below the spontaneous level and decreasing the probability of neoplastic transformation (Redpath and Elmore 2007). Such exposures may also enhance immune reactions of the organism and attenuate harmful effects of higher doses of radiation (Safwat, 2000, Safwat et al., 2003). These mechanisms may explain that nuclear workers and in the survivors of the Hiroshima and Nagasaki bombings whose absorbed doses did not exceed 0.25 Gy, the inc-

Table 1. Effect of fermented milk by *L. helveticus* and gamma radiation on GSH level, GPx, CAT and SOD activities in liver (n=8).

Group	GSH (mg GSH/g)	GPx (µmol oxidized GSH/min/g)	CAT (µM H_2O_2/g).	SOD (µg/g)
Control	30.6±1.4	148±2.1	101±2.8	9.3±0.5
FM	32.9±1.2[a]	146±2.5	98.8±3.0	9.2±0.5
Rad	32.7±1.6[a]	147±2.0	98.6±4.4	9.1±0.5
E2	26.3±1.4[a]	171±2.6[a]	86.6±2.9[a]	12.2±0.4[a]
E2+FM	28.5±1.8[ab]	156 ±2.2[ab]	91.6±2.7[ab]	11.4±0.4[ab]
E2+Rad	28.9±1.3[b]	155±1.2[ab]	92.7±1.8[ab]	10.9±0.2[ab]
E2+Rad+FM	29.8±0.4[b]	150±1.7[b]	94.2±2.7[ab]	10.1±0.3[ab]

Values represented are in the format means±SD. FM: fermented milk with *L. helveticus*, Rad: gamma radiation, E2: estradiol. **a** :significant compared to control (P≤0.05), **b** :significant compared to estradiol (E2) (P≤0.05).

Table 2. Effect of fermented milk by *L. helveticus* and gamma radiation on lipid peroxidation and nitric oxide levels in liver (n=8).

Group	MDA (µM MDA/g)	NO (nM sodium nitrite/g)
Control	85.8±2.8	6.3±0.40
FM	84.6±4.2	5.4±0.35[a]
Rad	89.2±4.0	5.5±0.38[a]
E2	160.7±4.1[a]	14.6±0.40[a]
E2+ FM	107.6±4.1[ab]	7.5±0.40[ab]
E2+Rad	139.0±3.8[ab]	9.6±0.38[ab]
E2+Rad+ FM	94.0 ±3.8[ab]	7.8±0.40[ab]

Values represented are in the format means±SD. FM: fermented milk with L. helveticus, Rad: gamma radiation, E2: estradiol. a :significant compared to control (P≤0.05), b :significant compared to estradiol (E2) (P≤0.05).

idence of leukemia and some solid tumors has been reported to be lower compared to the respective control groups (Matanoski et al, 1990**,** Katayama et al., 2002).

In the present study, E2 caused significant changes in the measured antioxidant markers as a reduction in GSH level and CAT activity with an increase in GPx and SOD activities accompanied with a marked increase in oxidative stress markers MDA and NO levels. E2 induced changes in antioxidant status may be the result of a mechanism through which a controlled increase in ROS may act to potentiate growth at the expense of increasing sensitivity to DNA damage (Davies, 1999**)** and induce oxidative stress results in elevated MDA and NO levels (Karabi et al., 2012**).** The decrease in the level of GSH following estradiol injection might be due to formation of GSH conjugates with estrogen metabolites in human breast tissue (Rogan et al., 2003**).** A decrease in peroxide metabolism was the result of a change in antioxidant enzyme activities or antioxidant substrates, total reduced and oxidized glutathione levels and the activities of CAT, SOD and GPx was reported **(**Mobley and Brueggemeier, 2004**,** Mense et al., 2009**)**. Also they found that estrogen receptor (ER)-mediated mechanism is responsible for changes in all of the enzymes studied. There was a statistically significant increase in SOD, GPx and G6PD activities also significant decrease in total GSH levels, a decrease in CAT activity was consistent with the observed decrease in cellular peroxide metabolism **(**Nishio and Watanabe,1997)**.**

It is well documented, that antioxidants play an important role in ameliorating the damaging effects of oxidative stress on cells. According to the results obtained in our study, oral administration of FM before and after E2 injection accompanied with low dose of irradiation normalized antioxidant enzymes of GPx, CAT and SOD activities, GSH level and also normalized stress

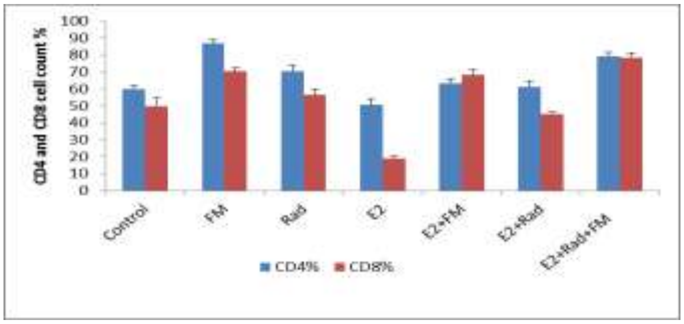

Figure 1. Immuno-modulatory effect of fermented milk by *L. helveticus* and low dose of gamma radiation on CD4 and CD8 percentage in blood (n=8).

Table 3. Effect of fermented milk by *L. helveticus* and gamma radiation on cell cycle analysis of estradiol injected group mammary gland (n=8).

Group	Go%	S %	M %
Control	82.7±0.8	5.8±0.9	1.9±0.9
FM	84.7±0.8[a]	9.6±0.7[a]	3.8±0.8[a]
Rad	82.4±0.6	7.5±0.6[a]	1.2±0.5
E2	52.4±0.6[a]	19.5±0.6[a]	8.8±0.8[a]
E2+FM	79.4±0.6[ab]	10.3±0.5[ab]	5.5±0.6[ab]
E2+Rad	67.8±0.8[ab]	8.8±0.9[ab]	4.3±0.6[ab]
E2+Rad+FM	84.6±0.7[ab]	8.7±0.8[ab]	3.6±0.5[ab]

Values represented are in the format means±SD. FM: fermented milk with L. helveticus, Rad: gamma radiation, E2: estradiol. a :significant compared to control (P≤0.05), b :significant compared to estradiol (E2) (P≤0.05).

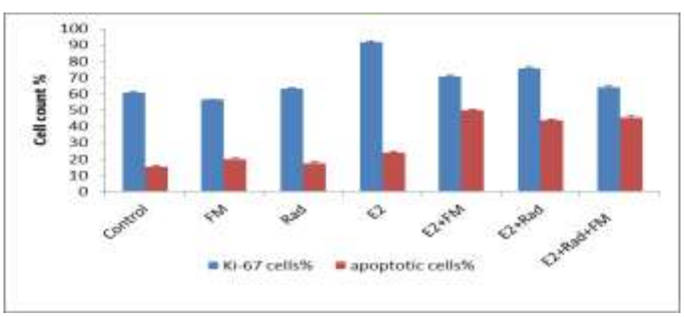

Figure 2. Effect of fermented milk by *L. helveticus* and gamma radiation on ki-67 and apoptotic cell count percentage.

markers of MDA and NO levels. This could be attributed to potent antioxidant activity and free radical scavenging capability of FM due to antioxidant ability of LAB. LAB strains are not only able to decrease the risk of ROS accumulation through oral ingestion but can also degrade the superoxide anion and hydrogen peroxide (Liu and Pan 2010). The results support the role of FM in scavenging free radicals and antioxidant properties. *Lactobacillus* species have been reported to play a significant role in the production of bioactive peptides in fermented dairy products showed a significant increase in radical scavenging activity. *Lactobacillus* may be used to combat oxidative stress, possess several anti-oxidative mechanisms: catalase, glutathione-system-related compounds, and Mn-SOD, attenuation of proliferation caused by reactive oxygen species **(**Ramesh et al., 2012) and reduced oxidative stress parameters as NO (Fernanda et al., 2012).

Exposure to low dose of gamma radiation stimulates cellular metabolic activities such as antioxidant activities; a phenomenon termed radiation hormesis caused increase in GSH level in the spleen 4 hours after irradiation in mice **(**Liu, 1996**)** and might be effective for the prevention of various reactive oxygen species-related diseases and exerts a protective effect upon cells and whole animals **(**Otsuka et al., 2006**,** Mansour, 2009).

In the present study, E2 markedly inhibited the production of T cells represented by CD4 and CD8 count which was ameliorated by FM pretreatment or exposure to gamma radiation, and combined treatment of FM and gamma radiation markedly normalized them.

It is documented that E2 may cause a reduction in CD4 and CD8 T cells number in rats (Kim et al., 2013). Oral administration of fermented milk induces CD4 and CD8 percentage **(**De Le-Blanc et al., 2005**)**, and exposures to low doses of ionizing radiation also enhanced CD4, CD8 production in the thymus and CD8 T cells in the spleen **(**Ina and Sakai 2004**,** 2005 a, 2005b) and increase natural killer activity post irradiation with reduction in the percentage of B cells in blood lymphocytes concomitant with an increase in helper T cell population (Kojima, 2006**).**

In this study, analysis of cell cycle by flow cytometry showed that, E2 markedly increased cell population at S and M phases which was inhibited by pretreatment with FM, this was observed as cell cycle arrest at S and M. Exposure to gamma- radiation causes cell cycle arrest at S phase. These effects were markedly ameliorated by pretreatment by FM accompanied with gamma-irradiation. The mechanism of cancer cell growth inhibition by lactic acid bacteria (LAB) is related to modulation of apoptotic signaling regulated proteins (Liu and Pan 2010).

Acquired resistance to the programmed cell death mechanism, apoptosis, is an important hallmark of cancer (Lowe et al., 2004). Lactic acid bacteria used in this study exerted its antiproliferative effects via the induction of apoptosis and necrosis as LAB exerts growth inhibitory effects both *in vitro* and *in vivo* (Kim et al., 2003). Fermented milk by *L. helveticus* has an antiproliferative effect on breast cancer cells. However, the precise mech-

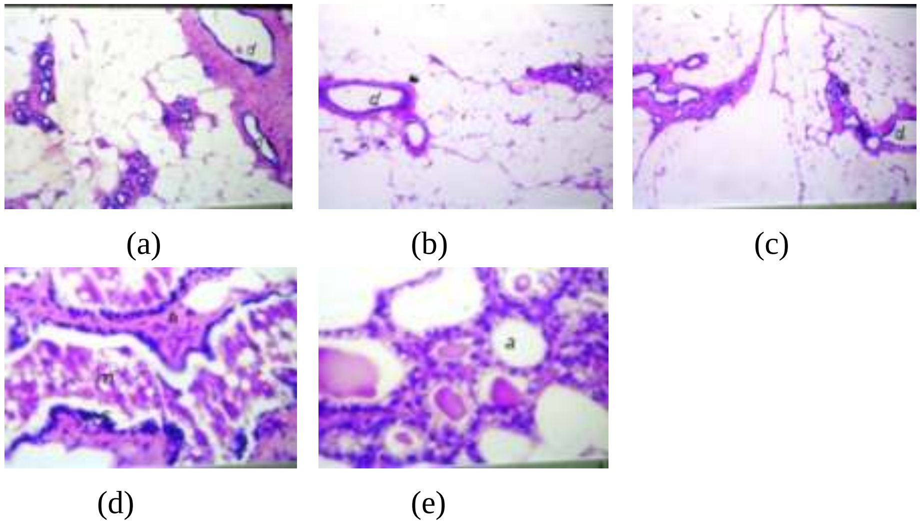

Figure 3. Histological study of female rats mammary gland tissue of: (a) control group (40), (b) fermented milk treated rats (40), (c) gamma irradiated rats (40), (d): estradiol treated rats (160), (e) fermented milk + gamma irradiated +estradiol treated rats (160). (a): acini and (d): lactiferous ducts.

anism by which LAB fractions exert anticancer effects remains unknown (Liu and Pan 2010). Combined treatment of FM with exposures to low level of gamma rays may suppress the development and progression of tumors and these effects can be associated with stimulation by such irradiations of anti-neoplastic functions of the immune system (Nowosielska et al., 2012).

Ki-67 marker is frequently measured as a marker of proliferative activity and a possible dynamic marker of treatment efficacy. In the present study ki-67 was evaluated as prognostic factor and monitoring marker during the treatment. We have demonstrated that rats treated with E2 induced preneoplastic changes in breast as demonstrated by elevated ki-67 in addition to histological analysis as preneoplastic and neoplastic changes in the breast, which were ameliorated by FM or gamma -radiation or combined treatment of both.

Apoptosis has been consistently reported to be positively correlated with ki-67 (Lipponen, 1999). In the present study, tumor growth index was markedly reduced by FM, gamma radiation and combined treatment of FM and gamma radiation, which is marker for treatment progression and effeciency. The cell turnover or growth index is an index based on the ki-67 apoptosis ratio developed to approximate the contribution that these two factors may make to tumor growth utility, it is used as an early marker of response to treatment in primary therapy of breast cancer (Bundred et al, 2002**,** Cleator et al., 2002**).** Low dose total body irradiation showed antitumor effects which could be explained by immune enhancement, induction of apoptosis, and intrinsic hypersensitivity to low dose of radiation and it is effective as chemotherapy **(**Hosoi, 2006**).**

In conclusion our results suggest that *Lactobacillus helveticus* can be used as adjuncts in fermentation of food and are potential candidate for breast cancer prevention combined with low doses of gamma radiation leading to inhibition of tumor growth.

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