

Inhibition of Human Breast Cancer Cell Growth and Enzymatic Activity by a Fermented Nutraceutical

An *in Vitro* and *in Vivo* Study

F. Marotta,^{a,d} H. Yadav,^b S. Pathak,^c E. Minelli,^d
P. Signorelli,^a A. Lorenzetti,^a and P. Marandola^a

^aNutraceutical–Nutrigenomic Unit, G.A.I.A. Age-Management Foundation, Pavia, Italy

^bNIDDK, National Institutes of Health, Bethesda, Maryland, 20892

^cCytogenetics & Molecular Biology Lab, University of Kalyani, Kalyani, India

^dWHO-cntr for Biotech & Nat. Medicine, University of Milan, Milan, Italy

Human breast cancer cell lines MCF-7 (ER-positive) and Hs578T (ER-negative) were cultured and one lot incubated for 48 h with 5–50 µg/ml of a fermented phyto compound (MK: Manda-Koso, Innoshima, Japan). *In vitro*, it appeared a dose-dependent decrease of cell viability (5–57%) in MK group in both cell lines ($P < 0.001$, plateau: 30 µg/ml), decreased beta-galactosidase activity, enhanced apoptosis, and inversely increased Bax/Bcl2 ratio ($P < 0.01$) with an upregulation of p53 ($P < 0.05$). In the *in vivo* model, Balb-c mice were inoculated with tumor cells and the treatment group was fed with 20 mg of MK. Tumor weight in MK-fed group was time-course reduced by 22% to 51% at 2 and 4 weeks, respectively ($P < 0.05$) with increased survival ($P < 0.05$). Tumour tissue of MK-fed mice showed a downregulated Bcl-2 with increased Bax/Bcl-2 ratio, reduced PCNA, and activated caspase 3. Although more studies are ongoing to foster the clinical applicability of MK integrated within a rational chemopreventive and therapeutic strategy, a p53-mediated mechanism is likely to play a relevant role, besides its reported antioxidant capacity, NK cell activity enhancement, cancer-cytostatic activity properties.

Key words: breast cancer cell lines; *in vivo* breast cancer model; Manda-Koso

Introduction

The improved treatment options for breast cancer still are met by the growing incidence of this cancer, thus prompting the need to develop mechanism-based novel agents for its prevention. It has been recognized that estrogen receptor (ER)-dependent breast cancers generally have a better prognosis and are often responsive to antiestrogen therapy. However, 40% of human breast cancers are ER-

independent and are more aggressive and unresponsive to antiestrogens. Apoptosis is one of the main targets of anticancer regimes since it plays a crucial role in eliminating mutated neoplastic and hyperproliferating cells from the system. Bcl-2 is overexpressed in a high percentage of human breast cancer cells.¹ Indeed, the proteins of Bcl-2 family play an important role in induction of apoptosis; antiapoptotic protein Bcl-2 has been associated with cell survival and shown to inhibit programmed cell death, whereas increase in proapoptotic protein Bax results in apoptosis.² Thus, several studies have also shown that the ratio of Bax to Bcl-2 proteins increases during apoptosis as

Address for correspondence: F. Marotta, M.D., Ph.D., Piazza Firenze, 12, 20154 Milano, Italy. fmarchimede@libero.it

a sign of effective cancer cell clearance. Several experimental and epidemiological studies have shown that antioxidant-endowed micronutrients present in food can inhibit carcinogenesis by affecting the molecular events either in the initiation, promotion, or progression phase.³⁻⁵ This has given rise to a number of studies testing the potential role of specific nutraceuticals to be applied either in the preventative manner or in integrated therapeutic anticancer treatment.⁶⁻⁸

Thus, in the quest of searching a multimodality approach to breast cancer we investigated the potential application of an ISO-controlled phytofermented compound (50 fruits and vegetables grown in non-OGM crops and fermented for 3 years; Manda-Koso, Innoshima, Japan) endowed by a number of cancer-fighting reports. The present study was focused on an *in vitro* test followed by an *in vivo* test.

Materials and Methods

The human breast cancer cell lines MCF-7 (ER-positive) and Hs578T (ER-negative) were maintained in RPMI medium 1640 supplemented with 2 mM glutamine, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified atmosphere of 950 mL/L air and 50 mL/L CO₂. Cells were maintained as monolayer cultures and medium was changed every two days. Before starting the test treatment the cells were grown to 80–90% confluency and synchronized by incubating in the basal medium (100% MEM) for 24 h so that confluent cell cultures were used for all experiments.

Preparation of Test Compound for Culture Test

Hot-water-extracted fermented phytocompound (MK: Manda-Koso), kindly donated by the Manda Research Institute, Innoshima Japan, was prepared and concentrated to 1 g

of compound in 1 ml of distilled water. After it was dissolved and mixed at 37°C for 30 min in DMEM, the concentration of MK was adjusted to 100 mg/ml. This solution was centrifuged (1200 × g, 30 min) to remove insoluble ingredients. The supernatant was sequentially passed through 0.45 μm and 0.22 μm filters for sterilization.

In Vitro Assay of Proliferation and Viability

Samples containing 200 μl cell suspensions were dispersed with 0.02% edetic acid to prepare the suspension of a cell density of 3×10^7 /L. Then cell suspension was fractionated and incubated in a 5% CO₂ atmosphere at 37°C for 4 h. Cultures were incubated for 48 h, graded concentrations (5–50 μg/ml) of MK solution. Then, 1 mCi of [methyl-³H]thymidine (Amersham, Piscataway, New Jersey) was added to each well, and the radioactivity of cell lysates was counted in scintillation solution. *In vitro* assays were performed in triplicates. After 24 h incubation cells were treated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfofenyl)-2H-tetrazolium (MTT) solution, 5 g/L (20 mL per well), and 25 μmol/L phenazine methosulfate. After another 4-h incubation, the supernatant were discarded and isopropanol hychloride 0.04 mol/L were added (100 mL per well). Then the suspension was vibrated on a micro-vibrator for 5 min and the absorbance of soluble formazan was measured at 570 nm using an ELISA reader. All assays were completed in triplicate, and results were confirmed by direct cell counting using a hemocytometer.

Apoptosis Assay by Flow Cytometry

Induction of apoptosis in both cell lines caused by MK was quantitatively determined by flow cytometry using an Annexin V-FITC kit binding assay and analyzed by cytometry using a fluorescence-activated cell sorter. Cell

suspensions (2 ml), prepared as above mentioned, were re-suspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂), and Annexin V-FITC was added and incubated for 30 min in 96 well plates at room temperature in the dark. After 24 h of incubation at 37°C with 5% CO₂, graded MK solutions were added. After another 72-h incubation, cells were collected by centrifugation and stained in tris-buffered saline containing 50 µg/ml PI, 10 µg/ml RNase or DNase, and 0.1% Igepal CA-630 for 1 h at 4°C. FACS analysis was carried out by a flow cytometer at 488 nm. Data from 4,000 cells were collected and analyzed in triplicate.

Western Blot Analysis

After being maintained in estrogen-free conditions for 48 h, cells were treated with 5–50 µg/ml of MK for 48 h. The protein expression was detected by enhanced chemiluminescence detection systems and autoradiography with HXR-film. Approximately 50 mg of protein was separated by a 7.5% SDS-PAGE, and transferred on to a nitrocellulose membrane by electrophoretic blotting. The membranes were developed using the Western blotting detection system according to the manufacturer's instructions using mouse monoclonal anti-p53 (Pab 421) antibody, anti-Bcl-xL, and Bcl-2. The relative intensity of each protein band in a blot was calculated by using a software program.

β-Galactosidase Activity

A separate batch of MCF-7 cell line was tested also with the addition of 10⁻¹⁰ M 17β-estradiol to examine the possible antagonistic activity of MK was assessed by the inhibition of β-galactosidase activity. The activity of MK was evaluated using a specific assay system and X-gal (X-gal 5-bromo-4-chloro-3-indolylβ-D-galactopyranoside) staining.

In Vivo Model

Both cell lines were separately inoculated s.c. with either 1 × 10⁶ viable cells in the preshaved back skin of or 10 Balb-c mice. The growth of tumor was monitored throughout the experiment. Treated groups were concomitantly fed with 20 mg/day of MK for 4 weeks.

Parameters assessed were: time-course change of tumor weight; survival; and, based on the assessment of four fields, Bcl-2, Bax/Bcl-2 ratio, proliferating cell nuclear antigen (PCNA) by using mouse anti-PCNA polyclonal anti-serum, and active caspase-3, which was detected by anti-active caspase-3 polyclonal antibody staining, which specifically recognizes amino acids 163–175 of caspase-3 but not the precursor form.

Results

In Vitro Assay of Proliferation and Viability

The *in vitro* assays showed that MK inhibited proliferation of either human ER-positive MCF-7 and Hs578T (ER-negative) breast cancer cells (30–70%, *P* < 0.05, data not shown). The inhibitory effects of this compound were dose-dependent with a plateau at 30 µg/ml.

Apoptosis Assay and Western Blot

As determined by Western blot analysis, treatment of both cell lines with MK resulted in dose-dependent reduction of Bcl-2 protein expression after 24 and 48 h of treatment, as seen by the relative intensity of each band below the blot. The protein expression of Bax was correspondingly upregulated from 1.2 untreated cells to 5.4-fold with increasing concentrations (5–30 µg/ml) and period (24 and 48 h) of MK treatment (data not shown, *P* < 0.001). As shown in Figure 1, the ratio of Bax/Bcl-2 was significantly increased dose- and time-dependently after MK

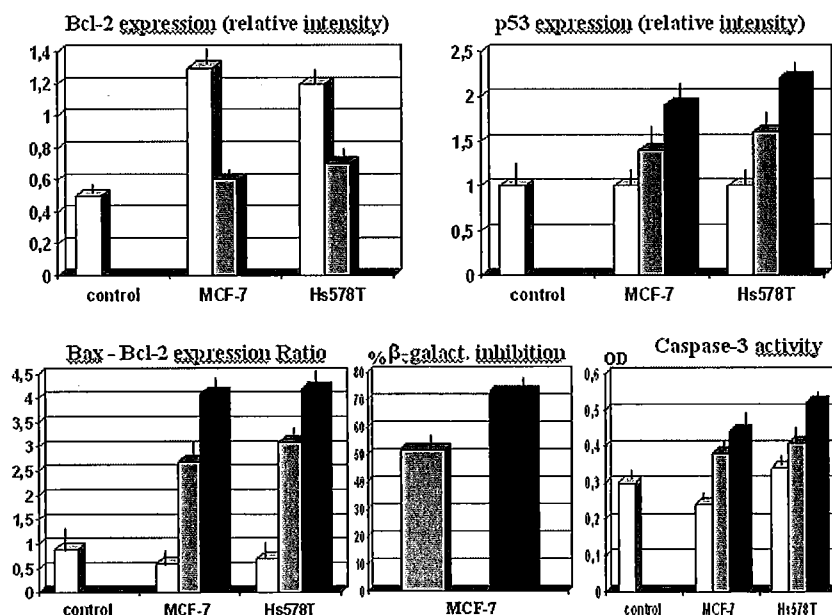


Figure 1. Effect of Manda nutraceutical on apoptotic and enzymatic variables in *in vitro* and *in vivo* model of breast cancer. Grey bar: 24 h incubation; black bar: 48 h incubation.

treatment together with enhanced p53 expression ($P < 0.01$ and $P < 0.001$).

β -Galactosidase Activity

MK incubation brought about a time-course and time-dependent decrease of β -galactosidase activity in MCF-7 cell line ($>70\%$ inhibition at 48 h, $P < 0.01$, Fig. 1).

In Vivo Model

There was no significant difference in body weight among groups. After subcutaneous inoculation of the tumor cells, it was observed that the tumor's volume was inhibited in MK-fed animals and the tumor weight was reduced by 22 to 51% at 2 and 4 weeks, respectively (data not shown, $P < 0.05$ vs. untreated). Oral administration of MK also increased 60-day survival in a separate group of the animals (ER-positive/ER-negative: +17%–+12%, $P < 0.05$). We observed that dietary MK resulted in decreased expression of the antiapoptotic protein Bcl-2 while increasing the expression of Bax and of

cleaved caspase 3 ($P < 0.05$). Further, the induction of apoptosis in *in vivo* tumors was confirmed by immunohistochemical detection of cleaved caspase 3+ cells in tumors from untreated mice. The percent of cleaved caspase 3+ cells in Manda-Koso treated tumors were 3.4-fold in comparison to untreated tumors (data not shown, $P < 0.01$).

Discussion

The administration of natural compounds capable of inducing the apoptotic death of cancer cell and with potential anticancer effect is gaining a widespread interest.⁶⁻⁸ In the present study we found that treatment of Manda-Koso to either MCF-7 (ER-positive) or Hs578T (ER-negative) human breast cancer cell lines enabled a reduction of Bcl-2 protein expression, but increased the expression of Bax. The Bcl-2 protein by binding to proapoptotic protein Bax form heterodimers and the increased molar ratio of the two was related to the apoptotic phenomenon either *in vitro* or *in vivo* experiment, where also the cleaved caspase-3, a key

executioner of apoptosis, was significantly increased. From our *in vitro* testing, it appeared that, irrespective of the ER-sensitivity cell lines, MK significantly upregulated p53, a major player in the apoptotic response to genotoxins being a negative transcriptional regulator of Bcl-2 gene. Further, p53 specifically targets Bax protein,⁹ which controls cell death through the disruption of mitochondria and subsequent release of cytochrome c in cytosols.¹⁰ Our results suggest that MK might possess an effective anticarcinogenic potential on the ER-negative human breast cancer cells as well. Although it has been reported that there are no structural abnormalities of the Bcl-2 locus in breast cancers,¹¹ and that invasive or metastatic (mostly ER-negative) breast cancers often have lower expression of Bcl-2 protein,¹² it would seem that MK triggered apoptosis in both cell lines in a p53-dependent manner.

Further mechanisms of actions might be its reported antioxidant properties together with NK cell activity enhancement and cancer-cytostatic activity promoted by its angiotensin-inhibitor activity.¹³⁻¹⁵ Further studies are in progress in order to assess the clinical applicability of MK within a chemopreventive and therapeutic strategy.

Conflicts of Interest

The authors declare no conflicts of interest.

References

1. Duenas-Gonzalez, A., M.M. Abad-Hernandez, J.J. Cruz-Hernandez & R. Gonzalez-Sarmiento. 1997. Analysis of bcl-2 in sporadic breast carcinoma. *Cancer* **80**: 2100–2108.
2. Fukui, M. & B.T. Zhu. 2008. Mechanism of 2-methoxyestradiol-induced apoptosis and growth arrest in human breast cancer cells. *Mol. Carcinog.* 2008 Jun 2. [Epub ahead of print].
3. Smiechowska, A., A. Bartoszek & J. Namieśnik. 2008. Cancer chemopreventive agents: Glucosinolates and their decomposition products in white cabbage (*Brassica oleracea* var. capitata). *Postępy Hig. Med. Dosw.* **62**: 125–140.
4. Herberg, S., P. Galan, P. Preziosi, et al. 2004. The SU.VI.MAX Study: A randomized, placebo-controlled trial of the health effects of antioxidant vitamins and minerals. *Arch. Intern. Med.* **164**: 2335–2342.
5. Davis, C.D. & E.O. Uthus. 2004. DNA methylation, cancer susceptibility, and nutrient interactions. *Exp. Biol. Med. (Maywood)* **229**: 988–995.
6. Prasad, S., N. Kalra & Y. Shukla. 2008. Induction of apoptosis by lupeol and mango extract in mouse prostate and LNCaP cells. *Nutr. Cancer* **60**: 120–130.
7. Eggler, A.L., K.A. Gay & A.D. Mesecar. 2008. Molecular mechanisms of natural products in chemoprevention: Induction of cytoprotective enzymes by Nrf2. *Mol. Nutr. Food Res.* **52**(suppl. 1): S84–94.
8. Srinivasan, P., S. Suchalatha, P.V. Babu, et al. 2008. Chemopreventive and therapeutic modulation of green tea polyphenols on drug metabolizing enzymes in 4-nitroquinoline 1-oxide induced oral cancer. *Chem. Biol. Interact.* **172**: 224–234.
9. Yin, C., C.M. Knudson, S.J. Korsmeyer & T. Van Dyke. 1997. Bax suppresses tumorigenesis and stimulates apoptosis in vivo. *Nature* **385**: 637–640.
10. Marzo, I., C. Brenner, N. Zamzami, et al. 1998. Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science* **281**: 2027–2031.
11. Leek, R.D., L. Kaklamanis, F. Pezzella, et al. 1994. Bcl-2 in normal human breast and carcinoma, association with oestrogen receptor-positive, epidermal growth factor receptor-negative tumours and in situ cancer. *Br. J. Cancer* **69**: 135–139.
12. van Slooten, H.J., M.J. van de Vijve, C.J. van de Velde & J.H. van Dierendonck. 1998. Loss of Bcl-2 in invasive breast cancer is associated with high rates of cell death, but also with increased proliferative activity. *Br. J. Cancer* **77**: 789–796.
13. Okuda, H., Y. Matsuura & M. Takara. 1994. Basic and clinical studies on Manda enzyme. In Satellite Symposium “Therapeutic Approaches in Pathophysiology” of the 2nd International Congress of Pathophysiology. Tokyo, Nov. 25, 1994.
14. Kim, J.H., M.K. Park, J.Y. Lee, et al. 1998. Antioxidant and antitumor effect of Manda. *Biochem. Arch.* **14**: 211–219.
15. Kim, D.C., K. Hwuang & M.J. In. 2003. In vitro antioxidant and anticancer activities of extracts from a fermented food. *J. Food Biochem.* **27**: 449–459.