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Abstract. *Background: There is an increased interest in alternative treatments that reduce the toxicity of chemotherapy by lowering the drug concentration, whilst maintaining potency against cancer cells. Previous studies have demonstrated that arabinoxylan from rice bran, MGN-3/Biobran, sensitizes human breast cancer cells (BCC) to daunorubicin (DNR). In the present study, we further evaluated the ability of MGN-3 to sensitize cells to another chemotherapy agent, paclitaxel. Materials and Methods: Non-metastatic MCF-7 (human BCC) and metastatic 4T1 (murine BCC) cells were cultured with different concentrations of paclitaxel in the presence or absence of MGN-3. Cell survival, DNA damage, and cell proliferation were examined. Results: MGN-3 increased the susceptibility of both types of cancer cells to paclitaxel by over 100-fold. Mechanistically, MGN-3 works synergistically with paclitaxel by causing DNA damage, enhancing apoptosis, and inhibiting cell proliferation in 4T1 cells. Conclusion: Our data demonstrate that MGN-3 is an effective chemosensitizer and may represent a novel adjuvant for the treatment of metastatic breast cancer.*

Cancer remains the largest cause of mortality in the world, claiming over six million lives each year. Chemotherapy is considered the cornerstone of treatment for many types of cancers. However, many chemotherapeutic agents exhibit dose-limiting toxicities (1-5). Therefore, there is increasing interest in identifying compounds that may increase the sensitivity of cancer cells to conventional chemotherapeutic agents, thus reducing chemotherapeutic-related toxicity (6-9). Early studies from our laboratory have shown that arabinoxylan rice bran, MGN-3/Biobran, sensitizes human leukemia HUT 78 cells to apoptosis induced by antibody to cluster of differentiation 95 (CD95) (10), and human breast cancer cells (BCCs) to daunorubicin *in vitro* (11). Further studies have revealed the synergistic effect of MGN-3 with transarterial oily chemoembolization in patients with hepatocellular carcinoma (12). In the present study, we thought it would be of particular interest to further investigate the chemosensitizing ability of MGN-3 towards another chemotherapeutic agent, paclitaxel, commonly known as taxol.

Several rice bran products have been examined for their role as antitumor agents, including polysaccharide RBS (13), lipoprotein fraction (14), and agglutinin (RBA) (15). In addition, a recent study showed that rice (*Oryza sativa* L.) inhibits the growth of human leukemia U937 cells through activation of peripheral blood mononuclear cells (16). MGN-3 is a natural product that is obtained by reacting rice bran hemicellulose with multiple carbohydrate-hydrolyzing enzymes from Shiitake mushrooms. The main chemical component of MGN-3 is arabinoxylan with a xylose in its main chain and an arabinose polymer in its side chain (17). We presented evidence elsewhere for the role of MGN-3 as a potent biological response modifier of human natural killer (NK) cells (18-20). It also activates human dendritic cells (21, 22), enhances the proliferation of T-cells and B-cells (17), and augments the phagocytic function of macrophages (23). In addition, further studies revealed that administration of MGN-3 to tumor-bearing mice resulted in significant reduction in tumor volume (24). Based on our earlier findings, we initiated this study to further investigate the chemosensitizing ability of MGN-3 towards another chemotherapeutic agent, paclitaxel, in metastatic breast cancer cells and to elucidate its mode of action.

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Key Words: MGN-3, Biobran, MTT, 4T1, MCF-7, paclitaxel.

Materials and Methods

Drugs and chemicals. Paclitaxel was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). RPMI-1640 supplemented with 10% fetal calf serum (FCS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) from Sigma-Aldrich.

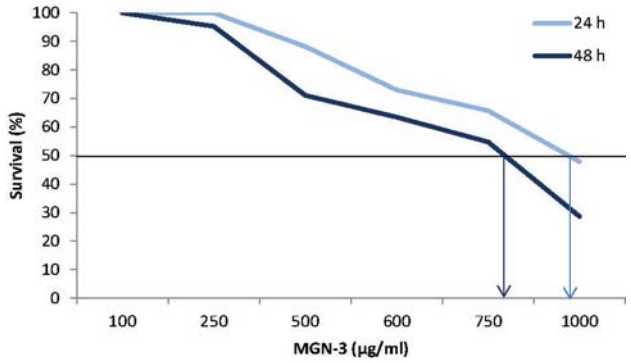

Figure 1. *MGN-3 alone reduces the survival rate of MCF-7 cells. Using MTT assay method, MCF-7 cells were incubated with MGN-3 (100-1000 µg/ml) for 24 and 48 h. The half-maximal inhibitory concentration (IC_{50}) is indicated by arrows.*

MGN-3 was provided by Daiwa Pharmaceutical Co. Ltd. (Tokyo, Japan) and was dissolved in complete medium (CM) at a concentration of 30 mg/ml.

Tumor cell lines and culture conditions. The human BCC line MCF-7 and murine 4T1 cells BCC, which metastasize to the lung was used in the present study. Cells were purchased from the American Tissue and Culture Collection (ATCC; Manassas, VA, USA). Tumor cells were maintained in our laboratory in a CM that consisted of RPMI-1640, supplemented with 10% FCS, 2 mM glutamine, and 100 µg/ml streptomycin and penicillin.

Effect of chemotherapy plus MGN-3 on growth of breast cancer cells. drug sensitivity assay. Drug sensitivity was determined by using a colorimetric MTT assay. Cancer cells (1×10^4/well) were seeded in 96-well plates and cultured in triplicate in the presence or absence of different concentrations of MGN-3 (100-1000 mg/ml) and in the presence or absence of selected concentrations of paclitaxel (1×10^{-1} to 1×10^{-6} M). The final volume of medium in each well after the addition of MGN-3 or paclitaxel was 200 µl. The cultures were incubated at 37˚C for 24 and 48 h after which 50 mg of MTT were added to each well and the cultures were incubated for an additional 4 h. The plates were then centrifuged, the medium was carefully removed, the formazan crystals solubilized with acid alcohol and the plates were read at 590 nm using an ELISA plate reader (Molecular Devices, Menlo Park, CA, USA). The 50% inhibitory concentration (IC_{50}) was determined as the drug concentration resulting in a 50% reduction of cell viability. The IC_{50} was determined by plotting the logarithm of the drug concentration *versus* the survival rate of the treated cells.

Trypan blue exclusion method. In sterile test tubes, the cells and chemicals were added with different concentrations of MGN-3, paclitaxel, and both MGN-3 plus paclitaxel in triplicates. Cells were incubated for 24 and 48 h at 37˚C in a humidified atmosphere of 5% CO_2 in sterile medium. Viable cells were counted by trypan blue exclusion using hemocytometer. Then the percentage of live cells was obtained by dividing the viable cells by the total number cells. All experiments were repeated in triplicates.

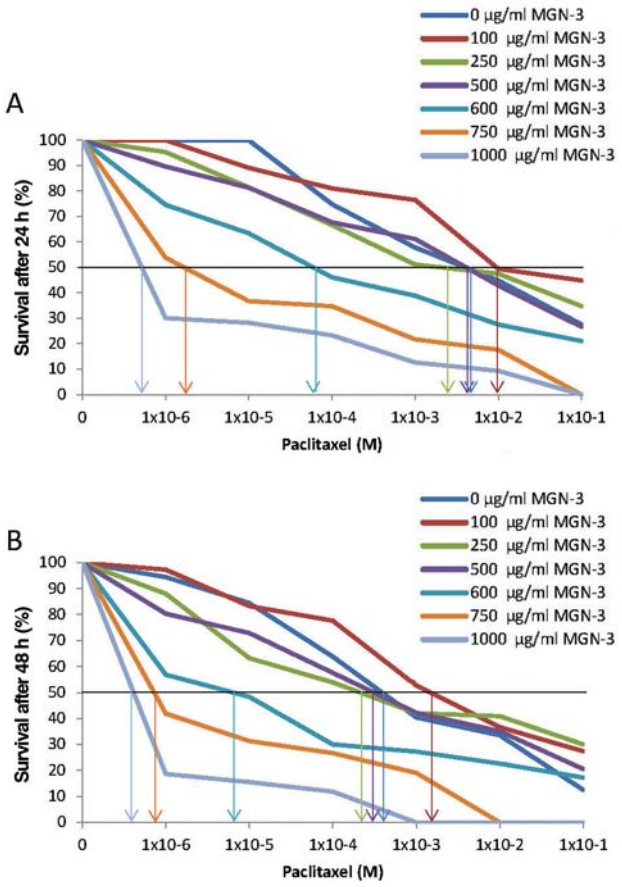

Figure 2. *Co-culture of MGN-3 sensitized MCF-7 cells to paclitaxel causing an even greater decrease in cell survival. MCF-7 cells were co-cultured with different concentrations of MGN-3 and paclitaxel for 24 h (A) and 48 h (B). The half-maximal inhibitory concentration (IC_{50}) for each combination is indicated with arrows.*

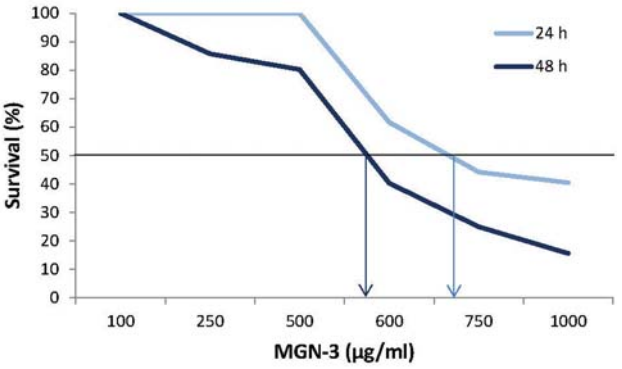

Figure 3. *MGN-3 alone reduces the survival rate of 4T1 cells. Using MTT assay method, 4T1 cells were incubated with MGN-3 (100-1000 µg/ml) for 24 and 48 h. The half-maximal inhibitory concentration (IC_{50}) is indicated by arrows.*

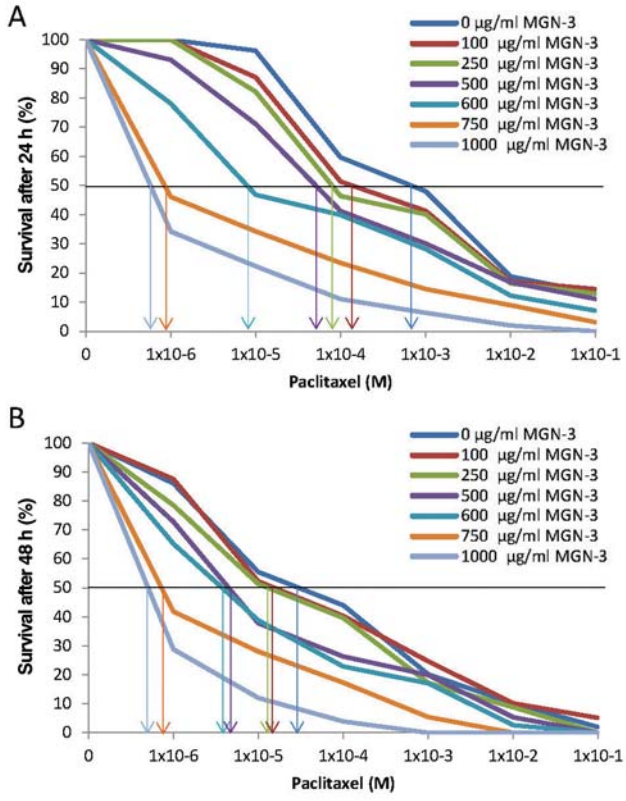

Figure 4. *Co-culture of MGN-3 sensitized 4T1 cells to paclitaxel causing an even greater decrease in cell survival. 4T1 cells were co-cultured with varying concentrations of MGN-3 and paclitaxel for (A) 24 h and (B) 48 h. The half-maximal inhibitory concentration (IC_{50}) for each concentration combination is indicated with arrows.*

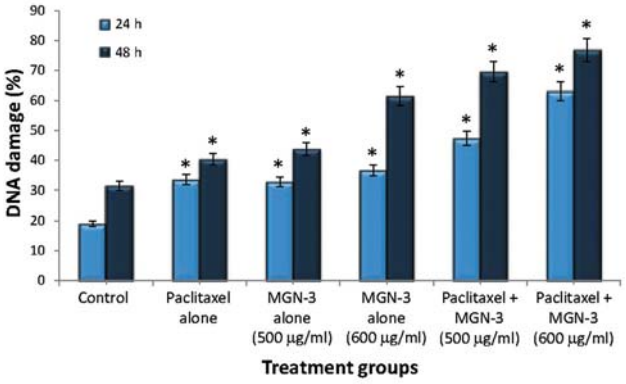

Figure 5. *DNA damage to 4T1 cells. The effect of paclitaxel (1×10^{-3} M), and MGN-3 (500 and 600 µg/ml) and paclitaxel plus MGN-3 on DNA damage to 4T1 cells was examined. DNA damage in 4T1 cells was assessed using flow cytometry. Data represent the mean±SD of experiments performed in triplicate. *p<0.01 as compared with control untreated 4T1 cells.*

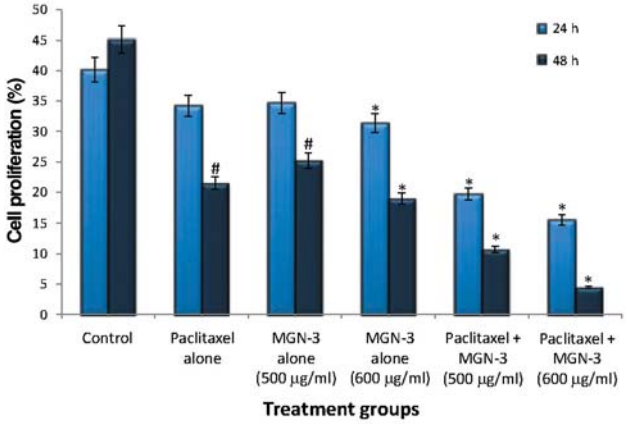

Figure 6. *Proliferation of 4T1 cells. The effect of paclitaxel alone (1×10^{-3} M), and in combination with MGN-3 (500 and 600 µg/ml) on the proliferation of 4T1 cells was examined. The percentage proliferation of 4T1 cells was assessed using flow cytometry. Data represent the mean±SD of experiments performed in triplicate. #p<0.05, *p<0.01 as compared with control untreated 4T1 cells.*

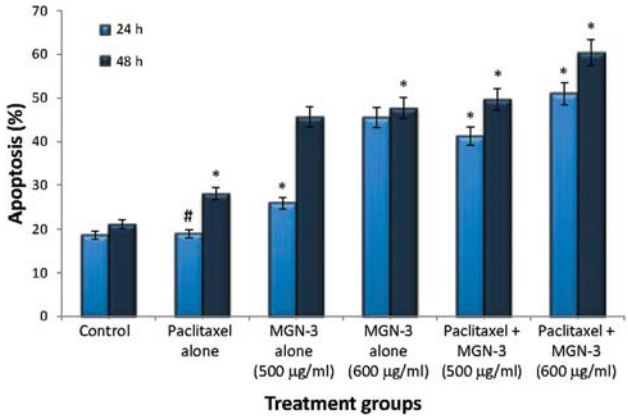

Figure 7. *Apoptosis of 4T1 cells. The effect of paclitaxel alone (1×10^{-3} M), and MGN-3 alone (500 and 600 µg/ml) and paclitaxel plus MGN-3 on apoptosis of 4T1 cells at 24 and 48 h was examined. The percentage of apoptosis of 4T1 cells was assessed using flow cytometry. Data represent the mean±SD of experiments performed in triplicate. p<0.05, #*p<0.01 level as compared with control untreated 4T1 cells.*

Flow cytometric analysis for apoptosis, DNA damage and cell proliferation. Quantitative detection of apoptosis, DNA damage, and cell proliferation in 4T1 cells treated with MGN-3 with and without paclitaxel was simultaneously determined by multicolor flow cytometric analysis using the Apoptosis, DNA Damage and Cell Proliferation Kit specific for incorporated bromodeoxyuridine (BrdU), phosphorylated H2AX (γH2AX) and cleaved poly ADP ribose polymerase (PARP) (BD Biosciences Pharmingen, San Diego CA, USA). Following the manufacturer's instructions, cells were cultured in a CM or with different concentrations of MGN-3 (500 µg/ml and 600 µg/ml) with and without paclitaxel (1×10^{-3}M) for 24 or 48 h. Ten

microliters of BrdU working solution [1 mM BrdU in 1× (DPBS)] was added to each milliliter of tissue culture medium (the cell culture density was approximately 1×10^6 cells/ml), following this, the cells were incubated for 30 min on ice. Cells were washed by adding 1 ml of staining buffer/tube and centrifuged (5 min) at 250 ×g, and the supernatant was discarded. Cells were fixed with 100 μl of BD Cytofix/Cytoperm Fixation/Permeabilization Solution per tube, and incubated for 30 min at room temperature. Afterwards, cells were washed with 1 ml of 1× BD Perm/Wash Buffer, centrifuged, and the supernatant was discarded. Cells were incubated in 100 μl of BD Cytofix/Cytoperm Plus Permeabilization Buffer/ tube for 10 min in ice, washed, and then re-fixed for 5 min. One hundred microliters of diluted DNase were added to cells which were incubated for 1 h at 37˚C and then washed. Cells were resuspended with 20 μl wash buffer plus PerCP-Cy™ 5.5 mouse anti-BrdU (5 μl/test), Alexa Fluor® 647 mouse anti-H2AX (pS139) (5 μl/test), PE anti-cleaved PARP (Asp214) (5 μl/test) for 20 min in the dark and then washed. Cells were resuspended in staining buffer for analysis by fluorescence-activated cell sorting (FACSCalibur; BD Biosciences, San Jose, CA, USA) using CellQuest 3.3 software (25, 26).

Statistical analysis. Values are reported as the mean±SD and data were analyzed using one way analysis of variance followed by post hoc tests for multiples comparisons. A *p*-value of less than 0.05 was considered statistically significant.

Results

Effects of MGN-3 alone on MCF-7 cell survival. The effect of MGN-3 alone on MCF-7 cell survival was examined at 24 and 48 h post-culture of cancer cells with MGN-3. Data using the MTT assay are depicted in Figure 1 and show that treatment with MGN-3 resulted in a decrease in the percentage of viable cancer cells at 24 h. The IC_{50} value was 1000 μg/ml. The cytotoxic effect became more remarkable at 48 h, where the IC_{50} value was approximately 800 μg/ml. Similar results were noted by the trypan blue assay (data not shown).

Effects of MGN-3 on the sensitivity of MCF-7 to paclitaxel. Data of MTT assay in Figure 2 show that the survival of MCF-7 cells was inhibited post-culture with paclitaxel. On the other hand, co-culture of cells with MGN-3 plus paclitaxel caused a more marked reduction than paclitaxel alone. The sensitizing effect of MGN-3 follows a dose-dependent pattern. At 24 h, the paclitaxel IC_{50} value decreased by a factor of over 100 at MGN-3 concentrations of 600, 750, and 1000 μg/ml, as compared with paclitaxel alone (Figure 2A). Further reduction of IC_{50} can be seen at 48 h (Figure 2B). Similar results were noted by trypan blue assay (data not shown).

Effects of MGN-3 alone on 4T1 cell survival. Data in Figure 3 showed that MGN-3 reduced 4T1 cell survival in a dose-dependent fashion as examined by MTT assay. We noted a remarkable cytotoxic effect by MGN-3 at 24 h: the IC_{50} was approximately 700 μg/ml. At 48 h post-culture of 4T1 cells with MGN-3, the IC_{50} value further decreased to approximately 580 μg/ml. Similar trends were observed using the trypan blue assay (data not shown).

Effects of MGN-3 on the sensitivity of 4T1 cells to paclitaxel. The survival of 4T1 cells post-culture with paclitaxel alone was inhibited; however, further inhibition was noticed post-culture with MGN-3-plus-paclitaxel. The sensitizing effect of MGN-3 follows a dose-dependent pattern. Data in Figure 4A shows that at 24 h, the IC_{50} value for paclitaxel decreased by a factor of approximately 3 at an MGN-3 concentration of 600 μg/ml, up to a factor of approximately 100 at an MGN-3 concentration of 1000 μg/ml, as compared with paclitaxel alone. More marked cytotoxic effects of the co-treatment can be seen at 48 h (Figure 4B). Results were further confirmed by trypan blue assay (data not shown).

Differential sensitivity of 4T1 and MCF-7 cells to paclitaxel with and without MGN-3. We compared the sensitivity of the non-metastatic MCF-7 and metastatic 4T1 cells to paclitaxel with and without MGN-3. The results showed that 4T1 cells are more sensitive than MCF-7 cells to the toxicity of paclitaxel both alone and in combination with MGN-3, as indicated by the cell survival at 24 and 48 h using the MTT assay. Exposure of both cancer cell types to paclitaxel alone and in combination with MGN-3 shows the IC_{50} for 4T1 cells to be lower than those of MCF-7 cells at 24 and 48 h. Investigation of DNA damage, cell proliferation, and apoptosis were, therefore, carried out using 4T1 cells alone to elucidate the mechanisms of action of MGN-3.

DNA damage of 4T1 cells. The effect of MGN-3 (500 and 600 μg/ml) and paclitaxel (1×10^{-3}M) on percentage of DNA damage of 4T1 cells was examined. Data in Figure 5 show that treatment of 4T1 cells with paclitaxel alone significantly increased the percentage of DNA damage ($p<0.01$) as compared with control untreated 4T1 cells. A similar pattern was observed on treatment of 4T1 cells with MGN-3 alone ($p<0.01$) as compared with control untreated 4T1 cells. On the other hand, exposure of 4T1 cells to both paclitaxel plus MGN-3 resulted in a marked increase in the percentage of DNA damage that was higher than that with either agent alone. The chemosensitizing effect of MGN-3 was significant at 24 h and was further increased at 48 h ($p<0.01$ level).

Proliferation of 4T1 cells. Figure 6 shows the effect of MGN-3 (500 and 600 μg/ml) and paclitaxel (1×10^{-3}M) on the percentage of 4T1 cell proliferation. Treatment of 4T1 cells with paclitaxel resulted in inhibition of cell proliferation at 24 h which further decreased at 48 h ($p<0.05$) as compared with control untreated 4T1 cells. Similarly, 4T1 cell proliferation decreased upon exposure to MGN-3 alone at concentration of 500 μg/ml ($p<0.05$) and

600 μg/ml ($p<0.01$) 24 and 48 h as compared with control untreated 4T1 cells. However, co-culture of 4T1 cells in the presence MGN-3 and paclitaxel showed that the inhibition of cell proliferation was greater than that with either agent alone ($p<0.01$).

Apoptosis of 4T1 cells. The effect of MGN-3 at concentrations of 500 and 600 μg/ml and paclitaxel (1×10^{-3}M) on the percentage of 4T1 cell apoptosis was examined. Data in Figure 7 show that treatment of 4T1 cells with paclitaxel alone increased 4T1 cell apoptosis at 24 h ($p<0.05$) and further increased it at 48 h ($p<0.01$ level). In a dose-dependent manner, MGN-3 significantly enhanced apoptosis of 4T1 cells ($p<0.01$ level). However, exposure of 4T1 cells to MGN-3 plus paclitaxel resulted in a higher percentage of 4T1 cell apoptosis than that with either agent alone ($p<0.01$ level).

Discussion

Paclitaxel, a natural product from Yew trees, is considered to be a very powerful chemotherapeutic drug for the treatment of a number of cancer types, such as those of breast, ovary and prostate, and esophageal, non-small cell lung carcinoma, and melanoma (27, 28). The apoptotic effect of paclitaxel on cells has been shown to be dose-dependent (29, 30) and low concentrations (5-25 nM) can induce apoptosis in anaplastic thyroid cancer (ATC) cells *in vitro* (30). However, a higher concentration is required to induce an apoptotic effect on cancer in clinical practice (31-34). Such a high dose of paclitaxel is associated with severe side-effects including neutropenia, neuralgia and gastrointestinal toxicity (28, 35, 36). Thus, investigations that would allow choosing an optimal concentration of paclitaxel that causes death of cancer cells yet resulting in minimal damage to normal tissue, are urgently required.

Data of this study indicate the potential of MGN-3 in reducing the chemotoxic effects of paclitaxel *via* reducing the concentration required for killing cancer cells. The IC_{50} value for paclitaxel was reduced by over 100-fold for both MCF-7 and 4T1 cells in the presence of MGN-3. MGN-3 has the ability to sensitize cancer cells to other types of chemotherapy, such as DNR (11). Furthermore, in animal studies, MGN-3 has beneficial effects by protecting against severe weight loss due to cisplatin (37). This protection was demonstrated in some gross gastrointestinal pathological changes, as well as in the prevention of death induced by chemotherapy (38). In addition, results of clinical trials on hepatocellular carcinoma and other types of progressive cancers have shown that chemotherapy treatment in the presence of MGN-3 resulted in a higher survival rate, a lower percentage of recurrence and a marked improvement in appetite as compared with chemotherapy alone (12, 39).

We previously showed the mechanism(s) by which MGN-3 induces its daunorubicin-sensitizing effect involves the ability of MGN-3 to increase the accumulation of this chemotherapy in human BCCs (11). Results of the current study showed that MGN-3 synergizes with paclitaxel in causing greater DNA damage in 4T1 cells, enhancing their apoptosis and inhibiting their proliferation. The combined effects of both paclitaxel and MGN-3 were higher than that of either treatment alone. Several agents enhance the cytotoxic effect of chemotherapeutics in cancer cells *via* increasing intracellular drug accumulation and reversing multidrug resistance (MDR) in cancer cells, including the calcium channel blocker diltiazem and the biscoclaurine alkaloid cepharanthine (40-42), anti-arrhythmic agent quinidine (43, 44), and synthetic isothiocyanate derivative E-4IB (45). In addition, nutritional intervention toward tumor responsiveness to chemotherapy has been examined. Omega-3 polyunsaturated fatty acids have been used to increase intracellular drug accumulation in cancer cells (9). Furthermore, our earlier study showed that MGN-3 reverses MDR in HL60/AR cells (46).

Research over the past two decades has revealed that many anticancer drugs function by inducing apoptosis (47-49). We have examined the role of MGN-3 in caspase activation. Treatment with MGN-3 resulted in an increase in the number of human BCCs with active caspase-8, and -9 in MCF-7 cells and -3,-8, and -9 in HCC70 cells (50), in addition, the sensitizing effect of MGN-3 on human leukemia HUT 78 cells to CD95 antibody-induced apoptosis, was correlated with an increased number of cells with active caspase-3, -8, and -9 (10). This suggests that MGN-3 sensitizes cancer cells to daunorubicin by a mechanism that involves caspase cascades. Similar findings were reported by Bodo *et al*., whereby increased intracellular platinum accumulation post-treatment with a synthetic isothiocyanate derivative E-4IB, was accompanied by the stimulation of caspase-3 activity (45). In this study, we observed that 4T1 cells treated with paclitaxel exhibited DNA damage that was associated with an inhibition of cell proliferation. This effect may be due to the ability of paclitaxel to bind to microtubules, thus preventing cell proliferation. Paclitaxel induces cell cycle arrest and apoptosis in most types of cancer cells (51).

The promise of anticancer activity by rice and rice bran derivatives has been the focus of many studies. MGN-3 is an arabinoxylan extracted from rice bran (17) and possesses immunomodulatory function for different immune cells, such as dendritic cells (DC), NK, T- and B-cells and macrophages (17-23), and increases the production of cytokines such as tumor necrotic factor-α and interferon-γ (52). In addition, MGN-3 has demonstrated characteristics as a novel antitumor agent able to sensitize human leukemia cells to death receptor (CD95)-induced apoptosis (10), yeast-induced apoptosis of cancer cells (50), and human BCCs to daunorubicin (11). Moreover, the results of this study showed that MGN-3 exerts

a paclitaxel-sensitizing effect on metastatic 4T1 cells. These data may suggest that the food supplementation of MGN-3, in conjunction with paclitaxel chemotherapy may be useful for the treatment of metastatic breast cancer.

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