

Synergistic apoptotic effect of Arabinoxylan rice bran (MGN-3/Biobran) and Curcumin (Turmeric) on human multiple myeloma cell line U266 *in vitro*

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The present study was carried out to investigate the synergistic apoptotic potential of arabinoxylan rice bran (MGN-3/Biobran) and curcumin (turmeric) on human multiple myeloma (MM) cell line U266, *in vitro*. U266 cells were cultured with MGN-3 (50 or 100 µg/ml) and curcumin (2.5-10 µM) for 3 days. The effects of MGN-3 and curcumin on the growth and survival of the U266 cells were determined by trypan blue, MTT assay, flow cytometry analysis of cancer cell cycle, and apoptosis. Expression of proapoptotic Bax, and antiapoptotic Bcl2 was determined by Western blot analysis. Treatment with MGN-3 alone or curcumin alone caused a dose-dependent inhibition in the proliferation of U266 cells. However, a synergistic effect was noticed post-treatment with both agents that maximized at 100 µg/ml MGN-3 plus 10 µM curcumin. This synergy was characterized by an 87% decrease in cell number and a 2.6 fold increase in the percentage of apoptotic U266 cells. Cell cycle analysis showed a 53% decrease in the percentage of cells in the G0-G1 phase treated with MGN-3 and curcumin (from 36% to 17%). Analysis of the expression of the pro and antiapoptotic molecules Bax and Bcl-2 revealed synergistic effects of these agents, as the expression of Bcl-2 was decreased and Bax was increased. This resulted in a cellular microenvironment favorable for apoptosis. We conclude that MGN-3 and curcumin synergize in the induction of U266 cell apoptosis. This data may establish the foundation for *in vivo* studies that could have therapeutic implications.

Key words: U266, MGN-3, curcumin, cancer cell survival, synergistic effect

Multiple myeloma (MM) is a B-cell malignancy characterized by the latent accumulation in bone marrow of secretory plasma cells with a low proliferative index and an extended life span [1]. MM accounts for more than 10% of all hematologic cancers. Various agents used for the treatment of myeloma include combinations of vincristine, Bis-2-chloroethylnitrosourea (BCNU), melphalan, cyclophosphamide, adriamycin, and prednisone or dexamethasone [2]. Usually, patients younger than 65 years are treated with high-dose melphalan with autologous stem-cell support, and older patients who cannot tolerate such intensive treatment receive standard-dose oral melphalan and prednisone. Despite these treatments, this malignancy remains incurable, with a complete remission rate of only 5% and a median survival of 30 to 36 months [3, 4]. It is therefore of particular interest to explore new therapeutic approaches for fighting MM.

Arabinoxylan rice bran (MGN-3) and curcumin are natural products that have been demonstrated to be promising anti cancer agents in animal models [5-9]. MGN-3 is obtained by reacting rice bran hemicellulose with multiple carbohydrate

hydrolyzing enzymes from shiitake mushrooms. The main chemical structure of MGN-3 is arabinoxylan, with a xylose in its main chain and an arabinose polymer in its side chain [10]. MGN-3 has been demonstrated to be a potent activator of natural killer (NK) cells [11, 12]. Previously, we presented evidence for the role of MGN-3 as a sensitizer of human leukemic HUT78 cells to anti-CD95 antibody-induced apoptosis [13]. Turmeric (curcuma longa), which is widely used as a spice and coloring agent in several foods, has recently been shown to possess anticancer activity [5-6, 8]. The active ingredient of curcumin is diferuloylmethane. In this study, we examined the ability of MGN-3 to synergize with curcumin to induce apoptosis of U266 cells, a human MM cell line, and examined the possible mechanism(s) underlying this effect.

Materials and methods

Tumor cell line and culture condition. The human multiple myeloma cell line, U266, is a plasmacytoma of B-cell origin and they were obtained from the American

Type Culture Collection (ATCC; Rockville, MD). Tumor cells were maintained in our laboratory in a complete medium (CM) that consisted of RPMI-1640 (Irvine Scientific, Irvine) supplemented with 10% fetal calf serum (FCS, Invitrogen, Carlsbad, CA), 2 mM glutamine, and 100 mg/ml streptomycin and 100 U/mol penicillin (Sigma Chemicals, St. Louis, MO).

MGN-3. MGN-3 is a denatured hemicellulose obtained by reacting rice bran hemicellulose with multiple carbohydrate hydrolyzing enzymes from Shiitake mushrooms. MGN-3 was dissolved in CM at a concentration of 30 mg/ml. MGN-3 was provided by Daiwa Pharmaceutical Co. Ltd., Tokyo, Japan.

Curcumin (turmeric). Curcumin was purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Curcumin was prepared as a 20-mM solution in dimethyl sulfoxide and then further diluted in CM.

Cell growth and survival assays. U266 cells (1×10^4 /well) were seeded in 96-well plates and cultured in triplicate in the presence or absence of various concentrations of curcumin (2.5, 5 and 10 μ M) and/or MGN-3 (50 and 100 μ g/ml). The final volume of medium in each well after addition of curcumin and/or MGN-3 was 200 μ l. Blank wells containing medium only and cells treated with DMSO (0.05%) were used as additional controls. The plates were incubated at 37°C in humidified 5% CO₂. 72 hours after treatment, the antiproliferative effects of curcumin, MGN-3 and their combination against U266 cells were determined by standard trypan blue dye exclusion and MTT dye uptake methods as described earlier [14,15]. In this study, we terminated experiments 72 hours post-treatment based on previous work on MGN-3 and curcumin by ourselves and others [14,16-18].

Trypan blue dye exclusion method. Trypan blue was used to determine the U266 cell growth and survival after culturing with MGN-3 (50 and 100 μ g/ml) and curcumin (2.5-10 μ M) for 3 days. 100 μ l of trypan blue (0.4% solution) was added to an equal volume of treated and untreated cells. After five minutes following the addition of trypan blue, the percentages of viable (unstained) and non-viable (stained) cells were determined using a hemacytometer.

MTT assay. This assay is based on the reduction of tetrazolium salt MTT [3-(4,5-dimethylthiazol-3-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich Chemicals, St. Louis, MO] by a mitochondrial dehydrogenase in viable cells, resulting in a change from a colorless to a blue-colored formazan product that can be measured spectrometrically. The amount of formazan produced is proportional to the number of living cells. Briefly, U266 cells were treated with or without curcumin in the presence or absence of MGN-3 as described above. 72 hours post-treatment, MTT (50 μ g/well) was added to each well and the cultures were incubated for an additional 4 h. The plates were then centrifuged and the medium carefully removed. The formazan crystals were solubilized with acidic alcohol, and the plates were read at $\lambda=590$ nm using an ELISA plate reader (Molecular Devices, Menlo Park, CA). The following formula was used: Percentage cell viability = (OD

of the experiment samples/OD of the control) \times 100, where OD is optical density.

Cell cycle analysis. Flow cytometry was used to determine the effect of MGN-3 (50 μ g/ml), curcumin (10 μ M), and MGN-3 plus curcumin on the cell cycle and apoptosis. U266 cells that were treated with MGN-3 and/or curcumin for 72 hours, washed, and fixed with 70% ethanol. After incubation overnight at -20°C, cells were washed with PBS, then stained with propidium iodide (PI) and suspended in staining buffer (10 μ g/mL PI; 0.5% Tween-20; 0.1% RNase in PBS). The cells were analyzed by means of a fluorescence-activated cell sorted (FACS) caliber flow cytometer (Becton Dickinson, San Jose, CA). Cells were gated to exclude cell debris, cell doublets, and cell clumps. The proportion of cells with hypodiploid DNA as well as cell cycle phases were analyzed by CellQuest and Modfit analysis programs.

Western blot analysis. The expression of Bax and Bcl-2 was determined using Western blot analysis. The cells were cultured with MGN-3 (50 μ g/ml), curcumin (5 μ M), and MGN-3 plus curcumin for 1 day. The 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 ethylene glycol tetraacetic acid [EGTA], 1 mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na₃VO₄, 1% NP40, 1 mmol/L Lphenylmethylsulfonyl fluoride, 10 Ag/mL aprotinin, and 10 Ag/mL leupeptin [pH 7.4]) for 30 minutes and centrifuged at 14,000.g for 20 minutes at 4°C as detailed previously. The supernatant was collected and used immediately. The protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. Seventy-five μ g of the cell lysate in lysis buffer were subjected to Western blot analysis by 4%-12% SDS polyacrylamide gel electrophoresis using 1:500 anti-Bax antibody or anti-Bcl-2 antibody (BD Biosciences, San Jose, CA, USA) as primary antibody. The washed polyvinylidene fluoride (PVDF) membranes were incubated with a 1:2000 dilution of monoclonal secondary antibodies. The immunoreactive bands were visualized by using an ECL Western Blotting Kit (Amersham, Buckinghamshire, UK). To verify equal protein loading and transfer, the blots were stripped and re-probed with β -actin using an anti-actin rabbit polyclonal antibody; thereafter the same protocol was followed as detailed above. The relative intensity of each protein band in a blot was measured by using the computerized software program OPTIMAS 6.2 (Media Cybernetics, Silver Spring, MD, USA) and the ratio of Bcl-2 to Bax was analyzed. The band intensities were determined using Quantity One software from Bio-Rad.

Statistical analysis. Statistical significance was determined by Student's t test. Differences were considered significant at the $p < 0.05$ level.

Results

Effects of MGN-3 and curcumin on U266 cell survival. The effects of MGN-3 and curcumin treatments on the growth

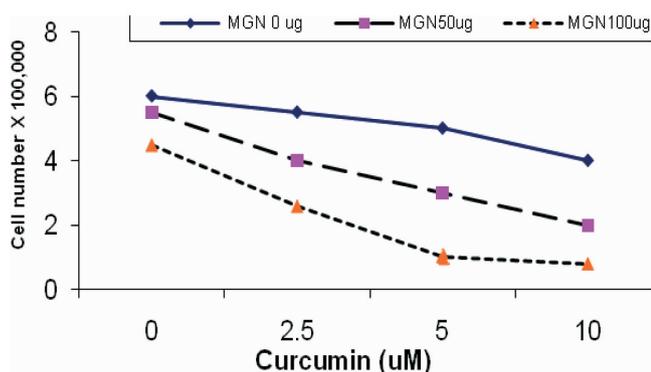


Figure 1. Synergistic effect of MGN-3 and curcumin on U266 cell growth via Trypan blue method. U266 cells were cultured with MGN-3 (50 and 100µg/ml) and curcumin (2.5-10µM) for 3 days. Cell survival was determined by trypan blue dye exclusion.

and survival of U266 cells was determined by dye exclusion and MTT assays.

Trypan blue dye. Trypan blue was used to determine the U266 cell growth and survival after culturing with MGN-3 and curcumin for 3 days. Data in Figure 1 shows that treatment of U266 cells with curcumin alone (2.5-10µM) and MGN-3 alone (50 and 100µg/ml) resulted in a decrease in cell survival that followed a dose-dependent manner. On the other hand, the synergistic effect of MGN-3 plus curcumin caused a significant decrease in cell survival that also followed a dose-dependent manner that maximized (87% decrease in cell number) at a concentration of 100µg/ml MGN-3 and 10 µM curcumin.

MTT assay. Table 1 displays the percent U266 cell survival, determined via MTT assay, post-treatment with MGN-3 (50, 100 µg/ml) and/or curcumin (2.5-10 µM). Results show each agent suppresses tumor cell growth in a dose dependent manner. However, a combination of the two agents displays a synergistic effect on cell survival, in a dose-dependent manner ($p \leq 0.0005$).

Apoptosis. U266 cells were cultured with curcumin (2.5-10µM), with or without MGN-3 (50 µg/ml). Apoptosis was determined by measuring hypodiploid DNA by FACScalibur. Data in Figure 2 shows that apoptosis induced by MGN-3 alone was similar to the background (10.0%). Curcumin alone

Table 1. Cell viability (%) via the MTT assay

Curcumin (µM)	MGN-3 (µg/ml)		
	0	50	100
0	100	88 ± 9	75 ± 8
2.5	86 ± 9	57 ± 7	51 ± 6
5	82 ± 8	54 ± 6	49 ± 6
10	76 ± 6	49 ± 7	43 ± 5*

U266 cells were treated with curcumin (2.5-10 µM) in the presence or absence of MGN-3 (50-100 µg/ml). 72 hours post treatment MTT was added and cell survival was determined. Percentage cell viability = (OD of the experiment samples/OD of the control) × 100, (* $p \leq 0.0005$).

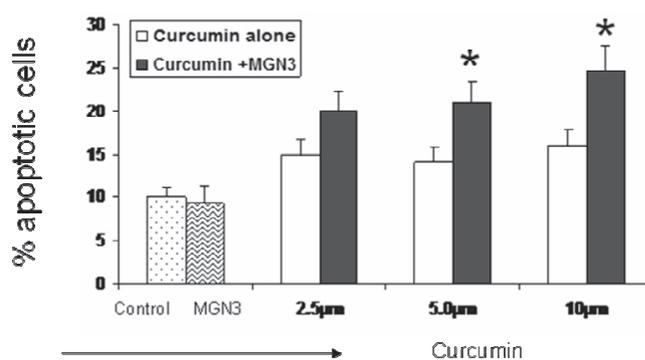


Figure 2. Apoptotic effect of MGN-3 and curcumin on U266 cells. U266 cells were cultured with MGN-3 (50µg/ml) and curcumin (2.5, 5 or 10µM) for 3 days. Apoptosis was determined by measuring hypodiploid DNA by FACScalibur. * $p \leq 0.05$

increased apoptosis in U266 cells. However, when MGN-3 was combined with curcumin, the levels of apoptosis were markedly increased to 20.0, 22.0, and 24.7% at curcumin concentrations of 2.5, 5, and 10µM, representing a 200%, 220%, and 247% increase in cancer cell apoptosis, respectively ($p \leq 0.05$), as compared to the control untreated cells with either agent.

Cell cycle. The effects of MGN-3 and curcumin treatments on the cell cycle of U266 cells were examined. Data depicted in Figure 3 shows that treatment of U266 cells with either MGN-3 (50 µg/ml) alone or curcumin (10µM) alone caused no significant change in the percentages of cells in G0-G1, S phase and G2-M phase. While the treatment of U266 cells with MGN-3 plus curcumin caused a significant decrease in the G0 phase (53%) from 36% to 17% ($p \leq 0.0005$), a 16% decrease in the S phase (from 15.5% to 13%) and a 20% decrease in the G2-M phase (from 15% to 12%) were also observed.

Pro and antiapoptotic molecules. The effect of MGN-3 and curcumin treatment on Bcl-2 and Bax expression was examined. Figure 4 shows that both MGN-3 and curcumin upregulated the proapoptotic molecule Bax. In addition, curcumin also down regulated the antiapoptotic molecule Bcl-2. However, the combination of MGN-3 plus curcumin resulted in a substantial decrease in Bcl-2 expression. This resulted in alteration of Bax to Bcl-2 ratio. The Bax to Bcl2 ratio in control, MGN-3 treated, curcumin treated and MGN-3 plus curcumin treated cells was 0.9, 1.5, 1.6 and 2.5, respectively.

Discussion

Multiple myeloma (MM) is an aggressive B-cell malignancy, and more than 90% of MM patients become chemoresistant. Therefore, several drugs have been tested in the search for a more effective treatment for MM, including PS341 (a proteasome inhibitor) and thalidomide (an inhibitor of TNF production) [19, 20]. In this study, we selected two dietary agents, arabinoxylan rice bran (MGN-3) and

curcumin, to explore their synergy in inducing apoptosis in human MM cells. Both agents have shown chemopreventive characteristics in animal models [5-9], and possess potent antioxidant potential [21-24]. The results of the current study showed that MGN-3 alone and curcumin alone have the ability to partially suppress the proliferation of U266 cells. Interestingly, a synergistic effect of both agents on U266 cell survival was noted. Combining both treatments resulted in a significant decrease in cell growth and an increase in cancer cell apoptosis.

Cell growth and apoptosis are independent, but linked, processes. In cancer cells, signaling pathways of both phenomena are deregulated. Bcl-2 family proteins are important regulators of cell survival and apoptosis. They are comprised of pro and anti apoptotic proteins. The members form heterodimers to inactivate each other. The upregulation of proapoptotic Bax expression and downregulation of antiapoptotic Bcl-2 expression have been demonstrated during both mitochondrial and death-receptor based apoptosis [25]. The mitochondrial pathway of apoptosis is dependent on the process of mitochondrial outer membrane permeabilization. Mitochondrial based apoptosis can be stimulated by caspase dependent and caspase independent pathways and is regulated by members of Bcl-2 family [26-28]. Results of this study show that curcumin downregulated antiapoptotic Bcl-2 and MGN-3 upregulated proapoptotic Bax of U266 cells. In addition, combination of MGN-3 plus curcumin resulted in a substantial decrease in Bcl-2 expression. This would suggest that the observed synergistic effect of MGN-3 and curcumin may be partially due to modulation of signaling pathways mediated by Bcl-2 family members.

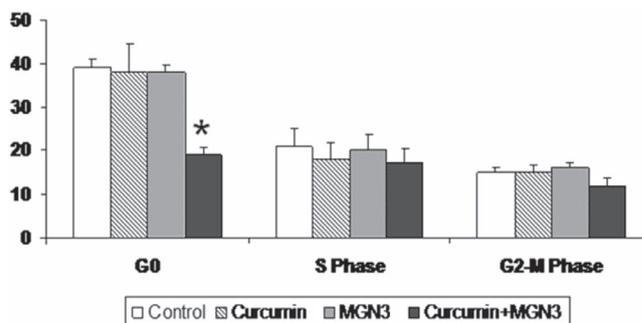


Figure 3. Cell cycle analysis of U266 cells post-treatment with MGN-3 and curcumin. U266 cells were cultured with MGN-3 (50µg/ml) and curcumin (10µM) for 3 days. Cell cycle analysis was performed using flow cytometry. *p≤0.0005

In this study we showed that the combination of curcumin and MGN-3 decreased the proportion of U266 cells in G0-G1, suggesting that these agents halt cell cycle progression and induce apoptosis. Several drugs and plant extracts have been shown to arrest cell growth at the G0-G1 phase and induce apoptosis in different cancer cell lines. These include: Gypenosides, a component of *Gynostemma pentaphyllum* Makino [29], Adiponectin [30], Terfenadine, a histamine H1 receptor antagonist [31] and Aucubin, an extract from the leaves of *Aucuba japonica* [32]. Curcumin has been reported to induce G1/S or G2/M arrest and apoptosis in other cancer cell lines [33] suggesting that the effect of curcumin is cancer cell type dependent.

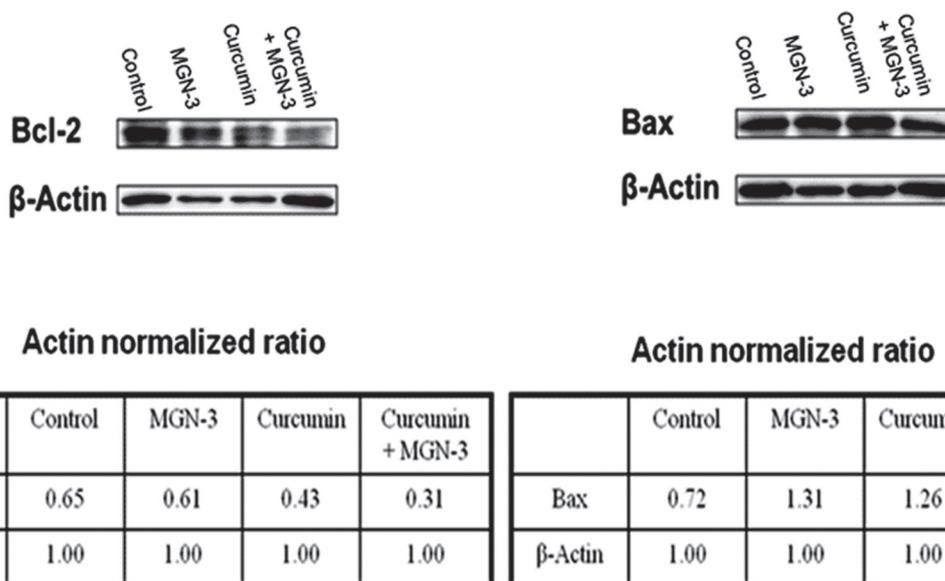


Figure 4. Effect of MGN-3 and curcumin on the expression of Bax and Bcl-2. U266 cells were cultured with MGN-3 (50 µg/ml) and curcumin (5µM) for 1 day. Bax and Bcl-2 expression was visualized via Western blot.

Nonspecific drug toxicity is a major problem in drug development. Discovery of a variety of therapeutic agents with less toxicity will lead to more effective therapies toward specific tumors. MGN-3 is an arabinoxylan extracted from rice bran [10] that has been shown to be a safe and nontoxic agent as manifested by the following: the LD50 (lethal dose, 50%) of MGN-3 is greater than 36g/kg; the Ames test for mutagenicity was negative; and the studies of subchronic toxicity, antigenicity and genotoxic testing in rats and guinea pig demonstrate that MGN-3 is nontoxic [34, 35]. In addition, absence of toxicity in humans was reported using blood chemistry analysis [11]. Similarly, curcumin is widely used as a spice and coloring agent in several foods such as curry, mustard, bean cake, cassava paste, and potato chips as well as cosmetics and drugs. Earlier studies have shown that curcumin (diferuloylmethane) is pharmacologically safe and known to have very little or no toxicity in humans [36]. Phase I clinical trials showed that humans can tolerate up to 8 g curcumin per day when it is taken orally [37].

The immune modulatory effects by both MGN-3 and curcumin have been well documented. Earlier studies have shown that both agents are potent biological response modifiers that have the ability to boost different arms of the immune cells, such as NK cells, T cells, B cells, macrophages, dendritic cells and Treg cells [11-14, 38-41]. These characteristics, in addition to their ability to synergize in inducing apoptosis of cancer cells may add an additional weapon for fighting cancer. This may suggest a rationale for considering the combination of MGN-3 and curcumin in future studies for the treatment of multiple myeloma in clinical trials.

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