RESEARCH ARTICLE

High Efficiency Apoptosis Induction in Breast Cancer Cell Lines by MLN4924/2DG Co-Treatment

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Abstract

2-deoxy-D-Glucose (2DG) causes cytotoxicity in cancer cells by disrupting thiol metabolism. It is an effective component in therapeutic strategies. It targets the metabolism of cancer cells with glycolysis inhibitory activity. On the other hand, MLN4924, a newly discovered investigational small molecule inhibitor of NAE (NEDD8 activating enzyme), inactivates SCF E3 ligase and causes accumulation of its substrates which triggers apoptosis. Combination of these components might provide a more efficient approach to treatment. In this research, 2DG and MLN4924 were co-applied to breast cancer cells (MCF-7 and SKBR-3) and cytotoxic and apoptotic activity were evaluated the by Micro culture tetrazolium test (MTT), TUNEL and ELISA methods. Caspase3 and Bcl2 genes expression were evaluated by real time Q-PCR methods. The results showed that MLN4924 and MLN4924/2DG dose-dependently suppressed the proliferation of MCF7 and SKBR-3 cells. Cell survival of breast cancer cells exposed to the combination of 2DG/MLN4924 was decreased significantly compared to controls ($p<0.05$), while 2DG and MLN4924 alone had less pronounced effects on the cells. The obtained results suggest that 2DG/MLN4924 is much more efficient in breast cancer cell lines with enhanced cytotoxicity via inducing a apoptosis cell signaling gene, caspase-3.

Keywords: Caspase 3 - bcl2 - MLN4924 - 2DG - cancer therapy

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Introduction

It was estimated that about 232,670 new cases of invasive breast cancer induced among women in the US during 2014 in comparison with 2360 new cases that were diagnosed for men. Despite advances in diagnosis and treatment, breast cancer remains a major public health problem and a major cause of death for women worldwide. Unfortunately, currently available chemotherapeutic agents for cancer diseases including breast cancer give serious side effects and cause excessive damage to normal cells (Subarnas et al., 2012). One of the biochemical characteristics related to the cancer cells is an increased utilization of glucose. The rate of glucose utilization in tumors is mostly correlated with the rate of proliferation and the degree of aggressiveness, thus cancer cells use more glucose than normal cells (Ahmad et al., 2010). Also, accelerated glucose uptake for aerobic glycolysis is one of the major metabolic changes found in malignant cells. Thus, using the nonmetabolizable glucose analog, 2-deoxy-D-glucose (2DG) interferes with glucose metabolism leading to breast cancer cell death (Zhang and Aft, 2009; Aghaee et al., 2012). 2DG is an inhibitor of glucose metabolism by inhibiting hexokinase that is the first rate-limiting enzyme of glycolysis (Ben Sahra et al., 2010). 2DG competitively inhibits glucose transport and phosphorylation by hexokinase to form 2DG-6-phosphate, which is minimally metabolized , thereby reducing the output from glycolysis (ATP) and the pentose-phosphate pathway (NAPDH) (Dwarakanath, 2009). 2DG changes N-linked glycosylation and lead s to unfolded protein responses and induces changes in gene expression and phosphorylation status of proteins involved in signaling, cell cycle control, DNA repair, Calcium influx and apoptosis (Dwarakanath, 2009). This leads to multiple related mechanisms which may involve in glucose-deprivation-induced signaling including the activation of kinases, changes in the redox state of the cell, or generation of free radicals (Zhang and Aft, 2009; Ahmad et al., 2010). Some studies *in vitro* have

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also shown that 2DG at non-toxic doses in combination with radiation therapy act as a radiosensitizer in various cancer cell types (Lin et al., 2003; Heminger et al., 2006; Dwarakanath, 2009). Furthermore, it has been reported that treatment of breast cancer cell lines with 2DG causes cell death (Aft et al., 2002; Zhang and Aft, 2009). 2DG also has been most widely investigated for targeting glucose metabolism and to be developed as a therapeutic agent or as an adjuvant in cancer therapy. Because of the tumor dependence on glycolysis, 2DG has been considered as a potential anticancer agent and association of chemotherapeutic agents and has been used successfully in mice (DiPaola et al., 2008; Simons et al., 2009). The ubiquitin-proteasome system (UPS) is responsible for the planned degradation of most intracellular proteins and NEDD8 Activating Enzyme (NAE) has been identified as an essential regulator of the NEDD8 conjugation pathway to cullin that called Neddylation (Swords et al., 2010). MLN4924 is an investigational and a newly discovered small molecule that binds to NAE at its active site to create a covalent NEDD8–MLN4924 adduct that cannot be further utilized in subsequent intra enzyme reactions. In this way, it leads to inhibiting NAE activity and prevents the neddylation (Edelmann et al., 2011; Liao et al., 2011; Wei et al., 2012). By blocking cullin neddylation, MLN4924 inactivates CRLs/SCF E3 ubiquitin ligase (SKP1, Cullins, and F-box protein). This causes accumulation of its substrates (Zhao et al., 2012), which causes DNA rereplication stress and DNA damage response (DDR), apoptosis and/or senescence (Milhollen et al., 2011; Luo et al., 2012b; Yang et al., 2012a). Various substrates accumulate upon MLN4924 treatment, including cell-cycle regulators, DNA licensing proteins , and apoptosis regulators, oncogenic proteins in a cell line–dependent manner (Lin et al., 2010b; Tan et al., 2011; Wei et al., 2012; Yang et al., 2012a; Zhao et al., 2012; Sun and Li, 2013; Yao et al., 2014). Generally MLN4924 effectively inhibits tumor cell growth by inducing all three common types of death, namely apoptosis (Soucy et al., 2009; Milhollen et al., 2010; Mackintosh et al., 2013), autophagy (Duan et al., 2011; Luo et al., 2012a; Zhao et al., 2012), and senescence (Lin et al., 2010a; Duan et al., 2011; Jia et al., 2011). Autophagy may play a dual role, prosurvival or prodeath, depend on different cellular kinds and stresses (Yang et al., 2012b). MLN4924 through inactivation of NF-κB can generates ROS, which is required for DNA damage-induced apoptosis (Emanuele et al., 2011). The cytotoxic effect of MLN4924 is found to be heterogeneous among different tumor cell lines. While profound growth inhibition and cell death have been found in same cells, additive effects on viability have been reported elsewhere (Swords et al., 2010). Because of its significant anticancer efficacy in preclinical studies, MLN4924 has been advanced into several phase I / II clinical trials for several solid tumors and hematologic malignancies (Luo et al., 2012b; Nawrocki et al., 2012). In contrast to the proteasome inhibitors that causes side effects, MLN4924 is more specific because it does not inhibit bulk proteasomal degradation (Basler et al., 2009; Edelmann et al., 2011). 2DG by itself sensitizes cancer cells to the action of other chemotherapeutics agents (Edelmann et al., 2011). Therefore, in this study, we have evaluated the effect of combined treatment of 2DG and MLN4924 on the breast cancer cells treatment.

Materials and Methods

Cell culture

MCF-7 and SKBR-3 cells (Human breast carcinoma cell line), purchased from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran), were cultured in RPMI 1640 and 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, US). At 80% confluence, cells were sub-cultured into 96-well plates or 6-well plates (Nunc, Denmark) according to the experiments. The experiments were performed in triplicate.

Drug treatment chemicals

2DG were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and MLN4924 (Activebiochem, USA). Stock solution of 2DG was dissolved in phosphate buffer saline (PBS).

MLN4924 was dissolved in dimethyl sulfoxide (DMSO) and kept in Room temperature before use or in -20ºC. The chemicals were added to cells at a final concentration of (500μM) 2DG, (30, 100, 200, 300 nM) MLN4924.

Cell viability assay

The cells were plated in triplicate and treated with different concentrations of 2DG and MLN4924 and incubated at 37°C for 48 hours in a 5% CO_2. the Cells were then treated with MTT reagent (3-(4, 5-dimetylthiazol-2-yl)-2, diphenyltetrazolium bromide) (Sigma-Aldrich, St. Louis, MO, US) according to the manufacturer's protocol. Briefly, (20μl) of 5 mg/ml MTT was added to the wells and incubated at 37°C for 4 hours in a 5% CO_2. Then 200 μl of DMSO was added to each well and were solubilized by (25μl) Sorenson buffer. Absorbandance was read at 570(nm) with ELISA plate reader.

TUNEL assay

TdT-mediated dUTP nick-end labeling (TUNEL) assay was performed by using an in situ cell death detection kit, POD (Roche Diagnostics GmbH, Germany) as described by the manufacturer's protocol. Briefly, the Cells, 48 hours after treatment, were fixed with 4% (w/v) paraformaldehyde in PBS (pH 7.4) for 1 hour at room temperature then rinsed twice with PBS. The fixed cells were incubated with blocking solution (3% H_2O_2 in methanol) for 15 min and rinsed with PBS. The cells were then incubated in permeabilisationsolution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. Subsequently, 50 μl of reaction mixture containing TdT enzyme and nucleotide was added to the cells and incubated for 1 hour. After washing three times with PBS, the slides were incubated with 50 μl converter-POD streptavidin for 30 min and rinsed three times with PBS. Finally, the cells incubated with DAB and were analyzed with a light microscopy.

Cell death detection by ELISA

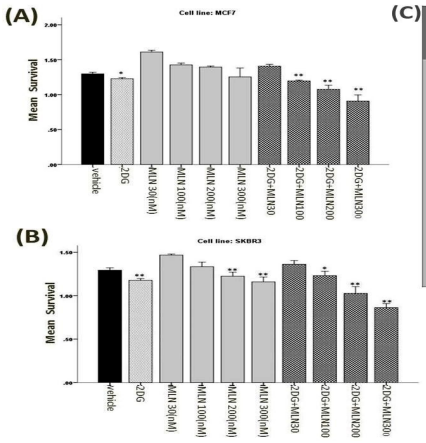
The frequency of and necrosis of the cells were measured by the Cell Death Detection ELISAPlus kit (Roche Diagnostics GmbH, Germany). Briefly, after incubation with the chemicals (at concentrations determined by MTT assay) for 48 hours, the culture supernatants and lysate of cells were prepared and incubated in the microtiter plate coated with a mixture of Anti-histone-biotin and Anti-DNA-POD anti-histone antibody. During the incubation period, the Anti-histone antibody binds to the histone-component of the nucleosomes and simultaneously captures the immune complex to the streptavidin-coated MP via its biotinylation. Additionally, the Anti-DNA-POD antibody reacts with the DNA-component of the nucleosomes. The remaining steps were carried out according to the instructions recommended by the manufacturer. The resulting color development, which was proportional to the amount of nucleosomes captured in the antibody sandwich, was measured at 405 nm wavelength using a Benchmark microtiter plate reader (BIO-RAD). The specific enrichment of mono- and oligo-nucleosomes released into the cytoplasm from these values were calculated using the following formula: mU = absorbance [10^{-3}]

$$\text{Enrichment factor} = \frac{\text{mU of the sample (dying/dead cells)}}{\text{mU of the correspanding negative control}}$$

RT-PCR and q-PCR

Following the treatment, total RNA was extracted using RNX-Plus reagent (Cinagen Co., Iran.) according to the manufacturer's protocol and Concentration of the purified RNA was determined by optical density at 260 and 280 nm wavelengths. Reverse transcriptase reaction was performed using Revert AidTM First Strand cDNA synthesis kit (MBI, Fermentas, Lithuania). Briefly, RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) before cDNA synthesis to avoid DNA contamination.

Specific primer sequences were designed for caspase-3 and bcl2 and β-actin as housekeeping gene. The primers were included; bcl2 primers, 5´-GAGACAGCCAGGAGAAATCA-3´(forward primer) and 5´-CCTGTGGATGACTGAGTACC-3´ (reverse primer), P53 primers: 5´- TGGGCGTGAGCGCTTCGAGA-3´ (forward primer) and 5´- GGTGGCTGG AGTGAGCCCTGC-3´ (reverse primer), β-actin primers: 5´- TCCCTGGAGAAGAGCTACG-3´ (forward primer) and 5´- GTAGTTTCGTGGATGCCACA-3´ (reverse primer). The sequence were done by an initial denaturation step at 95°C for 10 min was followed by 45 cycles at 95°C for 20 sec and 60°C for 1 min. Relative bcl2 and caspase-3 mRNA expression was calculated with the 2 -(ΔΔCT), (Livak and Schmittgen, 2001) using β-actin as a reference gene.



(C)

Treatment	Viable cells(%)	
	MCF7	SKBR-3
Control	100¹	100¹
2DG 500(μM)	94.6	90.9
MLN30(nM)	124	113.5
MLN100(nM)	109.8	103
MLN200(nM)	107.5	94.5
MLN300(nM)	99.2	89.6
2DG/MLN30	108.4	105.3
2DG/MLN100	92.1	95.1
2DG/MLN200	82.8	79.3
2DG/MLN300	69.93	66.6

Figure 1. 2DG Sensitizes breast Cancer Cells to the MLN4924 Drug Treatment. **(A)** MCF7 and **(B)** SKBR3 cell lines were treated with the different concentrations of MLN4924 and 2DG (500 μM). The cells were seeded in 96-well plates in triplicates and treated with increased concentrations of MLN4924 and 2DG alone and in combination for 48 hours. Cell Viability was assessed by the MTT assay. *p<0.05; ** p<0. 01. **(C)** Percentage of viable cells induced by the different treatments on the two cell lines. Results are expressed as the mean percentage of the dead cells in triplicate experiments with three wells each. 1a control sample of 100 is considered and numbers were normalized to 100

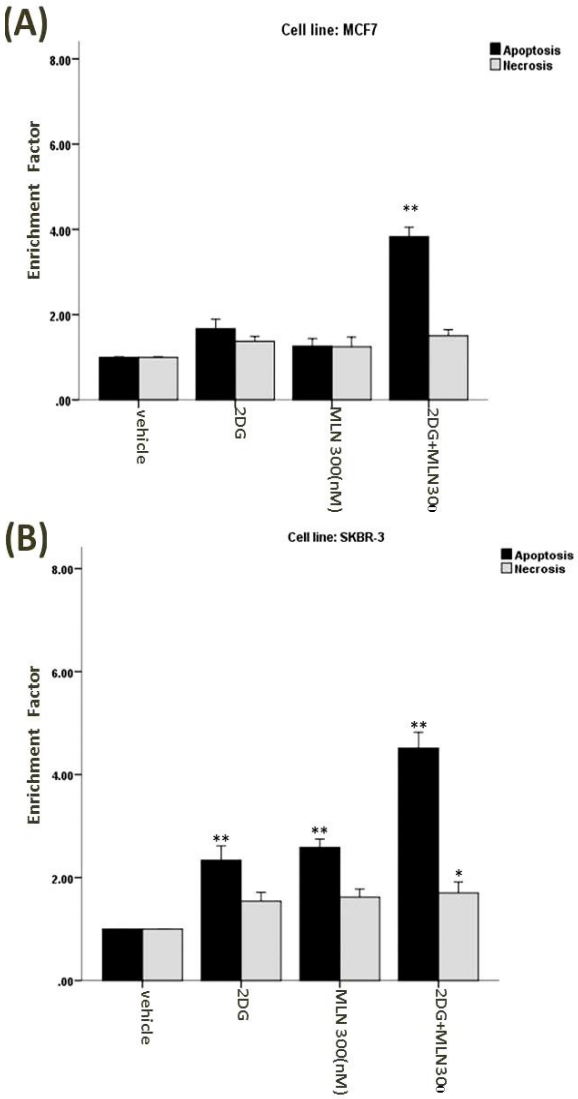


Figure 2. The enrichment of Apoptosis and Necrosis Compared to Controlled Sample by 2DG and/ or MLN4924 treatments. Measured by cell death ELIZA assay. **(A)** MCF7. **(B)** SKBR-3. Data presented are themean±SEM of two independent experiments. *, p<0.05; **p<0.001

Statistical analysis

The data were expressed as mean ± standard deviation (SD) for at least three independent determinations in the triplicates for each experimental point. One-way ANOVA were used with SPSS software for statistical comparisons involving multiple groups, followed by Tukey test to determine significance of each groups ($p<0.05$).

Results

Cytotoxic effects of 2DG/ MLN4924 on cancer cell lines

SKBR-3 and MCF7 human breast cancer cell lines were incubated with several concentrations of MLN4924 (30,100,200,300 nM) and 2DG (500 μM) for 48 hours, alone and in combination. Cell viability was measured with MTT assay. The results of these experiments are shown in Figure 1. The treated cells with 2DG/MLN4924 in comparison to the untreated control cells were shown a significant decline in viability, whereas treated cells with 2DG alone had little cytotoxic effect so the percentage of cell viability for MCF7 and SKBR-3 cells found to be 94.6 and 90.9, respectively. But MLN4924 in low concentration did not show cytotoxic activity. With higher concentrations of MLN4924 treatment on SKBR-3 cells, the cytotoxicity effect was achieved. 2DG in combination with MLN4924 (300 nM) caused greater effect on cell cytotoxicity in each two cell lines. Therefore, anti-proliferative effect of drugs was determined by MTT method showed a dose-dependent inhibition of the cell growth.

Induction of apoptosis by2DG/ MLN4924 on breast cancer cell lines

As determined by MTT assay, effect of exposure of the both cell lines with MLN4924 (300 nM) and 2DG (500 μM) for 48 hours were chosen for ELISA to determine the mechanism of the cytotoxic effects of 2DG, MLN4924 and 2DG/ MLN4924. Apoptosis and necrosis of cells were measured by cell death detection ELISA kit. Obtained results revealed that in comparison to the treatment alone with 2DG and MLN4924, 2DG/MLN4924 were dominantly induced apoptosis activity in the both cell lines. The results of these experiments are shown in Figure 2. Also since DNA fragmentation is one of the hallmarks of apoptotic cell death, in the current study, we confirmed the presence of nucleosomal DNA fragments in cells that treated with 2DG/MLN4924 by TUNEL assay. As shown in Figure 3, the apoptotic cells produced brown stained nuclei, whereas the nonapoptotic cells were not stained with similar observation was found in the control cells. Therefore, the results showed the potential of the treatment as an anticancer agent to inhibit cell growth and trigger apoptosis.

2DG/ MLN4924 cause more expression of caspase-3 in breast cancer cell lines

ELISA and TUNEL examinations clearly showed occurrences of apoptosis in both cell lines following the treatments. Therefore, to determine the apoptosis pathway and expression levels of caspase3 and Bcl2 mRNA in the cell lines treated with 2DG, MLN4924 and 2DG/ MLN4924 quantitative RT-PCR assay was used (Figure 4). Interestingly, in the cells were treated with drug

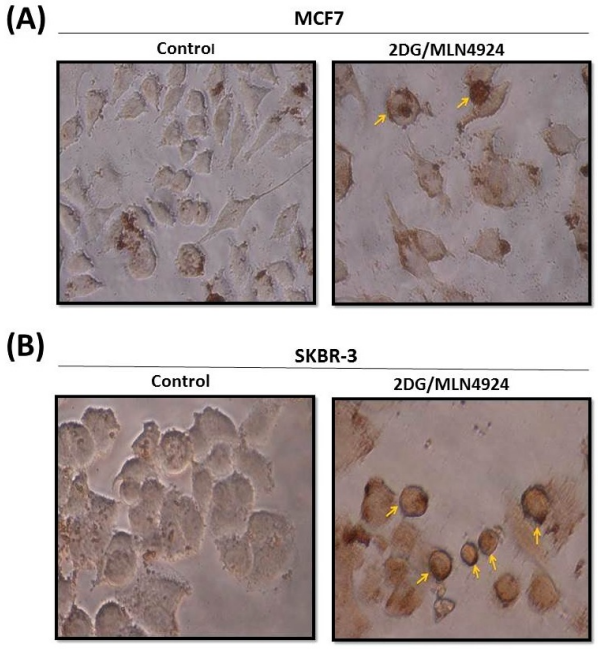


Figure 3. The Effect of 2DG/MLN4924 Treatment on: (A) MCF-7 Cell Lines and (B) SKBR-3 Cell Lines. The images show characteristic apoptotic morphology in TUNEL assay. Arrows indicate representative apoptotic cells

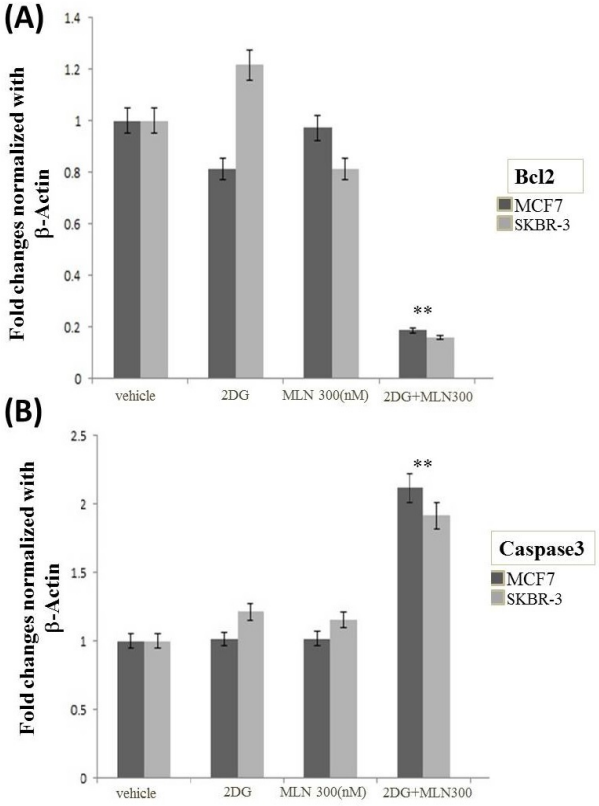


Figure 4. The mRNA Expression Levels of Bcl2 and Caspase-3 Following 2DG and/or MLN264 Treatments were Detected by Real Time PCR Assay. The mRNA levels for Bcl2 and Caspase-3 were normalized by the mRNA level of β-actin. (A) Expression of Bcl2 gene in both cell lines. (B) Expression of Caspase-3 gene in both cell lines. *p<0.05

combination compared to controls as well as cells treated with 2DG and MLN4924 alone, caspase-3 expression in both cell lines increased. Also expression level of Bcl2 reduced in this treated group.

Discussion

Breast cancers, like other cancers, depend on increased glucose uptake to sustain cell growth (Dwarakanath, 2009). Studies have shown that 2DG increases the efficacy of other chemotherapeutics agents, for example (DOX and Pacitaxel) in human osteosarcoma and non-small cell lung cancers *in vivo* (Kern and Norton, 1987). Also, 2DG plus DOX and BSO significantly enhances cytotoxicity via oxidative stress and via disruptions to thiol metabolism in breast cancer cells (Ahmad et al., 2010). recently, the effort to discover small-molecule inhibitors against CRL/SCF led to the discovery of MLN4924. MLN4924 potently reduces the viability of AML cells and disrupted the ability of AML cells to form colonies and led to a dose-dependent induction of apoptosis (Swords et al., 2010). Therefore using 2DG, as an Inhibitor of glucose metabolism, may enhance the chemo-sensitivity effect in human cancers. In the present study, as a first step to provide a scientific evidence for anti-cancer property of 2DG and MLN4924, the related cytotoxic activities on human breast cancer cell lines were evaluated. In breast cancer, MCF7 and SKBR-3 cell lines viability were decreased with increasing treatment concentration of MLN4924 (30-300 nM) (from 124 % to 99.2 % for MCF7, from 113.5% to 89.6% for SKBR-3) and with increasing treatment concentration of 2DG/MLN4924 (from 108.4 % to 69.93 % for MCF7, from 105.3% to 66.6% for SKBR-3) (Figure 1C). It appears that that the combination treatment by the two drugs has a much stronger deleterious effect than either drug. These findings support previous studies on 2DG that was increased the effects of other chemotherapy drugs (DOX) and reduced the viability of breast cancer cell lines (Ahmad et al., 2010). But unlike previous studies, we did not find a very significant impact on the treatment of MCF7 cell lines by 2DG alone at 500 μM (Ahmad et al., 2010). We also observed a significant reduction in survival and apoptosis induction in SKBR-3 cells compared to MCF7 cells that treated with 2DG alone and the combination therapy. These results confirmed the results of a study on the effect 2DG alone and in combination with DOX, CP, 5-FU, Herceptin, Cyclophosphamide, in the viability of the SKBR3 cells (Zhang and Aft, 2009). The observed increased viability in breast cancer cells in low concentrations of MLN4924 might be due to autophagy (Figure 1A-B). This is in accordance with other studies that investigate the effect of MLN4924 on several cancer cell lines of AML (Swords et al., 2010). These results showed that 2DG/MLN4924 inhibited the growth of MCF-7 and SKBR-3 cell lines, particularly in high concentrations of MLN4924 combined with 2DG. Microscopic studies showed morphological changes of the cells too. Chromatin condensation, cell shrinkage and other alterations, characteristics of apoptotic cells, caused the morphology changes of the treated cells, particularly cells that are treated with the 2DG/MLN4924 (Figure 3). In order to determine the anti-proliferative activity of the drugs through induction of apoptosis, cell death detection ELISA was used to quantify the nucleosome production during nuclear DNA denaturation of apoptotic cells. These results indicate that the antitumor effect of 2DG / MLN4924 in this breast cancer cell lines accrues via an apoptosis dependent pathway. Therefore, further studies are required to evaluate whether the treatment cause the cell cycle arrest or apoptosis induction. We have shown that the combination of 2DG and MLN4924 induce apoptosis as evidenced by the processing of caspase-3 to its active form (Figure 4). The results on SKBR-3 cells showed that the expression of caspase-3 became greater after combined treatment in compare with the control. It has been reported that glucose deprivation triggers apoptosis in breast cancer cells and Bcl2 inhibits apoptotic death (Lee et al., 1997). Also it has been shown that induction of apoptosis by 2DG has been independent of Bcl2 (Zhang and Aft, 2009). In our study there wasn't also find a significant change in the level of bcl2 expression in both cell lines that have been treated with 2DG alone compared to 2DG / MLN4924. These results provide clear-cut evidence that 2DG in combination with MLN4924 can be considered as an effective method to inhibit oncogene expression and activate apoptotic and tumor suppressor genes. In conclusion, the results of current research demonstrate the *in vitro* cytotoxic and apoptotic activity of 2DG/MLN4924 in human breast carcinoma cell lines. Therefore, a new potential chemotherapeutic approach for the treatment of breast cancer is suggested in this study. Also, a dose-dependent inhibition of the breast cancer cell proliferation with MLN4924, possibly via an apoptosis-dependent pathway, were found. The obtained data well established the anti-proliferative effects of 2DG/MLN4924 by induction of apoptosis as evidenced by the processing of caspase-3 to its active form.

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