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PI3K/Akt Pathway Activation Attenuates the Cytotoxic Effect of Methyl Jasmonate Toward Sarcoma Cells[1]

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Abstract

Methyl jasmonate (MJ) acts both *in vitro* and *in vivo* against various cancer cell lines. Activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway results in decreased susceptibility to cytotoxic agents in many types of cancer cells. We found a strong inverse correlation between the basal level of phospho-Akt (pAkt) and the sensitivity to MJ among sarcoma cell lines. Nevertheless, levels of pAkt increased in two sarcoma cell lines, MCA-105 and SaOS-2, after MJ treatment. Treatment of both cell lines with PI3K/Akt pathway inhibitors in combination with MJ resulted in a synergistic cytotoxic effect. Moreover, cells transfected with a constitutively active Akt were less susceptible to MJ-induced cytotoxicity in comparison with cells transfected with an inactive form of Akt. Taken together, these data suggest that the increase in pAkt after treatment with MJ played a protective role. Because it has been shown that the antiapoptotic effects of Akt are dependent on glycolysis, we examined the role of glucose metabolism in activation of Akt and the subsequent resistance of the cell lines to MJ. 2-Deoxy-D-glucose, a glycolysis inhibitor, decreased the levels of pAkt and was able to attenuate the MJ-induced elevation in pAkt. Accordingly, the presence of glucose attenuated MJ-induced cytotoxicity. Moreover, treatment with 2-deoxy-D-glucose in combination with MJ resulted in a synergistic cytotoxic effect. In conclusion, the PI3K/Akt pathway plays a critical role in the resistance of MCA-105 and SaOS-2 sarcoma cell lines toward MJ-induced cytotoxicity.

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Introduction

Jasmonates are a group of plant stress hormones that are produced in plants upon exposure to various types of stress. Several groups have reported in recent years that members of the plant stress hormones family of jasmonates, and some of their synthetic derivatives, exhibit anticancer activity *in vitro* and *in vivo*. The initial report [1] indicated that jasmonates can induce both death and suppression of cellular proliferation in various human and mouse cancer cell lines, including breast, prostate, melanoma, lymphoblastic leukemia, and lymphoma cells. Jasmonates were also found to suppress the proliferation or kill various other cancer cells including lung carcinoma and myeloid leukemia cells [2,3]. Furthermore, jasmonates increased the life span of EL-4 lymphoma-bearing mice [1] and exhibited selective cytotoxicity toward cancer cells while sparing normal blood lymphocytes, even when the latter were part of a mixed population of leukemic and normal cells drawn from the blood of chronic lymphocytic leukemia patients [1,4].

The phosphatidylinositol 3-kinase (PI3K)/Akt (also known as protein kinase B) pathway regulates fundamental cellular functions, such as transcription, translation, proliferation, growth, and survival, and is often overactivated in a wide range of tumor types [5–7]. Akt is a serine/threonine kinase that is recruited to the plasma membrane and is activated by phosphorylation in response to growth factor or stress signaling. Once activated, Akt modulates the function of numerous downstream substrates involved in the regulation of survival, growth, and cell cycle progression. This modulation includes inhibition of

Abbreviations: MJ, methyl jasmonate; 2-DG, 2-deoxy-D-glucose; STS, soft-tissue sarcoma; PI3K, phosphatidylinositol 3-kinase; pAkt, phospho-Akt

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proapoptotic factors such as BAD [8] and caspase-9 [9] and activation of antiapoptotic factors such as IKK [10] and CREB [11].

In addition to its role in the biology of human tumors, this pathway also plays a major part in the resistance of tumor cells to conventional anticancer therapies [12]. It has been shown in several studies that elevated Akt activity attenuates the sensitivity of cancer cell lines toward different chemotherapeutic agents such as vincristine, staurosporine, and TRAIL [13–15]. In addition, phospho-Akt (pAkt) expression level has been found to be a significant prognosticator in patients with different types of cancers such as breast cancer [16], gastric carcinoma [17], and soft-tissue sarcomas (STSs) [18]. Blocking the PI3K/Akt pathway could therefore simultaneously inhibit the proliferation and growth of tumor cells and sensitize them toward different cytotoxic agents.

Sarcoma is a general class of cancers that originate from connective tissues such as fat, muscle, nerve, bones, and cartilage. Although a relatively less common type of cancer, the incidence of sarcoma is not negligible, with 15,000 new STS and osteosarcoma (OS) cases diagnosed in the United States every year [19]. First-line chemotherapy yields disappointing results in advanced STS. Doxorubicin and ifosfamide are the most active drugs in the treatment of patients with STS; however, failure of these drugs leaves patients with very few therapeutic options [20].

Like other cancers, sarcomas seem to have abnormally activated growth factor signaling pathways. The most common growth factor pathways that seem to be activated in a variety of sarcomas include the insulin-like growth factor 1 receptor pathway in rhabdomyosarcomas and leiomyosarcomas, the PDGFR pathway in desmoplastic round cell tumors and OSs, the c-KIT receptor pathway in Ewing's sarcomas, and the c-MET–receptor pathway in synovial sarcomas and rhabdomyosarcomas.

A convergence point of activation of these growth factor receptors is downstream activation of PI3K-AKT [21]. Several studies have indicated that Akt functions as a predominant molecule in different sarcoma tumors [22,23]. In addition, Akt expression has been shown to possess a significant prognostic value in STSs [18]. In that study, elevated pAkt levels were in correlation with poor disease-free and overall survival. Moreover, a recent study showed that Akt inhibition results in significant antitumor activity against human STS *in vitro* and *in vivo* [24].

Hexokinase (HK) is the initial enzyme in the glycolytic pathway. Hexokinase types I and II can associate with the mitochondrial membrane protein voltage-dependent anion channel (VDAC). Dissociation of HK from VDAC leads to mitochondrial perturbation and promotes cell death [25,26]. In cancer cells, HK binding to mitochondria and VDAC expression are elevated [27]. The elevated levels of mitochondria-bound HK in cancer cells, as well as its glucose phosphorylation activity, are suggested to play a pivotal role in cancer cell growth rate and survival by both improving energy supplies and protecting against mitochondria-mediated cell death [28,29]. Indeed, HK is considered as an attractive target for anticancer therapy [30,31]. Furthermore, it has been shown that mitochondria-bound HK is found in sarcoma cells [32].

The specific connection between Akt signaling and mitochondrial HK in the regulation of cell death has been studied in rat fibroblasts. Prevention of apoptosis by Akt depends on mitochondrial HK [26,33,34]. In a recent paper published by our group, methyl jasmonate (MJ) was shown to bind to and detach HK from the mitochondria of various cancer cells [35]. In addition, MJ induces the detachment of HK from mitochondria isolated from MCA-105 cells (results not shown). Thus, the interaction between MJ and HK, taken together with the data regarding the Akt-HK axis, led us to investigate the effect of MJ on the PI3K/Akt pathway.

The aim of our study was to examine the involvement of the PI3K/Akt pathway in the cytotoxic effect of MJ toward various cancer cell lines, particularly sarcoma cell lines. We found that the reduced susceptibility to MJ was in correlation to pAkt levels in the sarcoma cell lines. Methyl jasmonate treatment led to an increase in pAkt levels in several cell lines, which could be attenuated using PI3K/Akt inhibitors, compromising the cells' ability to resist the cytotoxic effect of MJ. Expression of a constitutively active Akt protected the cells from MJ-induced cytotoxicity. Taken together, these data suggest that the increase in pAkt after treatment with MJ played a protective role.

In addition, we examined the relationship between Akt and glucose metabolism in the susceptibility of the cell lines to MJ. The signaling between Akt and glucose metabolism is bidirectional: Akt regulates glucose metabolism by various mechanisms such as increased glycolysis and glucose uptake, whereas glucose is required for the antiapoptotic functions of both native and constitutive forms of Akt [33,36,37].

We found that the presence of glucose attenuated MJ-induced cytotoxicity. Moreover, we found that treatment with 2-deoxy-D-glucose (2-DG), a glycolysis inhibitor, in combination with MJ resulted in a synergistic cytotoxic effect. 2-Deoxy-D-glucose decreased the levels of pAkt and was able to attenuate the MJ-induced elevation in pAkt. Thus, blocking glycolysis is an additional approach to inhibit the activity of Akt and to sensitize the cells to the cytotoxic effect of MJ.

Materials and Methods

Reagents

Methyl jasmonate [methyl 3-oxo-2-(2-pentenyl) cyclopentaneacetic acid] and 2-DG were purchased from Sigma-Aldrich Chemie GmbH Steinheim, Germany. Akt inhibitor 1 (1L6-hydroxymethyl-chiro-inositol-2-(*R*)-2-*O*-methyl-3-*O*-octadecyl-*sn*-glycerocarbonate) and the PI3K inhibitor, wortmannin, were purchased from Calbiochem, San Diego, CA. Methyl jasmonate was dissolved in ethanol to give a stock solution of 500 mM. Akt inhibitor 1 and wortmannin were dissolved in DMSO to give stock solutions of 25 and 50 mM, respectively. 2-Deoxy-D-glucose was dissolved in culture medium. Further dilutions were performed in culture medium. The final concentrations of ethanol and DMSO did not exceed 0.6% and 0.1%, respectively.

Cell Lines and Growth Conditions

CT-26 murine colon carcinoma, A-549 human lung carcinoma and B-16 murine melanoma were purchased from the American Type Culture Collection (Rockville, MD).

MCA-105 murine fibrosarcoma cells were kindly provided by Prof. Ronit Sagi Eisenberg (Tel Aviv University, Israel). SK human leiomyosarcoma cells were kindly provided by Prof. Lea Eisenbach (Weizmann Institute of Science, Rehovot, Israel). SaOS-2 human OS cells were kindly provided by Prof. Levana Sherman (Tel Aviv University, Israel). HCT-116 human colon carcinoma cells were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). RD human rhabdomyosarcoma cells were kindly provided by Prof. Haim Werner (Tel Aviv University, Israel). All cell lines were maintained in Dulbecco's modified Eagle's medium (Biological Industries, Beit-Haemek, Israel) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate,

and 1:100 dilution of nonessential amino acids (all purchased from Biological Industries). For experiments of cytotoxicity in the absence of glucose, Dulbecco's modified Eagle's medium without glucose was used (GIBCO-Invitrogen, Paisley, Scotland, UK).

Cytotoxicity Assay

Cells were plated into 96-well microtiter plates (Corning, Inc., Corning, NY) at a density of 3×10^3 cells per well and were allowed to adhere before treatment. The cells were exposed to MJ, Akt inhibitor 1, wortmannin, 2-DG, or their combinations at different concentrations for 16 hours. Akt inhibitor 1, wortmannin, and 2-DG were added 1 hour before MJ addition.

Cell proliferation was determined using the 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) kit. The assay is based on the ability of metabolic active cells to reduce the tetrazolium salt XTT to orange compounds of formazan. The dye formed is water-soluble, and the dye intensity is read at a given wavelength (490 nm) with a VERSAmax microplate ELISA reader (Molecular Devices, Sunnyvale, CA). Optical density is directly proportional to the number of living cells in culture. Cytotoxicity (%) was calculated as follows: [(absorbance of control cells − absorbance of drug-treated cells)/absorbance of control cells] × 100.

Detection of Protein Levels—Western Blot

Cells were seeded in six-well plates at 0.7×10^6 cells per well and allowed to adhere. After treatment with the relevant substances, cells were trypsinized and centrifuged at 1500 rpm for 5 minutes. Cells were washed with PBS and incubated with 60 µl of lysis buffer (50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 0.5% deoxycholic acid, 1% NP-40, 1 mM sodium orthovanadate, and 0.2% protease inhibitor cocktail; Sigma-Aldrich Co., St. Louis, MO) for 1 hour on ice. The protein concentration of the sample was determined by BioRad Dc Protein Assay (Bio-Rad Laboratories Inc., Hercules, CA). Protein extracts were mixed with sample buffer (5:1), consisting of 60 mM Tris, pH 6.8, 3% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, and 0.05% bromophenol blue, and were boiled for 5 minutes. Protein samples (30 µg of whole-cell lysate) were subjected to SDS–polyacrylamide gel (12%) electrophoresis (SDS-PAGE) followed by immunoblot analysis at 4°C overnight using the following primary antibodies: anti-pAkt (Ser473), anti–phospho-GSK-3α/β (Ser21/9), anti–total-Akt (all at 1:1000; Cell Signaling, Beverly, MA), and antiactin (at 1:500; Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibodies were detected using horseradish peroxidase–conjugated secondary antibodies (1:10,000) for 1 hour. The targeted protein was revealed by enhanced chemiluminescence (ECL). The membrane was incubated with an ECL solution (Biological Industries) and exposed to ECL film (Eastman Kodak, Rochester, NY).

Transient Transfection

The expression constructs pCMV5-myr wt PKB and myr KD (inactive) PKB K179A containing a myristoylation sequence were a kind gift from Prof. Alex Levitzki (Hebrew University, Jerusalem, Israel). Permuted enhanced green fluorescent protein was purchased from Clontech, Mountain View, CA. For transfections, cells were seeded into 24-well plates at a density of 0.4×10^5 cells per well. A mix of preincubated Metafectene transfection reagent (Metafectene Biontex Laboratories, Germany) and DNA (ratio of 5:2) was added to the wells. After overnight incubation, medium in wells was replaced with a fresh medium, and cells were incubated for 48 additional hours, during which cells were subjected to different treatments.

To evaluate transfection efficiency, cotransfection of the relevant plasmid and the EGFP plasmid was performed. The fraction of transfected cells was evaluated using a fluorescent microscope (IX51; Olympus, Tokyo, Japan).

Flow Cytometric Analysis of Cell Death

Cells were seeded in 24-well plates at a density of 0.4×10^5 cells per well and were allowed to adhere. After transfection and treatment with the relevant substances, cells were harvested and stained with propidium iodide (PI) to discriminate between live and dead cells. Live cells show a strongly reduced uptake of the charged cationic dye PI compared to dead cells [38]. The percentage of dead cells was assessed using the flow cytometer apparatus (FACScan; Beckton-Dickinson, San Jose, CA) and the WinMDI 2.8 software WinMDI v. 2.7 (Scripps Research Institute, La Jolla, CA).

Protein Quantification

Immunoblot images were digitized, and the optical densities of specific antigen-antibody complexes were quantified using the Gene Gnome Imaging System supported by the Gene Tools software package (both from Syngene, Frederick, MD).

Statistical Analysis

Results are presented as mean ± SD of *n* independent experiments. Statistical significance was assessed using the 2-tailed Student's *t* test. $P < .05$ was considered statistically significant.

The Bliss additivism model [39] was used to calculate the expected effect of combining MJ and wortmannin, Akt inhibitor 1, or 2-DG. Calculations were done using the following formula: $E_{\text{bliss}} = E_A + E_B - E_A \times E_B$, where E_A and E_B are the fractional inhibitions obtained by drug A alone and drug B alone at specific concentrations. Here, E_{bliss} is the fractional inhibition that would be expected if the combination of the two drugs was exactly additive. If the experimentally measured fractional inhibition is greater than E_{bliss}, the combination was said to be synergistic.

Results

Cytotoxicity of MJs Toward Sarcoma and Carcinoma Cell Lines

The cytotoxic activity of MJ was tested *in vitro* against four sarcoma (MCA-105, SaOS-2, SK-LMS, and RD) and four carcinoma (A-549, B-16, CT-26, and HCT-116) cell lines. Each cell line was exposed to MJ for 16 hours at concentrations ranging from 0.5 to 3 mM, and cytotoxicity was determined as described in the Materials and Methods section. Methyl jasmonate is a plant stress hormone, and we chose its concentration based on the plasma concentrations achieved upon administration of a well-studied plant stress hormone, salicylic acid. Salicylic acid is a nonsteroidal anti-inflammatory drug. Whereas most nonsteroidal anti-inflammatory drugs (such as indomethacin) act in the micromolar range, salicylates act in the low millimolar range. The highest nontoxic pharmacological concentration of salicylate used in humans is approximately 3 mM [40]. As can be seen in Figure 1, all cell lines responded in a dose-dependent fashion to MJ.

Basal Akt Expression Is Correlated with Sensitivity to MJ in Sarcoma But Not Carcinoma Cell Lines

Because Akt is known to be involved in the resistance of cancer cell lines toward numerous chemotherapies and cytotoxic agents [41,42], we hypothesized that this kinase could confer resistance to MJ as well. To evaluate the basal Akt expression in the different cell lines, we used Western blot analysis using specific antibodies and characterized the expression levels of pAkt, indicative of Akt activation, and Akt. As can be seen in Figure 2*A*, differential basal levels of pAkt were observed in the different cell lines. In contrast, basal total Akt levels were similar in all cell lines.

To evaluate the relationship between basal pAkt expression and the sensitivity to MJ, we quantified the expression of pAkt and Akt and examined the correlation between the pAkt/Akt ratio and the cytotoxic effect of 1 mM MJ in each cell line. As can be seen in Figure 2*B*, a strong inverse correlation was observed between pAkt/Akt ratio and the cytotoxic effect of MJ in the sarcoma cell lines ($R^2 = 0.9028$). However, only poor correlation was observed in the carcinoma cell lines ($R^2 = 0.3281$).

Methyl Jasmonate Modulates the Levels of pAkt in Sarcoma Cell Lines

Previous studies have shown that exposure of cells to different chemotherapies and cytotoxic agents leads to changes in the expression levels of pAkt [43–45]. Thus, we next determined whether exposure to MJ will affect the activation of Akt in the sarcoma cell lines. As can be seen in Figure 2*C*, treatment with MJ led to a differential response in pAkt expression. Methyl jasmonate exposure led to an increase in pAkt levels in MCA-105 and SaOS-2 cell lines and a decrease in the SK-LMS cell line.

Inhibition of the PI3K/Akt Pathway Sensitizes MCA-105 and SaOS-2 Cell Lines to MJ

The increase seen in pAkt in MCA-105 and SaOS-2 cells after treatment with MJ led us to hypothesize that this elevation in pAkt levels might have a cytoprotective role, which confers relative resistance to the cytotoxic effect of MJ. To determine whether the MJ-induced increase in pAkt has a protective role, we used two different inhibitors of the PI3K/Akt pathway. Wortmannin is an irreversible and highly specific PI3K inhibitor [46]. Akt inhibitor 1 binds to the pleckstrin homology domain of Akt, disrupting the translocation of Akt to plasma membrane and its ability to be phosphorylated and activated by membrane-bound phosphatidylinositol-dependent kinases [47]. MCA-105 and SaOS-2 cells were treated with 0.5 to 1 mM MJ, 40 μM wortmannin, 25 μM Akt inhibitor 1, or combinations of MJ with either inhibitor for 16 hours, and cell viability was quantified. As can be seen in Figure 3*A*, in both cell lines, treatment with combinations of MJ and wortmannin yielded a cytotoxic effect that was greater than the predicted combined cytotoxic effect according to the Bliss additivism model [39]. Activity of wortmannin was verified by evaluating pAkt levels by Western blot (Figure 3*B*). In addition, treatment of MCA-105 and SaOS-2 cells with a combination of MJ and Akt inhibitor 1 also yielded a synergistic cytotoxic effect according to the Bliss model (Figure 3*C*).

To further investigate the hypothesis that Akt protects the cells from the cytotoxic effect of MJ, we next examined the changes in pAkt levels in response to exposure of MCA-105 cells to MJ and the Akt inhibitor. As can be seen in Figure 3*D*, treatment with MJ resulted in an increase in pAkt and its downstream substrates pGSK-3α/β levels, whereas, as expected, treatment with Akt inhibitor 1 resulted in a decrease in pAkt and pGSK-3α/β levels. Interestingly, the combination of MJ and the Akt inhibitor resulted in reduced levels of pAkt and pGSK-3α/β levels compared with MJ by itself. Thus, it can be concluded that treatment with the Akt inhibitor prevented the MJ-induced increase in pAkt in MCA-105 cells.

Overexpression of Constitutively Active Akt in MCA-105 Cells Confers Protection from the Cytotoxic Effect of MJ

Because inhibition of Akt sensitized MCA-105 cells to the cytotoxic effect of MJ, we sought to examine the effects of overexpression of constitutively active Akt on MJ-induced cytotoxicity. MCA-105 cells were transiently transfected with a GFP-encoding vector (used as control), myr-Akt (encoding a constitutively active Akt) or myr-KD-Akt K179A (encoding an inactive Akt), and Akt levels were

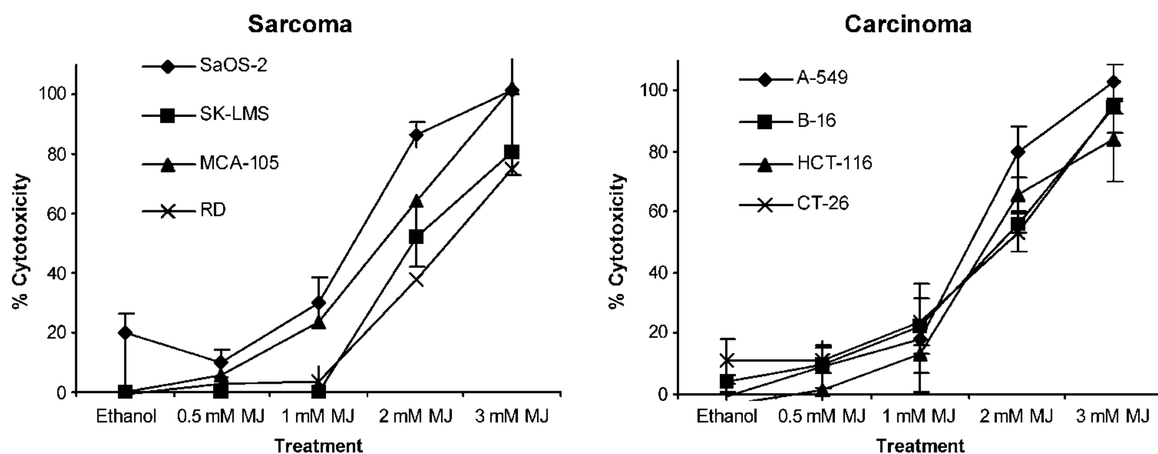


Figure 1. The cytotoxic effect of MJ toward sarcoma (left panel) and carcinoma (right panel) cell lines. SaOS-2, SK-LMS, MCA-105, RD, A-549, B-16, HCT-116, and CT26 cells were seeded into 96-well plates at a concentration of 3×10^3 cells per well and were allowed to adhere overnight. Cells were incubated for 16 additional hours in the presence of vehicle (ethanol) or different concentrations of MJ. The cytotoxic effect of MJ was measured using the XTT Cell Proliferation Kit as described in the Materials and Methods section. Values are means ± SD of at least three similar experiments.

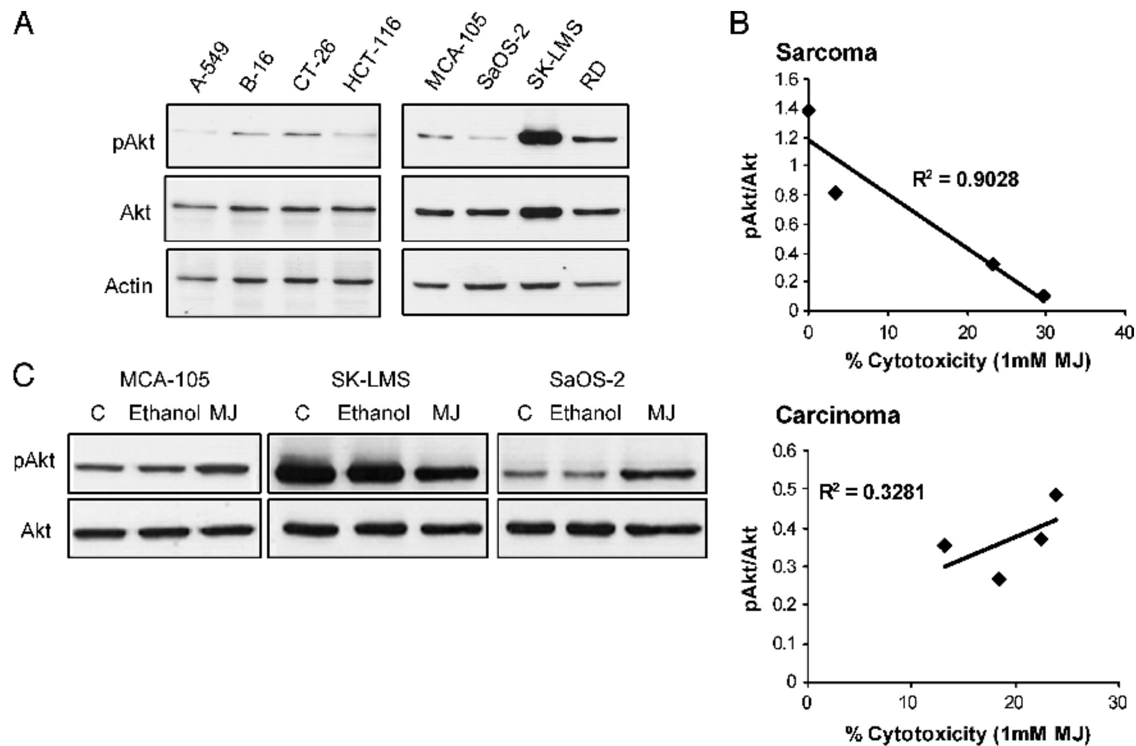


Figure 2. Basal pAkt levels in different cell lines, sensitivity to MJ, and modification of pAkt after treatment with MJ. (A) SaOS-2, SK-LMS, MCA-105, RD, A-549, B-16, HCT-116, and CT26 cells were seeded into six-well plates at a concentration of 0.7×10^6 cells per well and were allowed to adhere overnight. Total cell lysates were subjected to SDS-PAGE followed by immunoblot analysis using anti-pAkt, anti-Akt, and antiactin antibodies. (B) Correlation between basal pAkt levels and sensitivity to MJ in sarcoma (top panel) and carcinoma (bottom panel) cell lines. (C) MCA-105, SK-LMS, and SaOS-2 cells were seeded into six-well plates at a concentration of 0.7×10^6 cells per well and were allowed to adhere. After the overnight incubations, cells were incubated for 16 additional hours in the presence of vehicle (ethanol) or MJ (1 mM). Total cell lysates were subjected to SDS-PAGE followed by immunoblot analysis using anti-pAkt, anti-Akt, and antiactin antibodies.

measured by Western blot analysis (Figure 4*A*). Next, myr-Akt- and myr-KD-Akt K179A-transfected cells were treated with 3 mM MJ for 2.5 hours, and cytotoxicity was evaluated using PI exclusion by FACS analysis. As can be seen in Figure 4*B*, the cytotoxic effect of MJ was significantly greater in myr-KD-Akt K179A-transfected cells (47.36%) than in myr-Akt–transfected cells (25.2%).

Glucose Metabolism Plays a Significant Role in Resistance of MCA-105 Cells to MJ-Induced Cytotoxicity

The signaling between Akt and glucose metabolism is bidirectional. On the one hand, activation of Akt leads to increased glycolysis and glucose uptake. On the other, the presence of glucose and its metabolism through glycolysis is required for the antiapoptotic effects of Akt. Given the role of Akt in MJ-induced cytotoxicity, we sought to examine the involvement of glucose metabolism in the cytotoxic effect of MJ.

We have recently shown that combining MJ with 2-DG, an inhibitor of glycolysis, resulted in a synergistic cytotoxic effect in different cell lines [48]. We therefore studied the effect of this combination in MCA-105 cells. As can be seen in Figure 5, in accordance with our previous results, combination of MJ (0.5, 1 mM) and 2-DG (1, 2 mM) resulted in a synergistic cytotoxic effect. To further investigate the role of glucose in resistance to MJ, we evaluated the cytotoxic effect of MJ in cells that were grown in the presence (4.5 gr/L) or absence of glucose. As expected, cells that were grown in the absence of glucose exhibited increased sensitivity to the cytotoxic effect of MJ (Figure 6*A*).

2-Deoxy-D-Glucose Attenuates MJ-Induced Akt Activation and Functions in a Similar Manner to That of Akt Inhibitor

The fact that glycolysis is required for the activity of Akt, taken together with the synergistic cytotoxic effect of combining MJ with Akt inhibitor, as well as with 2-DG, led us to hypothesize that 2-DG functions in a similar manner to that of the Akt inhibitor. That is to say, we predicted that 2-DG would block MJ-induced Akt activation in the cells. As can be seen in Figure 6*B*, at its lower concentration (1 mM), 2-DG by itself led actually to an increase in levels of pAkt. This increase could represent a defense mechanism of the cell toward the metabolic stress induced by 2-DG. This defense is apparently insufficient, given that 2-DG at this concentration was shown to be toxic to the cells (Figure 5). At its higher concentration (5 mM), 2-DG by itself led to a decrease in levels of pAkt. Most importantly, in a similar fashion to the Akt inhibitor, 2-DG (1, 5 mM) abrogated the MJ-induced elevation in pAkt levels.

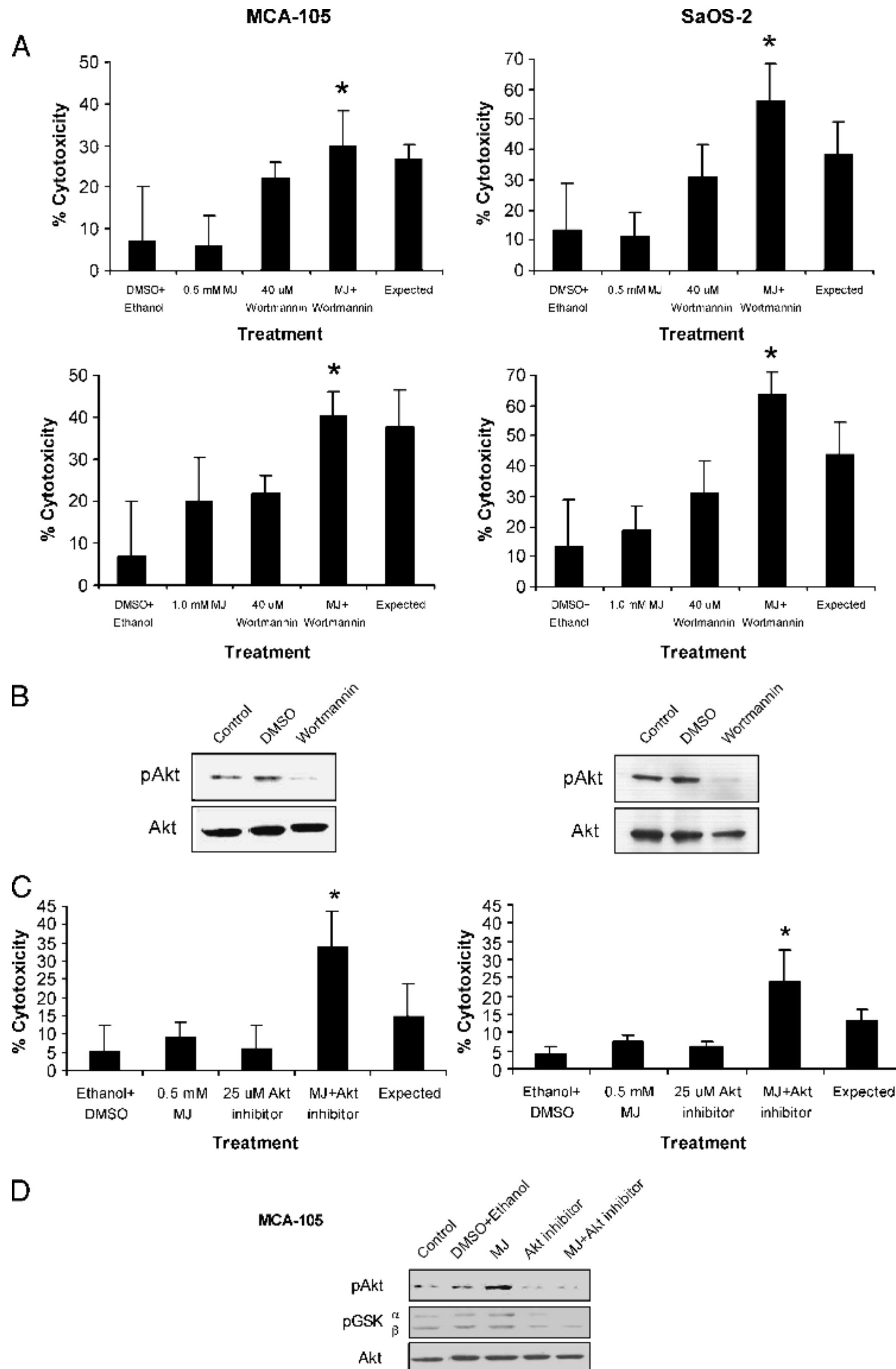
Discussion

In this study, we explored the involvement of the PI3K/Akt pathway in the cytotoxic effect of MJ. We identified a strong correlation between the susceptibility of cells to MJ and the basal pAkt levels in

sarcoma cell lines. In contrast, a rather weak correlation was found among the carcinoma cell lines.

We next evaluated MJ-treated sarcoma cells for phosphorylation of Akt, indicating activation of the kinase. We found that treatment with MJ resulted in an increase in levels of pAkt in two sarcoma cell lines: MCA-105 murine fibrosarcoma and SaOS-2 human OS. Because Akt functions in various survival pathways, the increase in pAkt levels after treatment with MJ may represent a cellular adaptive response, which protects the cell from the stress induced by MJ. Thus, although clearly inflicting cytotoxic effects and leading to cell death, MJ also seems to trigger a defense mechanism in the cell, which counteracts its own action. It is thus clear that the adaptive response to MJ needs to be overcome to maximize the potential cytotoxic effect of MJ.

By combining inhibitors of the PI3K/Akt pathway with MJ, we were able to block MJ-induced activation of Akt, thus sensitizing the cells toward the cytotoxic effect of MJ, resulting in a synergistic cytotoxic effect. These results are in accordance with previous studies, which report that inhibition of the PI3K/Akt pathway is an effective approach to sensitize cells to different chemotherapies and apoptotic stimuli [49–52].



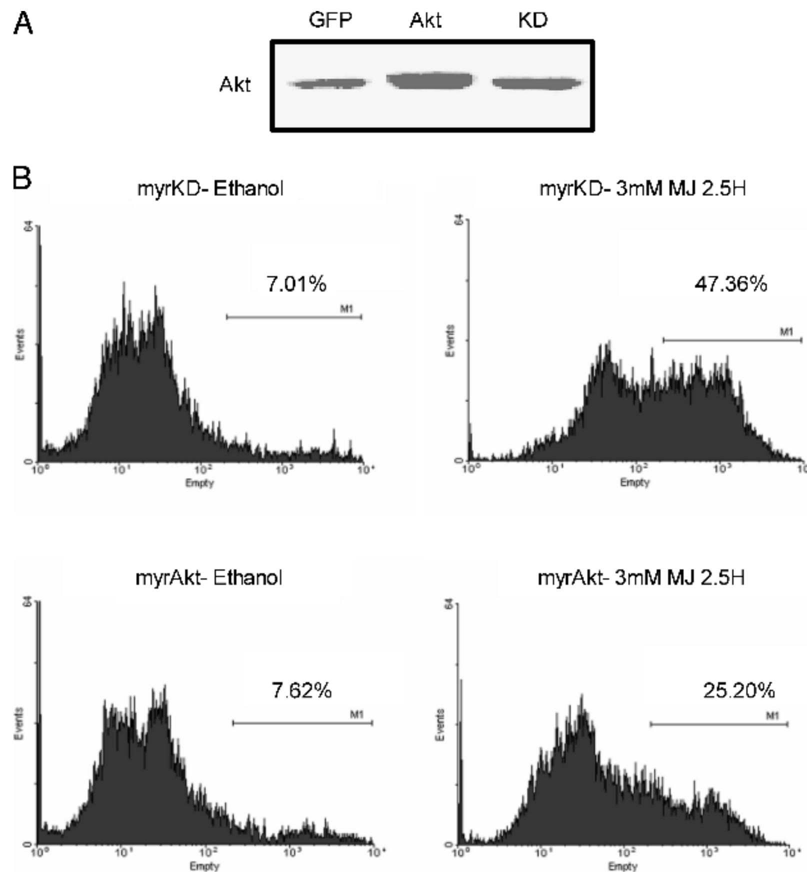


Figure 4. Constitutively active Akt protects MCA-105 cells from the cytotoxic effect of MJ. (A) MCA-105 cells were seeded into 24-well plates at a concentration of 0.4×10^5 cells per well and were transfected with GFP, myr-Akt, or myr-KD-Akt plasmids. Forty-eight hours after transfection, total cell lysates were subjected to SDS-PAGE followed by immunoblot analysis using anti-Akt antibodies. (B) MCA-105 cells were seeded into 24-well plates at a concentration of 0.4×10^5 cells per well and were transfected with either myr-Akt or myr-KD-Akt plasmids. Forty-eight hours after transfection, vehicle (ethanol) or MJ (3 mM) was added to the wells for 2.5 hours. Cells were collected for FACS analysis to assess cell death with the PI reagent as described in the Materials and Methods section.

In contrast to the activation of Akt in MCA-105 and SaOS-2 cells, pAkt levels were reduced in MJ-treated SK-LMS human leiomyosarcoma cells. This decrease may represent different subpopulations within sarcoma tumors, in which the MJ-induced stress leads to the inhibition of the activity of Akt. The decrease in pAkt levels after treatment with different agents has been demonstrated in several studies [52–56]. In these cases, the cytotoxic effect was mediated, at least in part, by inhibition of Akt.

Figure 3. Synergistic cytotoxic effect of MJ and PI3K/Akt pathway inhibitors toward MCA-105 and SaOS-2 cells. (A) MCA-105 (left panels) and SaOS-2 (right panels) cells were seeded into 96-well plates at a concentration of 3×10^3 cells per well and were allowed to adhere. After incubation, vehicles (ethanol and DMSO), wortmannin (40 μM), and MJ (0.5, 1 mM, 1 hour later) were added for 16 hours. The cytotoxic effect was measured using the XTT Cell Proliferation Kit as described in the Materials and Methods section. Values are means ± SD of at least three experiments. Statistical analysis: * Indicates that the experimentally measured combined cytotoxic effect is greater than the expected additive effect as calculated using the Bliss additivism model. (B) MCA-105 (left panels) and SaOS-2 (right panels) cells were seeded into six-well plates at a concentration of 0.7×10^6 cells per well and were allowed to adhere. After the overnight incubations, cells were incubated for 16 additional hours in the presence of vehicle (DMSO) or wortmannin (40 μM). Total cell lysates were subjected to SDS-PAGE followed by immunoblot analysis using anti-pAkt and anti-Akt antibodies. (C) MCA-105 (left panels) and SaOS-2 (right panels) cells were seeded into 96-well plates at a concentration of 3×10^3 cells per well and were allowed to adhere overnight. After the overnight incubations, cells were incubated for 16 additional hours in the presence of vehicles (ethanol and DMSO), Akt inhibitor (25 μM), and MJ (0.5 mM, added 1 hour later). The cytotoxic effect was measured using the XTT Cell Proliferation Kit as described in the Materials and Methods section. * Indicates that the experimentally measured combined cytotoxic effect is greater than the expected additive effect as calculated using the Bliss additivism model. (D) MCA-105 cells were seeded into six-well plates at a concentration of 0.7×10^6 cells per well and were serum-starved (0.5% FCS) for the duration of the experiment. After the overnight incubations, cells were incubated for 16 additional hours in the presence of vehicles (ethanol and DMSO), Akt inhibitor (50 μM), and MJ (1 mM, added 1 hour later). Total cell lysates were subjected to SDS-PAGE followed by immunoblot analysis using anti-pAkt, anti–pGSK-3α/β, and anti-Akt antibodies.

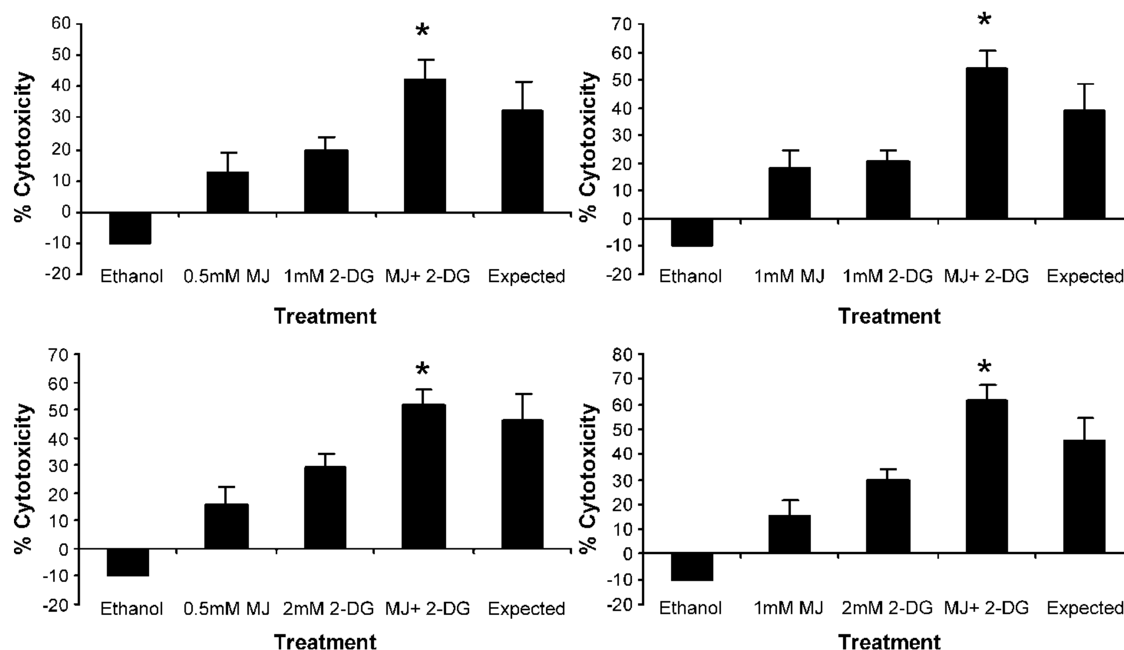


Figure 5. Synergistic cytotoxic effect of MJ and 2-DG toward MCA-105 cells. MCA-105 cells were seeded into 96-well plates at a concentration of 3×10^3 cells per well and were allowed to adhere overnight. After incubation, vehicle (ethanol), 2-DG (1, 2 mM), and MJ (0.5, 1 mM, added 1 hour later) were added to the wells for 16 hours. The cytotoxic effect was measured using the XTT Cell Proliferation Kit as described in the Materials and Methods section. Values are means ± SD of at least three experiments. * Indicates that the experimentally measured combined cytotoxic effect is greater than the expected additive effect as calculated using the Bliss additivism model.

Cancer cells show consistent alterations in metabolic regulation and, in most cases, have defects in regulation of cell death. Several studies have recently shown that there is an association between these two phenotypes. This association is maintained through the activity of Akt. Akt plays key roles in both maintaining glycolytic activity and promoting cell survival. These two activities are firmly linked, as the ability of Akt to promote survival and inhibit cell death is dependent on glucose availability [57]. Given the role of Akt in MJ-induced cytotoxicity, we decided to examine the involvement of glucose metabolism in this process. Interestingly, we found that modifying the glycolytic pathway resulted in similar effects to those induced by modifying the Akt pathway. First, combining MJ with the glycolysis inhibitor, 2-DG, yielded a synergistic cytotoxic effect compared to each agent by itself. In addition, cells grown in the absence of glucose were more susceptible to the cytotoxic effect of MJ than cells grown in the presence of glucose. Moreover, when evaluating levels of pAkt, we saw that 2-DG abrogated the MJ-induced elevation in pAkt levels. These results suggest that glucose metabolism is required for the antiapoptotic effects of Akt. Therefore, inhibiting this metabolism can sensitize the cells to the cytotoxic effect of MJ.

In 2001, Gottlob et al. [33] have shown that Akt was able to prevent apoptosis in the presence of 2-DG but not 5-thio-glucose. 2-Deoxy-D-glucose is a substrate of the first enzyme in the glycolytic pathway, HK, and can be phosphorylated but not further metabolized. In contrast, 5-thio-glucose is a competitive inhibitor of HK and cannot be phosphorylated by it. Thus, the results of that study suggest that the ability of Akt to inhibit apoptosis is dependent specifically on the first step of glycolysis. In our study, 2-DG was sufficient to inhibit the antiapoptotic effects of Akt, thus sensitizing the cells to MJ-induced cytotoxicity. Interestingly, we found a direct effect of 2-DG on pAkt levels, suggesting that the inhibition of the antiapoptotic effects of Akt was mediated, at least in part, by direct inhibition of Akt phosphorylation and subsequent activation. This inhibitory effect of 2-DG on the activation of Akt has been reported in a previous study [58] and adds to the growing body of evidence that suggests glucose metabolism as a crucial element in Akt-mediated antiapoptotic effects.

The data obtained in this study raise a number of questions: First, why was sensitivity to MJ poorly correlated with pAkt levels in carcinoma cell lines while strongly correlated in sarcoma cell lines? To answer this question, broad studies need to be performed, which will screen a wide variety of cell lines and their sensitivity to cytotoxic agents such as MJ. The next issue is the differential modification of pAkt levels after MJ treatment. What is the reason for the fact that some cells display increased Akt activity in response to cytotoxic stress, whereas in others, this pathway is attenuated? As mentioned before, we hypothesize that the elevation in pAkt represents an adaptive defense response of the cell toward MJ. The cell is activating survival pathways to overcome the stress induced by MJ. Conversely, MJ-induced decrease in pAkt levels may represent cells in which the cytotoxic effect of MJ is mediated, at least in part, by inhibiting the Akt pathway. This is not the case, however, in our study, because the decrease in pAkt levels in SK-LMS cells was not accompanied by any cytotoxic effect at the tested MJ concentration.

The SK-LMS cell line exhibited extensive Akt phosphorylation in comparison to other cell lines. This extensive Akt phosphorylation correlated well with the ability of this cell line to resist the cytotoxic effect of MJ. Recent data suggest that the PI3K/Akt pathway constitutes an important survival pathway in SK-LMS cell line [59]. Treatment of the cells with the PI3K inhibitor, LY 294002, led to dose- and time-dependent apoptosis. It is thus possible that the concentration of

MJ, which led to a decrease in pAkt levels, was insufficient to induce death in the cells, yet it was still capable to partially compromise survival signaling pathways.

The differential response of the three sarcoma cells lines toward MJ in the cytotoxic effect and modification of pAkt levels may be explained by the existence of a threshold of Akt activation that must be reached in order for the antiapoptotic effects of Akt to come into effect. The notion of the existence of an activation threshold of the PI3K/Akt pathway has been proposed by previous studies, which suggest that a sufficient level of activation of Akt might be necessary to mediate myocardial protection after ischemic preconditioning in diabetic rat hearts [60] and to maintain normal cell proliferation, growth, and differentiation [61]. Although MJ induces a decrease in pAkt levels in SK-LMS cells, these levels remain higher than the elevated levels of pAkt in MJ-treated MCA-105 and SaOS-2 cells, providing sufficient antiapoptotic signaling to protect SK cells from the cytotoxic effect of MJ.

Our observation that combination of MJ and Akt inhibitor yielded a synergistic cytotoxic effect (in cells in which pAkt increased after MJ treatment) suggests an important role for Akt in the response of cells to MJ. It would be of great interest to extensively investigate the effect of MJ on the numerous downstream effectors that constitute the Akt pathway, such as mTOR, BAD, IKK, etc. In this study, we found that both alpha and beta isoforms of the downstream kinase GSK-3 are altered in a similar manner to pAkt. The involvement of GSK-3α has been previously shown in several types of cancers, including colon cancer [62] and multiple myeloma [63]. Similarly, the beta isoform has been shown to be involved in

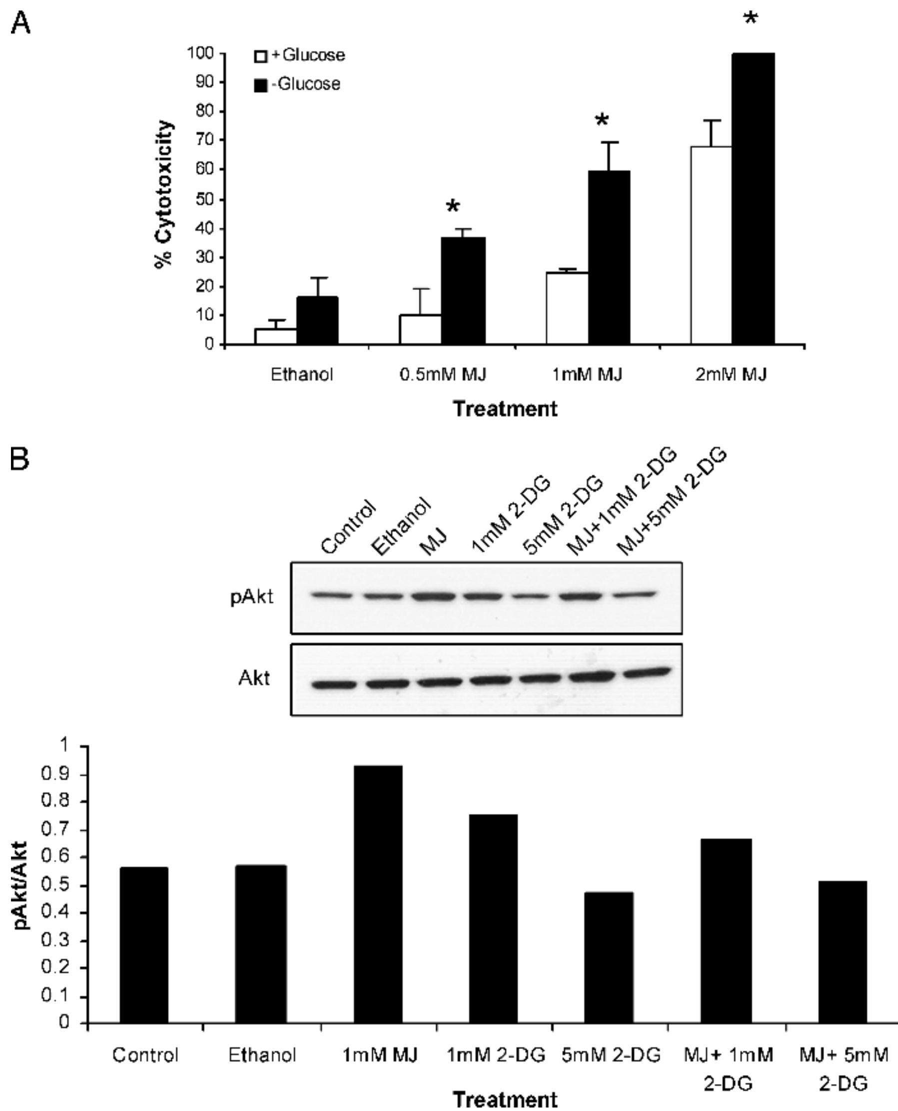


Figure 6. Glucose protects MCA-105 cells from the cytotoxic effect of MJ. (A) MCA-105 cells were seeded into 96-well plates at a concentration of 3×10^3 cells per well and were allowed to adhere overnight. After incubation, medium was replaced with fresh medium in the presence (4.5 g/L) or absence of glucose, and vehicle (ethanol) or MJ was added for 16 additional hours. The cytotoxic effect was measured using the XTT Cell Proliferation Kit as described in the Materials and Methods section. Values are means ± SD of at least three experiments. Statistical analysis: $^*P < .05$. (B) MCA-105 cells were seeded into six-well plates at a concentration of 0.7×10^6 cells per well and were allowed to adhere. After incubation, vehicle (ethanol), 2-DG (1, 5 mM), and MJ (1 mM, added 1 hour later) were added to the wells for 16 additional hours. Total cell lysates were subjected to SDS-PAGE followed by immunoblot analysis using anti-pAkt and anti-Akt antibodies. Lower panel: quantification of protein levels.

several types of cancers, including hepatoma [64] and colorectal cancer [65].

Our findings provide evidence to the involvement of GSK-3α/β in sarcoma and suggest a possible role for GSK-3α/β in MJ-induced cytotoxicity and/or the adaptive response of MCA-105 fibrosarcoma cells toward it.

In conclusion, our results raise the possibility of using combinations of MJ and Akt inhibitor/2-DG as a novel multicomponent anticancer therapeutic modality for sarcomas.

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