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IGF1R tyrosine kinase inhibitor enhances the cytotoxic effect of methyl jasmonate in endometrial cancer

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ABSTRACT

The present study evaluated the cytotoxic activity of methyl jasmonate (MJ) in endometrial cancer cells and examined the hypothesis that the apoptotic and anti-proliferative actions of MJ in these cell lines can be enhanced by co-targeting the insulin-like growth factor-1 receptor (IGF1R) signaling pathway. MJ had a potent pro-apoptotic effect and exhibited significant toxicity in all cell lines tested. MJ in combination with NVP-AEW541, a selective IGF1R tyrosine kinase inhibitor, had significantly increased cytotoxicity. MJ decreased IGF1R phosphorylation, however, it enhanced AKT phosphorylation and abolished the anti-apoptotic effect of IGF1. These findings suggest that combined IGF1R inhibitor and MJ administration may constitute an attractive modality for treating endometrial cancer.

Introduction

Endometrial cancer, a tumor of the endometrial lining of the uterine corpus, is the most common genital tract cancer and the fourth most common malignancy occurring in women in developed countries [1]. Endometrial cancers can be divided into two broad categories based on clinical, pathological and molecular characteristics. Type I cancers, which represent more than 80% of the cases, are estrogen-dependent, low-grade malignancies, with an endometrioid, well-differentiated morphology. Type I tumors are usually associated with a relatively good prognosis. Type II endometrial cancers have serous papillary or clear cell histology and a poor prognosis. Uterine serous carcinoma (USC), the predominant histological class among Type II tumors, represents about 10% of all endometrial carcinomas, is often diagnosed at an advanced stage, and accounts for 50% of all endometrial cancer relapses, with a 5-year survival rate of 55% [2]. Hence, there is an urgent need to identify new therapeutic regimens that will prolong survival of patients suffering from this tumor and that will transform response to treatment into a cure.

The insulin-like growth factors (IGFs) are a family of growth factors, binding proteins and receptors that play a key role in regulating growth, resistance to apoptosis and differentiation. The IGFs have been implicated in the etiology of a number of malignancies and, in recent years, the IGF1 receptor (IGF1R) has emerged as a potential target molecule for cancer treatment. Several studies have shown a correlation between some of the major components of the IGF system and endometrial cancer risk [3]. In recent studies, we investigated the effect of IGF1R targeting in Type I and Type II endometrial cancer cells. We reported that IGF1R targeting with a series of blocking monoclonal antibodies (IMC-A12, MK-0646) as well as with a specific IGF1R tyrosine kinase inhibitor (NVP-AEW541) inhibited the IGF1-induced proliferation of both types of endometrial cancer [4,5].

Methyl jasmonate (MJ), a plant stress hormone, has been shown to exhibit chemotherapeutic and pharmacological activities against several types of cancer [6]. Specifically, MJ has been reported to inhibit growth and to induce apoptosis in breast, prostate, bladder, liver, lung, and cervical cancers, as well as in

Abbreviations: IGF1, insulin-like growth factor-1; IGF1R, IGF1 receptor; MJ, methyl jasmonate; USC, uterine serous carcinoma; MAPK, mitogen-activated protein kinase; PCNA, proliferating cell nuclear antigen; ROS, reactive oxygen species; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide; HRP, horseradish peroxidase; PARP, poly-ADP ribose polymerase.

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[2] The authors wish to dedicate this paper to the memory of our beloved friend and colleague Professor Eliezer Flescher who pioneered research on methyl jasmonate in cancer.

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melanoma, neuroblastoma, lymphoblastic leukemia and lymphoma [7,8]. Of major interest, MJ has been shown to be selectively cytotoxic toward chronic lymphocytic leukemia cells without harming normal human lymphocytes [8]. The mechanism of action of jasmonates against cancer cells, however, is still not fully defined. Three mechanisms have been proposed to explain the anti-carcinogenic activity of jasmonates, including: (1) induction of cellular ATP depletion via mitochondrial perturbation; (2) induction of re-differentiation via mitogen-activated protein kinase (MAPK) activity; and (3) induction of reactive oxygen species (ROS)-mediated apoptosis via generation of hydrogen peroxide and pro-apoptotic proteins of the Bcl-2 family.

Given the potential overlap in the mechanisms of action and molecular targets of jasmonates and IGF1R inhibitors, and in view of the growing interest in the pharmacological applications of these compounds, the goals of the present study were to assess the cytotoxic activity of MJ in Type I and Type II endometrial cancer and to evaluate the hypothesis that the apoptotic and anti-proliferative actions of MJ in these cells can be enhanced by co-targeting the IGF1R signaling pathway.

Material and methods

Cell lines and treatments

The human endometrioid Ishikawa and ECC-1 cell lines (Type I) were obtained from Dr. Y. Sharoni (Ben Gurion University, Beer Sheba, Israel). Uterine serous papillary (USPC-1 and USPC-2; Type II) endometrial cancer cell lines were provided by Dr. A. Santin (Yale University School of Medicine, New Haven, CT, USA). Ishikawa and ECC-1 cells were grown in DMEM and USPC cells in RPMI-1640 media. Both media were supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 μg/ml gentamicin sulfate and 5.6 mg/l amphotericin B. Media and reagents were purchased from Biological Industries Ltd., Kibbutz Beit Haemek, Israel. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. MJ [methyl 3-oxo-2-(2-pentenyl) cyclopentaneacetic acid] was obtained from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Cells were serum-starved for 24 h, after which they were treated with 0.25–3 mM MJ, separately or in combination with 1–2 μM NVP-AEW541, a selective IGF1R inhibitor (Novartis Pharma, Basel, Switzerland), for 24–72 h, in the presence or absence of IGF1 (50 ng/ml) (PeproTech Ltd., Rocky Hill, NJ, USA).

Cytotoxicity assays

Cells were plated in 96-well plates (1×10^4 cells/well in complete medium). After 24 h, the cells were treated with MJ and NVP-AEW541, separately or in combination, at varying concentrations, for 24, 48 or 72 h, in quadruplicate wells. Cell viability was assessed using a standard 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reagent kit (Biological Industries Ltd.) [9]. Cytotoxicity was expressed as percentage of optical density values obtained after drug treatment, relative to untreated control cells.

Western immunoblots

Cells were starved overnight and then incubated with MJ and NVP-AEW541, separately or in combination, in the presence or absence of IGF1. Cells were then harvested and whole cell lysates were prepared. Samples (80 μg protein) were electrophoresed through 5%, 10% or 15% SDS–PAGE gels, followed by blotting of the proteins onto nitrocellulose membranes. After blocking with either 5% skim milk and/or 3% bovine serum albumin, the blots were incubated overnight with the antibodies listed below, washed and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. Antibodies against phospho-IGF1R (#3024), IGF1R β-subunit (#3027), phospho-Akt (#9271), Akt (#9272), phospho-ERK1/2 (#9106), ERK1/2 (#9102), poly-ADP ribose polymerase (PARP; (#9542), cyclin D1 (#2978) and caspase 9 (#9502) were obtained from Cell Signaling Technology (Beverly, MA, USA). An antibody against PCNA (FL-261) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary antibodies were HRP-conjugated goat anti-rabbit IgG (1:50,000) and donkey anti-mouse IgG (1:25,000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Proteins were detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). ERK1/2 expression was used as a loading control.

Statistical analysis

The statistical significance of the differences between groups was assessed using the Student's t test (two samples, equal variance). $P < 0.05$ was considered statistically significant.

Results

Cytotoxic effect of MJ on endometrial cancer cells

To evaluate the cytotoxic effect of MJ on different types of endometrial carcinoma, Type I (Ishikawa, ECC-1) and Type II (USPC-1, USPC-2) cancer cells were exposed to MJ for 24 h at concentrations ranging from 0.25 to 3 mM and cytotoxicity was determined using the XTT method. As illustrated in Fig. 1, all cell lines responded to MJ treatment in a dose-dependent fashion. Doses of 1–3 mM MJ induced significant cytotoxicity in all cells tested.

Cytotoxic effect of MJ on USC cells

Given the aggressive nature of USC malignancies and the need to develop better-targeted therapies, further analyses focused on this form of endometrial cancer. USPC-1 cells express a wild type p53 including two polymorphisms (deletion of 16 bp in intron 3 [c.96+41del16bp] and a C > A transition in exon 4, position 29). USPC-2 cells, on the other hand, exhibit a mutation in the p53 gene exon 5 (position c.493), which results in the formation of a stop codon at position p.165 [10]. To further assess the cytotoxic effects of MJ on USC, we evaluated the time- and dose-responsiveness of this effect using increasing doses of MJ (0.25–3 mM) for 24 h, 48 h and 72 h. Results obtained showed that both cell lines responded in a time- and dose-dependent fashion (Fig. 2). Thus, low doses of MJ (0.25–0.5 mM) exhibited low cytotoxicity whereas cells exposed to higher doses of MJ (2–3 mM for USPC-1, 1–3 mM for USPC-2) displayed time- and dose-dependent cytotoxic responses.

Effect of MJ on apoptosis

To examine the potential effect of MJ on apoptosis, USPC-1 and USPC-2 cells were serum-starved for 24 h and then treated with IGF1 (50 ng/ml) for an additional 24 h in the presence or absence of MJ. Caspase-9 is an initiator caspase which, following activation, cleaves pro-caspases-3 and -7, with ensuing cleavage of several cellular targets, including PARP1. Values of cleaved PARP1 and caspase-9 constitute *bona fide* markers of apoptosis. Western blot analysis revealed that treatment of USPC-1 cells with 2 mM MJ markedly enhanced apoptosis as reflected by the large induction of PARP1 cleavage (86 kDa). A modest increase in PARP1 cleavage and decrease in full-length PARP1 levels was seen in USPC-2 cells (Fig. 3). Furthermore, MJ in the presence or absence of IGF1 enhanced the levels of cleaved caspase-9 (37 and 35 kDa) in USPC-1 cells (Fig. 3). Finally, MJ also exerted a pro-apoptotic effect in USPC-2 cells as indicated by the diminished level of full-length caspase-9.

Effect of MJ on IGF1R phosphorylation

To evaluate the potential regulation of the expression and activation of IGF1R by MJ, USPC-1 and USPC-2 cells were treated with MJ for 24 h, in the presence or absence of the selective IGF1R tyrosine kinase inhibitor NVP-AEW541, and/or IGF1 (50 ng/ml) during the last 10 min of the incubation (Fig. 4). Western blots using an antibody against phospho-IGF1R revealed that IGF1 stimulated phosphorylation of the mature IGF1R (97 kDa) as well as the IGF1R precursor (~250 kDa) in USPC-1 cells. MJ treatment decreased the

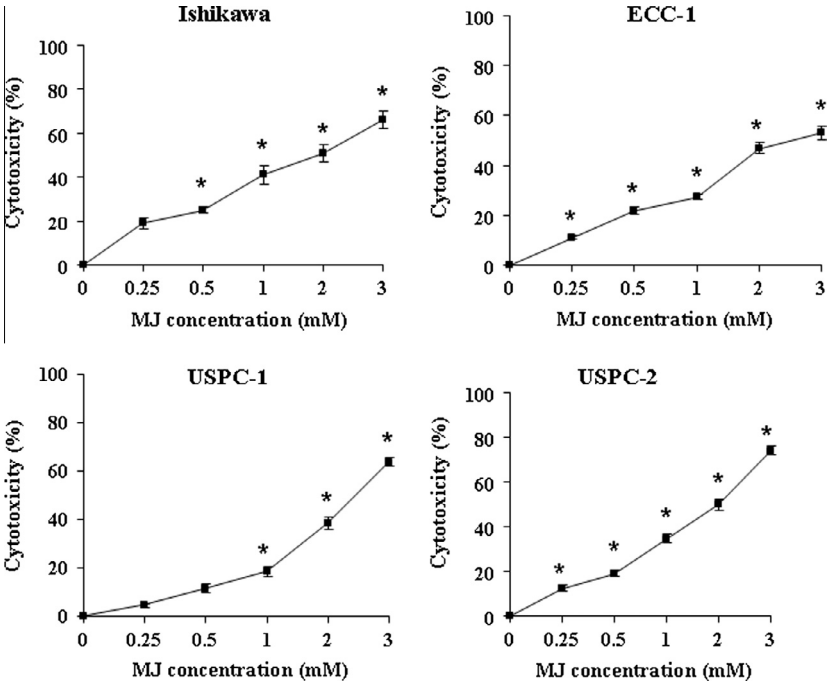

Fig. 1. Cytotoxic effect of MJ on endometrial cancer cells. Cells were plated in 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h in the presence of vehicle (ethanol) or different concentrations of MJ. Cytotoxicity was assessed by XTT assays. Results are mean ± SE (triplicate samples of four independent experiments) $^*p < 0.01$ vs. ethanol-treated cells.

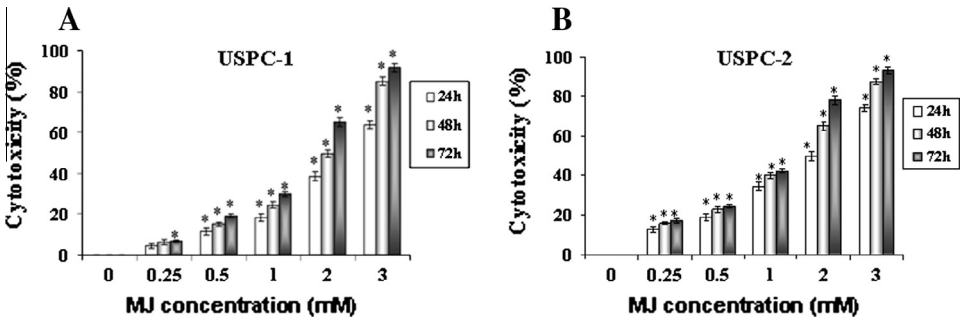

Fig. 2. Dose-dependent MJ cytotoxic effect on USC cells. USPC-1 (A) and USPC-2 (B) cells were seeded into 96-well plates at a concentration of 1×10^4 cells/well. Cells were incubated in the presence of increasing doses (0.25–3 mM) of MJ for 24 h, 48 h and 72 h. The cytotoxic effect of MJ was determined by XTT assays. The bars represent the mean ± SE of four independent experiments, each performed in triplicate. $^*p < 0.01$ vs. ethanol-treated cells.

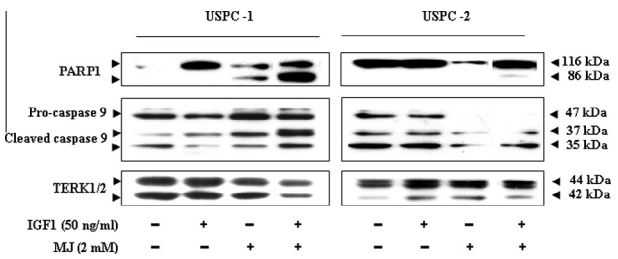

Fig. 3. Effect of MJ on apoptosis. Western blot analysis of PARP1 and caspase-9 in USPC-1 and USPC-2 cells. Cells were treated with IGF1 for 24 h in the presence or absence of MJ. Whole-cell lysates (100 μg) were resolved by SDS–PAGE and immunoblotted with the indicated antibodies. Results are representative of three independent experiments.

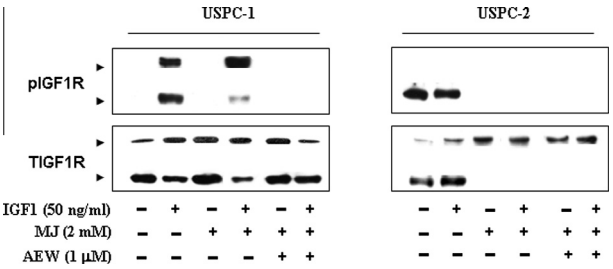

Fig. 4. Effect of MJ on IGF1-stimulated IGF1R phosphorylation. USPC-1 and USPC-2 cells were treated with MJ for 24 h (or left untreated) in the presence of absence of NVP-AEW541 for 2 h, followed by IGF1 treatment for 10 min. Whole cell lysates (100 μg) were resolved by SDS–PAGE and immunoblotted with antibodies against phospho-IGF1R and total-IGF1R. The figure shows the result of a typical experiment, repeated three times with similar results. Due to differences in IGF1R levels between cells, the exposure time for the autoradiogram shown in panel A was 30 s whereas the autoradiogram in panel B was exposed for 1 h.

IGF1-stimulated phosphorylation of the mature, but not precursor, IGF1R. MJ in combination with NVP-AEW541 exhibited an enhanced effect by inhibiting phosphorylation of both the precursor and mature IGF1R. Neither MJ nor NVP-AEW541, alone or in combination, altered IGF1R expression levels in USPC-1 cells. In contrast, high basal phospho-IGF1R levels were seen in USPC-2 cells, without any stimulatory effect of exogenous IGF1. Of interest, MJ, alone or in combination with NVP-AEW541, down-regulated expression of mature, but not precursor, IGF1R levels, with ensuing reduction in phospho-IGF1R values.

Effect of MJ on AKT and ERK1/2 phosphorylation

Next, we investigated the effects of MJ and NVP-AEW541 on signaling pathways downstream of IGF1R (Fig. 5A and B). As expected, IGF1 stimulated AKT and ERK1/2 phosphorylation in USPC-1 cells. Of interest, MJ (2 mM) significantly enhanced the IGF1-stimulated phospho-AKT and phospho–ERK1/2 levels. Addition of NVP-AEW541 (1 μM) led to a marked decrease in AKT and ERK1/2 phosphorylations in comparison with USPC-1 cells treated with IGF1 and MJ alone. In accordance with the lack of IGF1R activation in USPC-2 cells, only minimal IGF1-induced AKT and ERK1/2 phosphorylations were seen in these cells. MJ was shown to decrease the IGF1-stimulated phosphorylation of AKT and ERK1/2 in USPC-2 cells whereas combined MJ and NVP-AEW541 treatment led to a further reduction in phospho-AKT, but not phospho-ERK1/2, level.

Antiproliferative effect of MJ in combination with NVP-AEW541

Finally, the effect of combined treatment with MJ and NVP-AEW541 was studied in USC cell lines. For this purpose, USPC-1 (Fig. 6A) and USPC-2 (Fig. 6B) cells were treated with NVP-AEW541 at a concentration of 2 μM in the presence or absence of MJ (3 mM), for 72 h. Proliferation rates were analyzed by the XTT method. Treatment with MJ inhibited USPC-1 and USPC-2 cells proliferation by 60 ± 0.26% and 66 ± 0.03%, respectively, compared with untreated control cells. NVP-AEW541 decreased cell viability by 28 ± 0.05% and 23 ± 0.45% in USPC-1 and USPC-2 cells, respectively, compared with controls. Combined treatment led to additive decreases in proliferation rates in both cell lines (90.83 ± 0.005% in USPC-1 and 77.54 ± 0.01% in USPC-2). Finally, we investigated the capacity of MJ and NVP-AEW541 to inhibit AKT downstream mediators responsible for the anti-proliferative effect of these compounds. Specifically, we measured PCNA and cyclin D1 expression in USPC-1 cells, in view of previous studies showing that MJ downregulated PCNA, but not cyclin D1, expression [11,12]. Results of Western blots showed that MJ and NVP-AEW541, separately, decreased PCNA expression whereas, in combination, they completely inhibited cyclin D1 expression.

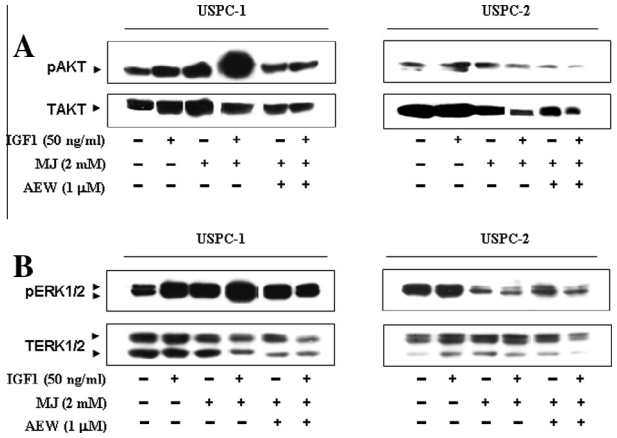

Fig. 5. Effect of MJ on AKT and ERK1/2 phosphorylations. USPC-1 and USPC-2 cells were exposed to MJ, NVP-AEW541 and IGF1, as described in Fig. 4. At the end of incubation period, cells were lysed and the phosphorylated and total AKT and ERK1/2 levels were measured by Western blot analysis.

Discussion

Conflicting retrospective data continue to emerge regarding optimal therapy for USC, especially for aggressive, advanced stage disease. Fingrut and Flescher were the first to show that jasmonates exhibited anti-cancer activity, inhibited cell proliferation, and had a cytotoxic effect on various cancer cell types [8]. A recent study [13] reported that MJ demonstrated higher cytotoxicity compared to cisplatin in ovarian cancer cell lines and fresh *ex vivo* ovarian cancer cells. MJ has also been shown to lower the IC_{50} of various chemotherapy drugs, including cisplatin, paclitaxel and doxorubicin, increasing their cytotoxicity towards breast, lung, prostate, and pancreatic cancer cells, as well as leukemia cells [14]. In addition, MJ was shown to induce cell death and down-regulate ectopic expression of human papillomavirus E6 and E67 in cervical cancer cells [15]. Another recent study showed that jasmonates inhibited the growth of human androgen sensitive and insensitive prostate cancer cells [16]. Furthermore, an *in vivo* study revealed that the combination of MJ and doxorubicin was synergistically effective for treatment of BCL1 leukemia in mice [14].

In this study we showed that MJ is cytotoxic to both Type I and Type II endometrial cancer cells. However, the extent of this effect differed between cell lines. Furthermore, MJ was shown to suppress proliferation of cultured USC cells in a time- and dose-dependent manner. Thus, at high concentrations (2–3 mM) and longer exposure time (72 h), MJ exhibited a significant cytotoxic effect by killing ~90% of USC cells, whereas at low doses (0.25–1 mM) and short exposure time (48 h) it had a minor cytotoxic effect. In addition, our data revealed that apoptosis induced by MJ in USPC-1 and USPC-2 cells were correlated with activation of PARP1 and caspase-9 cleavage. These results are consistent with previous studies demonstrating that effectiveness of this reagent as a chemotherapeutic drug was detected only at doses in the millimolar range [17].

IGF1R emerged as a target for novel oncological therapeutics, specifically monoclonal antibodies and small molecular weight IGF1R tyrosine kinase inhibitors. A number of reports have shown a correlation between components of the IGF system and endometrial cancer risk [3,18,19]. Moreover, studies reported significant increases in IGF1R expression in endometrial cancer [20–22] and correlation to disease stage and grade [23]. Recently, we showed that human monoclonal antibodies directed against IGF1R (IMC-A12, MK-0646), as well as a specific tyrosine kinase inhibitor (NVP-AEW541), successfully blocked IGF1R activity in endometrial cancer cell lines and abolished the IGF1-stimulated signaling cascades [4,5]. Unfortunately, mixed results were obtained when these drug candidates were evaluated in Phase III clinical trials, either as monotherapy or in combination with other reagents. It was suggested that combination as opposed to single anti-IGF1R therapy should improve clinical responses. In the current study, we demonstrated that combination of MJ with NVP-AEW541 had a significant additive effect in USC cells.

Elia and Flescher [24] investigated the involvement of the PI3K/AKT pathway in the cytotoxic mechanism of MJ. They showed that treatment with MJ resulted in a paradoxical increase in levels of pAKT in sarcoma cells. Consistent with these results, an additional study by Yeruva et al. established that MJ enhanced ERK1/2 activity in breast cancer cells [6]. Given that AKT and ERK1/2 play key roles in IGF1R signaling, we speculated that the mechanism of action of MJ might involve the IGF1R pathway. Our results revealed that MJ,

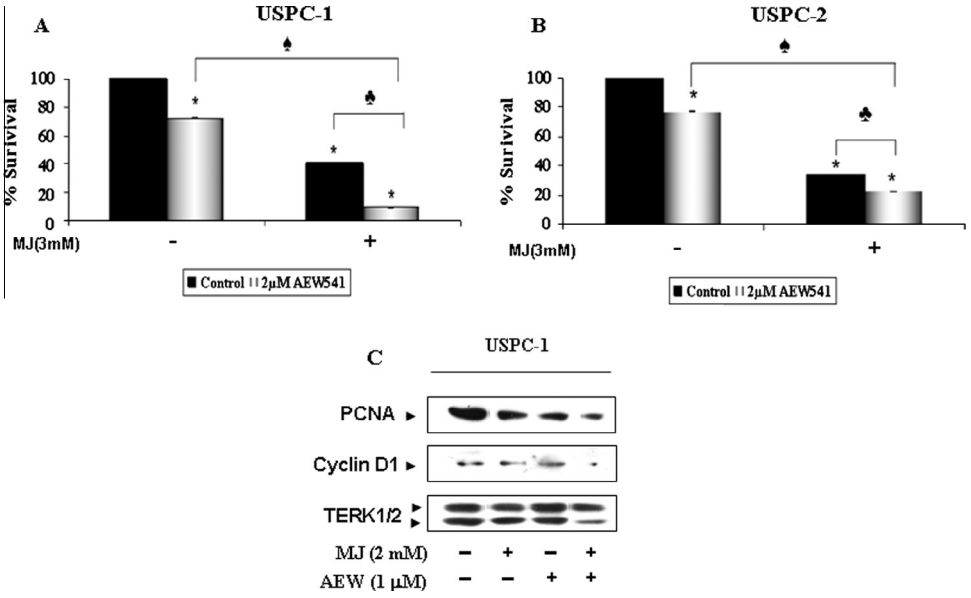

Fig. 6. Proliferation effect of MJ combined with NVP-AEW541 in USC cells. USPC-1 (A) and USPC-2 (B) cells were exposed to MJ (3 mM) (or left untreated), NVP-AEW541 (2 μM), or both for 72 h. Proliferation was determined by XTT assays. A value of 100% was assigned to the untreated cells. Values are presented as mean ± SE of three independent experiments in triplicate samples. $^{*}p < 0.01$ vs. ethanol-treated cells; $^{\spadesuit}p < 0.01$ vs. NVP-AEW541-treated cells or $p < 0.01$ vs. MJ-treated cells. Effect of MJ and/or NVP-AEW541 on PCNA and cyclin D1 levels (C). USPC-1 cells were processed as described in Fig. 4, lysed, electrophoresed, and immunoblotted with anti-PCNA or cyclin D1.

per se, inhibited the IGF1-induced IGF1R phosphorylation. These results are particularly intriguing in view of the fact that MJ is not directed against any specific component of the IGF1R signaling pathway. Future studies will address the mechanism responsible for IGF1R de-activation by MJ.

MJ is an anti-cancer agent that exhibits cytotoxicity towards endometrioid and USC endometrial carcinoma. Despite the fact that the cytotoxic activity of MJ is, most likely, not mediated through the IGF1R, its antiproliferative action was markedly enhanced when used in combination with a specific IGF1R inhibitor. Furthermore, and given that MJ exhibited pro-apoptotic and anti-mitogenic activities in cells containing a wild-type p53 (USPC-1) as well as in cells including a mutant form of p53 (USPC-2), our data is consistent with the fact that the inhibition of cell growth in USC is mediated via the AKT/p53 pathway in a p53-independent manner. In addition, our results indicate that inhibition of cell proliferation by MJ and/or NVP-AEW541 involves PCNA and cyclin D1 downregulation. We assume that MJ acts through the Ras/Raf/MAPK (ERK) signaling pathway to induce cell death. ERK activity has been associated with classical markers of apoptosis execution, including effect or caspase-3 activation, PARP cleavage, release of cytochrome C by Bcl-2 from mitochondria and activation of initiator caspase-9. Both caspase-9 activation and PARP cleavage were detected in both USC cell lines.

In summary, MJ is revealing itself as a novel potent anti-tumor reagent in several types of cancer, including endometrial tumors. Our study suggests that the use of MJ in combination with IGF1R inhibitor might prove to be more effective than MJ alone.

Conflict of Interest

None.

Acknowledgment

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