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Resveratrol Chemosensitizes Breast Cancer Cells to Melphalan by Cell Cycle Arrest

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ABSTRACT

Melphalan (MEL) is a chemotherapeutic agent used in breast cancer therapy; however, MEL's side effects limit its clinical applications. In the last 20 years, resveratrol (RSV), a polyphenol found in grape skins, has been proposed to reduce the risk of cancer development. The aim of this study was to investigate whether RSV would be able to enhance the antitumor effects of MEL in MCF-7 and MDA-MB-231 cells. RSV potentiated the cytotoxic effects of MEL in human breast cancer cells. This finding was related to the ability of RSV to sensitize MCF-7 cells to MEL-induced apoptosis. The sensitization by RSV involved the enhancement of p53 levels, the decrease of procaspase 8 and the activation of caspases 7 and 9. Another proposed mechanism for the chemosensitization effect of MCF-7 cells to MEL by RSV was the cell cycle arrest in the S phase. The treatment with RSV or MEL increased the levels of p-Chk2. The increase became pronounced in the combined treatments of the compounds. The expression of cyclin A was decreased by treatment with RSV and by the combination of RSV with MEL. While the levels of cyclin dependent kinase 2 (CDK2) remained unchanged by treatments, its active form (Thr^{160}-phosphorylated CDK2) was decreased by treatment with RSV and by the combination of RSV with MEL. The activity of CDK7, kinase that phosphorylates CDK2 at Thr^{160}, was inhibited by RSV and by the combination of RSV with MEL. These results indicate that RSV could be used as an adjuvant agent during breast cancer therapy with MEL. J. Cell. Biochem. 113: 2586–2596, 2012.

KEY WORDS: APOPTOSIS; CANCER; CELL CYCLE; MELPHALAN; RESVERATROL

Cancer is a growing health problem around the world particularly with the steady rise in life expectancy, increasing urbanization, and the subsequent changes in environmental conditions including lifestyle. According to a recent report [Jemal et al., 2011], cancer accounted for 7.6 million deaths (around 13% of all deaths) in 2008, and deaths from cancer worldwide are projected to continue to rise to over 11 million in 2030.

The therapies available to date for cancer treatment are surgery, radiotherapy, and chemotherapy. Chemotherapy is often used as the main regimen in the treatment of most cancers. However, the development of tumor resistance to chemotherapy (chemoresistance) presents a major hurdle in cancer therapy [Higgins, 2007]. The use of cancer chemopreventive phytochemicals in combination with chemotherapeutic agents has been shown to be a pragmatic approach to overcome chemoresistance and sensitize cancer cells to apoptosis or growth arrest, while minimizing the side effects arising from the conventional therapy [Garg et al., 2005]. Among the potential chemosensitizers are bioactive compounds such as resveratrol (RSV).

RSV is a natural phytoalexin that is present in especially high concentrations in grape skins and, as a consequence, in red wine [Gusman et al., 2001]. Its beneficial health effects include its anti-infective, antioxidant, and cardioprotective functions in addition to its anticancer potential [Baur and Sinclair, 2006; Marques et al., 2009]. Research from in vitro and in vivo studies indicate that RSV can sensitize tumor cells to chemotherapeutic agents by modulating

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apoptotic pathways, down-regulating drug transporters, and down-modulating proteins involved in tumor cell proliferation. In addition, RSV has also been shown to overcome chemoresistance by inhibiting the NF-κB and STAT3 pathways [Athar et al., 2009; Gupta et al., 2011].

Chan et al. [2008], using HCT116 human colon cancer cells, showed that RSV exerted synergistic effects with 5-FU in a caspase-6 dependent manner. RSV has also been shown to enhance the chemosensitivity of tumor cells by arresting the cells at different stages of the cell cycle and by downregulating genes involved in cell proliferation. Gatouillat et al. [2010] showed that RSV enhanced doxorubicin induced cytotoxicity in a chemoresistant B16 melanoma by downregulating cyclin D1. Combined treatment with RSV was also associated with an increase in cell cycle arrest at the G(1)-S phase.

Interestingly, this bioactive compound has also been reported to suppress apoptosis induced by paclitaxel, vincristine, and daunorubicin in some tumor cells [Ahmad et al., 2004; Mao et al., 2010]. The potential mechanisms underlying this dual effect are discussed. Overall, studies suggest that RSV can be used to sensitize tumors to specific cancer chemotherapeutics. No study has been conducted demonstrating the effect of the combination of RSV with melphalan (MEL) in breast cancer cells.

MEL is a well-known alkylating agent presently employed as an antineoplastic agent in humans, and is often the drug of choice in the treatment of metastatic melanoma, ovarian, and breast cancer. MEL, especially when used at a high dose, shows a diversity of toxic side effects. The most common side effect occurring during therapy is bone marrow suppression leading to leukopenia and thrombocytopenia [Dollery, 1991]. Therefore, the aim of this study was to investigate whether RSV would be able to enhance the cytotoxic effects of MEL in breast cancer cells. The improvement of the cytotoxic effects of MEL on tumor cells could result in clinical practice in reduction of the dose used of this compound, and this would provide a reduction of side effects.

MATERIALS AND METHODS

MATERIALS

RSV (>99% pure), MEL, protease inhibitor cocktail, propidium iodide, RNase A and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco's minimal essential medium (DMEM) and fetal bovine serum (FBS) used in the cell culture methods were products of Gibco (USA). All other chemicals were purchased in the purest form commercially available.

CELL CULTURES

The human breast epithelial cell lines MCF-7, an estrogen receptor-positive cell line derived from an in situ carcinoma, and MDA-MB-231, an estrogen receptor-negative cell line derived from a metastatic carcinoma, were used in this study. Both were obtained from American Type Culture Collection (ATCC) and were grown in DMEM supplemented with 10% FBS, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 5 μg/ml of insulin; the cells were kept at 37°C in a humidified atmosphere of 5% CO_2 in air according to ATCC recommendations. For all experiments, the cells were subjected to no more than 20 passages. For treatment, the cells were counted and plated at the same initial density. When the cultures reached 70–80% confluence, the cells were treated with various concentrations of *trans*-RSV, MEL, or both compounds at the indicated times.

CELL VIABILITY ASSAY

The cell viability assay was performed using MTT according to the method described by Carmichael et al. [1987]. After treatment, the cells were washed with phosphate buffered saline (PBS) and then incubated for 3 h in 0.5 ml of MTT solution (0.5 mg/ml of PBS) at 37°C in 5% CO_2 in an incubator. The medium was removed, and 0.5 ml of 0.04 mol/L HCl in absolute isopropanol was added to the attached cells. The absorbance of the converted dye in living cells was measured at a wavelength of 570 nm. The cell viability of breast cancer cells cultured in the presence of the studied compounds was calculated as a percent of the control cells. The experiments were performed in triplicate. The IC_{50} values were calculated from dose–response curves; the IC_{50} was defined as the concentration of drugs that reduced the number of viable cells to 50% of the control. GraphPad Software 5.0 was used for the IC_{50} calculations.

CELL CYCLE AND CELL DEATH ANALYSIS

Cell cycle distribution was analyzed by flow cytometry as described previously [Pozo-Guisado et al., 2002]. After treatment, the cells (5×10^5) were harvested, washed twice with PBS, and incubated with 0.5 ml of PBS containing 1 mg/ml RNase A, 0.1% Triton X-100, and 50 μg/ml propidium iodide for 30 min at room temperature in the dark. The stained cells were analyzed using a FACScan laser flow cytometer equipped with Cell Quest software (Becton Dickinson, San Jose, CA). The percentage of cells in the G1, S, G2/M, and sub-G1 phases was quantified using the WinMDI version 2.9 Software.

PREPARATION OF CELL LYSATES

After treatment for 24 h, the cells were washed with PBS and lysed in liquid nitrogen. The cells were then scraped in ice-cold lysis buffer [5 mM Tris–HCl, 10 mM ethylenediamine tetraacetic acid, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylarsine oxide, 1 μM okadaic acid, and 1 mM phenylmethylsulfonyl fluoride (pH 7.4)] with freshly added protease inhibitor cocktail (1.04 mM 4-2-aminoethylbenzenesulfonyl fluoride, 15 μM pepstatin A, 14 μM E-64, 40 μM bestatin, 20 μM leupeptin, and 0.8 μM aprotinin). The lysate was collected, sonicated and cleared by centrifugation at 8,000 rpm for 5 min at 4°C; the supernatant (total cell lysate) was then collected, aliquoted, and stored at −80°C. The protein concentration was determined according to Lowry's method [1951]; bovine serum albumin was used as standard.

WESTERN BLOT ANALYSIS

Equal amounts of total cellular proteins (100 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore, Bedford, MA). The membranes were blocked overnight at 4°C in Tris-buffered saline containing 1% Tween 20 (TBS-T), and 5% nonfat milk and incubated for 2 h with

the primary antibody (1:1,000). The membranes were then washed with TBS-T and incubated with a peroxidase-conjugated secondary antibody (1:5,000) for 1 h. The antibodies used were as follows: anti-p53 (DO-1), anti-procaspase 8 (SC7890), and anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 0411), from Santa Cruz Biotechnology, and anti-caspase 9 (#9502), anti-caspase 7 (#9492), anti-phospho-Chk2 (Thr68) (#2661), anti-p21 (#2947), anti-Cdc25A (#3252), anti-CDK7 (#2916), anti-cyclin dependent kinase 2 (CDK2) (#2546), anti-phospho-CDK2(#2561), anti-cyclin A (#4656), anti-cyclin E (#4132), from Cell Signaling Technology. The immunocomplexes were visualized with the enhanced chemiluminescence (ECL) kit (Amersham, UK). For every immunoblot assay, equal loading of protein was confirmed by stripping the blot and re-probing with the GAPDH antibody. The quantification of protein was performed by densitometric analysis of protein bands using ImageJ 1.42q Software.

IMMUNOPRECIPITATION

Two hundred microliters of cell lysate (containing 200 μg total cellular proteins) were first precleared by incubation with protein A/G-agarose (20 μl, 50% slurry, Santa Cruz Biotechnology) for 30 min. The clarified supernatants were collected by microcentrifugation and then incubated overnight with the primary antibody at 4°C. Then, 20 μl of protein A/G-agarose were added to the reaction mixtures for 3 h at 4°C to absorb the immunocomplexes. Immunoprecipitated proteins were subjected to SDS–PAGE and then transferred onto a PVDF membrane. The resulting proteins were visualized by immunoblotting.

IN VITRO CDK7 KINASE ASSAY

CDK7 kinase activity was determined by an in vitro method [Agarwal et al., 2004]. Briefly, 200 μg of total protein from cell lysates were precleared with protein A/G-agarose, and the CDK7 protein was then immunoprecipitated using an anti-CDK7 antibody with protein A/G-agarose beads. After an overnight incubation at 4°C, the immunocomplexes were washed three times with washing buffer containing 50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 80 mM β-glycerophosphate, 1 mM NaF, 0.1 mM sodium orthovanadate, 0.1% Tween 20, 10% glycerol, 1 mM PMSF, and 10 μg/ml of each aprotinin and leupeptin and were then rinsed with kinase buffer containing 50 mM HEPES-KOH, pH 7.5, 2.5 mM EGTA, 1 mM DTT, 10 mM β-glycerophosphate, 10 mM $MgCl_2$, 1 mM NaF, and 0.1 mM sodium orthovanadate. CDK2 phosphorylation was determined by incubating the immunocomplexes with 30 μl of the CDK2 kinase solution containing 1 μg of CDK2 substrate protein (Santa Cruz Biotechnology) and 0.1 mM ATP in kinase buffer for 30 min at 37°C. Then, the phosphorylated CDK2 was subjected to SDS–PAGE, transferred, and then visualized by immunoblotting with antibodies against specific phosphorylated sites of the CDK2 substrate.

STATISTICAL ANALYSIS

Quantitative data represent the mean values with the respective standard error (SE) of the mean corresponding to three or more replicates. Data were analyzed by one-way analysis of variance (ANOVA) using the post-hoc multiple comparisons Tukey's test. Data were considered to be statistically significant at $P < 0.05$.

RESULTS

CYTOTOXIC EFFECTS OF RSV AND MEL

We first investigated the effects of RSV and MEL on the viability of human breast cancer cells using the MTT assay as previously described. As shown in Figure 1A,B, RSV and MEL exhibited cytotoxic effects on MCF-7 and MDA-MB-231 cell growth in a dose- and time-dependent manner. The IC_{50} values of RSV and MEL in MCF-7 cells after 24 h of treatment were ~120 μM, and ~110 μM, respectively. In MDA-MB-231 cells, the IC_{50} values of RSV and MEL after 24 h of treatment were ~370 μM and ~305 μM, respectively. These results show that MCF-7 cells were more sensitive to RSV and MEL than MDA-MB-231 cells. To investigate the cytotoxic effect of RSV in non-malignant cells, the cell viability assay was performed in VERO cells, kidney epithelial cells extracted from an African green monkey, and BHK-21 cells, hamster kidney fibroblast cells (data not shown). The cytotoxic effect of RSV on the VERO cells was lower than on the MCF-7 cells, with an IC_{50} value of ~300 μM. The treatment of VERO cells with 200 μM of RSV reduced ~23% of cell viability, whereas treatment with the same concentration of RSV reduced the viability of the MCF-7 cells by ~68%. In BHK-21 cells, no cytotoxic effect was observed in treatments with up to 200 μM of RSV. Aziz et al. [2006] showed that treatment with RSV (0–50 μM) for 24 h resulted in a decrease of cell viability and an induction of apoptosis in human prostate carcinoma LNCaP cells. The same result was not observed in normal prostate epithelial HPEC cells. These observations suggested that RSV may have potential as a chemotherapeutic agent.

EFFECTS OF THE ASSOCIATION OF RSV WITH MEL ON THE CELL VIABILITY

Given the results obtained with the use of RSV against carcinoma cells, we analyzed the effect of RSV in combination with MEL on the MCF-7 and MDA-MB-231 cells (Fig. 2). After treatment for 24 h with a combination of 50 μM of RSV and 50 μM of MEL, MCF-7 and MDA-MB-231 cell viability decreased by 32.2% and 19.2%, respectively (Fig. 2A). This decrease in viability was greater than the sum of the cytotoxic effects of separate treatments with RSV and MEL at the same concentrations. Other combinations of RSV and MEL also produced similar results; for example, treatment with 100 μM of RSV and 25 μM of MEL led to a decrease of viability of MCF-7 and MDA-MB-231 cells that was 13% and 3%, respectively, greater than the sum of the effects of these compounds used individually at the same concentrations. After treatment for 48 h, combinations of 100 μM of RSV and either 25, 50, or 75 μM of MEL were able to reduce the viability of the MCF-7 cells by almost 100%. As shown by bright field microscopy, the combination of RSV with MEL promoted more modifications in the morphology of MCF-7 cells than treatment with RSV or MEL alone (Fig. 2B). These data show that RSV is able to potentiate the cytotoxic effects of MEL in MCF-7 and MDA-MB-231 cells. Although this increase of cytotoxic effects of MEL by RSV was greater in MCF-7 cells than MDA-MB-231 cells. Therefore, in the investigation of enhanced MEL

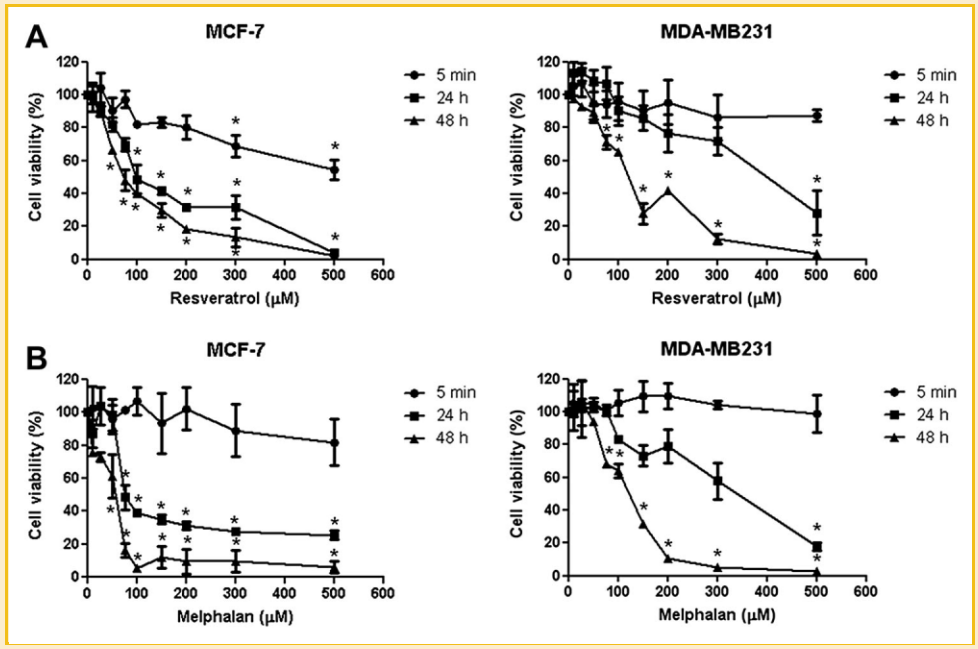

Fig. 1. The effects of RSV and MEL on the cell viability of MCF-7 and MDA-MB-231 cells. Cells were treated with 0, 10, 25, 50, 75, 100, 150, 200, 300, or 500 μM concentrations of RSV (A) or with the same concentrations of MEL for the indicated times (B). Cell viability was then determined by the MTT assay as described in the Materials and Methods Section. The percentage of cell viability was calculated as the ratio of treated cells to control cells. Data represent the mean ± SE of three independent experiments. MEL, melphalan; RSV, resveratrol.

cytotoxity by RSV, others experiments were carried out in this study using only MCF-7 cells.

Since a combination of cytotoxic agents may give rise to different effects depending on timing during a fixed treatment schedule, we tested whether the sequence of treatment with RSV and MEL might influence the cooperative effect (Fig. 2C). Interestingly, sequential treatment with RSV followed by MEL yielded more cytotoxic effects than treatment with MEL followed by RSV. These findings indicate that pretreatment with RSV conferred a state of increased responsiveness to MEL-induced cytotoxicity.

SENSITIZATION FOR MEL-INDUCED APOPTOSIS BY RSV

To explore the effect of RSV on apoptosis, the fraction of MCF-7 cells undergoing apoptosis after treatment with RSV was determined by FACScan (Fig. 3A). Treatments with 50 μM, 100 μM, 150 μM, and 200 μM of RSV increased the apoptosis in MCF-7 cells by 1.0%, 6.5%, 9.3%, and 8.0%, respectively. This increase was significant only in treatments in which the RSV concentration was greater than 100 μM. Treatment with 50 μM of MEL increased the apoptosis in MCF-7 cells by 4.6% (Fig. 3B). When 50 μM of MEL was combined with 50 μM or 100 μM of RSV, the mean amount of MCF-7 cells in the sub-G1 phase was increased by 21.9% and 24.7%, respectively. These results suggest that the increased apoptotic effect on MCF-7 cells with combination RSV and MEL treatment was achieved through the action of RSV, which enhanced the MEL-induced apoptosis. The induction of apoptosis by these compounds contributed to reduction of cell viability observed. Although others molecular mechanisms may be related to this reduced cell viability, such as necrosis and decreased cell proliferation. Pozo-Guisado et al. [2002] showed that treatment with RSV in similar concentrations used in this study inhibited cell proliferation and decreased cell number in a dose-dependent manner in MCF-7 cells. Scarlatti et al. [2008] demonstrated that, in response to RSV treatment, MCF-7 cells exhibited a significant increase in necrotic death within the first 24 h of incubation. The cell counting in combination treatment with RSV and MEL was performed to determine if, beside apoptosis, other mechanism is also involved in the reduction of cell viability (data not shown). It was observed that when 50 μM of MEL was combined with 50 μM or 100 μM of RSV, the MCF-7 cell number was decreased by 19.3% and 47.8%, respectively.

To determine if the sensitization by RSV involved p53 and caspases, p53 levels and caspase activation were analyzed by Western blot analysis (Fig. 4A). Densitometric analysis of protein bands was conducted to quantify the amount of protein (Fig. 4B). The data show that the p53 level was increased more than twofold by treatment with RSV alone and with treatments of combinations of RSV with MEL. No change in the p53 level was observed after the treatment with MEL alone. The expression of procaspase 8 was decreased by a combination treatment with RSV and MEL. Besides treatment of MCF-7 cells with RSV and MEL resulted in enhanced cleavage of caspase-7 and caspase-9 indicating that RSV cooperated with MEL to activate these caspases.

CELL CYCLE PROGRESSION

After observing the effects of RSV and MEL on the cell viability and apoptosis, the effect of these drugs on the cell cycle distribution was

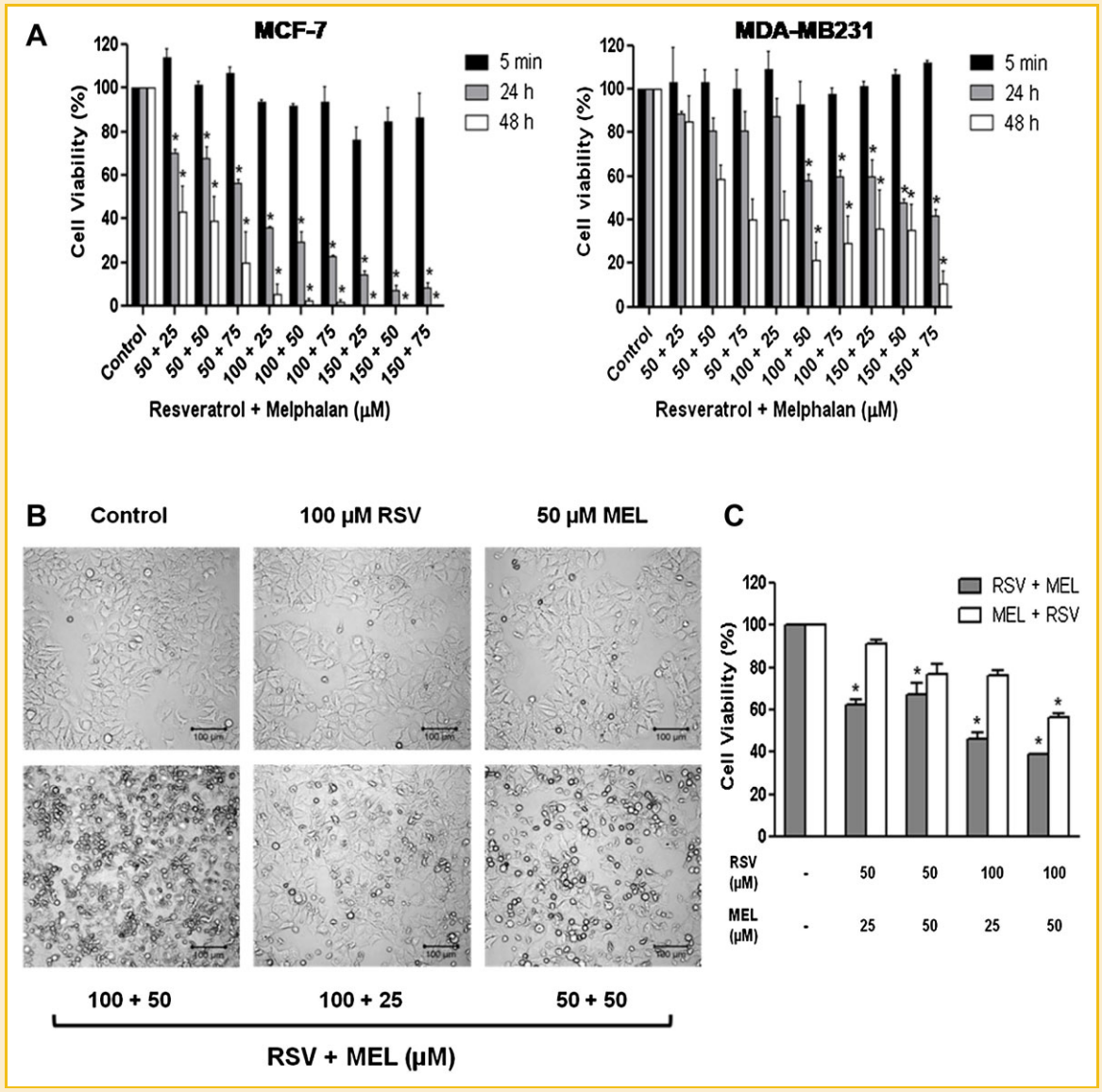

Fig. 2. The effects of the association of RSV with MEL in breast cancer cells. A: The effects of the association of RSV with MEL on the cell viability of MCF-7 and MDA-MB-231 cells for the indicated times. B: Representative bright field microscopy images of MCF-7 cells treated with RSV and MEL. C: Sequence-dependent effect of RSV and MEL on the cell viability. MCF-7 cells were treated with RSV for 24 h followed by MEL for 24 h (RSV + MEL) or with MEL for 24 h followed by RSV for 24 h (MEL + RSV). The cell viability was determined by the MTT assay as described in the Materials and Methods Section. The percentage of cell viability was calculated as the ratio of treated cells to control cells. Data represent the mean ± SE of three independent experiments. MEL, melphalan; RSV, resveratrol.

analyzed by flow cytometry (Fig. 5A,B). Treatment for 24 h with 50 μM or 200 μM of RSV induced a significant accumulation of cells in the S phase and a concomitant decrease in the number of cells the in G1 and G2/M phases (Fig. 5A). In accordance with these data, Pozo-Guisado et al. [2002] demonstrated that RSV, at the same concentrations used in this study, inhibited DNA synthesis activity in MCF-7 cells by measuring the rate of thymidine incorporation. Treatment for 48 h with 50 μM of RSV enhanced the accumulation of cells in the phase S. Treatment with 25 μM, 50 μM, or 75 μM of MEL also promoted the accumulation of cells in the S phase, but this result was not significant (data not shown). When the cells were treated with combinations of RSV and MEL, a significant increase of cells in the S phase was observed. This effect was significant only in treatments in which the concentration of RSV was 50 μM (Fig. 5B). It was not possible to perform the cell cycle distribution of treatment with 200 μM of RSV alone and with combination of 50 μM of RSV and 75 μM of MEL for 48 h due to low cell viability in these treatments.

To directly test the role of cell cycle progression in increasing MEL's cytotoxicity, cells were arrested in the G1, S, or G2/M phase using the specific cell cycle inhibitors mimosine, thymidine, or nocodazole and treated concomitantly with MEL (Fig. 5C). Similar to the treatment with both RSV and MEL, treatment with a combination of cell cycle inhibitors and MEL also potentiated the ability of MEL

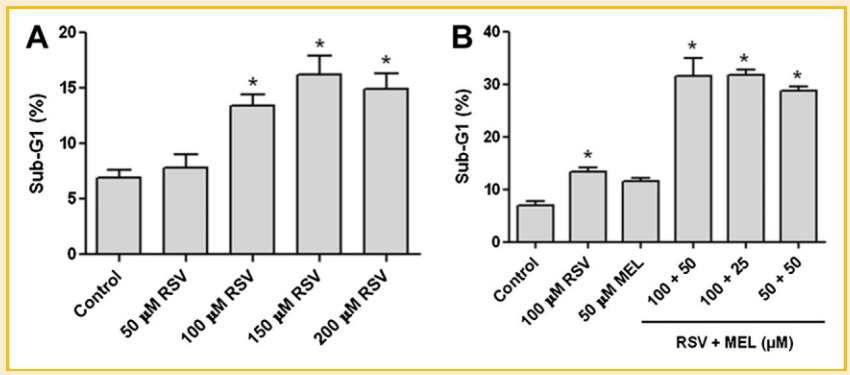

Fig. 3. The effects of RSV alone (A) and of the combination of RSV with MEL (B) on apoptosis in MCF-7 cells. After 24 h of treatment, cells were stained with propidium iodide for cytofluorimetric analyses. Percentages of apoptotic cells (sub-G1, apoptotic cells) are expressed as the mean ± SE (n = 3).

to decrease the viability of MCF-7 cells (Fig. 5D). These findings suggest that RSV-induced cell cycle arrest in S phase could be one of the mechanisms of RSV to sensitize MCF-7 cells for treatment with MEL.

EFFECTS OF RSV AND MEL ON THE EXPRESSION OF S CELL CYCLE CHECKPOINT-RELATED PROTEINS

Based on the results of the cell cycle distribution, we next investigated whether the cell cycle arrest at the S phase by RSV and/or MEL was related to the expression of cell cycle-regulatory proteins, which are essential for cell cycle progression at the S phase (Fig. 6A). MCF-7 cells were treated with RSV and MEL for 24 h and then harvested for immunoblotting. The levels of phospho-checkpoint kinase 2 (p-Chk2) were determined. Chk2 is a multifunctional enzyme that is involved in the induction of cell cycle arrest and apoptosis because it phosphorylates regulatory proteins such as Cdc25A and p53 [Ahn et al., 2004]. We observed that the treatment with RSV or MEL increased the levels of p-Chk2 expression. The increase became significantly pronounced in the combined treatments of RSV with MEL. No change was observed in the Cdc25A phosphatase levels. This phosphatase activates the CDK2.

While CDK2/cyclin E is known to play an important role in driving the cells in G1 phase to enter the S phase, CDK2/cyclin A is involved to drive cells in the late S phase to enter the G2 phase [Woo and Poon, 2003; Kaldis and Aleem, 2005]. The levels of cyclin E expression were increased by treatments, but this change was not significant. The expression of cyclin A was decreased by treatment with RSV and by a combination treatment with RSV and MEL. Moreover, the p21 protein levels, a CDK2 inhibitor, were significantly increased by treatment with RSV or MEL. No change was observed in p21 levels by combination treatment with RSV and MEL. Since p21 is in part regulated by p53 [Dotto, 2000] and the combination treatment with RSV and MEL increased the p53 levels,

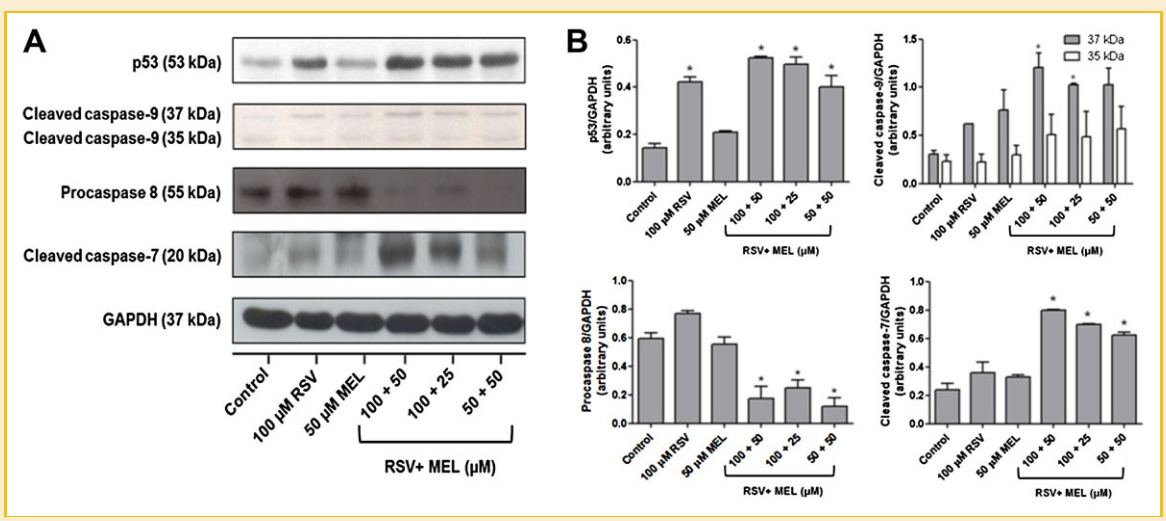

Fig. 4. The effects of RSV and MEL on p53 levels and activation of caspases. A: Equal amounts of total cellular proteins (100 μg) were loaded in each lane for the detection of p53, cleaved caspase-9, procaspase 8, and cleaved caspase-7. GAPDH was used as a loading control. The immunoblots are representative of three different experiments that gave similar results. B: Densitometric analysis of each lane was calculated using ImageJ Software, and the data are expressed as arbitrary units.

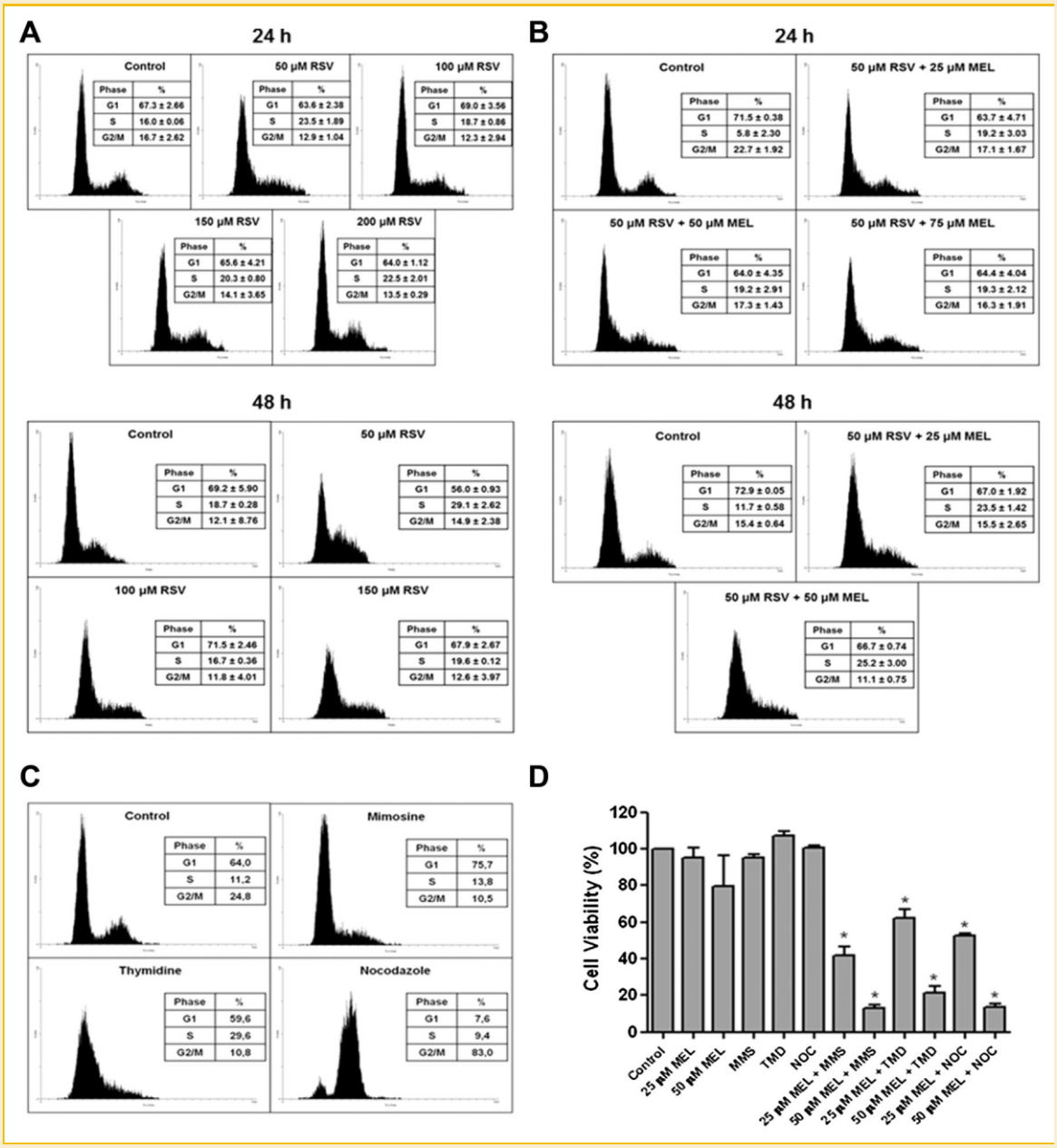

Fig. 5. The effects of RSV alone (A) and of the combination of RSV with MEL (B) on the cell cycle distribution in MCF-7 cells. After treatment, the cells were stained with propidium iodide, and their DNA content was determined by flow cytometry. C: Analysis of the cell cycle after 24 h of treatment with the cell cycle inhibitors mimosine, thymidine and nocodazole. The percentages of cells in G1, S or G2/M phase are shown. D: The effect of cell cycle inhibitors on MEL-induced cytotoxicity. Cell viability of MCF-7 cells was determined via the MTT assay after concomitant treatment of MEL with 2 mM thymidine, 0.4 mM mimosine, or 0.4 μg/ml nocodazole. The percentage of cell viability was calculated as the ratio of treated cells to control cells. Data represent the mean ± SE of three independent experiments. MEL, melphalan; MMS, mimosine; NOC, nocodazole; RSV, resveratrol; TMD, thymidine.

the results suggest that, under such experimental conditions, p21 is partly regulated in a p53-independent manner. The GAPDH expression was analyzed to confirm the equal loading of protein; a representative GAPDH blot is shown.

The effects of RSV and MEL on the expression of CDK2 were also determined. We found that while the total protein levels of CDK2 remained largely unchanged by treatments, its active form (Thr^{160}-phosphorylated CDK2) was decreased by treatment with RSV and by the combination treatment with RSV and MEL. This finding suggests that RSV is responsible for decreasing the phosphorylation of CDK2 at Thr^{160}. The treatment of MEL alone increased the levels of Thr^{160}-phosphorylated CDK2. Therefore, the activity of CDK7, the kinase that phosphorylates CDK2 at Thr^{160}, was analyzed by an in vitro kinase assay. Figure 6B shows a reduction in

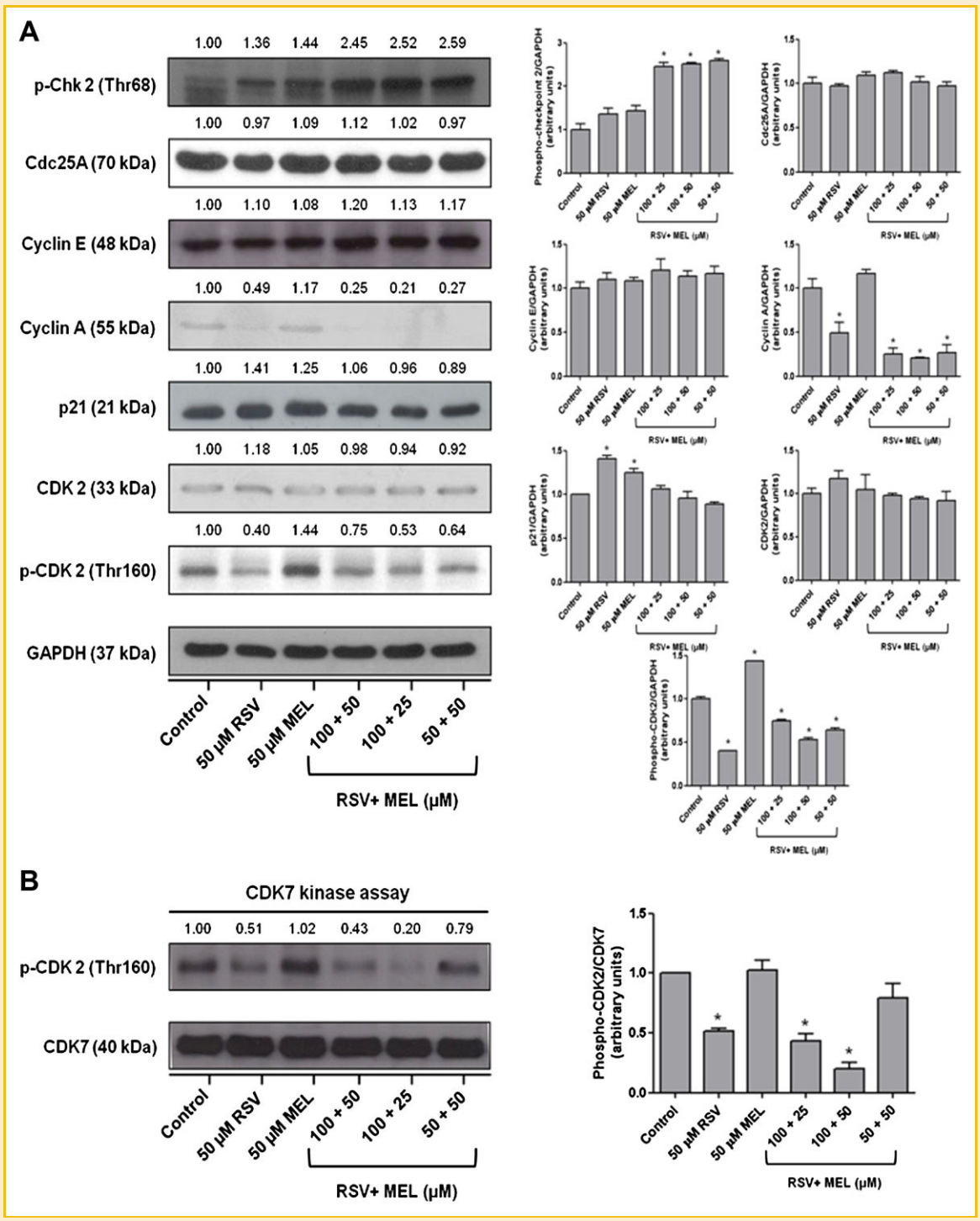

Fig. 6. The effects of RSV and MEL on the cell cycle-regulated proteins involved in the S phase progression and CDK7 kinase activity. A: Phospho-checkpoint 2, Cdc25A, cyclin A, cyclin E, p21, CDK2, and phospho-CDK2 levels were analyzed. After treatment for 24 h, total proteins in cellular extracts were collected and assayed by Western blotting using antibodies against the S cell cycle regulators as indicated. GAPDH was used as an internal control for equivalent protein loading. The GAPDH immunoblot is representative of all the different experiments. For CDK7 kinase activity (B), CDK7 was immunoprecipitated from treated cell lysates and then subjected to kinase assay in the presence of ATP and the CDK2 substrate protein as described in the Materials and Methods Section. The phosphothreonine site of the CDK2 substrate at Thr160 was determined by Western blotting. The immunoblots are representative of three different experiments that gave similar results. Densitometric analysis of each lane was calculated using ImageJ Software, and the data are expressed as arbitrary units. IP, immunoprecipitation.

the phosphorylation of CDK2 substrate at Thr^{160}; these data support the above suggestion that RSV inhibited the activity of the CDK7 kinase. The same effect was found in the cells treated with a combination of RSV and MEL but not in cells that were treated with MEL alone.

DISCUSSION

MEL is a phenylalanine derivative of nitrogen mustard used in cancer therapy; it was first synthesized in 1953. This cytotoxic drug is believed to exert its pharmacologic activity by inducing interstrand cross-links in the major groove of DNA; this mechanism represents the toxicity of all alkylation events [Rothbarth et al., 2004]. Initially, MEL was only used to treat multiple myeloma, but later, it is also shown to be effective in the treatment of patients with several other tumors, such as ovarian or breast cancer. MEL, especially when used at a high dose, shows a diversity of toxic side effects. The most common side effect occurring during therapy is bone marrow suppression leading to leukopenia and thrombocytopenia. The occurrence of resistance to antineoplastic agents such as MEL is a major problem in cancer treatment [Dollery, 1991]. Studies are necessary to improve the efficacy of MEL either by potentiating the cytotoxicity of MEL or by reducing the resistance against MEL.

Here, we investigated the effect of the combination treatment of RSV and MEL on human breast cancer MCF-7 and MDA-MB231 cells. RSV is a natural phytoalexin that is present in grape skins and, as a consequence, in red wine [Gusman et al., 2001]. As early as 1997, RSV was found to be a potent chemopreventive agent, which blocked the initiation, promotion, and progression of tumors [Jang et al., 1997]. Since that time, extensive research on its anticancer activity performed in a wide variety of cellular models suggests a potential antiproliferative and apoptogenic use of this compound [Goswami and Das, 2009].

Consistent with earlier observations [Nakagawa et al., 2001; Pozo-Guisado et al., 2002; Wesierska-Gadek et al., 2008], we found here that RSV inhibits the viability of human breast cancer MCF-7 and MDA-MB-231 cells while maintaining little cytotoxic effect in non-malignant cell lines.

We also showed that RSV enhanced the cytotoxic effects of MEL on MCF-7 and MDA-MB231 cells in vitro. The potentiation of cytotoxic effects of MEL by RSV was higher in MCF-7 cells than MDA-MB231 cells. This increase was dependent on the treatment sequence. Sequential treatment with RSV followed by MEL yielded more cytotoxic effects than treatment with MEL followed by RSV. Fulda and Debatin [2004] showed that pretreatment with RSV enhanced the ability of anticancer agents, such as VP16, doxorubicin, cytarabine, actinomycin D, taxol, or methotrexate, to induce apoptosis in SHEP neuroblastoma cells in a dose- and time-dependent manner. He et al. [2011] demonstrated that RSV enhanced the antitumor activity of rapamycin in multiple breast cancer cell lines.

Studies suggest that RSV could act as potent sensitizer for antitumor drug-induced apoptosis [Kubota et al., 2003; Fulda and Debatin, 2004; Jazirehi and Bonavida, 2004; Duraj et al., 2006]. A similar effect was observed in this study. To gain further insight into the molecular mechanism of RSV induced apoptosis sensitivity, we investigated key molecules known to regulate apoptosis. The combination treatment of RSV with MEL resulted in the upregulation of p53, downregulation of procaspase 8, and the activation of caspases 7 and 9. Gatouillat et al. [2010] reported that RSV enhanced the chemotherapeutic potential of doxorubicin in chemoresistant B16 melanoma cells through the upregulation of p53. Interestingly, in another study, an enhancement in the sensitization effect of RSV on apoptosis induced by various drugs in cancer cell lines was found to be p53 independent [Fulda and Debatin, 2004]. Bhardwaj et al. [2007] have shown that RSV enhanced the apoptotic and antiproliferative potential of velcade and thalidomide in multiple myeloma cells. Such an enhancement was associated with inhibition of NF-κB and STAT3 activation pathways and with an accumulation of the sub-G(1) population, an increase in Bax release, and the activation of caspase-3.

The analysis of cell cycle distribution showed that RSV, both alone and in combination treatment with MEL, inhibited cell cycle progression in the S phase. Treatment of MCF-7 cells with specific cell cycle inhibitors followed by MEL also potentiated the cytotoxic effect of MEL, suggesting that the cell cycle arrest is necessary for the chemosensitization of MCF-7 cells to MEL by RSV. The cell cycle arrest may increase MEL incorporation into the cells. This finding would explain the improved efficacy of MEL we observed here.

The effect on cell cycle progression was probably caused by downregulation of cyclin A and phospho-CDK2 (Thr160) by treatment with RSV and its association with MEL. The treatment of MEL alone increased the levels of Thr^{160}-phosphorylated CDK2. This observation is in agreement with the known function of activated CDK2 (phospo-CDK2) in driving cells in late G1 phase into the S-phase. Zhou et al. [2009] showed that activation of p-CDK2 is believed to play a role in the induction of a reversible, non-cytotoxic S-phase delay in HepG2 cells.

Figure 7 shows proteins related to cell cycle progression in the S phase. An important mechanism for regulating cell cycle progression is controlled by the activity of CDKs, which is regulated by interacting with their respective CDK subunits [Malumbres and Barbacid, 2005]. Cyclin E associates with CDK2 to regulate progression from G1 into S phase. Cyclin A binds with CDK2, and this complex is required during S phase. In addition to cyclin binding, CDK2 activity is also regulated by phosphorylation on conserved threonine and tyrosine residues. Dephosphorylation at tyrosine-15 and threonine-14 sites by the enzyme Cdc25A is necessary for activation of CDK2 and further progression through the cell cycle. Full activation of CDK2 requires phosphorylation of threonine 160, brought about by the CDK7 [Vermeulen et al., 2003].

Because hypophosphorylation of CDK2 protein was induced in MCF-7 cells by RSV treatment alone and by combination treatment with MEL, we analyzed the CDK7 activity and observed an inhibition of its activity. The CDK7 kinase inhibition maintained CDK2 in its inactive form and arrested the cell cycle in the S phase. Liang et al. [2003] demonstrated that RSV induced cell cycle arrest through the inhibition of CDK7 kinase activity in colon carcinoma HT29 cells. However, the cell cycle arrest observed by these researchers was at the G2 phase. CDK7, in addition to controlling the activity of CDK2,

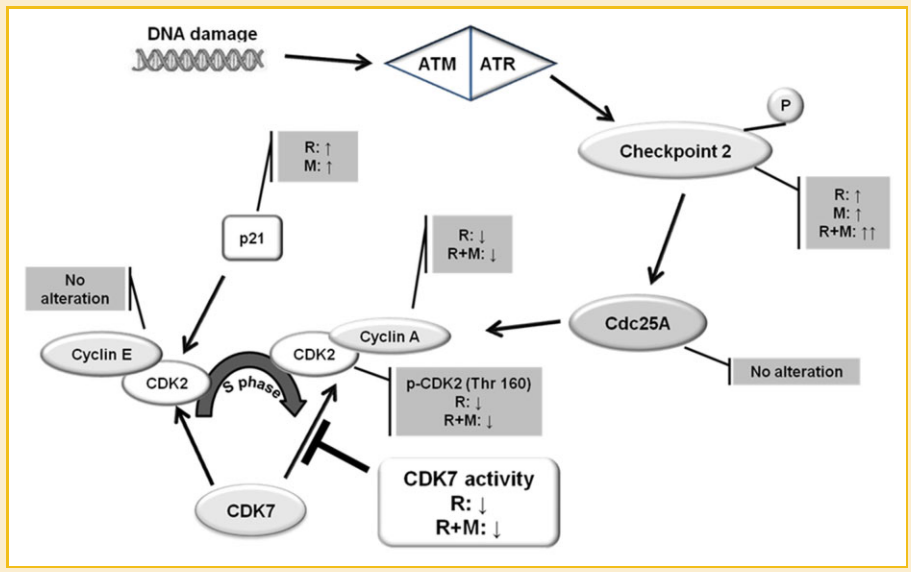

Fig. 7. The effects of RSV and its association with MEL on proteins related to the cell cycle progression in the S phase of MCF-7 cells. M, melphalan; R, resveratrol; R + M, association of RSV with MEL.

also controls the activity of CDK1, which is an enzyme responsible for progression at the G2 phase [Vermeulen et al., 2003].

In summary, our results indicate that RSV could be used as an adjuvant agent during breast cancer therapy with MEL. It is important to emphasize that this is the first study demonstrating the effect of the combination of RSV with MEL in human cancer cells. Despite more than 1,100 publications on the cancer chemotherapeutic potential of RSV, only about 20 reported on its chemosensitization potential. Thus, further studies should be performed in this area to determine effective combinations of RSV and chemotherapeutic agents, their effects on different types of cancers and possible mechanisms of action involved in chemosensitization by RSV.

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