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ORIGINAL ARTICLE

Resveratrol analog trans 3,4,5,4′-tetramethoxystilbene (DMU-212) mediates anti-tumor effects via mechanism different from that of resveratrol

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Abstract
Purpose Resveratrol is a well-known chemopreventive and chemotherapeutic agent. Among all of the resveratrol analogs synthesized, 3,4,5,4′-tetramethoxystilbene (DMU-212) shows high activity and selectivity against various cancer cell types. The objective of this study is to investigate why DMU-212 has higher anti-tumor activity than resveratrol.
Methods The effects of DMU-212 and resveratrol on cell viability, cell cycle, Stat3 activation, and microtubule dynamic were investigated and compared using MTT assay, cell cycle analysis, Western blot, tubulin polymerization assay, respectively, in MDA-MB-435 and MCF-7 human breast cancer cells.
Results Compared to resveratrol, DMU-212 exerted a significantly higher growth inhibition in both cell lines. Further studies demonstrated that DMU-212 acted via different mechanisms from resveratrol. First, DMU-212 induced predominantly G2/M arrest whereas resveratrol induced G0/G1 arrest in both cell lines. Correlating with these findings, resveratrol induced more dramatic changes in the expression of Cyclin D1 compared to DMU-212. Second, DMU-212 induced apoptosis and reduced the expression of multiple anti-apoptotic proteins more appreciably than resveratrol. Third, while both agents inhibited Stat3 phosphorylation, treatments of DMU-212 but not resveratrol led to a significant increase in tubulin polymerization. The higher sensitivity to DMU-122 in MDA-MB-435 correlated with the more prominent effects seen in these parameters in this cell line, as compared to MCF7.
Conclusion Compared to resveratrol, the novel stilbene derivative, DMU-212, had higher anti-tumor effects, which are likely owing to its modulation of multiple cellular targets.

Keywords DMU-212 · Resveratrol · Apoptosis · Cell cycle · Tubulin · Stat3

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Introduction

Resveratrol (*trans*-3,5,4′-trihydroxystilbene, Fig. 1a), a polyphenolic stilbene found in the skin of red grapes, various other fruits, and root extract of the weed *Polygonum cuspidatum*, has been an important constituent of Chinese and Japanese folk medicine [1]. Resveratrol has been extensively investigated as a cardioprotective, anti-inflammatory and anti-aging agent [2, 3]. Recent studies show that resveratrol has potent anti-cancer effects. This was evidenced by its in vitro and in vivo inhibitory effects on the growth of a number of tumor cell lines including lymphoma, myeloma, melanoma, breast, pancreatic, colorectal, hepatocellular and prostate carcinoma [4–10]. Resveratrol has been reported to have diverse effects on signaling molecules, such as downregulation of the expression of angiogenesis-associated genes [7], activation of the apoptotic mechanisms [5–7], and induction of cell cycle arrest [10]. Resveratrol was

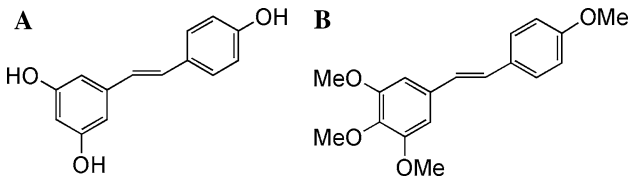

Fig. 1 Chemical structures of resveratrol (**a**) and DMU-212 (**b**)

also found to sensitize resistant tumor cell lines to a variety of chemotherapeutic agents, such as paclitaxel [4], thalidomide and Velcade [6].

In view of the great potential of resveratrol as a potent chemotherapeutic agent against a wide variety of cancers, the trihydroxystilbene scaffold of resveratrol has been the subject of synthetic manipulations with the aim of generating novel resveratrol analogs with improved anti-cancer activity. Among tested analogs, trans 3,4,5,4′-tetramethoxystilbene (DMU-212; Fig. 1b) has been shown to possess stronger anti-proliferative properties than resveratrol in HeLa cervical cancer cells, LnCaP prostate cancer cells, HepG2 hepatoma cells, as well as HCA-7, HCEC and HT-29 colon cancer cells [11–14]. In addition, DMU-212 was found to preferentially inhibit the growth of cancer cells, but not normal cells [11, 14]. Although it has been documented that the Bcl-2 family of protein was associated with the striking differential growth inhibitory effect of DMU-212 in cancer cells [14], the mechanism underlying the anti-tumor effects of DMU-212 is incompletely understood. In particular, why DMU-212 has superior anti-tumor effects compared to resveratrol is not known.

In this study, we aim to assess and compare the anti-tumor effects of DMU-212 with those of resveratrol, using two human breast cancer cell lines, MCF-7 and MDA-MB-435. We first established the superior anti-tumor effects of DMU-212 in these two cell lines. We then evaluated and compared the effects of these drugs on the expression of a number of cell cycle regulatory protein and apoptosis-regulatory proteins. Since resversatrol has been shown to suppress signal transducer and activator of transcription 3 (Stat3) [5], an oncogenic protein when it is aberrantly activated [15, 16], we abberrantly activated [15, 16], we contrasted the Stat3 suppressive effects of these two drugs.

Materials and methods

Chemicals and reagents

Resveratrol, propidium iodide (PI), ribonuclease A (RNase A), dimethyl sulfoxide (DMSO), paclitaxel, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT), and protease inhibitor cocktail were purchased from Sigma Chemical Co., St Louis, MO, USA; DMU-212 was synthesized and characterized in the laboratory of Dr. R. A. Gossage [17]; mouse anti-phosphorylated Stat3 (B7), rabbit anti-Stat3 (C-20), rabbit anti-Bcl-Xs/Xl (L-19), mouse anti-Bcl-2 (C2), rabbit anti-cyclin D1 (M20), rabbit anti-Actin (I-19), mouse anti-α-Tubulin (B7), and horseradish peroxidase-conjugated goat antimouse or anti-rabbit secondary antibodies were from Santa Cruz Biotechnology, CA; ECL chemiluminescent detection system was from Amersham Pharmacia Biotech., Buckinghamshire, UK; phenol red-free RPMI 1640 media and fetal bovine serum from Gibco, Grand Island, NY; Nunc® 96-well plates from Nalgene Nunc International, Rochester, NY.

Cell culture and cell viability studies

MCF-7 human breast cancer cell line was a generous gift from the laboratory of Dr. Susan Bates (National Cancer Institute, Bethesda, MD). Human breast cancer cell line, MDA-MB-435/LCC6 was a generous gift from the laboratory of Dr. R. Clarke (Georgetown University, Washington, DC). Cells maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin (growth medium) at 37°C in 5% CO_2 incubator. To study the effects of resveratrol and DMU-212 on cell viability, confluent stock cultures were trypsinized and seeded in 96-well plates at a density of 3–5 × 10^3 cells per well in growth medium. After 24 h, cell monolayers were treated with indicated concentrations of resveratrol and DMU-212 (dissolved in DMSO at a concentration of 100 mM/mL as a stock solution). The concentration of DMSO in the final cell treatment solutions was less than 0.1%. Control cells were incubated under identical conditions with vehicle (0.1% DMSO). At the end of treatments, cells were washed with PBS and 200 μL of MTT solution (0.5 mg/mL in serum-free RPMI-1640 medium) was added. Two hours later, medium was aspirated and the precipitated formazan was dissolved in 200 μL of DMSO. Cell viability was determined by measuring the optical absorbance at 570 nm with reference to 650 nm using a PowerWave$_x$340 microplate reader (BIO-TEK Instruments, Inc., Nepean, ON, Canada). In this study, the drug concentration required to inhibit cell growth by 50% (IC_{50}) was determined from a plot of percent cell viability from control untreated cells versus logarithm of concentration.

Cell cycle analysis

MCF-7 and MDA-MB-435 cells were cultured in 100 mm tissue culture plates until 80% confluency, and treated with vehicle (0.1% DMSO) or indicated concentrations of resveratrol and DMU-212. After 24 h incubation, cells were harvested by quick trypsinization, washed with PBS, and fixed with cold 70% ethanol. Two hours before fluorescence-activated cell sorting (FACS) analysis, cells were stained with

20 μg/mL propidium iodide in the presence of 200 μg/mL of RNase A. Stained cells were analyzed on a flow cytometer (Becton-Dickinson FACSort, Franklin Lakes, NJ) and data from 20,000 cells were collected for each data file.

Staining for apoptosis

To confirm DMU-212-induced apoptosis, MDA-MB-435 cells were stained with annexin V and propidium iodide for FACS analysis. Cells were cultured in 100 mm tissue culture plates until 80% confluency, and treated with vehicle (0.1% DMSO) or indicated concentrations of DMU-212. After 24 h incubation, cells were harvested by quick trypsinization, washed twice with cold PBS, and stained with annexin V-fluorescein 5(6)-isothiocyanate (FITC) and PI (Annexin V-FITC apoptosis detection kit, BD Pharmingen, San Diego, CA) in binding buffer (10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$). Stained cells were analyzed on flow cytometer (Becton-Dickinson FACSort) using Cell Quest analysis software with the laser excitation wavelength at 488 nm. The green signal from annexin V-FITC was measured at 525 nm, and the red signal from PI was measured at 620 nm. Cells that were Annexin V-positive and PI-negative were defined as early apoptotic cells; while those with both Annexin V- and PI-positive were defined as end stage apoptosis and death.

Caspase 3 activity assay

MDA-MB-435 cells were seeded in 100 mm cell culture dishes until 80% confluence, and treated with vehicle (0.1% DMSO) or indicated concentrations of DMU-212. After 4–20 h of incubation, cells were harvested by quick trypsinization, washed with cold PBS, and suspended in lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS and 5 mM DTT) at a concentration of 10^7 cells per μL. The Caspase 3 activity was determined using a colorimetric Caspase 3 assay kit (CASP-3-C, Sigma) according to the manufacturer's instruction, and the optical absorbance at 405 nm was measured using a PowerWave$_x$340 microplate reader (BIO-TEK Instruments).

Assessment of in vitro tubulin polymerization

The tubulin polymerization was detected by the use of CytoDYNAMIX Screen 01 kit (BK004, Cytoskeleton Inc., Denver, CO). Purified glycerol-free bovine brain tubulin proteins (>97% purity) were solubilized in ice-cold G-PEM buffer containing 80 mM PIPES, 2 mM $MgCl_2$, 0.5 mM EDTA, and 1.0 mM GTP (pH 6.9) and 5% glycerol with or without the tested compound. Then, the mixture was immediately transferred to a 96-well plate and the assembly of microtubule was monitored spectrophotometrically by following the change in absorbance at 340 nm at 37°C for 60 min using a PowerWave$_x$340 microplate reader (BIO-TEK Instruments).

Assessment of cellular tubulin polymerization

The effect of DMU-212 on cellular tubulin polymerization was performed as described by Giannakakou et al. [18]. In brief, breast cancer cells grown to 80% confluency were treated with indicated concentrations of test agents for 2 h. Cells were then washed twice with PBS and lysed at 37°C for 5 min with 400 μL of hypotonic buffer containing 20 mM Tris–HCl (pH 6.8), 1 mM $MgCl_2$, 2 mM EGTA, 2 μL/mL protease inhibitor cocktail, 1 mM Na_3VO_4, and 0.5% Nonidet. The cell lysate in which the cytosolic and cytoskeletal fractions containing soluble (S) and polymerized (P) tubulin, respectively, was separated by centrifugation, resolved by electrophoresis through 10% SDS–polyacrylamide gels, and immunoblotted with an antibody against α-tubulin.

Western blot analysis

Resveratrol- and DMU-212-treated MDA-MB-435 and MCF-7 cells were lyzed in lysis buffer (20 mM Tris pH 7.4, 250 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, 4 mM Na_3VO_4, and 0.1% protease inhibitor cocktail). Twenty μg of proteins were subjected to electrophoresis, transferred to a PVDF membrane, blocked with 5% skim milk, and probed with various antibodies (0.5–1 μg/mL) overnight at 4°C. The blots were then washed and exposed to horseradish peroxidase-conjugated donkey antimouse or antirabbit secondary antibody (1:5,000) and finally visualized with the ECL chemiluminescent detection system as described by the manufacturer. Optical densities of the bands were analyzed using Scion Image software (Scion, Frederick, MD, USA), and the relative levels were determined by normalization to β-actin expression.

Statistics

Experiments were repeated at least three times and the data were expressed as the mean ± SD. Comparison between different groups was performed by one-way ANOVA with the Tukey test applied post hoc for paired comparisons (SPSS 10) where P values of 0.05 or less were considered significant.

Results

Cell viability assay

The inhibitory effects of resveratrol and DMU-212 on MDA-MB-435 and MCF-7 breast cancer cells were evalu-

ated and compared using MTT assay. As shown in Fig. 2, continuous exposure to various concentrations of resveratrol and DMU-212 both resulted in dose-dependent decreases in MDA-MB-435 and MCF-7 cell viability relative to the negative control cultures, although MDA-MB-435 cells were more sensitive to these treatments than MCF-7. For MDA-MB-435 cells, DMU-212 was more potent than resveratrol, giving a six times lower IC_{50} than that of resveratrol at 48 h after treatment (9.9 vs. 69.3 μM, $P < 0.01$). For MCF-7 cells, DMU-212 at concentrations $\leq$ 50 μM was only slightly more active than resveratrol at 48 h after treatment ($P < 0.05$). At 100 μM, however, DMU-212 showed no superiority over resveratrol.

Cell cycle arrest and apoptosis

The effect of resveratrol and DMU-212 on cell cycle progression was investigated by cell cycle analysis, using MDA-MB-435 as well as MCF-7. In both tested cell lines, resveratrol induced a dramatic G0/G1 arrest while DMU-212 resulted in a dominant G2/M arrest (Fig. 3). MDA-MB-435 cells residing at G2/M phase were increased from 30.0% of control to 36.7, 40.6 and 63.7%, respectively, at 24 h after treatments with 5, 10 and 20 μM of DMU-212 (Fig. 3a). The DMU-212-induced G2/M arrest was coupled with a dose-dependent increase in apoptosis in MDA-MB-435, which was evidenced by formation of a sub-G1 population in FACS analysis. At DMU-212 concentrations of 5, 10, 20 μM, apoptotic cells accounted for 3.2, 4.5, and 11.9% of total cell population, respectively. The DMU-212 induced apoptosis in MDA-MB-435 was further confirmed by annexin V-FITC and PI double staining (Fig. 3c). In MCF-7 cells, DMU-212 induced a significant G2/M arrest at 20 μM; cells at G2/M phase were increased from 18.9% of control to 67.5% (Fig. 3b). However, DMU-212 induced G2/M arrest in MCF-7 was reversed with the increase of concentrations; cells at G2/M phase were decreased to 55.4 and 32.5% at 50 and 100 μM, respectively. In comparison to MDA-MB-435, MCF-7 was more resistant to DMU-212 induced apoptosis.

Cell cycle- and apoptosis-associated proteins

To define the molecular mechanisms by which resveratrol and DMU-212 induce cell cycle arrest and apoptosis, the expression of some cell cycle- and apoptosis-associated proteins, such as Cyclin D1, Bcl-xL and Bcl-2, were evaluated by Western blot analysis, and the enzymatic activity of Caspase 3 was determined using a colorimetric assay kit. Similar level of β-actin expression in each treatment ensured that equal amount of protein was loaded in each lane. Treatment with 100 μM of resveratrol significantly down regulated the expression level of Cyclin D1 to 4.9 and 11.4% of control in MDA-MB-435 and MCF-7, respectively, whereas the level of Bcl-xL was only reduced to 53.7 and 53.2% of control and Bcl-2 was not appreciably altered (Fig. 4a, b). By comparison, DMU-212 at tested high dose significantly decreased the levels of Bcl-xL and

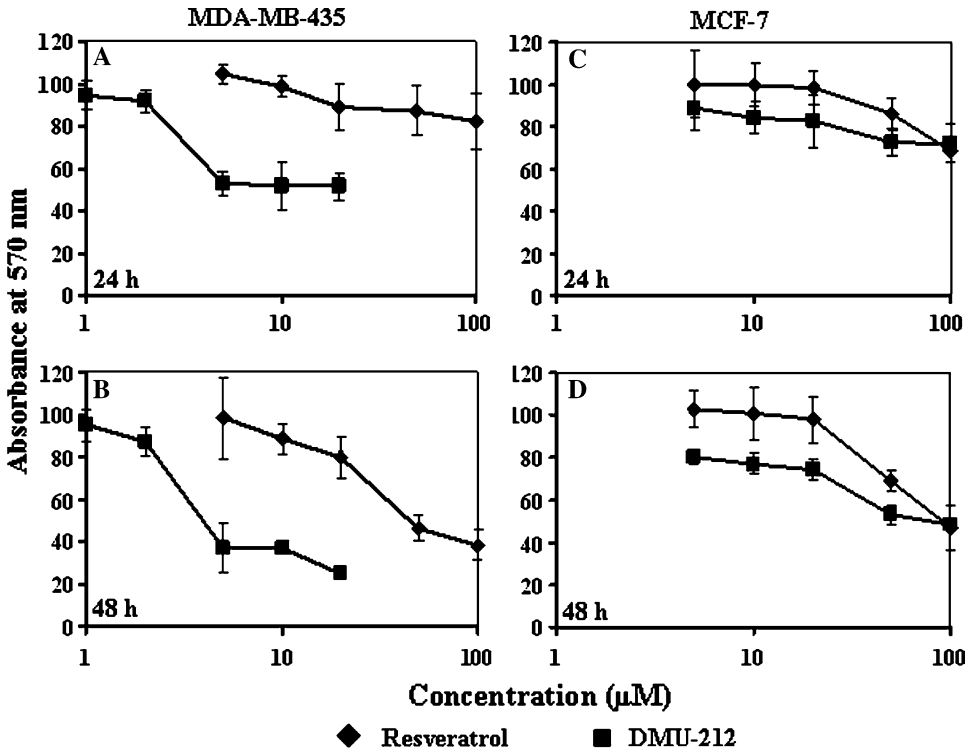

Fig. 2 Resveratrol and DMU-212 inhibit the cell viability. MDA-MB-435 (*left panels*) and MCF-7 (*right panels*) cells were seeded in 96-well plates at a density of 3–5 × 10^3 cells per well for 24 h, and then treated with vehicle (0.1% DMSO) or with various concentrations of resveratrol and DMU-212 for 24 and 48 h. Cell viability was finally determined by adding MTT reagent and measuring the optical absorbance at 570 nm as described in "Materials and methods". IC_{50} was determined from a plot of percent cell viability from control untreated cells versus logarithm of concentration. All results are expressed as mean ± SD from three independent experiments

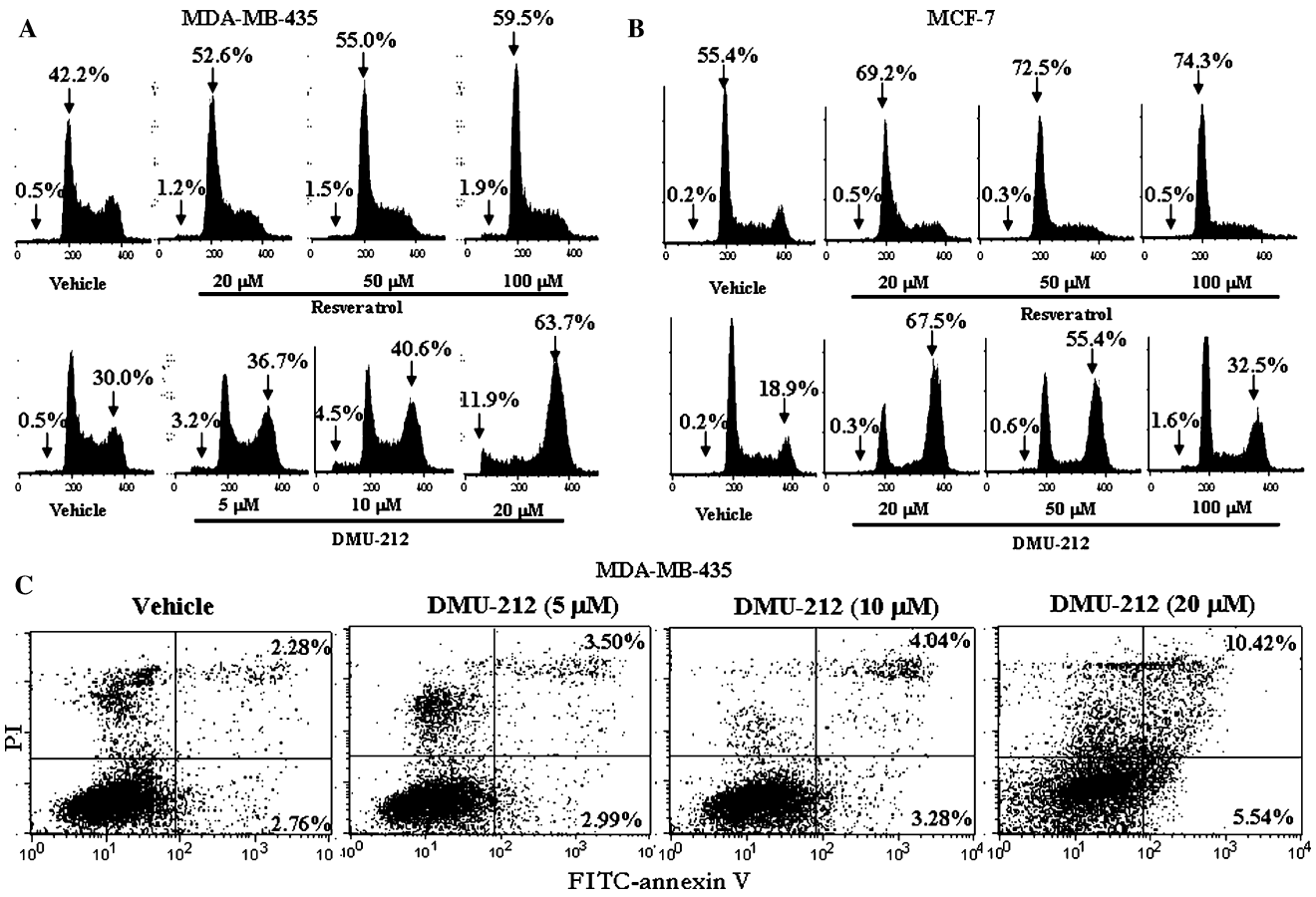

Fig. 3 Resveratrol and DMU-212 induces cell cycle arrest and apoptosis in MDA-MB-435 and MCF-7 cells. Effects on cell cycle phase distribution of MDA-MB-435 (**a**) and MCF-7 (**b**) cells after treatment with resveratrol and DMU-212. Cells in active growth were treated with vehicle or with various concentrations of resveratrol and DMU-212 for 24 h, then fixed and the DNA content determined by flow cytometric analysis as described in Materials and Methods. Effects of DMU-212 on apoptosis in MDA-MB-435 cells (**c**). Cells in active growth were treated with vehicle or with various concentrations of DMU-212 for 24 h, then stained and the apoptotic cells were determined using a flow cytometer as described in "Materials and methods". The figures are the representative of three independent experiments

Bcl-2 to less than 15% of control in both MDA-MB-435 and MCF-7. The expression level of Cyclin D1 was down regulated to a lower extent, i.e., 40.0 and 52.1% of control in MDA-MB-435 and MCF-7, respectively. Consistent with the alteration in Bcl-2 family proteins, DMU-212 significantly increased the Caspase 3 activity in MDA-MB-435 cells (Fig. 4c). At 20 h after treatment, the Caspase 3 activity was increased to 2.29-, 3.94- and 7.93-folds of that of vehicle at 5, 10 and 20 μM DMU-212 concentration, respectively. Functional Caspase 3 is deficient in MCF-7 cells [19], and thus, further assessment of Caspase 3 in this cell line is not possible.

Stat3 inhibition

The antitumor activity of resveratrol is, at least in part, ascribed to the blockade of Stat3-mediated dysregulation of growth and survival pathways [5, 20]. In this study the effect of DMU-212 on Stat3 expression and activation, as assessed by phosphorylation on tyrosine residue-705 (pStat3), was evaluated by Western blot analysis. As shown in Fig. 5a and b, the expressed level of pStat3 was significantly higher in MDA-MB-435 than in MCF-7 cells, which is consistent with previous report that the former harbors constitutively active Stat3 while the latter lacks constitutive Stat3 activity [21]. Similar to resveratrol, DMU-212 at tested concentrations was able to significantly inhibit the phosphorylation of Stat3 in both cell lines at 24 h after treatment, which was coupled with slight downregulation of Stat3 expression (Fig. 5a, b). The time-course alteration of Stat3 and pStat3 was also evaluated in MDA-MB-435 cells (Fig. 5c, d). At as early as 2 h after treatment, resveratrol and DMU-212 were both able to reduce the phosphorylation of Stat3 significantly; the level of pStat3 was reduced to 40.3 and 25.2% of control, respectively.

Tubulin polymerization

Since extensive structure/activity investigations have revealed analogues of resveratrol might function as potent

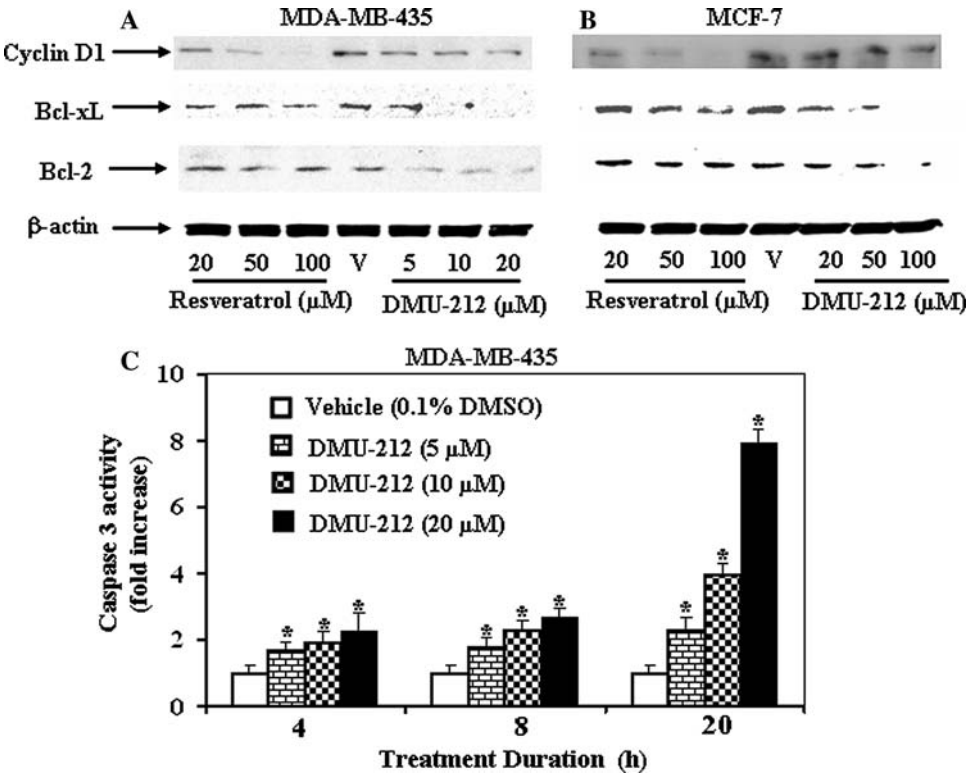

Fig. 4 The effect of resveratrol and DMU-212 on cell cycle- and apoptosis-associated proteins (Cyclin D1, Bcl-2 and Bcl-xL) in MDA-MB-435 (**a**) and MCF-7 (**b**) cells. Cells in active growth were treated with vehicle (*V*) or with various concentrations of resveratrol and DMU-212 for 24 h, and the levels of these proteins were determined by western blot as described in "Materials and methods". DMU-212 increases the enzymatic activity of Caspase 3 in MDA-MB-435 cells (**c**). Cells in active growth were treated with vehicle or with indicated concentrations of DMU-212 for 4–20 h, then quickly trypsinized, washed, and lysed. The Caspase 3 activity was determined using a colorimetric Caspase 3 assay kit as described in "Materials and methods". All results are expressed as the mean ± SD from three independent experiments. *Bars* with *asterisks* are significant different from vehicle at $P < 0.05$ as determined by one-way ANOVA

anti-mitotic agents [22, 23], the effects of resveratrol and DMU-212 on tubulin polymerization were evaluated in further studies. The addition of DMU-212 enhanced the in vitro tubulin polymerization in a dose-dependent manner while resveratrol failed to affect the assembly of microtubule (Fig. 6a). DMU-212 at a dose of 2.5 μM resulted in a microtubule-stabilizing effect comparable to that of 10 μM paclitaxel. To further confirm the antimicrotubule effect, the cytosolic (soluble) and cytoskeletal (polymerized) forms of tubulin were separated from whole cell lysate by centrifugation and measured as described above (Fig. 6b, c). Treatments of MDA-MB-435 cells with DMU-212 resulted in a dose-dependent shift in tubulin polymerization from soluble to polymerized form, which, at the concentration of 20 μM, was comparable to the alteration caused by paclitaxel at 200 nM. In MCF-7 cells, however, the effect of DMU-212 on tubulin polymerization was dose-independent, and it was less potent than in MDA-MB-435. On the other hand, resveratrol failed to modify tubulin polymerization in both cells at concentration up to 100 μM (data not shown).

Discussion

In this study, we investigated and compared the biological properties of resveratrol and its tetramethoxy derivative DMU-212 (Fig. 1). Our MTT results showed that DMU-212 was more potent than resveratrol in MDA-MB-435 and MCF-7 breast cancer cells (Fig. 2), consistent with the previous data derived from studies using cancer cell lines of the cervix, prostate, liver and colon [11–14]. Further studies revealed that the superior anti-cancer activity of DMU-212 over resveratrol was associated with several differences in the mechanism. First, DMU-212 induced predominantly G2/M arrest, as determined by cell cycle analysis (Fig. 3). In this study, we also have shown that DMU-212 treatment induced more apoptosis than resveratrol, and these findings correlates with the significant decrease in anti-apoptotic Bcl-2 and Bcl-xL in MDA-MB-435 and MCF-7 cells. Members of the Bcl-2 family play crucial roles in regulating apoptosis by controlling mitochondrial permeability [24]. These observations were in agreement with previous findings that specific activation of mitochondria-mediated

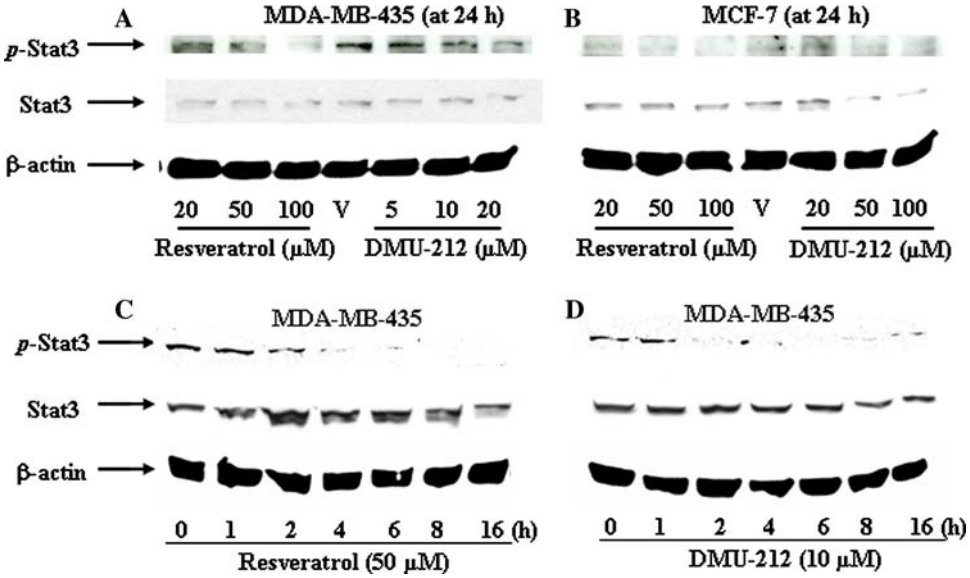

Fig. 5 The effect of resveratrol and DMU-212 on Stat3 activation in MDA-MB-435 and MCF-7 breast carcinoma cell lines. The dose dependence of Stat3 activation was determined in MDA-MB-435 (**a**) and MCF-7 cells (**b**) after 24 h incubation; the time-course alteration of Stat3 activation after treatment with resveratrol (**c**) and DMU-212 (**d**) was determined in MDA-MB-435 cells. Cells in active growth were treated with vehicle (*V*) or with indicated concentrations of resveratrol and DMU-212 for different durations, and the expression of Stat3 and pStat3 was determined by western blot as described in "Materials and methods". The image is the representative of three independent experiments

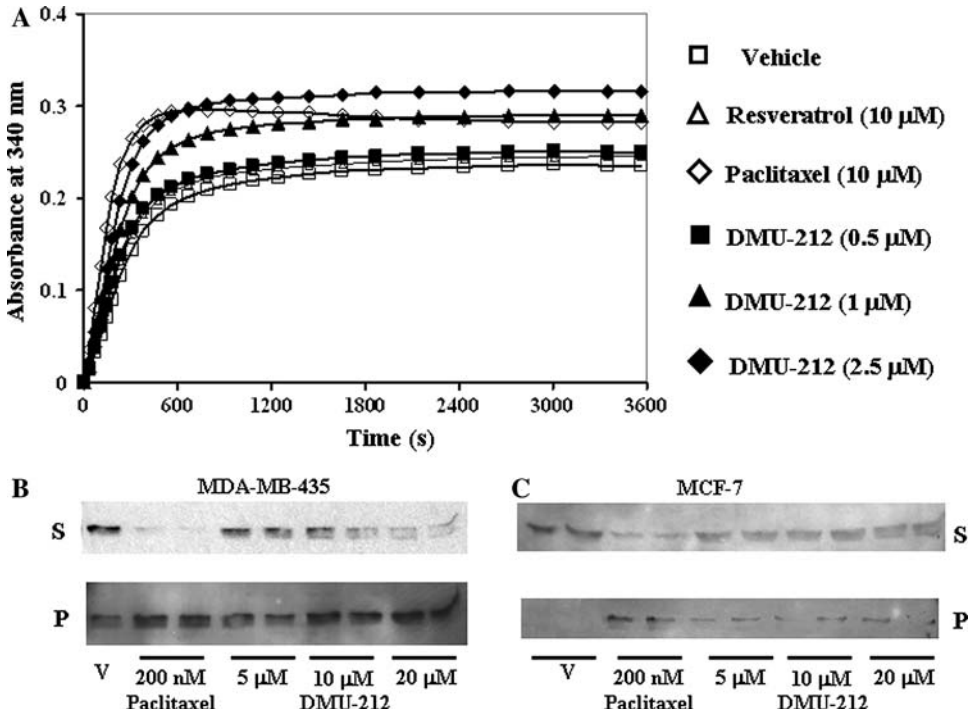

Fig. 6 DMU-212 enhances tubulin polymerization. The in vitro tubulin polymerization was detected by using a CytoDYNAMIX Screen 01 kit (**a**); the cellular tubulin polymerization was determined by Western blot in MDA-MB-435 (**b**) and MCF-7 cells (**c**). Cells in active growth were treated with vehicle (*V*) or with indicated concentrations of DMU-212 for 2 h, then quickly washed, and lysed in hypotonic lysis buffer. The cell lysate in which the cytosolic and cytoskeletal fractions containing soluble (*S*) and polymerized (*P*) tubulin, respectively, was separated, resolved by electrophoresis, and immunoblotted with α-tubulin antibody. All experiments were conducted three times

apoptotic pathway could be a major reason for the striking growth inhibitory effect of DMU-212 [14].

In contrast with DMU-212, resveratrol induced a dramatic G0/G1 arrest with minimal apoptosis in MDA-MB-435 and MCF-7 cells (Fig. 3). The transition from G1 to S phase is a crucial DNA damage checkpoint in mammalian cell cycle, which is initiated by the activation of cyclin D [25]. The level of cyclin D1 was significantly decreased after resveratrol treatments, which was consistent with the observed G0/G1 arrest in MDA-MB-435 and MCF-7 cells.

Since resveratrol was reported to be an inhibitor of Stat3-mediated growth and survival pathways [5, 20], we hypothesized that DMU-212 might also inhibit cell growth via blockade of Stat3 activation. In the present study, we dem-

onstrated that DMU-212, as well as resveratrol, resulted in a dose- and time-dependent inhibition in the phosphorylation and activation of Stat3 in MDA-MB-435 cells (Fig. 5). Accordingly, Stat3 regulated genes, such as cell cycle control gene Cyclin D1 and antiapoptotic genes Bcl-xL and Bcl-2, were also downregulated by DMU-212 (Fig. 4). These observations strongly supported the hypothesis that DMU-212, as well as resveratrol, is a Stat3 inhibitor.

However, inhibition of Stat3 pathway itself is not able to explain why DMU-212 has superior anti-tumor effects compared to resveratrol. In addition, a sizable number of studies have associated the Stat3 inhibition with a G0/G1 arrest [6, 26, 27]. DMU-212, however, induced a dominant cell cycle arrest at G2/M phase. These evidences suggested that there might be additional mechanisms involved in the anti-tumor activities of DMU-212.

The ability of a drug to block cells in G2/M is often associated with a dysregulation of microtubule dynamics [28–30]. Microtubules, the self assembly of α- and β-tubulin heterodimers, are major cytoskeletal components that play crucial roles in a lot of cellular functions, including the maintenance of cell shape, cell adhesion and movement, cell signaling, cell replication and cell division [31]. Antimicrotubule agents including polymerizing agents (paclitaxel and docetaxel) and depolymerizing drugs (vincristine, vinorelbine, and estramustine phosphate) are widely used in the treatment of cancer either alone or in combination with other anticancer drugs [31, 32]. These antimicrotubule agents disrupt the metaphase/anaphase transition, induce a G2/M cell-cycle arrest, and subsequently trigger the molecular signaling for the mitochondrial pathway of apoptosis [29, 30, 33]. In this study, DMU-212 was found to enhance the polymerization of tubulin and induce a shift in tubulin polymerization from soluble to polymerized form in both MDA-MB-435 and MCF-7 cells. A similar change has been induced by paclitaxel as the positive control at a concentration of 200 nM (Fig. 6). The results demonstrated DMU-212 is a novel microtubule-stabilizing agent. Consistent with previous study, resveratrol failed to affect the tubulin polymerization [34]. The anti-mitotic activities of resveratrol derivatives are often associated with methoxylation of polyhydroxystilbenes. A number of methoxy derivative of resveratrol analogues, such as *cis*-3,5,4′-trimethoxystilbene (R3), *trans*-2,3′,4,5′-tetramethoxystilbene and combretastatin A-4, have been identified as potent anti-mitotic agents [22, 23, 35]. Structure/activity relationship research demonstrated that demethylation at any position of R3 yielded compounds much less cytotoxic against cancer cells than R3 itself [22]. Parallel to the differences observed in relative cytotoxicity, demethylated R3 at position 4′ was 16-fold less active than R3 in the anti-mitotic activity.

Disruption of microtubules results in a cell cycle arrest at G2/M phase, after which it is cell type-dependent whether the cells undergo either cell death by apoptosis or necrosis or overcome the G2/M arrest and continue in the division cycle [36, 37]. In MCF-7 cells, the maximal G2/M arrest was obtained at 20 μM of DMU-212, beyond which the G2/M arrest was reversed (Fig. 3b). In addition, the DMU-212 induced microtubule-stabilization was not dose-dependent (Fig. 6c), indicating a saturated effect was possibly achieved at tested concentrations. It is consistent with the observation that the superiority of DMU-212 over resveratrol dissipated with the increase of concentrations in MCF-7 (Fig. 2c, d).

In this study, we demonstrated DMU-212 inhibited Stat3 activation and concomitantly increased microtubule assembly. Although Stat3 was reported to modulate microtubule dynamics and cell migration through a direct functional interaction with stathmin in the cytoplasm [38], the exact relationship between Stat3 activation and microtubule assembly remains largely uncharacterized.

Conclusion

DMU-212 has superior anti-tumor effects over resveratrol in MDA-MB-435 and MCF-7 cells since DMU-212 acted via different mechanisms from resveratrol. First, DMU-212 induced predominantly G2/M arrest whereas resveratrol induced G0/G1 arrest. Second, DMU-212 induced apoptosis and reduced the expression of multiple anti-apoptotic proteins more appreciably than resveratrol. Third, while both agents inhibited Stat3 phosphorylation, DMU-212 but not resveratrol led to a significant increase in tubulin polymerization. This is the first report showing that DMU-212 is an interesting anti-mitotic drug that increases tubulin polymerization. Targeting microtubule assembly leading to a subsequent apoptosis in cancer cells is an effective mechanism for cancer chemotherapy. These data suggested DMU-212 might be a promising multi-target anticancer agent for chemoprevention and chemotherapy.

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