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RESEARCH ARTICLE

Resveratrol confers resistance against taxol *via* induction of cell cycle arrest in human cancer cell lines

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Resveratrol, which is highly concentrated in the skin of grapes and is abundant in red wine, has been demonstrated to account for several beneficial properties, including antioxidant, anticoagulant, anti-inflammatory and anticancer effects. Taxol is a microtubule-stabilizing drug that has been extensively used as effective chemotherapeutic agents in the treatment of solid tumors. Here, we investigated whether the combination of the two compounds would yield increased antitumor efficacy in human cancer cells. Unexpectedly, resveratrol effectively prevented tumor cell death induced by taxol in 5637 bladder cancer cells. This pronounced antagonistic function of resveratrol against taxol was associated with changes in multiple signal transduction pathways, but not with tubulin polymerization. Importantly, cell cycle analysis showed that resveratrol prevented the cells from entering into mitosis, the phase in which taxol exerts its action. Furthermore, resveratrol blocked the cytotoxic effects of vinblastine but not cisplatin in 5637 cells. Interestingly, resveratrol pre-treatment followed by taxol resulted in synergistic cytotoxicity. Finally, we extended our studies to various human cancer cell lines. Taken together, our results indicate that resveratrol may have the potential to negate the therapeutic efficacy of taxol and suggest that consumption of resveratrol-related products may be contraindicated during cancer therapy with taxol.

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Apoptosis / Cancer / Cell cycle / Resveratrol / Taxol

1 Introduction

There is growing interest in using naturally occurring compounds as potential cancer chemopreventive agents in human populations. Resveratrol is a natural phytoalexin that is present in especially high concentrations in peanuts, mulberry skins and grape skins, and as a consequence, in red wine [1]. Keen interest has developed in resveratrol due to its chemopreventive, cardioprotective and possible anti-tumor attributes. The precise physiological role of resveratrol remains intangible, and it was shown to interact with numerous protein targets and to disrupt biological/biochemical reactions involved in all three stages of carcinogenesis (initiation, promotion and progression). Several phase I clinical trials are currently underway to evaluate the pharmacokinetics and safety of resveratrol [2].

Taxol (paclitaxel), one of the most clinically effective anti-neoplastic agents, has shown significant chemotherapeutic effects in the treatment of various cancers such as urothelial cancer, metastatic breast cancer and ovarian cancer [3, 4]. The biological activity of taxol is based on its ability to stabilize microtubules, bind to β-tubulin and promote tubulin polymerization, which interferes with the function of the mitotic spindle resulting in mitotic arrest at the metaphase/anaphase junction, and the resulting mitotic arrest triggers the mitotic spindle checkpoint, which somehow induces the mitochondrial permeability transition, release of prodeath molecules into the cytosol and caspase-dependent apoptosis of neoplastic cells [5].

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Abbreviation: NF-κB, nuclear factor-κB

As with many cancer therapeutic agents, the majority of cancer patients will eventually develop progressive disease after initially responding to taxol treatment. Tumor cells have adopted various mechanisms to resist taxol-induced apoptosis, such as overexpression of the multidrug transporter *P*-glycoprotein [6], decreased sensitivity to death-inducing stimuli [7], delayed G2/M transition [8] and alterations in microtubule dynamics [9]. Because resistance of many tumors to established treatment regimens still constitutes a major concern in oncology, attempts to improve the survival of cancer patients depend largely on strategies to target tumor cell resistance.

Resveratrol may hold cancer therapeutic potential when combined with established cancer treatments [10–12], while avoiding some of the debilitating side effects of conventional chemotherapy. Based on the encouraging earlier findings, we initiated a study to investigate whether resveratrol would be able to enhance the antitumor effects of taxol. However, to our surprise, we discovered that resveratrol effectively inhibited taxol-induced apoptosis in human cancer cell lines. Our results could be potentially important in light of the recent interest in the resveratrol for their possible use in combination chemotherapy regimens.

2 Materials and methods

2.1 Materials

Resveratrol and taxol (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in DMSO at 100 and 1 mM, respectively. Vinblastin and cisplatin (Sigma-Aldrich) were dissolved in normal saline at 1 mg/mL. SP600125 and LY294002 (Sigma-Aldrich) were dissolved in DMSO at 20 and 10 mM, respectively. The primary antibodies against Akt, p-Akt, β-actin, JNK, p-JNK, Erk, p-Erk, p38, p-p38, p-Bcl-2, p-CDK1, p-CDK2, cyclin A, cyclin B1, cyclin D1, cyclin E, histone H3 and Bid were purchased from Cell Signaling Technologies (Beverly, MA, USA), and the antibodies against PARP, CDK1, CDK2, CDK4, caspase-3, Bcl-2, p53, survivin, β-tubulin, Bcl-xl, Bax, *p*-IκB-α and NF-κB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2 Cell culture

The human bladder cancer cell line 5637, prostate cancer cell line PC3, breast cancer cell line MCF-7 and hepatocellular carcinoma cell line HepG2 were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere containing 5% CO_2 maintained at 37°C. The culture mediums containing different concentrations of drugs were all freshly prepared at the time of each experiment.

2.3 Cell viability assay

Approximately 10 000 cells were plated in each well of 96 well plates. After overnight incubation, the cells were treated with drugs for 48 h. 20 μL of the MTS solution (Promega, Madison, WI, USA) was added to each well, and the plates were incubated for another 2 h. The absorbance at 490 nm was measured using MRX II absorbance reader (DYNEX Technologies, Chantilly, VA, USA).

2.4 Colony formation assay

Cells were seeded into 6-well plates at 2500 cells *per* well. After complete cell adherence, the cells were exposed to drug treatment for 48 h. Thereafter, the drug was removed, and fresh growth medium was added. The cells were kept in culture undisturbed for 10 days, during which time the surviving cells spawned a colony of proliferating cells. Colony formation was analyzed by staining the cells with crystal violet.

2.5 Cell cycle analysis by flow cytometry

Cells were seeded in 6-well plates and incubated for 24 h before treatment. After harvest at 24 h following treatment, cells were washed twice with pre-chilled PBS and resuspended in 100 μL PBS at a concentration of 1×10^6 cells/mL. Cell cycle analysis was performed using the Coulter DNA Prep™ Reagents Kit (Beckman Coulter, Fullerton, CA, USA). Finally cell cycle analysis was performed by Beckman Coulter FC500 Flow Cytometry System with CXP Software (Beckman Coulter) within 1 h and the raw data was analyzed by Multicycle for Windows (Beckman Coulter).

2.6 Detection of apoptotic cells by flow cytometry

Cells were harvested 48 following drug treatment and washed twice with pre-chilled PBS and resuspended in 100 μL binding buffer at a concentration of 1×10^6 cells/mL. Annexin V and propidium iodide double-staining was performed using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) as described by the manufacturer's protocol. Cell apoptosis analysis was performed by Beckman Coulter FC500 Flow Cytometry System with CXP Software (Beckman Coulter) within 1 h.

2.7 Nuclear morphology

Cells were seeded in 24-well plates and exposed to drug treatment for 48 h. After washing twice with PBS, the cells were stained with Hoechst 33342 solution (final concentration 10 μg/mL) and then incubated at 37°C for 10 min.

The nuclear morphology was observed using ultraviolet light under an OLYMPUS microscope.

2.8 Western blotting analysis

Western immunoblotting was performed as previously described [13]. Protein concentration in the lysate was determined using the bicinchoninic acid protein assay Kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Thirty to fifty microgram of lysate from each sample was run in parallel. To determine nuclear factor-κB (NF-κB) cellular localization, nuclear proteins were isolated from cells using a cell fractionation kit (Keygen, Nanjing, China). NF-κB and histone H3 expression in the nuclear compartment were determined by immunoblot.

2.9 Western blotting for soluble and insoluble tubulin fractions

The soluble (unpolymerized) and insoluble (polymerized) tubulin fractions assays were done as previously described with some minor modifications [14].

2.10 Immunofluorescence

Briefly, cells were cultured in 24-well plate and subjected to drug treatment for 4 h, and then the cells were washed with cold PBS, immediately fixed using 4% paraformaldehyde in PBS for 10 min, and permeabilized with 0.3% Triton X-100 in PBS for 2 min. After blocking, the cells were incubated with β-tubulin antibody overnight at 4°C. After rinsing, the cells were incubated at 37°C for 1 h with diluted FITC-conjugated secondary antibody. Images were photographed with an OLYMPUS microscope.

2.11 Statistical analysis

All values are expressed as the mean ± SD. Statistical significance was compared between various treatment groups and controls using the one-way analysis of variance. Data were considered statistically significant when p values were <0.05.

3 Results

3.1 Resveratrol blocks the cytotoxic effects of taxol in 5637 bladder cancer cells

To investigate whether the cytotoxic effects of taxol could be affected by the addition of resveratrol, we used the 5637 bladder cancer cell line as representative of various cell lines for detailed investigation. Treatment of 5637 cells with taxol and resveratrol inhibited cell proliferation in a dose-dependent manner within 48 h, respectively (Fig. 1A). Because taxol concentrations ≥ 25 nmol/L yielded effects that did not differ from one another, we chose the 25 nmol/L dose in combination with different concentrations of resveratrol for subsequent studies. Surprisingly, MTS assay of 5637 cells revealed that resveratrol at concentrations of 10, 25, 50 and 100 μM signicantly prevented the cytotoxicity induced by taxol when the two compounds were given simultaneously (Fig. 1B). The 25 μM concentration was the most protective one. Thus, this concentration in combination with 25 nM taxol was used in subsequent experiments. To assess bladder cancer cell survival, clonogenicity assays were performed in 5637 cells. Figure 1C shows an example of the actual colonies that formed after drug treatment. Nearly no colonies were observed in taxol-treated cells; when cells were treated with taxol in the presence of resveratrol, a few colonies were observed but the number was significantly lower than those in the control or resveratrol group. The antagonistic characteristic of taxol and resveratrol treatment could also be documented at variable concentrations of each compound. For example, 10 nM taxol alone reduces viability by 49.6% after 48 h, but when combined with 10 μM resveratrol, viability is only reduced by 26.7% (Fig. 1D). Interestingly, even under conditions where resveratrol is toxic at the concentrations of 50 and 100 μM, it is still able to antagonize the cytotoxic effects of taxol, and viability of cells treated with combination of taxol and resveratrol at certain concentrations seems to be even higher than that of cells treated with resveratrol alone.

3.2 Resveratrol inhibits taxol-induced apoptosis in 5637 cells

Because treatment with taxol leads to apoptosis in targeted cells, we investigated whether the reduced cytotoxicity induced by resveratrol was due to its anti-apoptotic effect. Hoechst staining showed that taxol-induced chromatin condensation or fragmentation was reduced by resveratrol (Fig. 2A). Taxol-induced apoptosis was also determined by flow-cytometric analysis of 5637 cells labeled with propidium iodide and annexin V. Here, resveratrol alone caused no apoptosis on 5637 cells when applied at concentrations of 25 μM. Following treatment with taxol for 48 h, the number of early (LR) and late (UR) apoptotic cells increased to 11.6 and 36.8%, respectively, and this increase was effectively blocked by the addition of resveratrol (Fig. 2B). These results confirmed the protective effect of resveratrol on taxol-induced apoptosis.

To gain further insight into the way in which resveratrol is cytoprotective, we investigated several key molecules known to regulate apoptosis. Activation of caspase-3 plays a

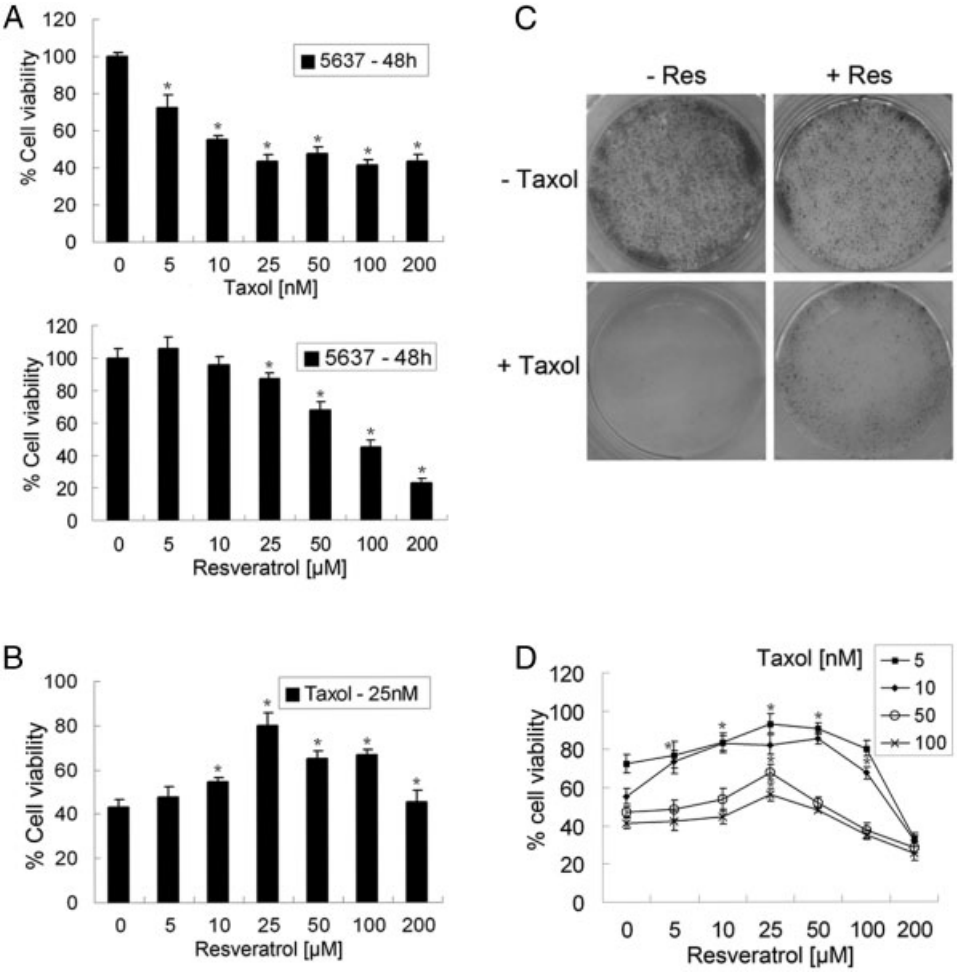

Figure 1. Resveratrol blocks the cytotoxic effects of taxol in 5637 cells. (A) Percent cell viability of 5637 cells was determined *via* MTS assay after 48 h of treatment with various concentrations of taxol (5, 10, 25, 50, 100 and 200 nM) and resveratrol (5, 10, 25, 50, 100 and 200 μM), respectively. (B) The percentage of viable cells was determined with the MTS assay after treatment with 25 nM taxol and various concentrations of resveratrol for 48 h. The percentage survival of non-treated cell cultures was set to 100%. (C) 5637 cells were treated with 25 nM taxol in the presence or absence of 25 μM resveratrol for 48 h and the number of long-term surviving cells (10 days) was determined by colony formation assays. Images were taken of representative 6-well plates. (D) Percent cell viability of 5637 cells treated for 48 h with increasing concentrations of taxol in the presence of increasing concentrations of resveratrol as determined *via* MTS assay. $^{*}p<0.05$ *versus* control. Res, resveratrol.

central role in apoptosis by initiating cell death [15]. As shown in Fig. 2C, the addition of 25 μM resveratrol significantly inhibited taxol-induced activation of caspase-3 and the consequent cleavage of PARP, which represents one of the final steps of the proteolytic caspase cascade and reliably indicates ongoing apoptosis [16].

Like apoptosis initiated by other stimuli, taxol-induced apoptosis is also regulated by members of the Bcl-2 family. It is inhibitable by the anti-apoptotic Bcl-2 and Bcl-X_L and can be enhanced by the pro-apoptotic Bcl-X_S, Bid, Bad and Bax [17]. As shown in Fig. 2D, Bcl-2, Bcl-xl, Bax and Bid expression were not substantially altered by treatment with resveratrol alone, while taxol upregulated the expression of pro-apoptotic proteins Bax and Bid and inhibited the expression of anti-apoptotic proteins Bcl-2 and Bcl-xl, thus there was an overall shift in the ratio of pro-apoptotic and anti-apoptotic following taxol treatment. The combination treatment, however, resulted in a significantly decrease in Bax and Bid expression and increase in Bcl-2 and Bcl-xl expression as compared to taxol alone, indicating that resveratrol reverses the shift in the ratio of pro-apoptotic/anti-apoptotic proteins of the Bcl-2 family induced by taxol and prevents cell death. Additionally, since phosphorylation of Bcl-2 has been reported to play a key role in taxol-induced apoptosis [18], we also examined the phosphorylation status of Bcl-2 after taxol exposure with or without the addition of 25 μM resveratrol. We found that the taxol-induced phosphorylation of Bcl-2 was evident, and cotreatment with resveratrol determined a significant reduction of the phosphorylated form of Bcl-2.

3.3 Resveratrol interferes with signal transduction pathways involved in taxol-induced apoptosis

Taxol-induced apoptosis is associated with changes in various intracellular transduction pathways [19], and it has been demonstrated that resveratrol also modulates changes in intracellular signaling pathways in a variety of human cells [20]. To determine the mechanism by which resveratrol inhibits apoptosis in taxol-treated 5637 cells, we analyzed PI3K/Akt, MAPK superfamily and NF-κB signaling pathways, which play the critical roles in regulating cell growth,

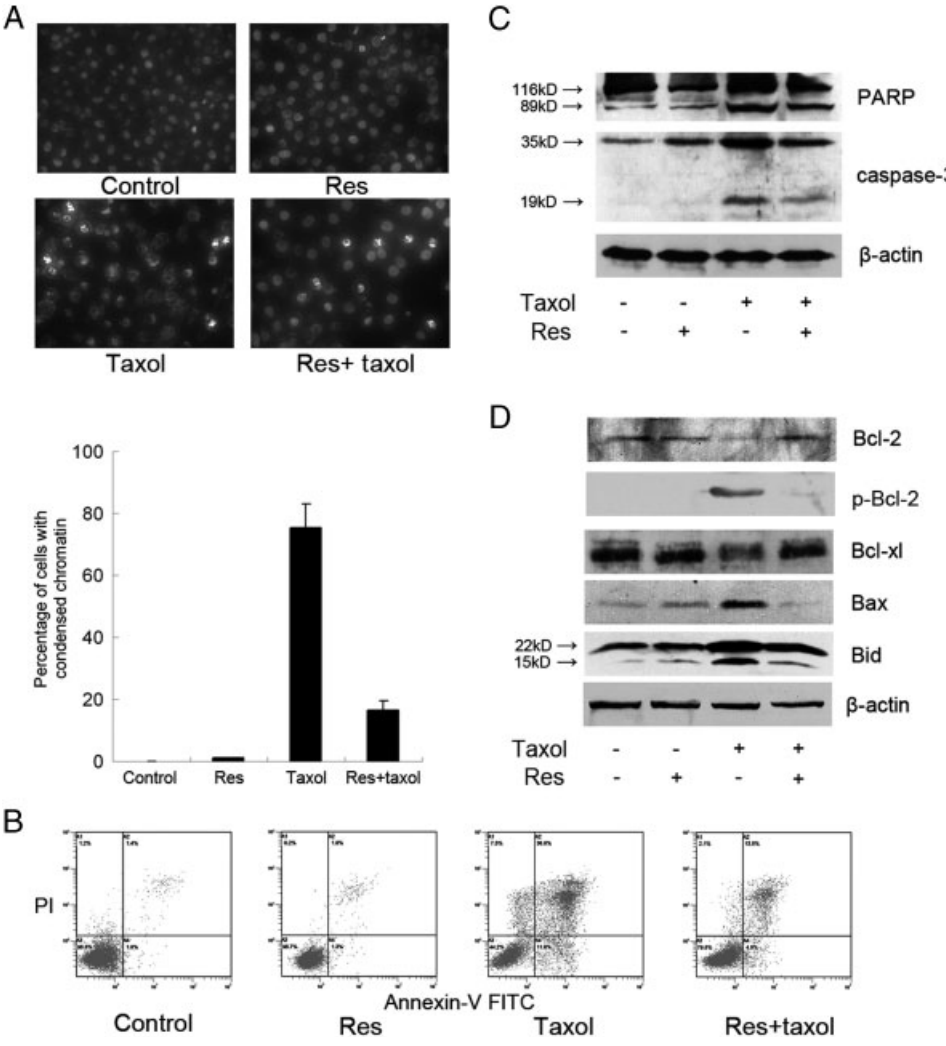

Figure 2. Resveratrol inhibits taxol-induced apoptosis in 5637 cells. Cells were treated for 48 h with 25 nM taxol in the presence or absence of 25 μM resveratrol. (A) Cells were stained with Hoechst 33342 for 10 min at 37°C. The nuclear morphology was observed under a fluorescence microscope. Representative images at × 100 magnifications. The proportion of cells with condensed chromatin was expressed as a percentage. (B) Percent apoptotic cells were detected by flow cytometry using a double-staining method with FITC-conjugated annexin V and propidium iodide. (C) Resveratrol inhibited taxol-induced activation of caspase-3 and the consequent cleavage of PARP. (D) Resveratrol reversed the shift in the ratio of pro-apoptotic/anti-apoptotic proteins of Bcl-2 family and inhibited Bcl-2 hyperphosphorylation induced by taxol. Res, resveratrol.

survival and apoptosis [21–23]. As shown in Fig. 3A, exposure of 5637 cells to 25 nM taxol induced the activation of JNK and p38, inhibited the phosphorylation of ERK1/2 and Akt, and prevented the nuclear accumulation of NF-κB. However, cotreatment of resveratrol with taxol was effective in reducing JNK and p38 activation, increasing ERK1/2 and Akt phosphorylation, and promoting NF-κB nuclear accumulation, showing its antagonistic manner against taxol. Our results suggest that these kinases could mediate the anti-apoptotic effect of resveratrol. We addressed this possibility by studying the effect of the specific inhibitors of Akt, JNK and NF-κB (LY294002, SP600125 and parthenolide respectively) because it has been reported that the induction of apoptosis by taxol is mainly mediated by the activation of JNK [17] and sustained Akt and NF-κB activity may attenuate drug-induced apoptosis, thereby promoting taxol resistance [24, 25]. However, inhibition of the Akt, JNK or NF-κB pathway with specific inhibitors did not confer any effect on the cytotoxic effects of taxol or the protective effect of resveratrol against taxol (Fig. 3B).

3.4 Resveratrol does not inhibit the tubulin polymerization induced by taxol

The β-tubulin subunit of microtubules are the target of taxol-induced apoptosis, and it has been demonstrated that a resveratrol analog exerts anti-neoplastic effects by altering microtubule polymerization [26], thus we investigated whether resveratrol inhibited the tubulin polymerization induced by taxol in 5637 cells. As demonstrated in Fig. 4A, immunofluorescent staining of 5637 cells, cultured under standard conditions with anti-β-tubulin antibody, revealed a well-organized microtubule network concentrated around the nucleus and extending throughout the cytoplasm. After 2 h of exposure, resveratrol alone seems to cause a change in β-tubulin distribution and taxol caused the expected pattern of condensed microtubule hyperpolymerization. In the presence of both taxol and resveratrol, the typical modification induced by taxol alone seems to be much more evident, indicating no functional reciprocal interference on tubulin polymerization between the two agents.

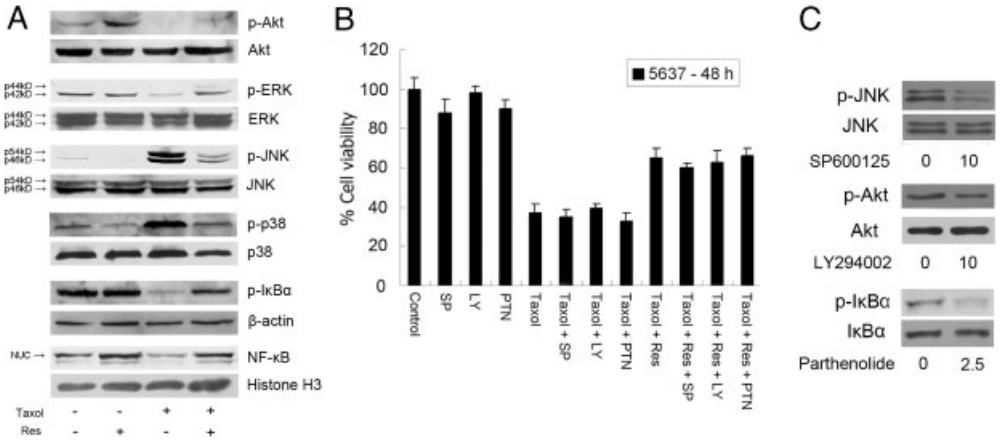

Figure 3. Resveratrol interferes with several signal transduction pathways involved in taxol-induced apoptosis in 5637 cells. Cells were incubated for 48 h with 25 nM taxol in the presence or absence of 25 μM resveratrol. (A) 5637 cells were harvested and used to prepare cell lysates. The lysates were subjected to SDS-PAGE and blotted with various antibodies. The PI3K/Akt, MAPK superfamily and NF-κB signaling pathways were analyzed. (B) 5637 cells were treated with taxol and resveratrol in the absence and presence of specific inhibitors. The percentage of viable cells was determined with the MTS assay after 48 h. Res, resveratrol; LY, LY294002; SP, SP600125; PTN, parthenolide. (C) The phosphorylation status of JNK, Akt and IκB-α upon 10 μM SP600125, 10 μM LY294002 or 2.5 μM parthenolide treatment for 48 h was measured by Western blotting to confirm the effectiveness of the specific inhibitors.

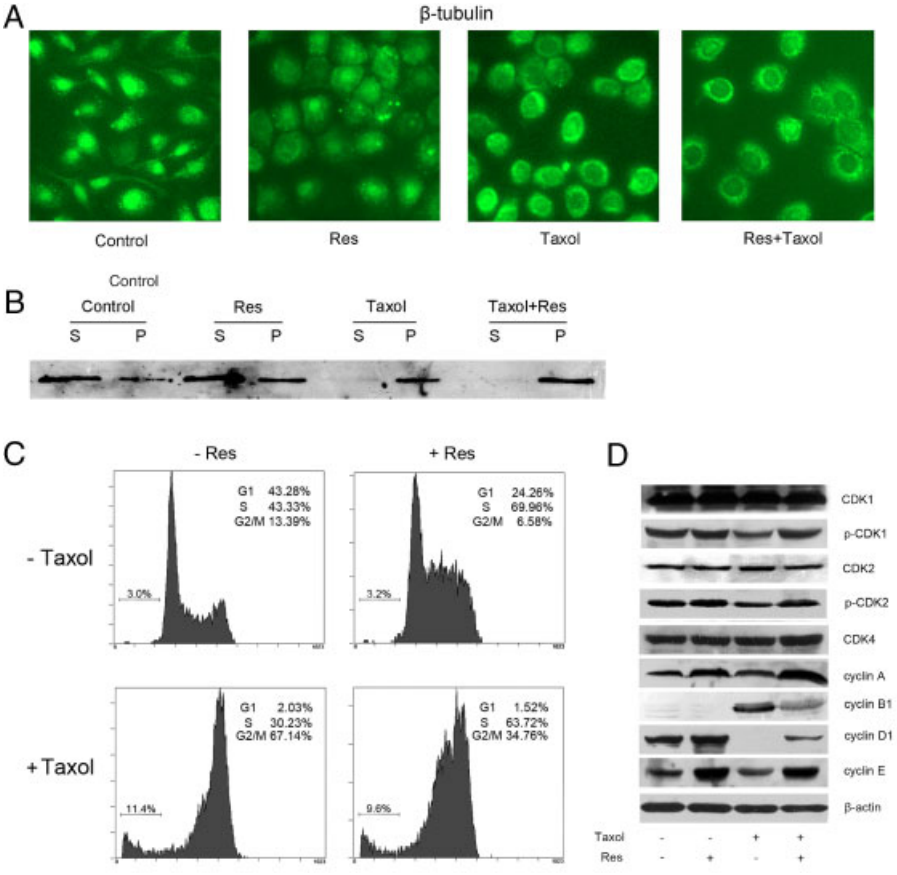

Figure 4. (A) 5637 cells were incubated for 2 h with 25 nM taxol in the presence or absence of 25 μM resveratrol. Immunofluorescence staining for β-tubulin (green) showed the organization of 5637 cellular microtubule network. Representative images at × 200 magnifications. (B) Cells were treated with the 25 nM taxol in the presence or absence of 25 μM resveratrol for 16 h. The fractions containing soluble and polymerized tubulin were collected and separated by SDS-PAGE. β-tubulin was detected by Western blot analysis. (C) Cell cycle distribution of resveratrol and taxol-treated 5637 cells. Cells were incubated for 24 h with 25 nM taxol in the presence or absence of 25 μM resveratrol. The percentages of cells in G1, S or G2/M phase are shown. (D) Effects of resveratrol and taxol on cell cycle-related proteins. Cell lysates were analyzed by immunoblotting using antibodies against the cycle-related proteins. Res, resveratrol.

Western blot analysis of soluble and polymerized tubulin fractions confirms the immunocytochemistry results (Fig. 4B). In untreated 5637 cells, there is a relatively even distribution of tubulin, with slightly more present in the soluble fraction. In cells treated with resveratrol, there is no distinct shift in the tubulin balance as compared with control. Taxol, as expected, caused complete tubulin polymerization, with almost all tubulin present in the polymerized fraction. However, addition of resveratrol failed to induce any significant effects on tubulin polymerization promoted by taxol.

3.5 Resveratrol inhibits taxol-induced G2/M phase cell cycle arrest in 5637 cells

Taxol binds microtubules and causes kinetic suppression of microtubule dynamics. The consequent arrest of the cell cycle at mitotic phase has been considered the cause of taxol-induced cytotoxicity [19]. To study the mechanism behind the resveratrol-induced antagonistic effect, we investigated if resveratrol could inhibit the taxol-induced G2/M cell cycle arrest in 5637 cell line. Because we were interested in evaluating the distribution of actively dividing cells before the induction of extensive apoptosis, we harvested cells at 24 h, rather than at 48 h. As shown in Fig. 4C, resveratrol induced the arrest of cell proliferation at the S phase, and taxol caused an increase of cells in the G2/M phase. A higher percentage of S and a corresponding lower percentage of G2/M phase cells were observed in the cells treated with taxol and resveratrol combination as compared to taxol alone. These results suggest that the antagonism of resveratrol against taxol may be the result of blocking cell cycle progression at the S phase and thus preventing the cells from entering into mitosis, the phase in which taxol exerts its action. Consistent with previous results, the percentage of sub-G1 phase cells, which indicate cells undergoing apoptosis, induced by taxol and resveratrol combination was significantly lower than the cells treated with taxol alone.

Next, we examined the expression of several cell cycle-related proteins. As shown in Fig. 4D, Resveratrol is able to induce S-phase cell arrest and this interference with the cell cycle is associated with an increase of cyclin A, cyclin D1 and cyclin E, and no alteration in cyclin B1 expression. Taxol determined a marked increase in cyclin B1 and a down-regulation of cyclin D1 expression at 24 h, while following taxol and resveratrol cotreatment, an increase in cyclin A, cyclin D1 and cyclin E, but a decrease in cyclin B1 protein level were observed in comparison to cells exposed to taxol alone. However, both resveratrol and taxol did not affect the protein levels of cyclin-dependent kinases such as Cdk1, Cdk2 and Cdk4. Since the function of Cdks is dependent on the phosphorylation status of the protein itself, especially dephosphorylation of Cdk1 appears to be the critical regulatory step during progression into mitosis [27], we also analyzed the phosphorylation of Cdk1 and Cdk2. Our results show that exposure of 5637 cells to 25 nM taxol reduced Cdk1 and Cdk2 phosphorylation, but addition of resveratrol was effective in restoring the phosphorylation of the two proteins.

3.6 Arresting 5637 cells in G1 or S phase inhibits taxol-induced cytotoxicity

To directly test the role of cell cycle progression in blocking taxol's cytotoxicity, we arrested 5637 cells in G1 or S phase using the specific cell cycle inhibitors mimosine or thymidine. As shown in Fig. 5A, both thymidine and mimosine significantly reduced taxol-induced cell death. Similar to treatment with resveratrol, cell cycle analysis revealed that cotreatment with thymidine or mimosine blocked the transition of 5637 cells into G2/M phase (Fig. 5B).

3.7 Resveratrol blocks the cytotoxic effects of vinblastine, but not cisplatin, in 5637 bladder cancer cells

Vinblastine is another type of microtubule-interfering agents used to treat certain kinds of cancer. Taxol stabilizes microtubules, whereas vinblastine inhibits microtubule polymerization. Although the two compounds exert opposite effects on microtubules, both vinblastine and taxol share the common property of suppressing microtubule dynamics and thereby microtubule function, leading to the disruption of the mitotic spindle function and blocking cell cycle progression at the transition from prometaphase/metaphase to anaphase [19]. Thus, we speculated that resveratrol might also abrogate vinblastine activity through a mechanism similar to that of taxol by altering cell cycle distribution. As shown in Fig. 5C, resveratrol blocks the cytotoxic effects of vinblastine in 5637 cells as expected. Furthermore, we tested the effects of resveratrol on apoptosis induced by cisplatin, a platinum-based chemotherapy drug. The generally accepted biological target of cisplatin is DNA, to which it binds and forms several types of adducts, primarily intrastrand purine cross-links. Our results show that resveratrol appears to increase the apoptotic effect on cisplatin-induced apoptosis (Fig. 5C). Cell cycle analysis confirmed that treatment with vinblastine arrested most of 5637 cells at G2/M phase while cisplatin alone mainly induced G1 phase arrest. When tumor cells were cotreated with resveratrol, the percentage of mitotic cells was decreased in the vinblastine group, while an increased percentage of G1 phase cell was observed in the cisplatin group (Fig. 5D).

3.8 Resveratrol sensitizes 5637 cells to taxol-induced cytotoxicity when given sequentially

The above experiments were all done under the conditions that taxol and resveratrol were concurrently added to the cell

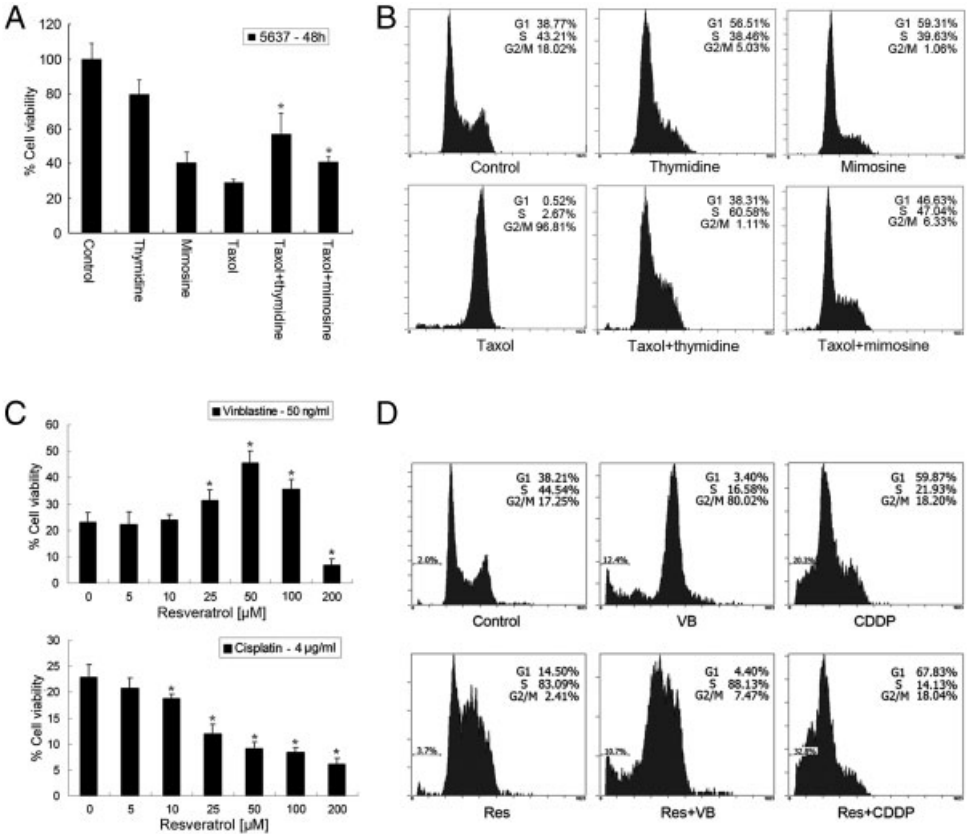

Figure 5. (A) Effect of cell cycle inhibitors on drug-induced cytotoxicity. Percent cell viability of 5637 cells was determined *via* MTS assay after 48 h of treatment with 2 mM thymidine, 0.4 mM mimosine, 25 nM taxol, 2 mM thymidine or 0.4 mM mimosine and 25 nM taxol cotreatment. $^{*}p < 0.05$ *versus* taxol. (B) Concomitant analysis of cell cycle after 24 h of treatment with drugs as described above. The percentages of cells in G1, S or G2/M phase are shown. (C) Percent cell viability of 5637 cells was determined *via* MTS assay after 48 h of treatment with 50 ng/mL vinblastine or 4 μg/mL cisplatin and various concentrations of resveratrol (5, 10, 25, 50, 100 and 200 μM). $^{*}p < 0.05$ *versus* control. (D) Effects of resveratrol on the cell cycle distribution of vinblastine and cisplatin-treated 5637 cells. Cells were left untreated or were treated with 50 μM resveratrol, or 50 ng/mL vinblastine alone or with 50 μM resveratrol, or 4 μg/mL cisplatin alone or with 50 μM resveratrol. The percentages of cells in G1, S or G2/M phase are shown. Res, resveratrol; VB, vinblasine; CDDP, cisplatin.

culture. Previous studies have demonstrated that pre-treatment with resveratrol and followed by taxol results in synergistic cytotoxicity [11, 12]. We further examined the effects of resveratrol on taxol-induced cytotoxicity when it was given before taxol treatment. When the 5637 cells were treated with resveratrol at 5 or 10 μM for 48 h first, had resveratrol washed away, and then exposed to taxol for another 48 h, we found that resveratrol showed synergistic cytotoxicity when combined with taxol in 5637 cells, and a concentration of 5 μM resveratrol was similarly effective compared to 10 μM resveratrol to sensitize 5637 cells (Fig. 6A).

3.9 Resveratrol acts as a survival factor in human cancer cells

To further test the potential of resveratrol as a survival factor, we extended our studies to various human cancer cell lines. Treatment with resveratrol reversed the reduction of viability in HepG2, MCF-7 and PC3 cells (Fig. 6B). These findings indicate that resveratrol antagonizes cytotoxicity induced by taxol in a variety of human cancer cell lines.

4 Discussion

As early as 1997, resveratrol was found to be a potent chemopreventive agent, blocking the initiation, promotion and progression of tumors [28]. Since that time, extensive literature on its anticancer activity, performed in a wide variety of cellular models, suggests a potential anti-proliferative and apoptogenic use of this compound. It has been also rediscovered for a plethora of beneficial properties such as anti-aging, antiviral, cardiovascular and neuroprotective effects [20], thereby making it one of the most sought after phytochemicals for supplementing human diet.

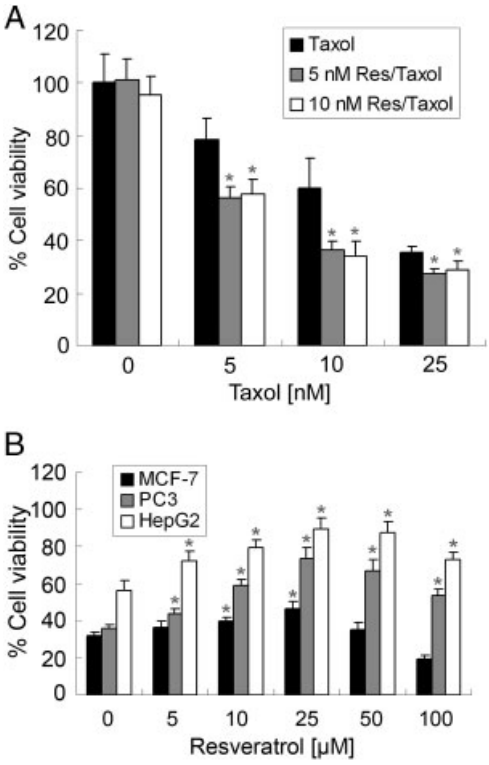

Figure 6. (A) Effect of resveratrol on taxol-induced cytotoxicity when given sequentially. In total, 5637 cells were pre-treated with 5 or 10 μmol/L resveratrol for 48 h, washed once with medium and then fed with fresh medium containing 0–25 nmol/L taxol. After 48 h, cells were subjected to MTS assay. $^*p<0.05$ *versus* taxol alone. (B) Protective effects of resveratrol on taxol-induced cytotoxicity in human cancer cell lines. MCF-7 breast carcinoma, PC3 prostate carcinoma and HepG2 hepatocellular carcinoma were treated with 25 nM taxol and various concentrations of resveratrol (5, 10, 25, 50 and 100 μM) for 48 h, and then the percentage of viable cells was determined with the MTS assay. $^*p<0.05$ *versus* control. Res, resveratrol.

Several studies have established that resveratrol could act as potent sensitizer for antitumor drug-induced apoptosis [10–12, 29]. Single agent taxol resulted in clinically significant responses in advanced metastatic bladder cancer and the combination of the doublets and triplets taxol and other chemotherapeutic drugs have led to response rates better than taxol alone, so we examined the potential effect of resveratrol on the anticancer activity of taxol in 5637 bladder caner cell line, but found that resveratrol effectively blocked the anticancer efficacy of taxol [30]. This is consistent with the previous study that resveratrol exerts its neuroprotective effects against taxol in neuroblastoma SH-SY5Y cells [31]. More importantly, Ahmad *et al.* [32] also reported the antagonism of resveratrol against vincristine and daunorubucine in human leukemia cells.

To investigate the possible mechanism by which resveratrol confers chemoresistance, we explored three different possibilities: (i) that the combination of the two drugs induces changes in signal transduction pathways or/and cellular proteins involved in controlling apoptotic death which are different from those induced by taxol alone; (ii) that resveratrol inhibits the tubulin polymerization induced by taxol; and (iii) that resveratrol arrests the cells early in its cell cycle and prevents cells entering the M phase when taxol can be toxic by hindering the formation of the mitotic spindle.

We approached the first possibility by studying the effect of resveratrol and taxol given separately or in combination on caspase 3, PARP and Bcl-2 family proteins. The concomitant exposure of 5637 cells to resveratrol and taxol markedly reduces the activation of caspase-3 and PARP cleavage induced by taxol alone. The relative amount of pro- and anti-apoptotic proteins of the Bcl-2 family determines the fate of the cell by modulating the activity of the caspases. Resveratrol also reverses the shift in the ratio of pro-apoptotic/anti-apoptotic proteins and inhibits the hyperphosphorylation of Bcl-2, which is generally believed to promote taxol-initiated apoptosis, induced by taxol. We noted that the expression of these apoptotic regulatory proteins were not substantially altered by treatment with resveratrol alone at the concentration of 25 μM, indicating that resveratrol may interact with them indirectly.

Next, our data show that the mechanism behind the antagonism of resveratrol seems to involve several intracellular signal pathways including Akt, MAPK and NF-κB. JNKs/SAPKs, a subfamily of the MAPK superfamily, are frequently associated with taxol-initiated apoptosis [17]. Another important survival signaling involves the activation of the serine/threonine kinase Akt, which inhibits apoptosis by phosphorylating a number of downstream genes. Activation of NF-κB pathway has also been reported to confer resistance to taxol [24]. Although resveratrol effectively prevented taxol-induced JNK activation, suppressed the taxol-mediated inhibition of Akt phosphorylation and promoted nuclear accumulation of NF-κB, the use of SP600125, LY294002 or parthenolide, a chemical inhibitor of JNK, Akt and NF-κB, respectively, did not induce any change in the cytotoxicity of taxol alone or in combination with resveratrol. These results suggest that except for the JNK, Akt and NF-κB pathways, other signaling pathways may be involved in resveratrol-mediated anti-apoptotic effect. However, we can not rule out another two possibilities: first, the antagonistic effect of resveratrol appears to involve multiple signal transduction pathways, thus inhibition of only one pathway may not be able to generate variation in cytotoxicity; second, resveratrol, when cotreated with taxol, inhibited all the tested biological activities caused by taxol, indicating that resveratrol may act upstream from the multiple signal transduction pathways on taxol-induced microtubule disarray or cell cycle changes induced by taxol.

To date, the microtubule is the only known cellular target that physically interacts with taxol; no cell membrane-associated

or cytoplasmic receptor has been identified. Taxol mediates its cytotoxic effects by binding to β-tubulin and promoting tubulin polymerization. The cytotoxicity of taxol against cancer cells can be affected by the status of tubulin expression in cancer cells [33]. It has been reported that the resveratrol analog (Z)-3,5,4′-trimethoxystilbene is a potent anti-mitotic drug inhibiting tubulin polymerization [26]. We next questioned whether resveratrol antagonizes taxol-induced tubulin polymerization in 5637 cells. By analyzing the tubulin distribution, our results indicate that resveratrol exerts no effect on tubulin polymerization induced by taxol. The observation that resveratrol also blocked the cytotoxic effects of vinblastine, which, contrary to taxol, inhibits tubulin polymerization further excludes the possibility that resveratrol interferes with taxol's capacity to polymerize tubulin.

It was reported that the delayed M-phase entry renders cells more resistant to taxol-induced apoptosis [8], and loss of normal p53 function has been demonstrated to sensitize tumor cells to taxol by promoting progression of cells to the G2/M phase [34]. By evaluating the third possibility, we performed flow cytometric analysis to evaluate the cell cycle distribution and determined the cell cycle-related proteins after exposure to drugs for 24 h. Our results show that resveratrol causes predominantly S phase arrest that subsequently results in preventing the cells from entering into mitosis, the phase in which taxol exert its action. Similarly, cell cycle arrest in G1 or S phase using the specific cell cycle inhibitors minosine and thymidine protects 5637 cells against taxol-induced cytotoxicity. We also find that resveratrol prevents vinblastine-induced cell death but enhances the cytotoxicity of cisplatin. A higher percentage of G2/M and G1 phase cells was observed in 5637 cells treated with vinbalsine and cisplatin, respectively. Addition of resveratrol abrogates the ability of vinblasine to induce G2/M arrest, but promotes the cisplatin-induced G1 arrest. These results suggest that resveratrol-mediated antagonism to chemotherapeutic drugs depends on cell cycle progression, and this would be the major antagonistic mechanism of resveratrol toward taxol. It still remains unclear, however, whether cell cycle arrest is responsible for all antagonistic effects of taxol or whether taxol also directly interferes with some other cellular proteins.

To establish chemoresistance by resveratrol, it was critical to ascertain that its effects are not restricted to a single cell line. Although we primarily used 5637 cells, resveratrol also antagonized taxol in PC3, MCF-7 as well as HepG2 cells. Generally, resveratrol arrests tumor cells in the G1 or S-phase. However, Jazirehi and Bonavida [12] found that the resveratrol-induced arrest in the G2/M phase made the human Burkitt's lymphoma B cells more sensitive to the effect of taxol. Thus, the effect of resveratrol on cell cycle progression is dependent on the cellular model examined and this could explain the opposite effects of resveratrol on the sensitization of the tumor cells to the taxol. Additionally, the schedule of cell treatment may also give rise to these two different paradigms. It has been reported that pretreatment with resveratrol sensitizes the neuroblastoma and lung cancer cells to taxol-induced apoptosis [11, 29]. Our results demonstrate that lower concentrations of resveratrol exerted a sensitizing effect on 5637 cells to cytotoxicity induced by taxol, underscoring the importance of the treatment regiment if to be applied to patients. Future research to delineate the underlying molecular mechanism of sensitization, and to compare the two treatment modalities, is warranted.

Because of the low bioavailability of resveratrol, plasma levels as high as 25 μM may not be physiologically attainable in man. However, our results suggest that resveratrol at relatively low concentrations (5–10 μM) also inhibits the cytotoxic effects of taxol in several cancer cell lines, and the drug accumulation in organs may achieve higher levels than those in serum. *In vivo* experiment therefore is urgently needed to confirm the antagonistic effects of resveratrol against taxol.

In conclusion, the present study demonstrates that resveratrol *via* induction of cell cycle arrest can render the chemoresistance phenotype of the tumor cell lines in an *in vitro* model system and may have the potential to negate the therapeutic efficacy of taxol when given concurrently. Further work will elucidate the *in vivo* significance of these findings, which in turn will inform the need for dietary advice on the intake of resveratrol for the patients undergoing taxol therapy and, moreover, cell cycle-sensitive drugs.

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