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ORIGINAL ARTICLE

Anticancer effect and enhanced chemotherapy potential of resveratrol in human pancreatic cancer cell lines*

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Abstract

Objective Gemcitabine, the only approved drug for the treatment of pancreatic cancer, is not very effective. Novel and effective cancer chemopreventive agents are urgently needed. Recently, emerging studies determined resveratrol possessed anticancer effects on various cancer cells. We explored the anticancer effect of resveratrol in pancreatic cancer cells and investigated the involved moleculars of action. We also examined whether resveratrol enhanced antitumor activity of gemcitabine *in vitro*.

Methods Proliferation inhibition was assessed by cell count kit-8 assay. Cell cycle phase distribution and apoptotic cells were measured by flow cytometric analysis. We determined the expression of bcl-2, cyclinD1, and activation of caspases-3 and poly (ADP-ribose) polymerase1 proteins used Western blot analysis.

Results Resveratrol inhibited the proliferation of three pancreatic cancer cell lines in a dose dependent fashion, and induced accumulation of cells at the G1 phase as well as apoptosis. Our data also demonstrated that resveratrol enhanced gemcitabine-induced apoptosis in pancreatic cancer cells. In addition, resveratrol inhibited the expression of cyclinD1, bcl-2, and induced activation of caspase-3 and poly (ADP-ribose) polymerase1.

Conclusion Our results suggested that resveratrol might be not only a potential regimen, but also an effective chemosensitizer for the chemotherapy of pancreatic cancer.

Key words: resveratrol; gemcitabine; pancreatic cancer; apoptosis; proliferation

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In the United States, it was estimated that 45,220 men and women would be diagnosed with pancreatic cancer (PaCa) and 38,460 would die from this disease in 2013. [1]. PaCa is ranked as the fourth leading cause of cancer-related deaths after lung, prostate (breast in women), and colorectal cancer in the United States since the 1970s, with a relative 5-year survival rate of only 5%–6% [2]. Metastatic disease present at the time of first diagnosis, aggressive progression, and limited effective therapies account for the high mortality. Surgical resection may provide the only chance for a cure or long-term survival in PaCa patients. However, most PaCa patients with locally advanced or incurable metastatic disease are diagnosed for the first time, and only 15%–20% of patients with potentially resectable disease have the chance for surgery at presentation. Furthermore, over 80% of patients experience recurrence within 2 years of surgery [3–4]. Median survival after recurrence is 7 months for local and 3 months for metastatic recurrence cases [5]. Thus, palliative treatment may be a good choice for patients with ad-

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vanced-stage PaCa. Gemcitabine, the only approved drug for the treatment of PaCa, offers a poor partial response (< 6%) in patients [6]. This disappointing outcome strongly suggests that novel, effective, and less toxic chemopreventive agents or chemosensitizers are urgently needed.

Resveratrol (trans-3, 4′,5-trihydroxy-trans-stilbene) is found in more than 70 plant species, including grapes, peanuts, berries, and pines, and is particularly abundant in the skin of red grapes (50 to 100 μg of resveratrol per gram wet weight) [7–8]. It is produced by plants in response to infection by the pathogen Botrytis cinerea [9]. This compound might account for the reduced risk of coronary heart disease in individuals with moderate red wine consumption [10–11].

Since 1997 when Jang *et al* [12] demonstrated that resveratrol had cancer-chemopreventive activity in three major phases of carcinogenesis, namely initiation, promotion, and progression, numerous studies about the anticancer effect of this compound have emerged. Extensive experimental data have shown that resveratrol induced growth inhibition, cell cycle arrest, apoptosis, blockade of angiogenesis, and inhibited metastasis of numerous cancers, such as gastric carcinoma [13], colorectal cancer [14], skin cancer [15], glioma [16], and lung carcinoma [17]. Despite the potential interest, very little data are available regarding the precise mechanism underlying the anticancer effect of this compound. It is also not known whether resveratrol can potentiate the effect of gemcitabine in PaCa cells.

Therefore, we investigated whether resveratrol induces an anticancer effect on human PaCa cells, and if it can potentiate the effect of gemcitabine in these cells. In addition, we further explored the macromolecules involved in the anticancer effect of this compound.

Materials and methods

Cell culture and experimental reagents

Three human PaCa cell lines (SW1990, PANC-1, and BxPC-3) were used. SW1990 and PANC-1 were stored by our laboratory (Shanghai Institute of Digestive Disease, Shanghai, China). BxPC-3 was a gift from Ruijin Hospital, Shanghai Jiaotong University School of Medicine (Shanghai, China). All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin (Gibco BRL, Waltham, MA, USA) in a 5% CO_2 incubator at 37 ℃. All experiments were performed during the exponential growth phase of the cells. Each treatment condition was a single dose of resveratrol at the indicated concentration or 0.1% dimethyl sulfoxide (DMSO; vehicle) as control.

Resveratrol (trans-3,4′,5-trihydroxystilbene) and propidium iodide (PI) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). A stock solution of resveratrol was prepared in DMSO at a concentration of 250 mmol/L and stored in the dark at –20 ℃. The DMSO concentration in all drug-treated cells was always less than 0.1% (v/v). Gemcitabine was purchased from Jiangsu Hansen Pharmaceutical Co., Ltd (Jiangsu Province, China). The annexin V/fluorescein isothiocyanate (FITC)-PI staining kit was obtained from BD Biosciences Inc. (Heidelberg, Germany). Cell Counting Kit-8 (CCK-8) was obtained from Dōjindo Laboratories (Kumamoto, Japan). The probes used were rabbit anti- BCL2, apoptosis regulator (Bcl-2), anti-procaspase-3, and anti-activated caspase-3 polyclonal antibodies; rabbit anti-α-tubulin monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA); and mouse anti-cyclin D1 antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Goat anti-rabbit/mouse horseradish peroxidase (HRP)-conjugated secondary antibody was from Cell Signaling Technology (Danvers, MA, USA).

Proliferation assay

Antiproliferative effect of resveratrol in human pancreatic cancer cells was analyzed by CCK-8 assay. Briefly, SW1990, PANC-1, and BXPC-3 cells (5000 per well) were incubated in 96-well plates overnight and treated with various doses of resveratrol, or the combination of resveratrol and gemcitabine for 24, 48, and 72 h. Treatment with each concentration was repeated three times in three replicates. After various treatments, the cells were incubated with 100 μL of DMEM supplemented with 10 μL of CCK-8 reagent for 0 to 4 h at 37 °C. Cell viability was determined by scanning the absorbance with a microplate reader at 450 nm. The results were expressed as the percentage of viable cells as follows:

Relative viability (%) = $(A_{450[\text{treated}]} - A_{450[\text{blank}]}) / (A_{450[\text{control}]} - A_{450[\text{blank}]}) \times 100\%$.

Apoptosis and cell cycle analysis by flow cytometry

To quantify the percentage of apoptosis, we performed a flow cytometry analysis. In the resveratrol-alone treatment, cells were seeded in 6-well culture plates overnight and subsequently treated with 50, 100, and 150 μM resveratrol, or were untreated (0.1% DMSO) for 24 h. In the combination treatment, pancreatic cancer cells were treated with resveratrol (50 μM), gemcitabine (2 μM), or a combination of the two compounds. Then, both floating and trypsinized adherent cells were collected and incubated in 500 μL of binding buffer containing 5 μL of annexin-V/FITC for 30 min at 37 °C, and were then treated with 5 μL of PI for 5 min in the dark at room temperature. Analysis was immediately performed using a flow cytometer (BD Biosciences, San Jose, CA, USA). Cells for the cell cycle analysis were washed in cold phosphate-

buffered saline (PBS) twice and incubated in 300 μL of PBS containing 0.5% saponin, 50 μg/mL PI, and RNase 0.1 mg/mL for 30 min at 4 °C. The cells were analyzed by a flow cytometer, and cell-cycle phase distribution was analyzed using the Cell-Fit program. Data acquisition was gated to exclude cell doublets.

Western blot analysis

Western blot analysis was performed using standard techniques, as described previously [18]. Briefly, equal protein aliquots in each sample were resolved in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and the proteins were transferred onto nitrocellulose membranes. After blocking with 5% skimmed dried milk, the membranes were incubated with a 1:2000 dilution of primary antibodies. The membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (1:2000; Pierce, Thermo Fisher Scientific Inc., Waltham, MA, USA). The proteins were detected by an enhanced chemiluminescence detection system (SuperSignal™ West Femto Substrate; Pierce, Thermo Fisher Scientific Inc., Waltham, MA, USA), and light emission was captured on Kodak X-ray films.

Statistical analysis

Unless otherwise indicated, all results were representative of three separate experiments. Values represented the mean ± standard deviation (SD) of these experiments. The statistical significance of differential findings between experimental groups and the control was determined by one-way analysis of variance (ANOVA) using SPSS 13.0 and significance was set at $P < 0.05$.

Results

Cell growth inhibition by resveratrol in pancreatic cancer cells

The cytotoxicity of resveratrol on pancreatic cancer cells was first determined using CCK-8 assay to investigate the effect of resveratrol on cell proliferation in three PaCa cell lines. The decrease in absorbance in this assay could either be a consequence of cell death or cell proliferation inhibition. Resveratrol inhibited growth in all the cell lines (SW1990, PANC-1, and BxPC-3) in a dose-dependent manner (Fig. 1). In addition, we calculated the IC50 in each cell line and found that the sensitivity of the three PaCa cell lines to resveratrol differed. The IC_{50} values of PANC-1, BxPC-3, and SW1990 cells were 118.21 ± 2.91, 117.09 ± 9.94, and 99.97 ± 2.03 μM, respectively.

Cell apoptosis induced by resveratrol

To investigate whether resveratrol induced apoptosis of PaCa cells, three cell lines were treated for 24 h with 50, 100, or 150 μM resveratrol, or 0.1% DMSO. In general, we found that resveratrol induced apoptosis in a dose-dependent fashion in the three cell lines (Fig. 2a). For example, 50 μM resveratrol did not significantly induce apoptosis compared with that in the control group, whereas the 100 μM and 150 μM groups showed a marked increase in apoptosis in PANC-1 cells (Fig. 2b). In the case of SW1990 cells, a similar result was obtained. Nevertheless, in BxPC-3 cells, only 150 μM resveratrol induced marked apoptosis.

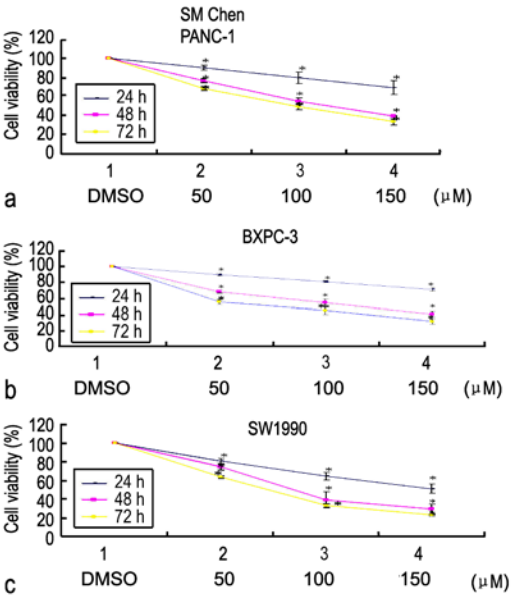

Fig. 1 Effect of resveratrol on PANC-1, BxPC-3, and SW1990 cell proliferation. Dose and time-dependent inhibition of cell proliferation by resveratrol in three pancreatic cancer cell lines. Each value represents the mean ± SD ($n = 4$) of three independent experiments. * $P < 0.05$, compared with the DMSO group

Effect of resveratrol on cell-cycle phase distribution

To investigate resveratrol induced cell proliferation inhibition further, cell-cycle phase distribution was examined using flow cytometry. Compared with control cells, the cell cycle profiles of PANC-1 (Fig. 3a), BxPC-3 (Fig. 3b), and SW1990 (Fig. 3c) changed significantly after incubation with 100 μM of resveratrol for 24 h. In general, resveratrol led to an arrest in the G1 phase, correspondingly, causing a decrease of cells in the S and G2 phases of the cell cycle. As shown in Table 1, the number of cells in the G1 phase significantly increased from 45.70% to 66.79% in PANC-1, from 47.23% to 72.35% in BxPC-3, and from 48.97% to 73.29% in SW1990. The number of cells in the S and G2 phases was decreased in the three PaCa cell lines; although in the PANC-1 cells, the reduction was not significant (Table 1).

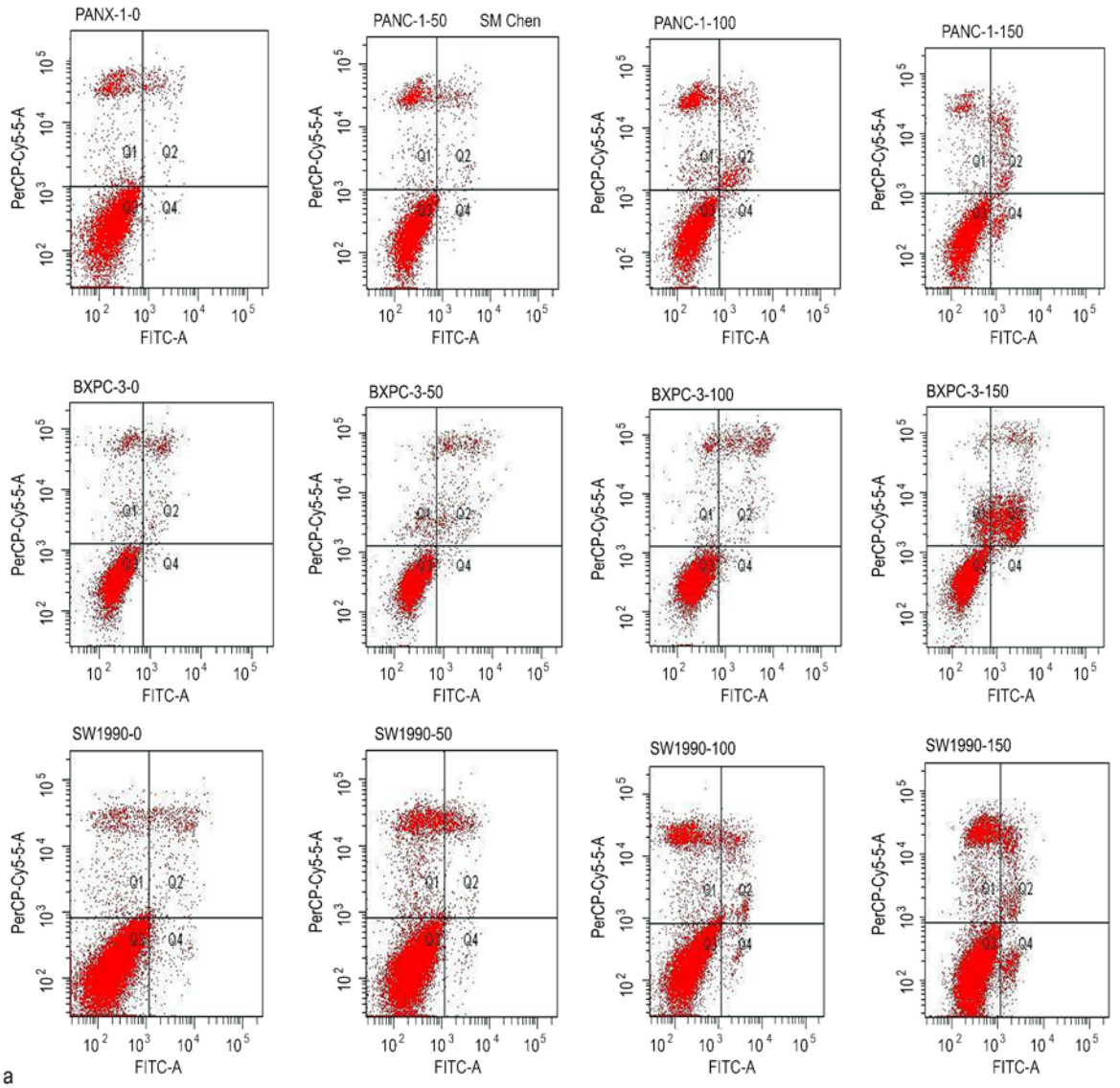

Fig. 2 Effect of resveratrol on PANC-1, BxPC-3, and SW1990 cell apoptosis. PANC-1, BxPC-3, and SW1990 cells were stained and analyzed by flow cytometry to detect Annexin V-FITC positive and/or PI positive cells. The unaffected, early apoptotic, late apoptotic, and necrotic cells are present in the lower left, lower right, upper right, and upper left quadrant, respectively. Representative data from one experiment are shown, and two additional experiments yielded similar results. * $P < 0.05$ and ** $P < 0.01$ compared with the control

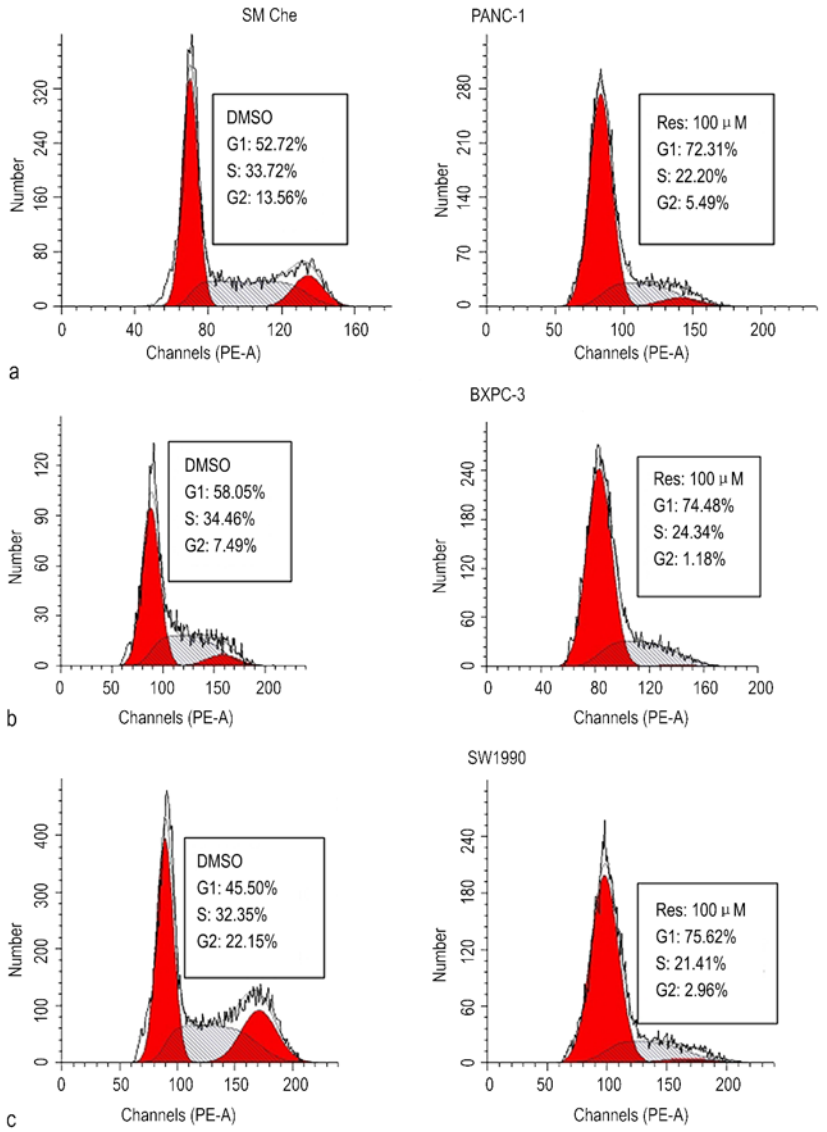

Fig. 3 Analysis of cell-cycle phase distribution by flow cytometry. PANC-1, BxPC-3, and SW1990 cells were treated with 0.1% DMSO, or 100 μM resveratrol for 24 h and incubated in 300 μL PBS containing 0.5% saponin, 50 μg/mL PI, and RNase 0.1 mg/mL for 30 min at 4 ℃ for flow cytometric analysis of DNA content. Representative data from one experiment are shown, and two additional experiments yielded similar results

Resveratrol blocks cyclin D1 and Bcl-2 expression and induces activation of procaspase-3 and poly (ADP-ribose) polymerase 1 in pancreatic cancer cells

To further understand the mechanism of resveratrol-induced cell proliferation inhibition and apoptosis, we examined the expression of cyclin D1, Bcl-2, caspase-3, and poly (ADP-ribose) polymerase 1 (PARP1) after resveratrol treatment. The PaCa cell lines were exposed to 50, 100, 150 μM resveratrol, or 0.1% DMSO for 48 h. Consistent with the results of the cell-cycle phase distribution, the level of cyclin D1 was reduced in a dose-dependent fashion in the PaCa cell lines. A major decrease was seen in the 150 μM group among all cell lines, but in the SW1990 cells 50 μM resveratrol did not significantly reduce protein expression (P = 0.055) (Fig. 4). We also observed that resveratrol repressed Bcl-2 expression, and induced the proteolytic cleavage of procaspase-3 into its 17-kD active form as well as the activation of its substrate PARP1, resulting in effector caspase and activated PARP1 up-regulation (Fig. 4). In conclusion, these data suggested that cell growth inhibition and apoptosis were at least in part related to the expression levels of cyclin D1 and Bcl-2 as well as activated caspase-3 and PARP1, although the sensitivity to resveratrol was dependent on the individual cell type.

Cell growth inhibition with a combination of resveratrol and gemcitabine

We also investigated whether resveratrol enhanced antitumor activity of gemcitabine in PANC-1 and BxPC-3 cells. As detected by the CCK-8 assay, data showed that

Table 1 Effects of resveratrol on PANC-1, BxPC-3, and SW1990 cell-cycle phase distribution

Cell	Treatments	G1 (%)	S (%)	G2 (%)
PANC-1	DMSO	45.70 ± 6.72	37.65 ± 7.83	16.65 ± 4.98
	100 μM	66.79 ± 6.93*	29.86 ± 8.93	3.35 ± 2.94*
BXPC-3	DMSO	48.97 ± 9.56	37.14 ± 3.42	13.89 ± 6.26
	100 μM	73.29 ± 1.1*	24.22 ± 1.97**	2.49 ± 2.61*
SW1990	DMSO	47.23 ± 2.46	37.25 ± 6.22	15.52 ± 6.3
	100 μM	72.35 ± 3.21**	20.85 ± 1.09*	3.46 ± 2.55*

* $P < 0.05$ and ** $P < 0.01$ versus the DMSO group. Values are the mean and SD of three independent assays

the resveratrol (50 μM), gemcitabine (2 μM), and combination (50 μM resveratrol and 2 μM gemcitabine) treatment decreased the growth of PaCa cells in a time-dependent manner (Fig. 5). However, the combination treated cells showed a more significant decrease ($P < 0.05$). After treatment for 24 h, 48 h, and 72 h, the inhibition in rate of cell growth in the combination group was 31.36 ± 7.34%, 61.03 ± 0.52%, and 67.26 ± 3.27%, respectively.

Cell apoptosis induced by resveratrol and gemcitabine

Cell apoptosis induced by resveratrol and gemcitabine was detected in PANC-1 and BxPC-3 cells. As shown in Fig. 6, 48 h after various treatments the apoptosis rates in the DMSO (0.1%), resveratrol (50 μM), gemcitabine (2 μM), and combination (50 μM and 2 μM) group were 4.34 ± 1.53%, 12.80 ± 1.71%, 16.07 ± 1.34%, and 23.3 ± 3.01% in PANC-1 cells, respectively; and in BxPC-3 cells were 7.87 ± 0.8%, 13.33 ± 1.21%, 16.50 ± 5.30%, and 29.07 ± 3.59%, respectively. This meant that resveratrol could significantly increase cell apoptosis induced by gemcitabine ($P < 0.05$). Taken together, our data demonstrate that resveratrol not only inhibited cell growth but also enhanced gemcitabine-induced apoptosis in PaCa cells, and that resveratrol markedly enhanced the chemosensitivity of these cells.

Discussion

Pancreatic cancer is a highly aggressive malignant disease, which is currently treated using available chemotherapy regimens but with limited success and dismal outcomes. Gemcitabine, the only approved drug, has not resulted in radically improved outcomes for the treatment of PaCa. Over several decades, numerous agents have been tested in combination with gemcitabine including erlotinib, platinum analogs, bevacizumab, and celecoxib, but all have afforded disappointing results [19]. Emerging evidence suggests that many dietary compounds exhibit beneficial effects for the prevention of cancer [20–22]. Resveratrol is derived from a Chinese traditional medicinal plant (Polygonum cuspidatum) as well as grapes, peanuts, berries, and pines, and is regarded as safe to consume [8]. Extensive data show that resveratrol affects tumor initiation, promotion, and progression, and suppresses angiogenesis and metastasis by involving multiple pathways [8]. Consistent with previous reports, this study demonstrated that resveratrol induced cell growth inhibition and apoptosis in a dose-dependent manner in SW1990, PANC-1, and BxPC-3 PaCa cell lines. The mechanism of resveratrol's anticancer effect is not fully understood. Yang Shu found that resveratrol elicits anti-colorectal cancer effect by activating miR-34c that targets KIT ligand *in vitro* and *in vivo* [23]. In this study, we found that resveratrol blocked cell cycle progression in three PaCa cell lines with cells remaining in the G1 phase, which resulted in cell proliferation inhibition. To explore which proteins may be involved in resveratrol-induced cell proliferation inhibition and apoptosis, we investigated the cell cycle regulator, cyclin D1, and apoptotic proteins Bcl-2, caspase-3, and PARP1. Western blot analysis showed that resveratrol repressed cyclin D1 and Bcl-2 expression in a dose-dependent fashion and upregulated activation of caspase-3. We also observed that the proteolytic cleavage of procaspase-3 was followed by proteolytic degradation of PARP1, a specific substrate of both effector caspases-

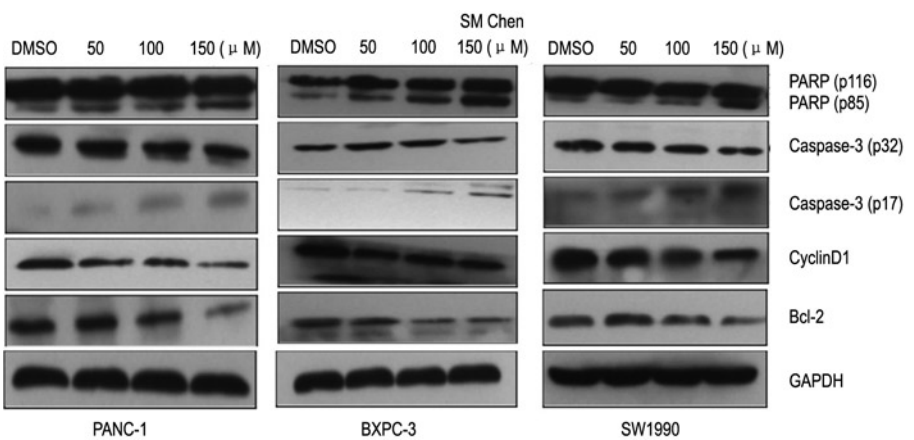

Fig. 4 Effect of resveratrol on expression of cell cycle- and apoptosis-related proteins. Pancreatic cancer cells were incubated with increasing concentrations of resveratrol for 48 h. Levels of Bcl-2, cyclin D1, caspase-3, and PARP1 proteins were detected by western blotting. GAPDH was inserted as a control. Results are representative of three independent experiments

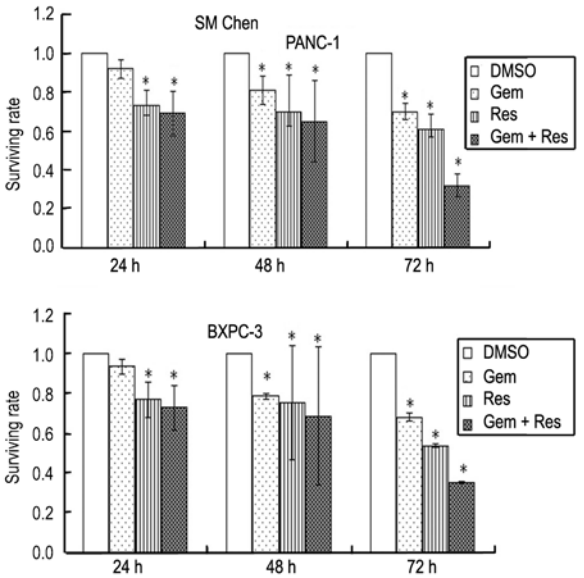

Fig. 5 Synergistic effect of resveratrol and gemcitabine on PANC-1 and BxPC-3 cells. Dose and time-dependent inhibition of cell proliferation were observed. Each value represents the mean ± SD (n = 4) of three independent experiments. * P < 0.05, compared with the DMSO group

3 and -7, suggesting that resveratrol-induced cleavage of procaspase-3 leads to activation of the protease. This down-regulation of cyclin D1 may be related with the G1 arrest observed. Cyclin D1 appears in the G1 phase and interacts with cyclin-dependent kinase (CKD) regulating the progression of cells from late G1 phase into the S phase. Cells accumulated in the G1 phase, and correspondingly, the percentage of cells in the S and G2/M phase decreased. The results suggest that resveratrol could reduce DNA synthesis and induce DNA fragmentation in all three examined cell lines.

In chemotherapeutic drug-induced apoptosis of tumor cells, three different death signaling pathways lead to apoptosis [22], namely the extrinsic death receptor-dependent pathway [23], the intrinsic mitochondria-dependent pathway [24], and the intrinsic endoplasmic reticulum (ER) stress-mediated pathway [25]. The mitochondria-dependent death signaling begins with the release of mitochondrial cytochrome c into the cytoplasm, which together with the apoptotic protease activating factor-1 (APAF-1) activates caspase-9 in the presence of dATP, and then activates caspase-3, leading to cell death [26–27]. In the ER stress-mediated apoptotic pathway, activation of caspase-12 can directly activate caspase-9 [28–29] .ER stress also triggers activation of caspase-8 and c-jun N-terminal kinase (JNK), both of which are known to cause mitochondrial cytochrome c release [30–31]. Thus, the caspases, a family of cysteine-dependent aspartate-directed proteases, appear to be involved in three different death signaling pathways, and caspase-3 is at the intersection of these pathways. We also found that the proteolytic cleavage of procaspase-3 was followed by proteolytic degradation of PARP1, a substrate of caspase-3, suggesting that resveratrol induced activation of procaspase-3 leads to activation of the protease. Proteolytic degradation of PARP1 results in DNA fragmentation in the process of apoptosis, which is known to play an important role in protecting chromosomal DNA [32].

The Bcl-2 family is another critical component involved in apoptosis. The family includes a number of

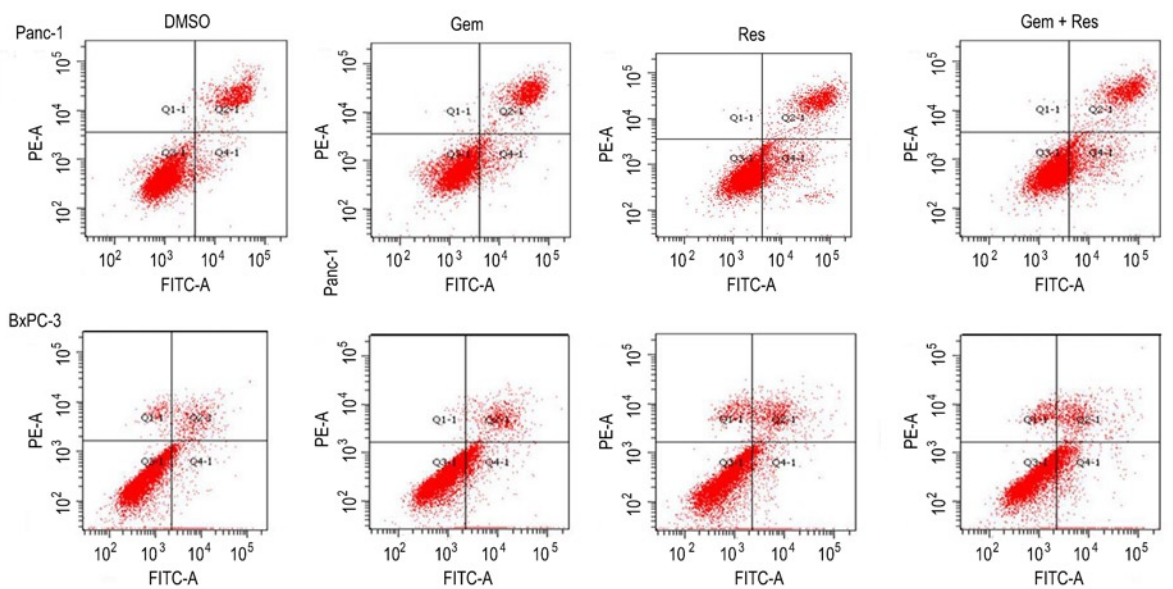

Fig. 6 Synergistic apoptotic effect of resveratrol and gemcitabine on PANC-1 and BxPC-3 cells. Representative data from one experiment are shown, and two additional experiments yielded similar results. * P < 0.05 and ** P < 0.01 compared with the control

proteins, which have homologous amino acid sequences, including antiapoptotic members such as Bcl-2, BCL2 like 1 (bcl-xL), and X-linked inhibitor of apoptosis (XIAP), as well as proapoptotic members including BCL2 associated X, apoptosis regulator (BAX). Interestingly, Kirsch [33] found that the Bcl-2 protein was cleaved at Asp-34 by caspases during apoptosis and by recombinant caspase-3 in vitro. Arnoult [34] also observed that antiapoptotic proteins, Bcl-2, bcl-xL, and XIAP, are cleaved by caspase-3 and are converted to proapoptotic proteins similar to BAX. The proapoptotic Bcl-2 cleavage product localizes on the mitochondrial membrane and causes a release of cytochrome c [35–36], and then triggers mitochondria-dependent death signaling and results in apoptosis. In this study, we examined the expression of Bcl-2 protein, to confirm the participation of caspases in cell death. The results showed that resveratrol induced activation of caspase-3 and repressed Bcl-2 expression. Therefore, it seems that the apoptotic effects of resveratrol are accomplished by a caspases signaling-dependent mechanism through the downregulation of Bcl-2.

We next tried to determine whether resveratrol has potential in combination with gemcitabine in the treatment of PaCa, one of the most lethal cancers. We found that resveratrol not only inhibited cell growth but also enhanced gemcitabine-induced apoptosis in the cells. Our results were in agreement with a previous report that resveratrol when used in combination with gemcitabine, was highly effective in inducing apoptosis in PaCa cells [37]. It has been speculated that downregulation of NF-κB, which was constitutively activated in PaCa, could be one of the mechanisms to explain this observation. However, the mechanism of the observed synergistic effects of the combinatorial treatment was not fully investigated. As far as we know NF-κB, which mediates inflammatory signaling pathway closely linked to drug resistance, also regulates Bcl-xL, Bcl-2, prostaglandin-endoperoxide synthase 2 (PTGS2 or COX2), cyclin D1, vascular endothelial growth factor A (VEGFA), and matrix metallopeptidase 9 (MMP-9). In the present study, we also observed that resveratrol inhibited protein expression of cyclin D1 and Bcl-2, and induced activation of caspase-3 and PARP1, which may explain why resveratrol enhanced anticancer activity of gemcitabine in pancreatic cancer.

Conclusions

Our results showed that resveratrol played an important role in cell proliferation inhibition, cell cycle arrest, and apoptosis in pancreatic cancer cells. In addition, resveratrol markedly increased the chemosensitivity of pancreatic cancer cells to gemcitabine. Nevertheless, we did not explore the anticancer effect of this compound *in vivo*. In the present study, we only investigated the expression level of Bcl-2, caspase-3, and PARP1, but did not explore the precise interaction of these proteins in the apoptosis progression. Therefore, to obtain a better understanding of its chemopreventive activity, the cell death signaling pathways of resveratrol-induced apoptosis in the PaCa cell should be fully explored. Furthermore, the potential biological activities of resveratrol *in vivo* should be investigated in future studies. Based on these results, further studies are required to explore the potential of resveratrol as an anticancer agent for pancreatic cancer treatment.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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