Original Paper

Synergistic Induction of Erlotinib-Mediated Apoptosis by Resveratrol in Human Non-Small-Cell Lung Cancer Cells by Down-Regulating Survivin and Up-Regulating PUMA

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Key Words

Erlotinib • Resveratrol • NSCLC • Apoptosis • Survivin • PUMA • Synergistic

Abstract

Background/Aim: Treatment of human non-small-cell lung cancer (NSCLC) often involves uses of multiple therapeutic strategies with different mechanisms of action. Here we found that resveratrol (RV) enhanced the anti-tumor effects of epidermal growth factor receptor (EGFR) inhibitor erlotinib in NSCLC cells. **Methods:** Cell viability was measured by MTT assay and clonogenicity assay. Western blot was applied to assess the protein expression levels of target genes. Cell apoptosis was monitored by AnnexinV-FITC assay and sub-G1 population assay. Intracellular ROS were measured by flow cytometric analysis. Cell caspase activities were carried out by fluorometric assays. **Results:** Exposure of H460, A549, PC-9 and H1975 cells to minimal or non-toxic concentrations of RV and erlotinib synergistically reduced cell viability, colony formation and induced cell apoptosis. Furthermore, RV synergistically enhanced erlotinib-induced apoptosis was involved in ROS production. Additionally, co-treatment with RV and erlotinib repressed the expressions of anti-apoptosis proteins, such as survivin and Mcl-1, whereas promoted p53 and PUMA expression and caspase 3 activity. Moreover, the combination was also more effective at inhibiting the AKT/mTOR/S6 kinase pathway. Subsequently, small interfering RNA (siRNA) depletion of PUMA and overexpression of survivin significantly attenuated NSCLC cells apoptosis induced by the combination of the two drugs. **Conclusion:** Our findings suggested that RV synergistically enhanced the anti-tumor effects of erlotinib in NSCLC cells were involved in decrease of survivin expression and

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induction of PUMA expression. In conclusion, based on the observations from our study, we indicated that the combined administration of these two drugs might be considered as a novel therapeutic regimen for treating NSCLC.

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Introduction

Lung cancer is one of the most commonly occurring malignancies and a major cause of cancer-related deaths worldwide, which accentuates the need for effective strategies to prevent and treat this tumor. Therapies available for treatment of lung cancer include surgery, radiation therapy, chemotherapy, immunomodulatory therapy, and molecularly targeted treatments such as anti-epidermal growth factor receptor (EGFR) and MET receptor tyrosine kinase therapy [1, 2]. However, chemotherapy is still not effective enough for patients with advanced non-small-cell lung cancer (NSCLC) and the response rate is only 20% to 35% with a median survival of 10 to 12 months [2].

By targeting critical molecules to cancer development, targeted treatment alone or in combination with other treatments was recently recognized as a promising strategy to conquer cancers including NSCLC. Among those molecularly targeted therapies, EGFR-targeted therapy as one of the most important clinical strategies has been increasingly widely applied in patients with NSCLC [3]. EGFR is a transmembrane glycoprotein with an extracellular EGF-binding domain and an intracellular region containing the tyrosine kinase domain that regulates signaling pathways to control cell proliferation. Constitutive activation of EGFR has been associated with a poorer prognosis in most NSCLC patients [4]. These strategies available for targeting of EGFR include using monoclonal antibodies such as cetuximab, and small molecular tyrosine kinase inhibitors (TKI) such as gefitinib and erlotinib [5]. Erlotinib is a synthetic anilinoquinazoline and orally selective EGFR-TKI that inhibits the signal pathway involved in the proliferation and survival of cancer cells [6, 7]. In a clinical setting, erlotinib treatment was approved for the treatment of NSCLC in 2004. However, the emerging clinical experience has disappointingly revealed that despite erlotinib demonstrating some antitumor activity in NSCLC, there is a high level of *de novo* resistance to such treatment [8]. Moreover, erlotinib has been shown to be ineffective for the majority of NSCLC patients because the patients are initially resistant to the inhibitor [9]. Statistics show that approximately 10–14 months after the primary therapy of erlotinib, the patients start to develop resistance to the drug, which results in reoccurring lung cancer [10].

The emergence of Chinese medicine monomer antitumor drugs has provided a new option for cancer treatment. Resveratrol (RV), extracted from *Polygonum cuspidatum*, is a small molecule natural antioxidant that has been shown to be a potential chemopreventive and anticancer agent [11]. It also has been shown that RV exhibit potential anticancer activity in pancreatic cancer cells, prostate cancer cells and other tumor models including breast, ovarian, liver, intestine and colorectal cancer [12]. Several studies show that RV block the proliferation of a variety of cancer cells via inhibition of various cell survival signaling pathways including the phosphatidylinositol 3-kinase (PI3-K)/AKT and mammalian target of rapamycin (mTOR) pathways [13, 14]. Moreover, it has been reported that RV treatment inhibits cell growth and induces cell senescence via reactive oxygen species (ROS) mediated DNA damage in lung cancer cells [15]. In addition, recent clinical trials have found that RV is well tolerated and relatively nontoxic drug for use in humans [16]. However, it has been reported that a major challenge for the use of RV is that the dose of RV required to induce cell death in cancer cells *in vitro* is too high to achieve *in vivo* in a clinical setting [17]. Therefore, efforts are ongoing for the development of anti-lung cancer regimens that would combine RV with other drugs. In this study, we sought to exploit the antitumor activities of RV plus erlotinib by testing the hypothesis that treatment with erlotinib in combination with RV will overcome the resistance to EGFR inhibitors in NSCLC cells.

In the current study, we showed that the combination of the erlotinib and RV was

synergistic at inhibiting cell proliferation and inducing apoptosis in cultured human NSCLC cells. Furthermore, we provided evidences that erlotinib combined with RV regulated cell apoptosis were involved in induction of PUMA expression and reduction of survivin. Taken together, these accumulating data might guide development of novel NSCLC therapies.

Materials and Methods

Chemicals and antibodies

Resveratrol (trans-3', 4', 5-trihydroxystilbene) and erlotinib were purchased from Selleck Chemicals LLC. MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide], dimethyl sulfoxide (DMSO), hoechst 33258, N-acetylcysteine (NAC), z-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk), necrostatin-1, necrostatin-5 were purchased from Sigma. All reagents were dissolved in DMSO respectively; aliquots were stored at -80 °C. Antibodies against p-mTOR (Ser2448) (2971s), mTOR (2983s), AKT (2938s), p-AKT (Ser473) (9271s), p-S6K (9205s), S6K (2708s), PUMA (12450s), survivin (2803s), BAX (2772s), BIM (2933s), Bcl-2 (2872s), Mcl-1 (4572s), Bcl-XL (2762S), cleaved PARP (Asp214) (9546s) and β -Actin antibodies (4967s) all from Cell Signaling Technologies. p53 (Abcam, ab28), Noxa (Abcam, ab36833) and γ H2AX (Abcam, ab26350) were purchased from Abcam.

Cell culture and drugs treatment

The lung cancer cell lines H460 [wild-type (wt) EGFR], A549 (wt EGFR), PC-9 (EGFR E746-A750 deletion) and H1975 (EGFR L858R/T790M) were obtained from the American Type Culture Collection (ATCC). The erlotinib-resistant PC-9 cells (PC-9/ERL1) were established from a parental PC-9 cell line after continuously exposing PC-9 cells to low dose of erlotinib. PC-9/ERL1 cells were 22-fold more resistant than the parental PC-9 cells to erlotinib. All cells were grown in DMEM medium (Gibco; Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco; Life Technologies) at 37°C in 5% CO₂ incubator. Cells were grown in monolayer and passaged routinely 2–4 times a week. For drugs treatment, stock solutions were diluted to the desired final concentrations with growth medium just before use. Prior to drugs treatment, cells were incubated for 12 to 18 h and thereafter replaced with media containing drugs; DMSO-treated cells were used as a mock control.

Cell viability

Cell viability was assessed using standard MTT assay. Briefly, cells were plated at a density of 0.5×10^4 cells per well in 96-well plates and incubated for 12 h in a 5% CO₂ atmosphere at 37 °C before treatment of exposed to drugs. The media were then removed, and the cells were treated with resveratrol and/or erlotinib. After the cells were incubated for 48 h, 100 μ L MTT solutions (2 mg/mL) were added to each well and the plate was incubated for another 4 h at 37 °C. The formed formazan crystals were dissolved in DMSO (200 μ L per well) with constant shaking for 5 min. Absorbance of the solution was then measured using a Micro-plate Reader (Bio-Rad) at 495 nm. This assay was conducted in triplicate.

Clonogenicity assay

For clonogenicity experiments, the attached cells from the same 10 cm culture dish that were trypsinized with 1 mL trypsin-EDTA (Gibco; Life Technologies) and inactivated with media containing 10% FBS. The cells were counted using a hemocytometer and plated at low density (1000 per well in six well plate). After 24 h, resveratrol and/or erlotinib were added at indicated concentration for 24 h. After drugs removal, cells were allowed to proliferate in a humidified 5% CO₂, 37°C environment for 15 days in fresh growth medium. Cells were fixed in 70% ethanol and stained with 0.005% crystal violet (sigma) for analysis of clonogenic cell survival as previously described. The colony forming units with more than 100 cells were counted using a light microscope.

Combination index

For combination treatment of resveratrol and/or erlotinib, MTT assay data were converted to fraction of growth affected by the individual drug or the combination treated cells compared with untreated cells and analysed using CalcuSyn software (Biosoft) to determine whether the combination was synergistic.

This program is based upon the Chou-Talalay equation [18], which calculates a combination index (CI). The general equation for the classic isobologram is given by: $CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$. Where Dx indicates the dose of one compound alone required to produce an effect, $(D)_1$ and $(D)_2$ are the doses of compounds 1 and 2, respectively, necessary to produce the same effect in combination. From this analysis, the combined effects of the two compounds can be summarized as follows: $CI < 1$, $CI = 1$, $CI > 1$ indicate synergistic, additive and antagonistic effects, respectively.

Apoptosis assays

Measurement of apoptosis was conducted by Annexin V-FITC (fluorescein isothiocyanate) analysis as described previously [19]. Briefly, cells were seeded and treated with resveratrol and/or erlotinib for 48 h. Afterward, the cells were washed twice with PBS and 1×10^6 cells were resuspended in 1 mL of $1 \times$ Annexin V binding buffer. Cells undergoing apoptotic cell death were analyzed by counting the cells that stained positive for Annexin V-FITC using FACS Calibur™ flow cytometer (BD Biosciences).

Sub-G1 population assay

Sub-G1 population was analyzed by flow cytometer. Every well of a 6-well plate was seeded with 5×10^5 /mL cells. Then cells were treated with the indicated concentration of drugs. After 48 h, cells were harvested and fixed using 70% ethanol at -20°C for 24 h. And then cells were stained with mixture of 50 $\mu\text{g/mL}$ propidium iodide, 0.2% Triton X-100, and 100 $\mu\text{g/mL}$ RNAase, and performed using a FACS flow cytometer equipped with Modfit LT for Mac V2.0 software (BD Biosciences).

Flow cytometric analysis of ROS

Intracellular ROS were measured by flow cytometric analysis. Briefly, cells were loaded with 20 μM of 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) and incubated at 37°C for 30 min. The peak excitation wavelength for oxidized DCF-DA was 488 nm and emission was 525 nm.

Western blot analysis

Cells were lysed on ice for at least 30 min in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% deoxycholic acid, 0.02% sodium azide, 1% NP-40, 2.0 mg/mL aprotinin, 1 mM phenylmethylsulfonylfluoride). The lysates were centrifuged at 12,000 rpm for 30 min at 4°C . The protein concentration was determined by Bradford dye method. Equal amounts (30 to 60 μg) of cell extract were subjected to electrophoresis in 6-12.5% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) and transferred to PVDF membranes (Millipore, Darmstadt, Germany) for antibody blotting. The membranes were blocked and then incubated with antibodies. Subsequently, the membranes were incubated with a HRP-conjugated secondary antibody (Protein Tech Group, Chicago, IL) at room temperature for 1 h. Detection was performed with the ECL kit (GE Healthcare), according to the manufacturer's instructions.

Caspase activity assay

Fluorometric assays of caspase activity were carried out by using the substrate Ac-DEVD-AMC (BD Pharmingen) for caspase 3 and Ac-IETD-AMC (BD Pharmingen) for caspase 8. Briefly, cells were lysed in lysis buffer (10 mM HEPES, 142 mM KCl, 5 mM MgCl_2 , 1 mM EDTA, 0.2% NP-40 and pH 7.2) with 10 mM DTT. Following incubation for 30 min on ice, samples were centrifuged at 12,000 rpm for 30 min at 4°C and the protein content in supernatants was determined by Bradford dye method. Aliquots of 10 mg/100 mL assay volume were incubated with 140 mM site-specific tetrapeptide substrates Ac-DEVD-AMC for caspase 3 and Ac-IETD-AMC for caspase 8 in a caspase assay buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 0.01% (w/v) CHAPS, 10% (w/v) sucrose and pH 7.2) with 10 mM DTT for 30 min. The release of the fluorogenic group AMC was determined at 37°C in a VersaFluor Fluorometer (Bio-Rad) with excitation at 380 nm and emission at 440 nm.

RNA interference

siRNAs for down-regulating PUMA gene expression were performed by transfection of RNA oligonucleotides with lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. One day before transfection, cells were plated on a six-well culture plate in growth medium. After cells reached 30%–50% confluence, they were transfected with siRNAs. Briefly, cells were placed in 1 mL of siRNA mixture

with 100 nM siRNA and 5 μ L lipofectamine 2000. After 8 h of transfection, 1 mL of fresh growth medium was added, and experiments were conducted 48 h after transfection. Protein levels were analyzed by Western blot. The negative control (NC) siRNA and siRNAs against PUMA were synthesized by Shanghai GenePharma Co.. For PUMA: 5'-GAGCGGCGGAGACAAGAAGA GUU-3';

Plasmid transient transfection

The survivin and control plasmid DNA (PCMV6XL4) used in this study were obtained from OriGene (Rockville). For transient transfection, 5×10^5 cells per well were seeded into 6-wells plates and transiently transfected with 2 μ g of survivin plasmids using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Briefly, plasmid DNA (2 μ g) was diluted into 100 μ L of RPMI 1640 media that lacked supplementation with FBS. Lipofectamine 2000 reagent (5 μ L) was diluted into 100 μ L of RPMI 1640 media that lacked supplementation with FBS. The two solutions were then mixed together and incubated at room temperature for 30 min. The total mix was added to each well (6-well plate) containing 800 μ L RPMI 1640 media that lacked supplementation with FBS. The cells were incubated for 8 h at 37 °C in 5% CO₂ incubator, after which time the media was replaced with DMEM growth media containing 10% (v/v) FBS.

Statistics

All experiments were repeated three times and were expressed as mean $\pm$ SD. *P* values were calculated using student's *t* test and two-way ANOVA and *P* value < 0.05 was considered significant. Statistical analysis was analyzed using the Statistical Package for Social Sciences (SPSS) software (version 16.0).

Results

Synergistic antineoplastic effects induced by resveratrol and erlotinib in NSCLC cells

To determine the effects of combination treatment of RV and EGFR inhibitor erlotinib on human NSCLC cell viability, H460, A549, PC-9 and H1975 cells which differed in their EGFR status, were treated with different concentrations of RV in the absence or presence of erlotinib for up to 48 h, followed by the determination of cell viability using MTT assay. As shown in Fig. 1A, B, C and D, H460, A549, PC-9 and H1975 cells were significantly inhibited by each drug alone. The combination of RV and erlotinib further decreased the proliferation rate of NSCLC cells, compared to single drug application. Similar results were found in erlotinib-resistant PC-9 (PC-9/ERL) cells (Fig. 1E).

To further determine whether RV and erlotinib synergize to inhibit cell viability in the four tested human NSCLC cell lines, we treated cells with a combination of the two agents in a constant ratio to one another and used CalcuSyn software to calculate the combination index (CI) following Chou and Talalay's formula as described under Methods. Plotting of isobolograms with data generated from the four NSCLC cells showed that the CI values were mostly < 1 (Fig. 1F-I), indicating that there was a synergistic interaction between RV and erlotinib in these four NSCLC cell lines. Additionally, we found that the combination of RV with erlotinib showed weak synergy (CI > 0.5) at low concentrations tested. However, the combination of RV with erlotinib displayed high synergistic effects (CI < 0.5) when both RV

Table 1. Statistical analysis of cell viability following treatment of RV and ERL (two-way ANOVA)

Source of variation	H460		A549		PC-9		H1975		PC9/ERL	
	F-Value	P-Value	F-Value	P-Value	F-Value	P-Value	F-Value	P-Value	F-Value	P-Value
RV	3447.2	0.0000	3066.3	0.0000	1427	0.0000	1092.3	0.0000	3635.6	0.0000
ERL	2763.1	0.0000	2765.2	0.0000	1574.4	0.0000	1015.4	0.0000	2048.7	0.0000
RV*ERL	27.2	0.0000	46.6	0.0000	17.5	0.0000	21.1	0.0000	81.4	0.0000

ANOVA, analysis of variance; RV, resveratrol; ERL, erlotinib; RV*ERL, interaction between RV and ERL.

Fig. 1. Synergistic antineoplastic effects of resveratrol and erlotinib against NSCLC cells. H460 (A), A549 (B), PC-9 (C), H1975 (D) and PC-9/ERL (E) cells were cultured in control conditions (DMSO) or in the presence of the indicated concentrations of resveratrol (RV) and erlotinib (ERL), alone or in combination, for 48 h, and then assessed for viability by MTT assay. Results were means of duplicate assessments from one out of three independent experiments. (F) H460, (G) A549, (H) PC-9, (I) H1975 and (J) PC-9/ERL cells were plated, treated, and processed as in A-E. The combination index (CI) values for RV and ERL were calculated according to the Chou-Talalay's

method at the 48 h time point, with the biological response being expressed as the fraction of affected (Fa) cells. Diamond symbol designated the CI value for each Fa, $CI < 1$, $CI = 1$, $CI > 1$ indicated synergistic, additive and antagonistic effects, respectively. The effect ranges from 0 (no inhibition) to 1 (complete inhibition). The data are representative of three independent experiments. (K) and (L) Influence of H460, A549, PC-9 and H1975 on the number of colony-forming cells, as evaluated by colony-forming assay. For clonogenic assay, the assay was done as described in materials and methods. Columns, means of three determinations; bars, SD. Results shown are representative of three independent experiments. **, $P < 0.01$, ***, $P < 0.001$, compared with DMSO-treated cells.

and erlotinib were used at higher doses (Fig. 1F-I). Similar synergistic effects between RV and erlotinib were found in PC-9/ERL cells (Fig. 1J). In addition, two-way ANOVA indicated that all interactions between RV and erlotinib were significantly positive (Table 1, $P < 0.001$).

A long-term clonogenicity assay was carried out to assess the capacity of RV plus erlotinib to cause irreversible growth arrest in NSCLC cells. We found decreases in the colony-forming capacities of the cells (Fig. 1K and L), suggesting irreversible growth arrest. Taken together, those results indicated that the RV could enhance the cytotoxic effects of erlotinib in NSCLC cells.

Resveratrol synergistically enhances erlotinib-induced apoptosis in NSCLC cells

To determine if the cytotoxic effects of RV combined with erlotinib are linked to induction of apoptosis, H460, A549, PC-9 and H1975 cells were treated with the two compounds, alone or in combination, for 48 h and then cell apoptosis was determined by Annexin V-FITC staining and flow cytometry analysis. We found that treatment with RV (20 μ M) or erlotinib (10 μ M) alone had weak effects on apoptosis in H460 cells. However, there was a significantly higher apoptosis rate found upon treatment with the combination of RV (20 μ M) and erlotinib (10 μ M) (Fig. 2A and B). Similar results were found in A549, PC-9 and H1975 cells (Fig. 2A, B and C). Additionally, we measured

cell apoptosis by detecting sub-G1 population with propidium iodide staining and flow cytometry analyses. As shown in Fig. 2D and E, the sub-G1 population percentages induced by the treatments with the RV plus erlotinib in combination were greater than those induced by the compounds individually. Moreover, by Hoechst 33258 staining, we observed that in NSCLC cells RV plus erlotinib caused markedly chromatin condensation and fragmentation which are typical apoptotic nuclear morphological changes (data not shown).

Resveratrol and erlotinib treatment induce cell apoptosis associated with ROS production in NSCLC cells

Many chemotherapeutic agents kill tumor cells through the induction of ROS [20]. Others have demonstrated that ROS plays a critical role in mediating RV-induced cell death [15]. Therefore, we hypothesized that RV plus erlotinib may cause cell death via increased ROS production in NSCLC cells. To test this hypothesis, we investigated if RV treatment has any impact on ROS induction in NSCLC cells. As shown in Fig. 3A, DCF-DA staining and flow cytometric assays showed that the levels of ROS were increased in RV-treated NSCLC cells compared with that of control cells. Moreover, Fig. 3A-C indicated that erlotinib markedly enhanced the levels of ROS induced by RV in NSCLC cells. However, erlotinib alone failed to induce the production of ROS in these cell lines. These results suggested that erlotinib enhanced the cytotoxic effects of RV might be involved in ROS-mediated cell death.

Although our data had shown that RV plus erlotinib increased ROS production in NSCLC cells (Fig. 3), it had yet to be determined if the cells apoptosis induced by the two drugs in combination is associated with increased ROS production. Thus, we pre-incubated

Fig. 2. Resveratrol synergistically enhances erlotinib-induced apoptosis in NSCLC cells. (A) H460, A549, PC-9 and H1975 cells were cultured in control conditions (DMSO) or in the presence of the indicated concentrations of resveratrol (RV) and erlotinib (ERL), alone or in combination, for 48 h. And then cells were stained with Annexin V-FITC and analyzed by flow cytometry. This experiment was done in triplicate and representative diagrams of Annexin V-FITC assays are shown. (B) and (C) Quantitative measurement of Annexin V-FITC flow cytometry analyses showed positive apoptotic cells in response to RV and ERL, alone or in combination. Columns, mean; bars, SD. ***, $P < 0.001$, compared with DMSO-treated cells. (D) and (E) H460, A549, PC-9 and H1975 cells were

treated with the indicated concentrations of RV and ERL either alone or in combination for 48 h. Apoptosis was measured by detecting sub-G1 population with propidium iodide (PI) staining and flow cytometry analyses as described in materials and methods. Columns, means of three determinations; bars, SD. Results shown are representative of three independent experiments. ***, $P < 0.001$, compared with DMSO-treated cells.

NSCLC cells with antioxidant N-acetylcysteine (NAC) prior to RV plus erlotinib treatment to determine whether NAC could attenuate cell apoptosis induced by the two drugs. As shown in Fig. 3D and E, pretreatment with NAC significantly decreased cells apoptosis in four NSCLC cell lines. Taken together, these findings supported that RV and erlotinib treatment induced cells apoptosis was associated with ROS production in NSCLC cells.

Resveratrol and erlotinib combination therapy alters the expression levels of apoptotic regulatory factors in NSCLC cells

Since apoptosis is tightly regulated by pro- and antiapoptotic members, the proapoptotic factor p53, BAX, Noxa, PUMA and BIM as well as the antiapoptotic protein Bcl-2, Mcl-1, Bcl-XL and survivin were studied in PC-9 and A549 cells after treatment with RV and erlotinib alone or in combination by Western blot analysis. As shown in Fig. 4A, PC-9 cells treated by RV plus erlotinib manifested increased amounts of the proapoptotic proteins p53 and PUMA as well as major reduction in the levels of the anti-apoptotic protein Mcl-1 and survivin. However, the two drugs in combination failed to affect the expression levels of other apoptosis associated proteins, including the proapoptotic proteins BAX and BIM as well as antiapoptotic protein Bcl-2 (data not shown). Similar results were found in A549 cells (Fig. 4B).

Apoptosis involves initiation, effector and execution phases. To better determine the progression of the apoptotic process in response to these drugs, we evaluated the activities of caspase 3 and caspase 8 by fluorogenic substrate cleavage. As shown in Fig. 4C-F, all NSCLC cells treated with two compounds in combination showed significant increase in caspase 3 activity. In addition, RV plus erlotinib exerted a time-dependent caspase 3 activity increase on NSCLC cells (Fig. 4C-F). However, no changes were found in caspase 8 activities in these NSCLC cells (Fig. 4G-J). Furthermore, PC-9 and A549 cells before treatment of RV and erlotinib were pretreated with z-VAD-fmk, a pan caspase inhibitor. We observed that z-VAD-fmk remarkably attenuated cell apoptosis induced by RV plus erlotinib (Fig. 5). However, the cell apoptosis triggered by the two agents in combination could not be blocked by necrostatin-1 or necrostatin-5, a novel class of potent small-molecule inhibitors of necrosis (Fig. 5). These data supported that the caspase cascade rather than necrosis pathway was involved in the cell death induced by RV combined with erlotinib in NSCLC cells.

Moreover, by Western blot analysis, we detected the status of PARP, which act at later stages of apoptosis. Significant increases in the amounts of cleaved PARP were found in NSCLC cells treated with two agents in combination compared to treatment with single drugs (Fig. 4A and B). Phosphorylated H2AX (γ H2AX) is a robust marker of DNA double strand breaks at later stages of apoptosis [21]. DNA double strand breaks in cells can be estimated by evaluating levels of γ H2AX. As shown in Fig. 4A and B, we observed a synergistic increase in the level of γ H2AX in PC-9 and A549 cells in response to RV plus erlotinib compared to RV or erlotinib alone.

Resveratrol and erlotinib combination treatment is more effective at inhibiting AKT and mTOR signaling pathways in NSCLC cells

RV and erlotinib significantly inhibited the growth of four types of NSCLC cells compared to treatment with either agent alone. Next, we sought to determine the molecular mechanisms by which RV plus erlotinib inhibit cell growth in NSCLC cells. AKT and mTOR

Fig. 3. Resveratrol and erlotinib treatment induce cell apoptosis associated with ROS production in NSCLC cells. (A) The levels of ROS were measured by DCF-DA staining and flow cytometric analyses at 48 h after resveratrol (RV) plus erlotinib (ERL) treatment in H460, A549, PC-9 and H1975 cells. (B) and (C) The levels of ROS were presented as fold change compared to the levels in control cells. Columns, mean of three determinations; bars, SD. Results shown are representative of three independent experiments. **, $P < 0.01$; ***, $P < 0.001$, compared with DMSO-treated cells. (D) and (E) Inhibition of ROS by 5 mM of N-acetylcysteine (NAC) diminishes cells apoptosis induced by RV plus ERL in H460, A549, PC-9 and H1975 cells. **, $P < 0.01$ vs. control; ***, $P < 0.001$ vs. control.

continued

signaling pathways play a critical role in cell survival. Moreover, a recent study reported that RV treatment inhibits PI3K/AKT signalling pathway [22]. Thus, we determined if RV combined with erlotinib is involved in the activation of these pathways. PC-9 and A549 cells were treated with RV and erlotinib either alone or in combination for 48 h. Subsequently, the cells were processed for detecting phosphorylation of AKT, mTOR and S6K by Western blot. Interestingly, as shown in Fig. 6A and B, low dose of RV (15 μ M for PC-9 cells and 20 μ M for

A549 cells) could not significantly suppress the AKT and mTOR activity, as assessed by Western blot for phosphorylation of AKT, mTOR and S6K. Additionally, we observed minimal decreases of phosphorylated AKT, mTOR and S6K with 10 μ M erlotinib in PC-9 and 16 μ M in A549 cells. Notably, the combination of the two agents completely abrogated the expression of phosphorylated AKT and phosphorylated mTOR in both two cells (Fig. 6A and B).

Up-regulation of PUMA and down-regulation of survivin are required for apoptosis triggered by resveratrol combined with erlotinib in NSCLC cells

The data shown above indicated that RV combined with erlotinib increased the PUMA levels and attenuated the survivin expression in PC-9 and A549 cells. To more directly examine the role of PUMA in the apoptotic activity of the RV combined with erlotinib, we knocked down expression of PUMA in PC-9 and A549 cells by using specific siRNA against PUMA, and analyzed cell apoptosis induced by RV and erlotinib alone or in combination by Annexin V-FITC staining. As shown in Fig. 7A, knockdown of PUMA significantly suppressed cell apoptosis induced by RV treatment in combination with erlotinib in NSCLC cells. To evaluate the role of survivin in the apoptotic activity of the two compounds in combination, PC-9 and A549 cells were transiently transfected with overexpressing survivin vector, and then cell apoptosis was analysed in the PC-9 and A549 cells treated with RV and erlotinib in combination. We showed that overexpression of survivin could rescue the cell apoptosis triggered by the two agents in combination (Fig. 7B). Together, these findings suggested that upregulation of PUMA along with reduction of survivin contributed to the sensitivity of NSCLC cells to apoptotic induction after combined treatment with RV and erlotinib.

Discussion

NSCLC is one of the most common malignant tumors in the world, with high rate of recurrence and metastasis. The development of various chemotherapeutic treatments directed against specific target molecules expressed in NSCLC cells is a promising strategy. There have been multiple studies of chemotherapeutics that target EGFR signaling pathway and thereby inhibiting cells viability in NSCLC [3]. However, therapeutic effects for single EGFR inhibitor remain relatively modest unless the EGFR inhibitor is combined with other chemotherapeutics [23]. Traditional Chinese medicine and its more recently derived extracted monomer treatment can improve cancer symptoms and reduce the risk of recurrence of cancer. Among these extracted monomers, RV is a perfect anticancer agent. In this study we sought to assess whether the efficacy of the targeted EGFR inhibitor, erlotinib, against NSCLC could be improved by concurrent treatment with the clinically available agent RV.

Fig. 4. Resveratrol and erlotinib combination therapy alters the expression levels of apoptotic regulatory factors. (A) and (B) PC-9 and A549 cells were plated, treated for 48 h with the indicated concentrations of resveratrol (RV) and erlotinib (ERL) either alone or in combination. Cell lysates were subjected to Western blot analysis with the indicated antibodies. Expression of β -Actin served as a loading control. This experiment was repeated thrice. The data are representative of three independent experiments. (C-F) and (G-J) The caspase 3 and caspase 8 activities were quantified respectively as described under methods. The experiments were repeated thrice. Columns, mean; bars, SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with DMSO-treated cells.

Previous studies revealed that RV treatment induces growth inhibition in breast cancer cells, gastric cancer, head and neck carcinomas [24-26]. Furthermore, RV may exert its

Fig. 5. Resveratrol and erlotinib combination therapy induces caspase-dependent cell apoptosis in NSCLC cells. (A) and (B) PC-9 and A549 cells were pre-treated with 10 mM z-VAD-fmk (zVAD) or 20 μ M necrostatin-1 (Nec1) or necrostatin-5 (Nec5) for 1 h followed by treatment with the indicated concentrations of resveratrol (RV) and erlotinib (ERL) alone or in combination, for additional 48 h. And then the apoptotic cells were determined by Annexin V-FITC staining and flow cytometry analysis. This experiment was repeated thrice. Columns, mean; bars, SD. **, $P < 0.01$.

Fig. 6. The combination of resveratrol and erlotinib inhibits AKT and mTOR signaling pathways. (A) and (B) PC-9 and A549 cells were plated, treated for 48 h with resveratrol (RV) and erlotinib (ERL) either alone or in combination, and the expression levels of AKT, mTOR, S6K, and phosphorylation were determined by Western blot analysis as described under Methods. Expression of β -Actin served as a loading control. This experiment was repeated thrice. The data are representative of three independent experiments.

antitumor and chemopreventive activities via the induction of cell cycle arrest in NSCLC cells [27]. In this study, consistent with previous findings, low doses of RV and erlotinib alone modestly inhibited the cell viability in NSCLC cells. However, RV combined with erlotinib was more effective to decrease cell viability than single agent alone. Combination index data analysis indicated that this combination was highly synergistic at inhibiting NSCLC cells viability. Moreover, clonogenic survival assay results showed the number of positive colonies was remarkably reduced in the cells treated with the two drugs in combination, suggesting that the damage inflicted by the interaction of treatments was chronic and the affected cells were not able to recover. In addition, by Annexin V assay and sub-G1 population analysis we showed that the combination of RV plus erlotinib was synergistic at inducing apoptosis in NSCLC cells. These results raised the possibility that the combination of RV and erlotinib exerted a dual anticancer action in NSCLC cells, which consisted in cell apoptosis induction plus cell-cycle blockade. Additionally, our results showed that the combined treatment

Fig. 7. Up-regulation of PUMA and down-regulation of survivin contribute cell apoptosis triggered by resveratrol combined with erlotinib in NSCLC cells. (A) PC-9 and A549 cells were treated with 100 nM siRNA against PUMA and 100 nM negative control (NC) siRNA for 12 h, and treated with the indicated concentrations of RV and ERL in combination for an additional 48 h. And then the apoptotic cells were determined by Annexin V-FITC staining and flow cytometry analysis. This experiment was repeated thrice. Columns, mean; bars, SD. ***, $P < 0.001$. The knockdown effects on PUMA were confirmed by Western blot analysis. The data are representative of three independent experiments. (B) PC-9 and A549 cells were transfected with control (CTR) vector and survivin-overexpressing vector. After 12 h, cells were treated with the indicated concentrations of RV and ERL in combination for an additional 48 h. And then the apoptotic cells were determined by Annexin V-FITC staining and flow cytometry analysis. This experiment was repeated thrice. Columns, mean; bars, SD. ***, $P < 0.001$. The overexpressing effects on survivin were confirmed by Western blot analysis. The data are representative of three independent experiments.

induced cytotoxic synergism in these EGFR mutants (PC-9 and H1975 cells) and EGFR wild-type (H460 and A549 cells) NSCLC cell lines, suggesting that this co-treatment may overcome different types of TKI resistance. Erlotinib is known to inhibit transmembrane transporters of the ABC family, including the P-gp, MRP1 and BCRP [28]. This raised a possibility that erlotinib incremented the cellular accumulation of RV correlating with its capacity to exacerbate RV effect.

Many chemotherapeutic agents can induce the generation of ROS in cancer cells [29]. Previous studies showed that RV induced the production of ROS in NSCLC cells [15]. To determine whether erlotinib increased the production of RV-induced ROS in NSCLC cells, we investigated the levels of ROS in RV plus erlotinib treated four NSCLC cell lines using DCF-DA staining and flow cytometric assays. Our results showed that erlotinib could increase RV-induced ROS generation. These observations were consistent with our hypothesis. ROS can trigger oxidative DNA damage and lead to DNA double strand breaks, thus causing cell apoptosis [20, 30]. Moreover, we observed that the two drugs in combination strikingly stimulated the expression of γ H2AX, a robust marker of DNA double strand breaks [21]. p53 plays a critical role in the regulation of DNA damage-dependent cell apoptosis. Our results revealed that RV plus erlotinib remarkably induced the expression of p53. This raised a possibility that cell apoptosis induced by RV plus erlotinib was associated with increased

ROS production and DNA damage in NSCLC cells. The important contribution of ROS to cells apoptosis triggered by the two agents was further confirmed by the observations that cells apoptosis were obviously prevented by NAC, an ROS scavenger.

Annexin-V assay and sub-G1 population analysis revealed that RV plus erlotinib induced NSCLC cells apoptosis. In addition, the two agents in combination exerted apoptotic effects, as indicated by the fact that cells death induced by the two agents could be prevented by z-VAD-fmk, a broad-spectrum caspase inhibitor. However, cells death increased by RV and erlotinib in combination failed to be blocked by necrostatin-1 or necrostatin-5, a novel class of potent small-molecule inhibitors of necroptosis [31]. To further address the underlying mechanism of this enhanced cell apoptosis, the activity of caspase 3 and expression of cleaved PARP were examined in NSCLC cells. The caspase 3 activity is induced and PARP is cleaved in the late “execution” apoptotic phase. We showed that the combination of RV and erlotinib synergistically induced the activity of caspase 3 and cleavage of PARP. Notably, caspase 8 activities could not be induced by these agents. Additionally, we found that the combination of RV and erlotinib could lead to marked inhibition of Mcl-1, which would serve to facilitate mitochondria-mediated apoptotic induction.

Our results showed that erlotinib inhibited the expression of p-AKT, p-mTOR and p-S6K, main mediators of AKT/mTOR pathway. Moreover, combined treatment with RV and erlotinib decreased the expression of p-AKT, p-mTOR and p-S6K relative to either agent alone, indicating synergistic suppression of AKT/mTOR pathway. It has been shown that AKT/mTOR pathway is critical for cell survival and resistant to apoptosis [32, 33]. Thus, cells apoptosis and inhibition of cells survival induced by two agents in combination could be involved in inhibiting the AKT/mTOR pathway.

The majority of cancers, including NSCLC, are known to overexpress survivin to protect cells from apoptosis [34]. Levels of survivin in tumors have been reported to be correlated with aggressive phenotypes and a poor prognosis [35]. In this study, we found that RV combined with erlotinib reduced the expression of antiapoptotic protein survivin. To determine the role of survivin in RV plus erlotinib co-treatment, we ectopically expressed plasmids encoding survivin in A549 and PC-9 cells. Results showed that overexpression of survivin reversed cell apoptosis induced by the two compounds combination treatment. Our findings hinted that RV combined with erlotinib induced apoptosis, at least in part, by reducing survivin expression in NSCLC cells. Survivin expression has been reported to be upregulated by AKT [36]. Our results showed that RV combined with erlotinib inhibited the activity of AKT. This raised a possibility that RV plus erlotinib decreased the survivin expression via AKT pathway. Further investigation is needed to study whether AKT upregulated the expression of survivin in this system.

Notably, p53 along with its transcriptional target PUMA were simultaneously upregulated by the two drugs in combination in NSCLC cells. This suggested that RV plus erlotinib potentially induced the p53-dependent PUMA expression. To ask whether the induction of apoptosis by RV in combination with erlotinib was involved in PUMA, PUMA was depleted by using specific siRNA in PC-9 and A549 cells. We found that depletion of PUMA by siRNA attenuated apoptosis of NSCLC cell induced by RV and erlotinib in combination. These results suggested PUMA contributed to the cell apoptosis triggered by the two agents co-treatment.

Here, we explored the effects of RV in combination with the EGFR inhibitor, erlotinib, using *in vitro* models. Synergy was consistently observed in a number of parameters, including cytotoxicity, inhibition of colony formation, cell apoptosis induction and apoptotic protein activation. Moreover, the combination of RV and erlotinib markedly inhibited AKT/mTOR pathway, perhaps accounting in part for these synergistic effects. Moreover, our study results suggested that PUMA-mediated apoptotic pathways, ROS-dependent DNA damage and survivin-dependent apoptotic pathways were involved in RV plus erlotinib-induced cell death in NSCLC cells. Taken together, this study demonstrated for the first time that RV and erlotinib acted synergistically to inhibit human NSCLC cells, suggesting that the two drugs in combination could be exploited as a novel NSCLC therapy strategy.

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Disclosure Statement

The authors have declared that no conflict of interest exists.

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