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ORIGINAL ARTICLE – CANCER RESEARCH

Synergic effects of artemisinin and resveratrol in cancer cells

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Abstract
Purpose The aim of this study was to investigate whether resveratrol (Res) combined with artemisinin (ART) possess synergistic effect on different cancer cells.
Materials and methods The viability of HepG2 and HeLa cells treated with ART and Res was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Combination index (CI) analysis and isobologram were used to assess the synergistic effect of ART and Res in different ratios. Wound-healing assay was used to investigate the migration rate. AO staining and fluorescent microscopy measurements were performed to detect the cell apoptosis. Reactive oxygen species (ROS) was measured with 2′7′-dichlorofluorescein diacetate (DCFH-DA).
Results MTT assay indicated that ART and Res inhibited the growth of HeLa and HepG2 cells in a dose-dependent manner. The combination of ART and Res exhibited the strongest anticancer effect at the ratio of 1:2 (ART to Res). The combination of the two drugs also markedly reduced the ability of cell migration. Apoptosis analysis showed that combination of ART and Res significantly increased the apoptosis and necrosis rather than use singly. Additionally, ROS levels were elevated by combining ART with Res.
Conclusions Taken together, the present study suggested that ART and Res possessed the synergistic anti-tumor effect. ART in combination with Res could be an effective therapeutic strategy for cancer.

Keywords Artemisinin · Resveratrol · Synergistic effect · Migration · Apoptosis · Reactive oxygen species

Introduction

Combination therapy with multiple drugs is becoming a common practice for cancer treatment because combining two or more anticancer agents may get better effect than a single one alone (Baxevanis et al. 2009). The potentially favorable outcomes for synergism include: minimizing or slowing down the development of drug resistance, providing selective synergism against cancer target (or efficacy synergism) versus host (or toxicity antagonism), increasing the efficacy of the therapeutic drugs, and reducing the dose of the drugs, but increasing or maintaining the same efficacy to avoid toxicity (Chou 2006). Therefore, it is necessary to evaluate the potential drug–drug interactions in cancer chemotherapy. A large number of untapped and potential therapeutic molecules can control human cancers with reduced side effects, which usually occur in plant compounds from traditional herbal medicines and the diet (Firestone and Sundar 2009).

Resveratrol (3, 4′, 5-trihydroxystilbene, Res), a phytoalexin, is found mainly in the skin of grapes and red wine. Recent studies showed that Res possessed potent anti-tumor activity against several malignant tumors (Yang et al. 2014; Zhou et al. 2014; Cakir et al. 2011). Res has been shown not only to suppress cell proliferation and induce apoptosis by inducing G0/G1 cell cycle arrest but also to suppress

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constitutively active STAT3 and JAK2 phosphorylation and inhibit BCL-2 expression by down-regulating miR-21 expression (Trung et al. 2013; Liu et al. 2013). In addition, it was suggested that Res blocked the hepatocellular carcinoma cells in the G1 and G2 phase of the cell cycle (Notas et al. 2006). Some studies have demonstrated that Res can serve as either an antioxidant or pro-oxidant relying on the specific microenvironment (Khan et al. 2013). Res inhibited the production of reactive oxygen species (ROS) and modulated the NO/NOS system, either at the protein (enzymatic) or transcriptional level (Notas et al. 2006). It also has been reported that Res can exert protective effects against VNR-induced venous toxicities in ECV-304 cell by increasing the activity of superoxide dismutase (SOD) and attenuating the generation of ROS (Zhang et al. 2013). However, the specifics of what make Res a radical generator with cytotoxicity against cancer cells, and a protective agent for normal cells, is still debatable (Muqbil et al. 2012).

Artemisinin (ART) is a natural product isolated from the plant Artemisia annual or sweet wormwood. ART has been widely used as an antimalarial compound. ART and its derivatives showed the anti-proliferative effects on various tumor cell lines, including cancers of the breast, prostate, colon, and liver (Nakase et al. 2009; Lai et al. 2009; Riganti et al. 2008). The primary mechanism of the anticancer effect of ART is thought to be induction of apoptosis. The activation of caspase-3, the increase in the Bax/Bcl-2 ratio and polyADP-ribose polymerase, and the down-regulation of MDM2 were involved in the apoptosis induced by ART (Tan et al. 2011). Several studies have reported that ART exerted its effect by impairing the cytokinesis, enhancing the levels of oxidative stress and inhibiting the tumor invasion, migration, and metastasis (Xu et al. 2011). Tumor cells showed enhanced vulnerability to ROS damage after treatment with ART. The lower expression of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase were found in cancer cells treated with ART compared to that in normal cells (May et al.1985; Bostwick et al. 2000).

Based on these findings, we hypothesized that combining ART with Res would generate better antitumor effects than treatment with either agent alone. Therefore, a series of studies were performed in vitro to verify this hypothesis. The results provide a scientific basis for the clinic usage of combining ART with Res in cancer therapy.

Materials and methods

Chemical

Resveratrol, Dimethyl sulfoxide (DMSO), trypsin, penicillin, streptomycin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide (MTT) and acridine orange (AO) were purchased from Sigma (St. Louis, MO, USA). Artemisinin was purchased from Aladdin Industrial Corporation (Shanghai, China). Fetal bovine serum was obtained from Tianhang Biotechnology Company (Zhejiang, China). FITC AnnexinV/PI apoptosis assay kit and cell cycle assay kit were purchased from Roche Diagnostics Co., Ltd. (Indianapolis, IN, USA).

Cell cultures

The human cervix carcinoma HeLa and human hepatoma HepG2 cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM supplemented with 10 % fetal bovine serum, at 37 °C in 5 % CO_2.

Cell viability assay

HepG2 or HeLa cells were seeded in 96-well plates, at an initial density of 4×10^4 cells per ml, with 0.1 ml medium per well. The stock solutions of ART and Res [both dissolved in Dimethyl sulfoxide (DMSO)] were then diluted in culture medium to obtain the desired concentrations, and the final concentration of DMSO is less than 0.2 % in culture medium. In order to evaluate the synergistic effects of ART and Res, HeLa cells were treated with Res (20, 40, 80, 160 and 320 μM), ART (20, 40, 80, 160, 320 μM), and ART combined with Res (5 + 10, 10 + 20, 20 + 40, 40 + 80, 80 + 160 μM) for 48 h. And HepG2 cells were treated with Res (10, 20, 40, 80 and 160 μM), ART (10, 20, 40, 80, 160 μM), and ART combined with Res (5 + 10, 10 + 20, 20 + 40, 40 + 80, 80 + 160 μM) for 48 h. Growth and viability of cells were measured by the tetrazolium salt assay. Cell viability was incubated for h with the tetrazolium salt (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide), and metabolically active cells reduced the dye to purple formazan. Dark blue crystals were dissolved with 150 μL DMSO. The absorbance was measured with a BIO RAD microplate reader (model 360, Hercules, California USA) at 490 nm. The percentage of control well was the relative cell viability.

Analysis of drug synergism using the CI method

The combination index (CI) was calculated for the analysis of the synergistic, antagonistic or additive effects of the two drugs (Chou and Talalay 1984). The CI is calculated using the formula: $CI = [(D)_1/(D_x)_1] + [(D)_2/(D_x)_2]$, in which $(D)_1$ is the concentration of the first drug required to achieve a particular effect in the combination; $(D_x)_1$ is the concentration of the first drug that causes an identical effect alone; $(D)_2$ is the concentration of the second drug that achieves a particular effect in the combination; $(D_x)_{2i}$s the concentration of the second drug that generates the same

effect alone. CI > 1 indicates antagonism, CI = 1 indicates an additive effect and CI < 1 indicates synergy.

Evaluation of the combination effect using the isobologram method

Isobologram is another mathematical approach that has been used to determine the drug interactive effects (Tallarida 2001). When the effect is not the goal and it is desired only to assess whether a combination dose is additive, then simple graphical methods may be used. A commonly used method is the isobologram, a graph of equally effective dose pairs (isoboles) for a single effect level. Specifically, a particular effect level is selected, such as 50 % of the maximum, and doses of drug A and drug B (each alone) that give this effect are plotted as axial points in a Cartesian plot (the doses are denoted by italicized letters that correspond to the respective drugs).The straight line connecting A and B is the locus of points (dose pairs) that will produce this effect in a simply additive combination. This line of additivity allows a comparison with the actual dose pair that produces this effect level experimentally. It is notable that some dose combinations may be subadditive while others are either superadditive or additive.

Measurement of apoptosis by fluorescent microscopy measurements

Acridine orange (AO) staining was used for detecting the cell apoptosis and necrosis (Leite et al. 1999). In the present study, HeLa cells were seeded in 6-well plates at a density of 80,000 cells per well. Then, cells were treated with different concentrations of ART and Res (ART: 20, 40, 80 μM; Res: 40, 80, 160 μM; ART + Res: 20 + 40, 40 + 80, 80 + 160 μM and the control group only incubated with culture medium containing less than 0.2 % DMSO) for 24 h. Ten microliters of prepared AO-working solution (100 μg/ml in PBS) was added into each well. The cells were immediately examined with a fluorescence microscope (Olympus U-RFLT50, Tokyo, Japan). Morphologically apoptotic and necrotic cells were counted from 10 visual fields of 5 different areas for each group.

Cell apoptosis analysis by flow cytometry

Annexin V-FITC/PI staining was used to investigate cell apoptosis. Briefly, HeLa cells were seeded in 6-well plates and exposed to different treatments (medium; 160 μM ART); 40 μM Res; 20 μM ART + 40 μM Res). After incubation for 48 h, the floating and trypsinized adherent cells were then collected and prepared for detection. Then, the cells were stained with PI and fluorescein isothiocyanate (FITC)-annexin V and analyzed by the manufacturer's instructions with a FACSCalibur machine (Becton–Dickinson, San Jose, CA, USA).

Wound-healing assay

A cell-based wound-healing assay was performed following well-established methods. Briefly, serum-starved HeLa cells were grown to 90 % confluence, and a linear wound was created in the confluent monolayer using a 200-μL micropipette tip. The cells were then washed with PBS to eliminate detached cells and diluted in serum-free DMEM. Then, HeLa cells were treated with 160 μM ART, 80 μM Res and 40 μM ART + 80 μM Res for 24 h, and the wound edge movement was monitored with a microscope (Olympus U-RFLT50, Tokyo, Japan).

Measurement of ROS production

Detection of ROS accumulation in HeLa cells was performed using oxidation-sensitive probe 2′, 7′-dichlorofluorescin diacetate (DCFH-DA), which can be used to detect oxidative activity in living cells. Following drug treatment, cells in the plates were incubated with 10 μM DCFH-DA at 37 °C for 20 min. After DCFH-DA was removed, the cells were washed and incubated with PBS. The fluorescence intensities of the cells were immediately examined with a fluorescence microscope (Olympus U-RFLT50, Tokyo, Japan). Then, 10 visual fields of 5 different areas for each group were chose the treatment status of each sample, and representative fields were photographed.

Statistical analysis

Data are expressed as the mean ± SD of at least three separate experiments. All statistical analysis was performed using Statistical SPSS software package (version 16.0, SPSS Inc., Chicago, IL). One-way ANOVA and the Student's *t* test were performed. $P < 0.05$ was considered to a statistically significant.

Results

Effects of combination of ART and Res on cell proliferation

To determine the effect of different concentrations of ART and Res on the cell viability in HepG2 and HeLa cells, MTT assay was performed. As shown in Fig. 1, ART and Res significantly inhibited cell viability in HeLa and HepG2 cell lines in a dose-dependent manner. Furthermore, the combination of ART and Res showed significantly higher inhibition rate compared with single-agent treatment in both cell lines. The IC_{50} of ART and

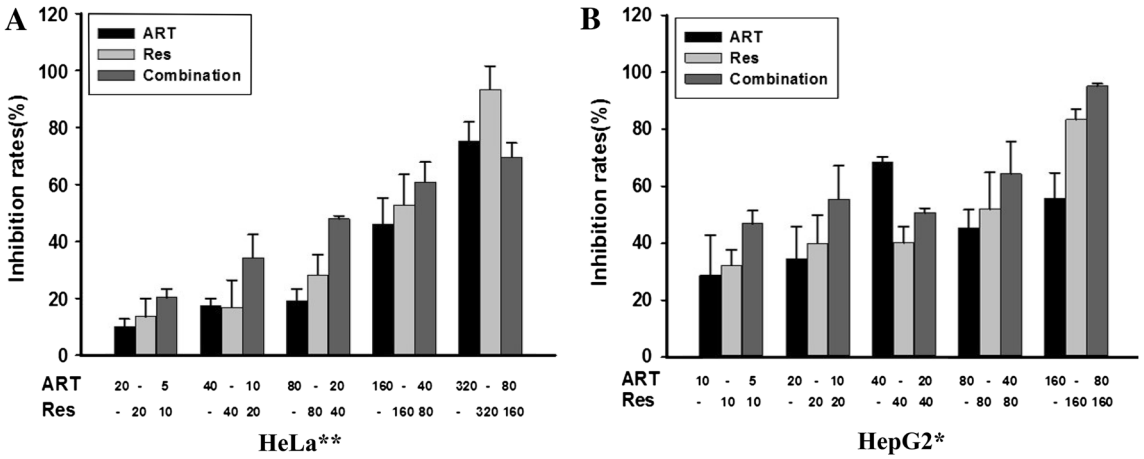

Fig. 1 Inhibition rates of combination of ART and Res on the cell proliferation in HeLa and HepG2 cells. **a** Effects of ART and Res on the proliferation of HeLa cells ($n = 48$). ART and Res, administered singly and in combination for 48 h, significantly inhibited cell proliferation in HeLa cells in a dose-dependent manner. $^{**}P < 0.01$, compared with the control group that was treated with no drugs. **b** The two drugs, administered singly and in combination for 48 h, significantly inhibited cell proliferation in HepG2 cells in a dose-dependent manner. $^{*}P < 0.05$, compared with the control group that was treated with no drugs. All data are mean ± SD from three independent experiments, and *error bars* was represented with (standard deviation) SD

Table 1 The IC_{50} of ART and Res were used alone or combination in Hela cells (a) and HepG2 cells (b)

	Alone	Combination
(A) HeLa		
IC_{50} (μM)		
ART	167.38 ± 3.26	24.65 ± 1.57
Res	98.52 ± 2.13	49.36 ± 2.05
(B) HepG2		
IC_{50} (μM)		
ART	108.15 ± 2.76	9.290 ± 1.33
Res	38.51 ± 1.97	18.59 ± 1.42

Data represent mean ± SD of three independent experiments from five samples for each group

Res in HeLa and HepG2 cells are shown in Table 1. The data suggested that the IC_{50} of the combination of ART and Res is lower than that of each drug used alone.

Synergistic action of ART combined with Res

Drug combination analysis with isobologram was carried out to investigate whether the combination of ART with Res could generate a synergistic effect. As shown in Fig. 2, the Y-axis represented the IC_{50} of Res and the X-axis represented the IC_{50} of ART. The straight line (additivity line) connected the IC_{50} values of ART and Res when the drugs were used alone. The point "a" and "b" represented the IC_{50} of ART and Res in combination group. In the present study, the point "a" and "b" was below the straight line, which indicated that combination of the two drugs might generate a synergistic anti-tumor effect in HeLa and HepG2 cells (Fig. 2). The CI was used to analyze the synergistic effect. The IC_{50} of ART and Res was used to calculate CI. The CI was (24.65/167.38) + (49.36/98.52) = 0.633 in the HeLa cells, indicating that combined ART and Res generated synergistic effect. In the HepG2 cells, the CI was (9.29/108.51) + (18.59/38.51) = 0 .569. These results suggested that combination of ART and Res had the synergistic action in HeLa and HepG2 cells.

Effects of different ratios of ART and Res on the synergistic action in HeLa cells

The classical CI and isobologram method were used to evaluate the drug–drug interaction between ART and Res. The CI values of ART:Res in HeLa cells were 1:2, 1:1, 2:1. The results showed that the value of CI was different with the change of the ratio (Table 2). As shown in Table 2, the CI in the ratio of 1:2 (A:R) was 0.633, indicating that combined ART and Res in the ratio generated stronger synergistic effect than others. Furthermore, we used the classical isobologram to evaluate the drug–drug interaction between ART and Res. The data points of A1R2 at the left of the line (data not shown) indicated that the combined effects of A1R2 have the strongest synergistic effect (Fig. 3).

Effects of ART and Res on cell apoptosis

To assess whether the decrease in proliferation was mediated by inducing apoptosis, AO staining was used. Cell apoptosis was found in HeLa cells after treatment with ART

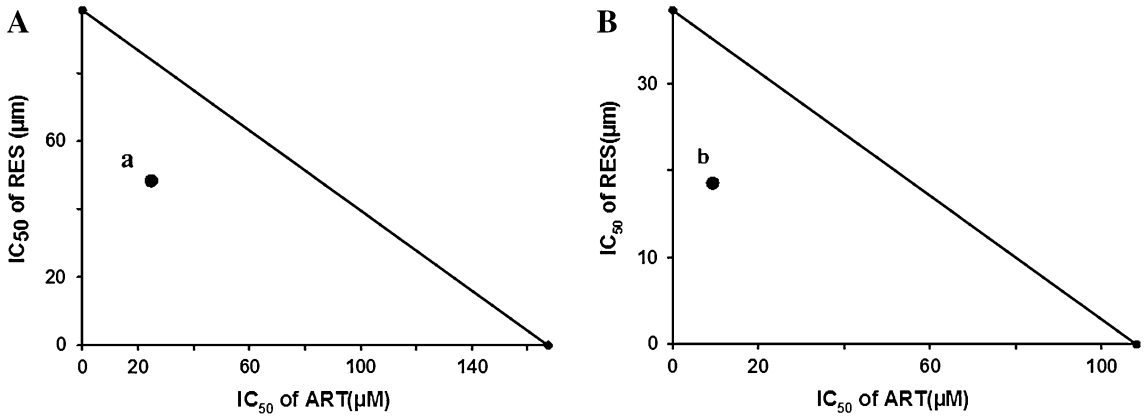

Fig. 2 The synergistic effects of ART and Res in HeLa and HepG2 cells were evaluated by Isobolograms. **a** The isobologram of ART and Res in HeLa cells. The points plotted on the isobolograms were based on the results of cytotoxicity assays from Table 1. **b** The isobologram of ART and Res in HepG2 cells. The points plotted on the isobolograms were based on the results of cytotoxicity assays from Table 1

Table 2 CI values of different ratios

Data represent mean ± SD of three independent experiments from five samples for each group

ART:Res (ratio)	1:2	1:1	2:1	Alone
IC_{50}(μM)				
ART	24.65 ± 1.57	56.86 ± 2.43	102.29 ± 3.15	167.38 ± 1.53
Res	49.36 ± 2.05	56.86 ± 2.43	51.15 ± 2.87	98.52 ± 3.06
Value of CI	0.633	0.917	1.13	

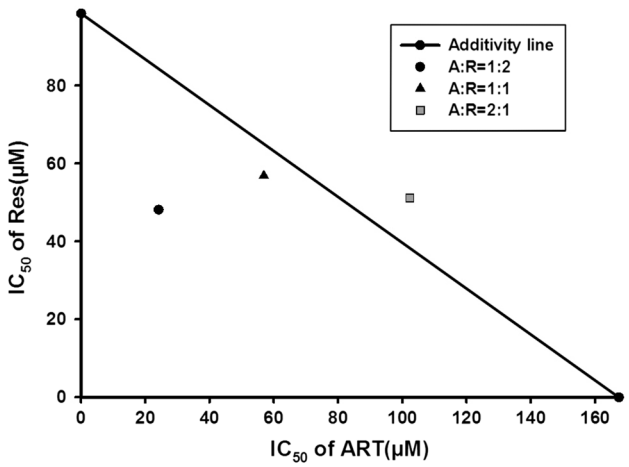

Fig. 3 The isobologram of ART and Res in different ratios. The *straight line* (additivity line) connects the IC_{50} values of ART and Res when the drugs are used alone, and the points plotted on the isobolograms were based on the results of cytotoxicity assays from Table 2

and Res alone or together (Fig. 4). AO staining showed that ART and Res resulted in morphological alterations of apoptosis, such as cytoplasmic vacuoles, nuclear condensation and fragmentation. As shown in Fig. 4, the apoptotic rate in the combination group was 68.74 %, and the single group was only 34.96 %. The results suggested that the number of apoptotic cells was increased significantly in the combination group compared with the single treatment group in a concentration- dependent manner.

Flow cytometric analysis of ART and Res-induced apoptosis

To further detect the ART and Res-induced cellular apoptosis, the flow cytometric analysis was performed. As shown in Fig. 5, the percentage of terminal apoptotic cells in the control group was 0.126, 0.863 % of apoptotic cells was found after treatment with 160 μM ART. Interestingly, 6.66 % of apoptotic was found in cells treated with 20 μM ART + 40 μM Res. Moreover, the early apoptotic rate of the combination group was also higher than that in the single drug treatment group (Fig. 5). These results indicated that combination treatment with ART and Res increased the apoptotic rate of cancer cells in different periods.

Combination of ART and Res suppressed the aggressive migration

To investigate the effect of combination of ART and Res on cancer cell migration, wound-healing assay was applied. The results showed that combination treatment with ART and Res for 12 h and 24 h significantly decreased cell migration than the single treatment group (Fig. 6). After treatment with drugs for 24 h, a wound in HeLa cells was almost closed, while

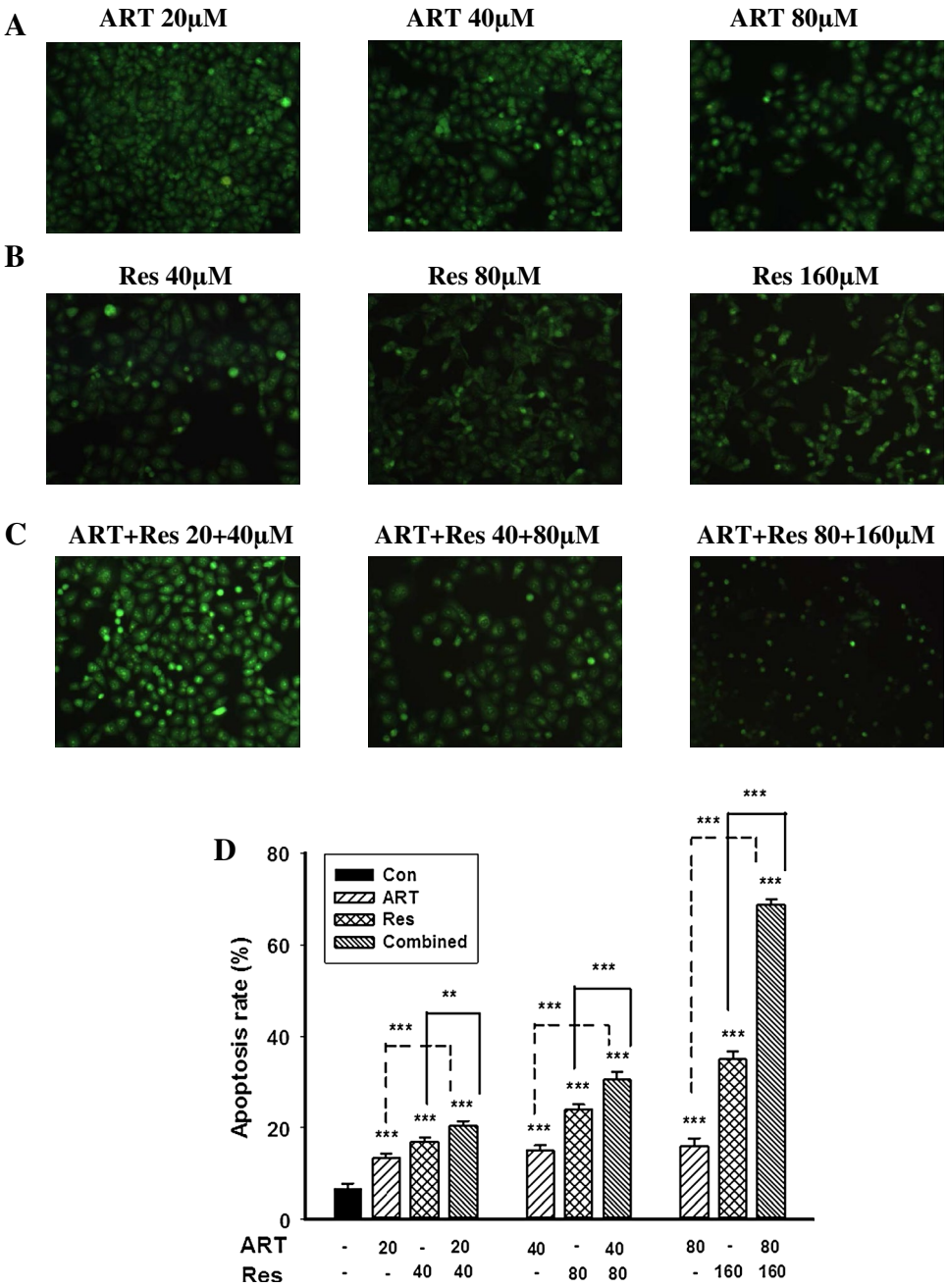

Fig. 4 Apoptosis was assessed in HeLa cells by AO staining. **a** HeLa cells were treated with ART (20, 40, 80 μM) for 48 h. **b** HeLa cells were treated with Res (40, 80, 160 μM) for 48 h. **c** HeLa cells were treated with ART + Res (20 + 40, 40 + 80, 80 + 160 μM) for 48 h. **d** Apoptosis induced in ART-, Res- or ART + Res-treated cells. In the combination group, the apoptosis rates were increased compared with the single treatment groups, $^{**}P < 0.01$, $^{***}P < 0.001$. Data are mean ± SD from three independent experiments, and *error bars* was represented with (standard deviation) SD

cells treated with combination of ART and Res still showed a noticeable wound. Results from both the wound-healing assays suggested that combination of ART and Res had a potentially inhibiting effect on metastasis in HeLa cells.

Combination of ART and Res increased the ROS production in HeLa cells

In order to investigate whether ROS was involved in drug-induced apoptosis, the intracellular ROS was detected in HeLa cells treated with 160 μM ART, 80 μM Res and 40 μM ART + 80 μM Res for 24 h, respectively. ROS production was monitored by detecting the fluorescence from the reaction of intracellular ROS with DCFH-DA using laser confocal microscopy. As shown in Fig. 7, the fluorescent intensity of the combination group was increased than that of ART or Res used alone, which indicated that combination treatment with ART- and Res-induced ROS formation.

Discussion

The present study suggested that artemisinin (ART) acted synergistically with resveratrol (Res) to inhibit the growth

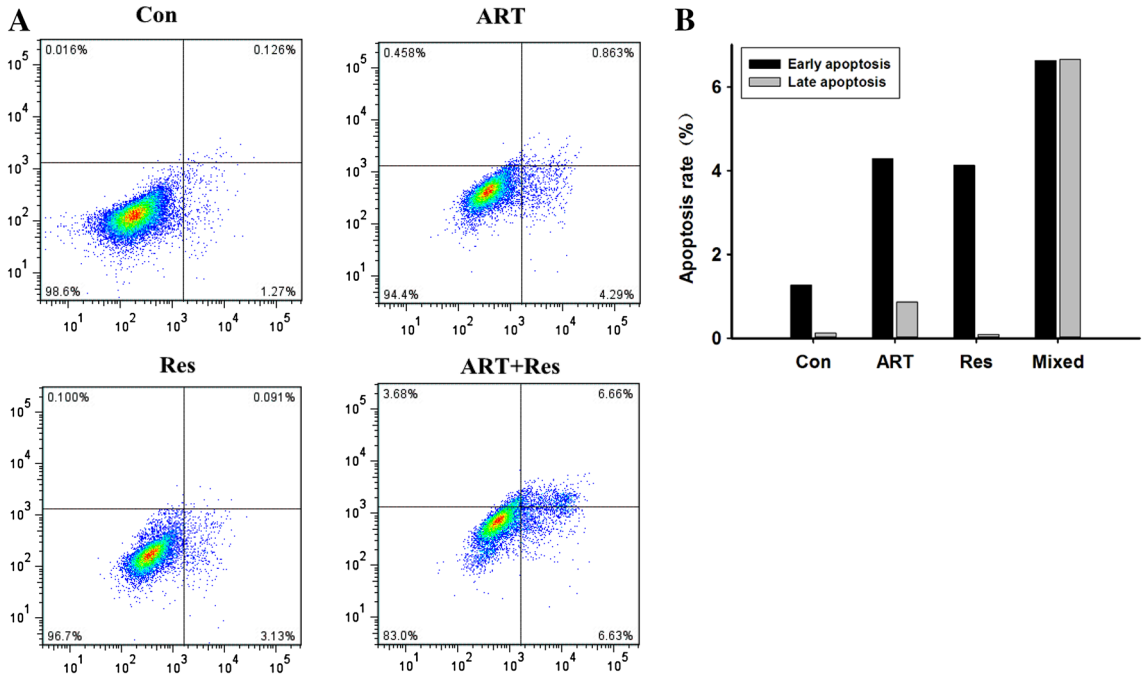

Fig. 5 Cell apoptosis analysis by flow cytometry. **a** Flow cytometric analysis of ART, Res and mixed drug-induced apoptosis in HeLa cells using FITC-annexin V/PI staining. HeLa cells were treated with ART (160 μM), Res (40 μM) and ART + Res (20 + 40 μM) for 48 h and stained with FITC-annexin V/PI. Apoptotic and necrotic cell populations were analyzed by flow cytometry. Cells in the lower right quadrant represent early apoptotic cells, and cells in the *upper right quadrant* represent late apoptotic cells undergoing secondary necrosis. **b** The early and late apoptotic rates of HOS cells induced by ART

of HeLa and HepG2 cancer cells. The cell proliferation assay showed that ART in combination with Res significantly increased the anti-proliferation effect on cancer cells in a dose-dependent manner. Furthermore, the isobologram and CI analysis indicated that combining ART with Res generated a synergistic effect. Moreover, ART and Res showed the strongest synergistic effect when the combination ratio was ART:Res = 2:1. The results from the fluorescent microscopy measurements and flow cytometry also demonstrated that combining ART with Res enhanced the apoptosis and necrosis in HeLa cells. DCF fluorescence staining revealed that ROS levels were enhanced by combining ART and Res. The migration rate was also inhibited in HeLa cells treated with ART and Res in wound-healing assays.

ART showed potent cytotoxic activity against the malaria-causing *Plasmodium sp*. ART was regarded as the first line therapeutic drug used in combination with other antimalarial compound (World Health Organization 2006). Recent researches mainly focussed on the anticancer potential of the ART. It has also been reported that ART was used successfully in patients with metastatic melanoma (Berger et al. 2005; Xu et al. 2007). As an anticancer drug, the advantage of ART is not only the potential to kill cancer cells but also the low toxicity to normal cells (Lai et al. 2013). Res possessed chemopreventive and cytostatic properties against a variety of human tumors, including leukemia. Res showed the antioxidant, anti-inflammatory, and anti-mutagenic properties. Furthermore, Res also inhibited the function of cyclooxygenase and hydroperoxidase (anti-promotion activity) and induced the cell differentiation (anti-progression activity) (Aggarwal et al. 2004; Aziz et al. 2003). In addition, Res significantly weakened QT prolongation induced by As_2O_3, abnormalities of structure and oxidative damage in the heart (Zhao et al. 2008). Nevertheless, a number of researches suggested that the natural chemopreventive agent (Res) was still unknown for its chemotherapeutic potential. These researches strongly suggested that Res merited further investigation as a chemopreventive or chemotherapeutic agent in cancer.

Here we reported for the first time that combined treatment with ART and Res caused a synergistic anti-proliferative effect in HeLa and HepG2 cells. Meanwhile, more apoptosis was found in the ART and Res treatment group. These drugs have established therapeutic roles in their respective indications. However, their potentiality in using together still not been explored. The present study investigated the anti-proliferative effects of ART and Res on cancer cell lines. Meanwhile, we assessed the effects of the combination usage of ART and Res on the cell growth, migration, apoptosis and ROS level. Additionally, the value of drug combination strategies was explored. Currently,

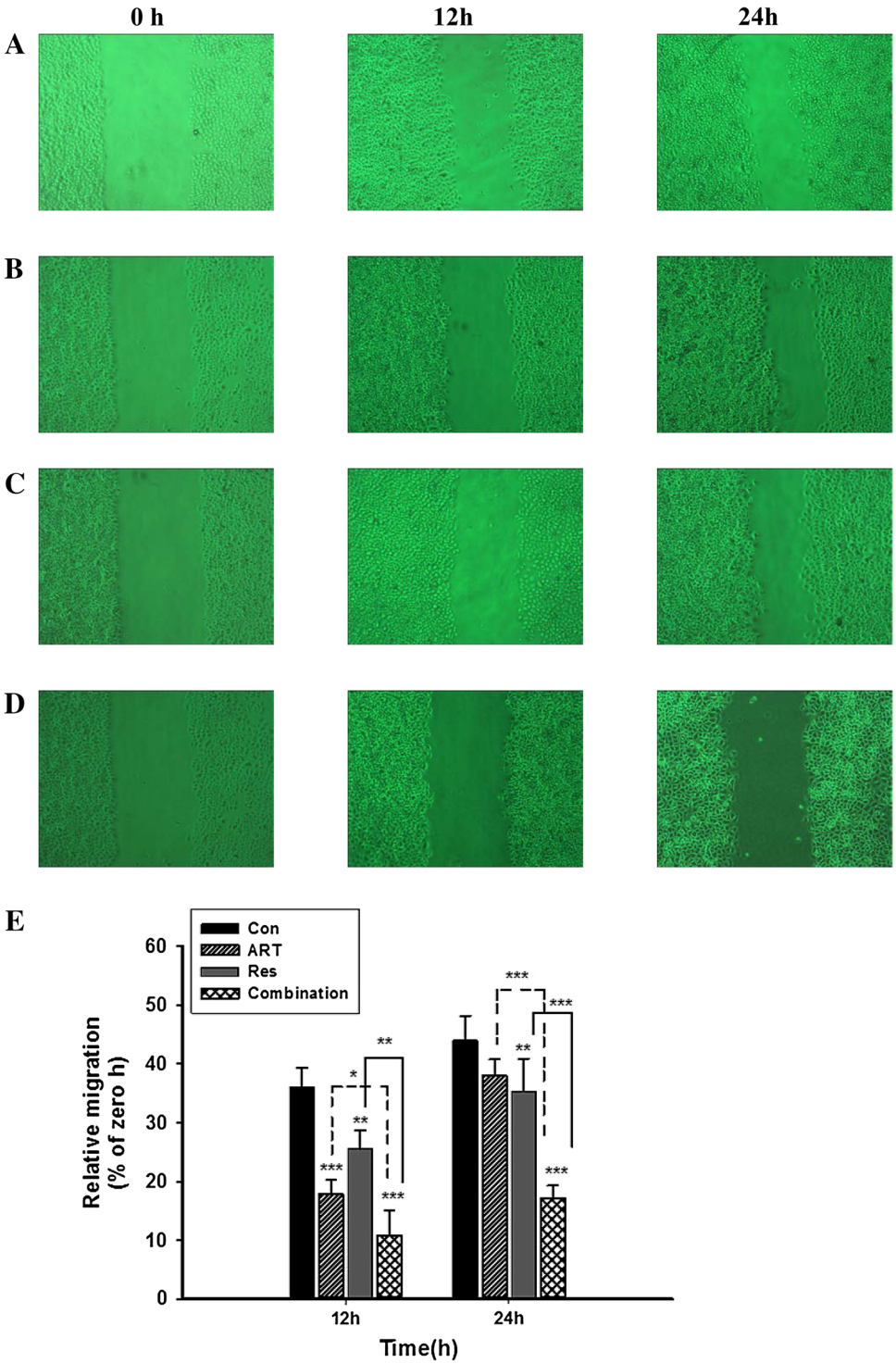

Fig. 6 Down-regulation of the migration phenotypes in HeLa cells by combination of ART and Res. For the wound-healing assay, 90 % confluent cultures were scraped to form a wound and then exposed to ART (160 μM), Res (80 μM) and ART + Res (40 + 80 μM) at the indicated concentrations for 12 and 24 h. **a** Blank control. **b** HeLa cells were preincubated with ART (160 μM). **c** Cells were treated with 80 μM Res. **d** HeLa cells were treated with ART combination with Res (40 + 80 μM). **e** Migration induced in ART-, Res- or ART + Res-treated cells. The combination group compared with the single treatment groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are mean ± SD from three independent experiments, and *error bars* were represented with (Standard deviation) SD

a number of phase I trials assessed the activity and tolerability of ART in patients with the colon and breast cancer. These studies endorsed the validity of this drug as a therapeutic candidate (Liu et al. 2011).

The present study suggested that the combination of ART and Res exhibited the anti-proliferative effect in HeLa and HepG2 cells by inducing apoptosis. MTT assays demonstrated that ART and Res treatment showed a dose-dependent reduction in cell viability in HeLa and HepG2 cells. The IC_{50} of the combination of ART and Res was lower than the IC_{50} of the drugs used singly. The primary aim of this study was to explore the therapeutic potential of ART and Res in combination in cancer. Thus, we evaluated the drug–drug interaction between ART and Res using the

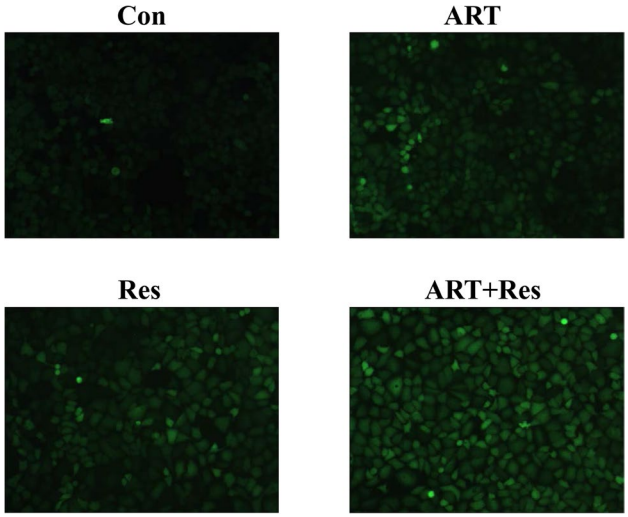

Fig. 7 Intracellular reactive oxygen species (ROS) changes after treatment with ART and Res in HeLa cells. Cells were treated with different concentrations of ART (160 μM), Res (80 μM) and ART + Res (40 + 80 μM) for 24 h and imaged by fluorescence microscope at a magnification of ×10. **a** Blank control. **b** HeLa cells were treated with ART (160 μM). **c** Cells were treated with 80 μM Res. **d** HeLa cells were treated with ART combination with Res (40 + 80 μM)

CI method and isobologram methodology. The foundation of both CI and isobologram was $E = D/(D_{50} + D)$, where D is the concentration, D_{50} is the concentration that produce 50 % survival, and E is the effect (Zhao et al. 2004). And, CI provided an exact value for comparing the synergy levels among different ratios, and isobologram was a qualitative method for judgment of the synergy, additive or antagonistic in the fix ratio. Notably, the present study demonstrated that combining ART with Res generated a synergistic anticancer effect in HeLa and HepG2 cells. Nevertheless, our data suggested that not all combination of ART and Res had the same synergistic effect. The optimal combination rate of drug–drug interaction was ART:Res = 1:2. Furthermore, we investigated the migration of single or combination group in HeLa cells. Combination of ART and Res inhibited the migration significantly than that in single group. Although the mechanism is still unclear, Res has received wide attention for its ability to induce cancer cell apoptosis and inhibit cell migration in many different cancer cell lines (Frazzi et al. 2013; Vishchuk et al. 2013). Currently, the activity of ART has been associated with interference of principal signaling pathways (Xu et al. 2007), anti-metastasis (Rasheed et al. 2010), DNA damage (Li et al. 2008), and cell cycle modifications (Hou et al. 2008). ART was able to induces a G1-phase arrest to inhibit cancer cell growth in part by down-regulating the transcript and protein levels of the CDK2 and CDK4 (Willoughby et al. 2009; Tin et al. 2012). Res, an effective chemopreventive agent, prevented different cancers by hindering the initiation, promotion, and progression of the tumors (Zhu and Huang 2011; Jang et al. 1997). Res treatment inhibits the hepatocellular carcinoma cells growth and induces the apoptosis, which is mainly through blocking cells in the G1 and G2 phase of the cell cycle (Notas et al. 2006). The present results showed that ART and Res synergistically accelerated the rate of cells apoptosis in various different periods (Fig. 4). Notably, these data suggested that the induction of apoptosis might be one of the mechanisms of the combination of ART and Res.

Excessive intracellular ROS production induced by a toxicant within mitochondria or the cytoplasm can damage many types of biological macromolecules, such as membrane lipids, DNA, and enzymes. Furthermore, ROS can also induce mitochondrial depolarization and permeability transition (Fatemi and Izadiyan 2011). ART contains an endoperoxide bridge, which reacts with ferrous iron. It can generate free radical such as reactive oxygen species (ROS), which leads to macromolecular damage and cell death (Wang et al. 2012). In the present study, DCF fluorescence revealed that ROS levels in combination-treated HeLa cells were much higher than those of the control group and single drug group, suggesting that combination of ART and Res caused oxidative stress, mitochondrial permeability transition, and then caused apoptosis in HeLa cells. Therefore, we can assume that excessive ROS induced by combination of ART and Res may be a key early factor of cellular damage and apoptosis in HeLa cells.

Several studies have reported that ART exerted its effect by inhibiting the tumor invasion, migration and metastasis (Xu et aL. 2011). The present study also suggested that combination of ART and Res inhibited the migration rate in HeLa cells. E-cadherin is a transmembrane molecule involved in cell-to-cell adhesion. Proteolytic enzymes such as MMPs also act on extracellular matrix degradation, which promotes tumor migration, invasion, and metastasis. Meanwhile, TIMPs can counterbalance the activities of MMPs to minimize matrix degradation and metastatic potential (Canel et al. 2013). It has been reported that ART inhibited metastasis of cancer cells through significantly up-regulating E-cadherin and down-regulating the levels of both MMP2 and TIMP-2 in hepato-carcinoma cells (Tan et al. 2011). Up-regulating E-cadherin and down-regulating the levels of both MMP2 and TIMP-2 may be one of the molecular mechanisms of the synergistic effects of ART and Res. Further studies would be performed to test this hypothesis.

In conclusion, the present study confirmed that combining ART with Res generated a synergism effect in vitro model of HeLa and HepG2 cells. Additionally, the following fluorescent microscopy measurements and cytometry demonstrated that ART and Res effectively inhibited the

proliferation of cancer cells and enhanced the migration, apoptosis, necrosis, and ROS level. Combining ART with Res is a hopeful strategy in clinical therapy of solid tumor. Further investigation will be performed on the molecular mechanism of combining ART with Res in vitro and in vivo.

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Conflict of interest The authors declare no conflicts of interest.

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