Original Article

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Combination treatment of ligustrazine piperazine derivate DLJ14 and adriamycin inhibits progression of resistant breast cancer through inhibition of the EGFR/PI3K/Akt survival pathway and induction of apoptosis

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Summary **A ligustrazine (TMP) derivative, (E)-2-(2, 4-dimethoxystyryl)-3,5,6-trimethylpyrazine (DLJ14) was synthesized for the improvement of low bioavailability and short half-life of ligustrazine. We have observed potential reversal effects of DLJ14 on adriamycin (Adr)-resistant human myelogenous leukemia cells (K562/A02) and Adr-resistant human breast cancer cells (MCF-7/A) *in vitro* or *in vivo* in previous studies. The aim of the present study was to investigate the underlying molecular mechanism of DLJ14 and Adr combination treatment on Adr-resistant human breast cancer. Inhibition of cancer cell growth was estimated by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cell cycle distribution was analyzed by flow cytometry and apoptosis determined using Annexin V-FITC/propidium iodide (PI) double staining and Hoechst 33258 nuclear staining. The expression of proteins in the epidermal growth factor receptor (EGFR)/phosphatidylinositol-3 kinase (PI3K)/Akt survival pathway and mitochondrial-mediated apoptosis pathway were measured by Western blotting analysis. Results showed that DLJ14 and Adr combination treatment exhibited stronger inhibition of the survival of MCF-7/A cells than Adr treatment alone. This effect might be associated with its role in cell cycle arrest and apoptosis induction. DLJ14 combined with Adr induced cell cycle arrest in the G2/M-phase by activating $p21^{wafl/cip1}$ and p53 in mitochondria and increased cleavage of caspase-9 and caspase-3, and Bax/Bcl-2 ratio. Mitochondrial membrane potential (MMP) disruption and cytochrome c (Cytc) release from mitochondria to cytosol suggested that apoptosis induction might be mediated by the mitochondrial pathway. Moreover, the combination of DLJ14 and Adr could down-regulate the expression of EGFR, p-EGFR, PI3K, and p-Akt in MCF-7/A cells. Overall, DLJ14 and Adr combination treatment may inhibit proliferation of Adr-resistant human breast cancer cells through inhibition of the EGFR/PI3K/Akt survival pathway and induction of apoptosis *via* the mitochondrial-mediated apoptosis pathway.**

Keywords: Ligustrazine piperazine derivative DLJ14, resistant human breast cancer, EGFR/PI3K/Akt, mitochondrial-mediated apoptosis pathway, cell cycle

1. Introduction

The development of multidrug resistance (MDR) represents a major obstacle for successful cancer chemotherapy (*1*). For example, the severe MDR of adriamycin (Adr), which is one of the most effective anticancer drugs for various types of cancers, has limited its therapeutic effectiveness (*2*). Great efforts have been made in development of agents which could improve the efficacy of Adr against Adr-resistant cancer cells.

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(E)-2-(2,4-dimethoxystyryl)-3,5,6-trimethylpyrazine (DLJ14), a novel ligustrazine (TMP) piperazine derivate, was synthesized for the improvement of low bioavailability and short half-life of ligustrazine. Our preliminary results showed that DLJ14 efficiently enhanced anti-proliferative properties of Adr against Adr-resistant human myelogenous leukemia cells (K562/A02) and Adr-resistant human breast cancer cells (MCF-7/A) *in vitro* and *in vivo* through modulating the glutathione S-transferase (GSTπ)-mediated pathway (*3,4*). However, the molecular mechanisms underlying the enhancement effects of DLJ14 and Adr combination treatment on cancer cells have not been thoroughly investigated.

In previous studies, we observed that DLJ14 significantly increased the expression of cellular c-Jun NH_2-terminal kinase (JNK) and p-JNK in both K562/A02 cells and MCF-7/A cells, especially in the presence of Adr. The JNK signaling pathway is mainly activated by epidermal growth factor receptor (EGFR) (*5*). EGFR is over-expressed in all subtypes of breast cancer and could activate the phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway. In cancer cells, dysregulation of PI3K/Akt signaling pathways is associated with deficiency of apoptosis and the phenotype of multidrug resistance (*6*). PI3K/Akt might exert many anti-apoptotic functions, such as Bax, Bad, and caspase-9 as well as inactivation of pro-apoptotic transcription factors like p53 (*7*). Apoptosis has been accepted as a fundamental component in the pathogenesis of cancer. It is mediated by caspases which can be activated through two pathways, the extrinsic pathway characterized by activation of cell-surface death receptors (tumor necrosis factor receptor, Fas) and the intrinsic pathway depending on release of mitochondrial factors (*8*).

In this study, we investigated the effects of DLJ14 on the survival pathway of EGFR/PI3K/Akt and the Adr-induced mitochondrial-mediated apoptosis pathway in MCF-7/A cells. Our results showed that DLJ14 and Adr combination treatment may inhibit proliferation of Adr-resistant human breast cancer cells through inhibition of the EGFR/PI3K/Akt survival pathway and induction of apoptosis *via* the mitochondrial-mediated apoptosis pathway.

2. Materials and Methods

2.1. *Chemical*

(E)-2-(2,4-dimethoxystyryl)-3,5,6-trimethylpyrazine (DLJ14, CAS1000672-75-2) was synthesized at the Institute of Medicinal Chemistry, School of Pharmaceutical Sciences, Shandong University, China. It was dissolved in dimethylsulfoxide (DMSO, St. Louis, MO, USA) for *in vitro* assays.

2.2. *Drugs and reagents*

Adriamycin (Adr) was purchased from Zhejiang Hisun Pharmaceutical Co. Ltd. (Zhejiang, China). RPMI-1640 medium was purchased from GIBCO BRL (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO), propidium iodide (PI), RNase, trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Hoechst 33258 was purchased from Sigma (St. Louis, MO, USA). Annexin V-FITC and PI apoptosis detection kit was purchased from Life Technologies Corporation (Grand Island, NY, USA). Mitochondria/cytosol kit and 5,5',6,6'-tetrachlo-1,1',3,3'-tetraethyl benzimidazolcarbocyanine (JC-1) kit were purchased from Beyotime Biotech Inc. (Shanghai, China). Monoclonal antibody of phosphor-JNK was purchased from Santa Cruz Biotechnology (Santa Cruz, Califonia, USA). Monoclonal antibodies against Bax, Bcl-2, caspase-9, cytochrome c, p53, $p21^{wafl/cip1}$, EGFR, phosphor-EGFR, Akt, phosphor-Akt, PI3K, and cyclin A were purchased from Cell Signaling Technology (CST, Boston, MA, USA). Monoclonal antibody against β-actin was purchased from ZS Bio (Beijing, China). Caspase-3 polyclonal antibody was purchased from Boster Biotech Inc. (Wuhan, Hubei, China).

2.3. *Cell lines and cell culture*

The Adr-resistant human breast cancer cells (MCF-7/A) were obtained from the Institute of Hematology of Chinese Academy of Medical Sciences (Tianjin, China). MCF-7/A cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humid atmosphere (5% CO_2 - 95% air) The cells were maintained in medium containing 1 μmol/L adriamycin and cultured in drug-free medium for 2 weeks prior to experiments.

2.4. *Cell proliferation assay*

Cell viability was measured by MTT assay as described elsewhere (*9*). Briefly, MCF-7/A cells were seeded into 96-well plates (3.0×10^3 /well) and cultured in the presence or absence of DLJ14 (10 μM, 20 μM) and/or Adr (5 μM) for 48 h. MTT solution (5 mg/mL) was added to each well for 4 h. Then 150 μL DMSO was added to dissolve the formazan precipitate before absorbance was measured at 570 nm using a THERMOmax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.5. *Analysis of cell apoptosis by change of nuclear morphology*

Hoechst 33258 staining was used to visualize change of nuclear morphology and apoptotic body formation. MCF-7/A cells were seeded into 96-well plates (3.0×10^3 /well) and cultured in the presence or absence of DLJ14 (10 μM, 20 μM) and/or Adr (5 μM) for 48 h.

Then cells were washed twice with PBS and fixed with methanol-glacial acetic acid (3:1) for 10 min, followed by staining with Hoechst 33258 in the dark at room temperature for 10 min. After three washes with PBS, cells were visualized with the fluorescence microscope (excitation, 340 nm; emission, 460 nm) (NIKON, Ti-U, Tokyo, Japan). Viable cells were identified by intact nuclei with blue fluorescence, apoptotic cells by chromosome condensation and fragmentation of nuclei, exhibiting highlight staining (*10*).

2.6. *Analysis of cell apoptosis by annexin V cell surface binding*

MCF-7/A cells seeded in 6-well plates (2.0×10^5/well) were incubated with DLJ14 (10 μM, 20 μM) and/or Adr (5 μM) for 48 h in the presence or absence of 40 μM SP600125 (a JNK inhibitor). Both floating and attached cells were harvested and washed with cold PBS. Cell surface phosphatidylserine in apoptotic cells was quantitatively estimated using an Annexin V/FITC and PI apoptosis detection kit according to the manufacturer's instructions with a FACSVantage flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA, USA) using emission filters of 525 and 575 nm, respectively (*11*).

2.7. *Cell cycle analysis*

MCF-7/A cells were synchronized by 24 h of growth in 0.5% serum medium, then exposed to 10% serum medium containing DLJ14 (10 μM, 20 μM) and/or Adr (5 μM) for 48 h. Cells were harvested, washed twice with cold PBS and fixed with precooled 70% ethanol at 20°C overnight. Fifty μg/mL DNase-free RNase A was then added for 30 min at 37°C before addition of PI, at a final concentration of 50 μg/mL for DNA staining in the dark at 4°C for 45 min. For each sample, more than 1×10^4 cells were analyzed using a FACScan Flow cytometer. The distribution of cell cycle stages was determined using ModFit LT software 3.0 (Varity Software House, Topsham, USA).

2.8. *Measurement of mitochondrial membrane potential (MMP)*

Mitochondrial membrane potential ($\Delta\Psi m$) was measured using the fluorescent, lipophilic and cationic probe JC-1 according to manufacture's directions. Cells seeded in 6-well plates (2.0×10^5/well) were treated with DLJ14 (10 μM, 20 μM) and/or Adr (5 μM) for 48 h. Cells were harvested and stained with JC-1 for 20 min at 37°C in the dark. Then stained cells were centrifuged and the pellet was washed with serum free medium and resuspended in JC-1 staining buffer. Intracellular fluorescence intensity of JC-1 was quantified by FACScan Flow cytometer.

2.9. *Preparation of mitochondria and cytosol*

A mitochondria/cytosol isolation kit was used to separate mitochondria and cytosol of MCF-7/A cells according to the manufacture's protocol. After treatment with DLJ14 (10 μM, 20 μM) and/or Adr (5 μM) for 48 h, cells (1.0×10^7) were collected and suspended in 400 μL of isolation buffer containing protease inhibitors and lysed on ice for 10 min. After mechanical homogenization with a Dunce grinder, a mixture containing unbroken cells, debris and nuclei was separated by centrifugation at 800× g for 10 min at 4°C. The supernatants were centrifuged at 12,000 g for 15 min at 4°C to obtain pellets of mitochondria which were dissolved in 30 μL of lysis buffer and cytosol supernatant. The mitochondria and cytosol were used for analysis of cytochrome c by Western blotting assay.

2.10. *Western blotting assay*

MCF-7/A cells seeded in 6-well plates (3.0×10^5/well) were treated with DLJ14 (10 μM, 20 μM) and/or Adr (5 μM) for 48 h. Cells were harvested and cell lysates were subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) followed by protein transfer to a PVDF membrane and probed with monoclonal antibodies against phosphor-JNK, Bax, Bcl-2, caspase-3, caspase-9, cytochrome c, p53, $p21^{wafl/cip1}$, EGFR, phosphor-EGFR, Akt, phosphor-Akt, PI3K, cyclin A, and β-actin. Immunoblots were developed with horseradish peroxidase-conjugated secondary antibodies, and visualized using an enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA) and quantified by densitometry using a ChemiDoc XRS (Bio-Rad, Berkeley, California, USA). The band density was normalized to β-actin. The percentage increase or decrease of the proteins was estimated by comparison to vehicle control (100%).

2.11. *Statistical analysis*

Data are expressed as mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple range tests using the SPSS/Win 13.0 software. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. *Anti-proliferation effect of DLJ14 and Adr combination treatment in vitro*

The inhibitory effect of DLJ14 and Adr combination treatment on MCF-7/A cells was evaluated using MTT assay. As shown in Figure 1A, combination treatment of DLJ14 (20 μM) and Adr (5 μM) on MCF-7/A cells resulted in significant inhibition of cell proliferation ($p <$

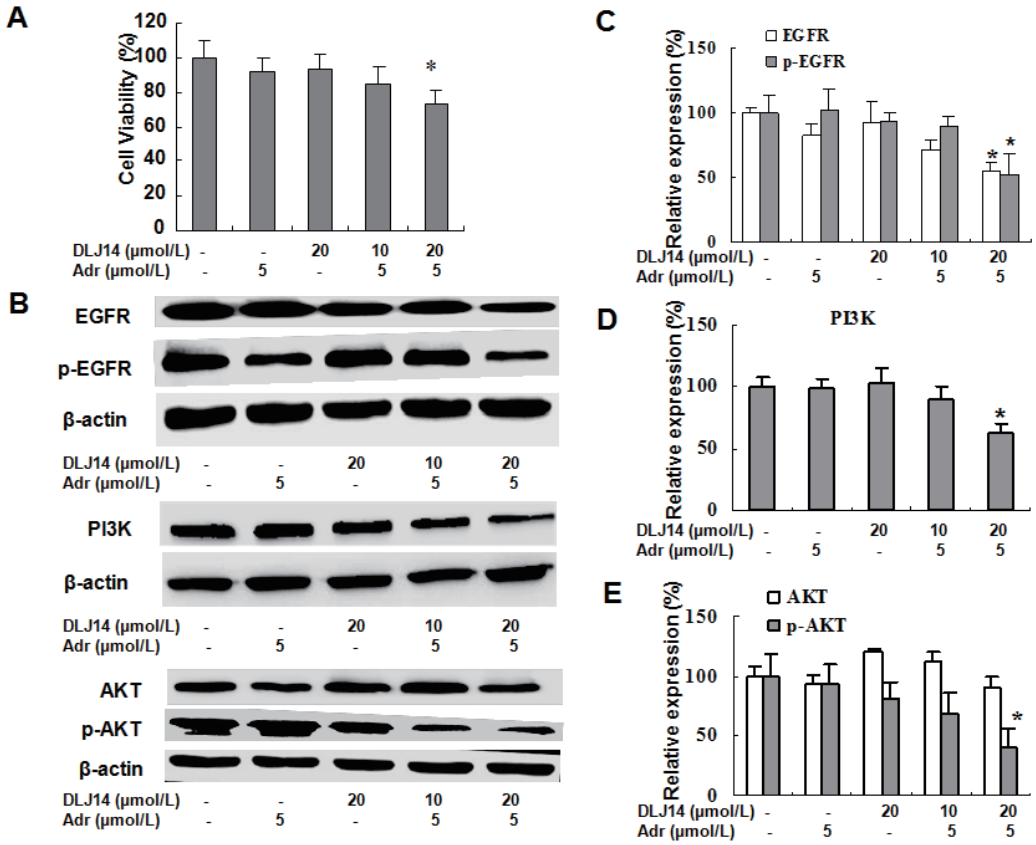

Figure 1. DLJ14 enhanced Adr-mediated anti-proliferation on MCF-7/A cells and regulated the expressions of EGFR, PI3K, and AKT. MCF-7/A cells were treated with DLJ14, Adr, or the combination treatment of DLJ14 and Adr for 48 h and cell viability was determined by MTT assay **(A)**. The expression of EGFR, p-EGFR, PI3K, total Akt, and p-Akt in MCF-7/A cells were detected by Western blotting method. Representative blots of three independent experiments are shown in **(B)**. Quantification of the protein levels of EGFR and p-EGFR, PI3K, and total Akt and p-Akt were normalized to the expression of β-actin and are shown respectively in **(C)**, **(D)**, and **(E)**. Data are expressed as means ± SEM ($n = 3$). * $p < 0.05$ *vs.* Adr treatment alone.

0.05 *vs*. the Adr treatment alone group).

3.2. *DLJ14 and Adr combination treatment inhibits the EGFR/PI3K/Akt survival pathway in MCF-7/A cells*

In order to investigate the effect of DLJ14 and Adr combination treatment on the EGFR/PI3K/Akt survival pathway in MCF-7/A cells, expression of related proteins was determined by Western blotting analysis. As shown in Figures 1B-1E, either DLJ14 or Adr has no significant effect on the expression of EGFR, p-EGFR, PI3K, Akt, and p-Akt. However, DLJ14 and Adr combined treatment could significantly decrease the expression of EGFR, p-EGFR, PI3K, and p-Akt when compared with the Adr treatment alone group except for the expression of total Akt.

3.3. *Arrest of MCF-7/A cells in the G2/M Phase*

The anti-proliferative effect of DLJ14 and Adr combination treatment on MCF-7/A cells was also determined by cell cycle analysis. Flow cytometry analysis showed that DLJ14 treatment alone at 20 μmol/L had no obvious effect on cell cycle progression. Adr treatment alone at 5 μmol/L slightly arrested MCF-7/A cells in the G2/M phase. However, combination treatment of DLJ14 (10 μmol/L, 20 μmol/L) and Adr (5 μmol/L) significantly arrested MCF-7/A cells in the G2/M phase compared to Adr treatment alone (Figures 2A and 2C).

The arrest of the MCF-7/A cell cycle by combination treatment of DLJ14 and Adr was associated with upregulation of $p21^{Waf1/Cip1}$ (a cell cycle regulator gene) expression, and downregulation of cyclin A expression. Moreover, we also observed that the expression of p53 was increased significantly by this combination treatment (Figure 2B and 2D). Activation of p53 could up-regulate transcription of $p21^{Waf1/Cip1}$.

3.4. *Induction of apoptosis by DLJ14 and Adr combination treatment*

The effect of DLJ14 and Adr combination treatment on the apoptosis of MCF-7/A cells was first determined by detecting cell morphology. Treatment of MCF-7/A cells with DLJ14 and Adr combination treatment for 48 h followed by nucleus staining with DNA-binding fluorochrome Hoechst 33258 showed an increased number of cells with reduced nuclear size, chromosome

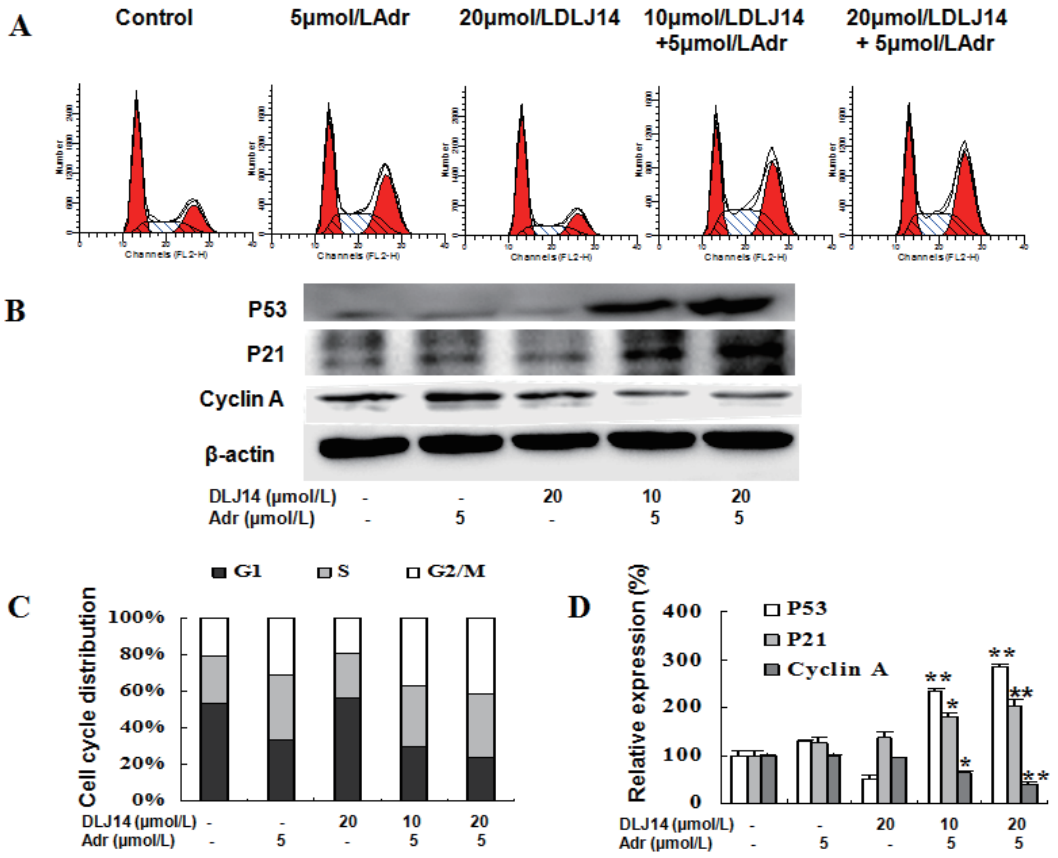

Figure 2. DLJ14 potentiates G2/M phase arrest of Adr in MCF-7/A cells by regulating the expression of p53, p21, and Cyclin A. MCF-7/A cells were treated with DLJ14, Adr, or the combination treatment of DLJ14 and Adr for 48 h before the cells were harvested, fixed, and stained with PI and analysed by flow cytometry **(A)**. Percentage of cells in different stages is shown in **(C)**. The expression of p53, p21, and Cyclin A in MCF-7/A cells were detected by Western blotting method. Representative blots of three independent experiments are shown in **(B)**. Quantification of the protein levels of p53, p21, and Cyclin A were normalized to the expression of β-actin and are shown in **(D)**. Data are expressed as mean ± SEM (n = 3). * $p < 0.05$, ** $p < 0.01$ *vs*. Adr treatment alone.

condensation, and nuclear fragmentation, which are characteristics of apoptosis in comparison to Adr-treated cells (Figure 3A).

Annexin V cell surface staining followed by flow cytometry analysis also showed a significant increase of annexin V positive cells following treatment of the cells with 20 μmol/L DLJ14 and 5 μmol/L Adr, while 5 μmol/L Adr alone had no obvious effect. Interestingly, 20 μmol/L DLJ14 treatment alone induced an obvious increase of early apoptotic cells (Annexin V^+/PI^- cells) in comparison to the control cells, while the combination treatment induced a dramatic increase of necrosis/late apoptotic cells (Annexin V^+/PI^+) (Figures 3B and 3C).

Further analysis suggested that the apoptotic effect of DLJ14 and Adr combination treatment was associated with alteration of apoptotic proteins in MCF-7/A cells. As shown in Figures 3D and 3F, this combination treatment caused a marked increase of the expression of caspase-3 and caspase-9 and also a marked increase of cleavage of caspase-3 and caspase-9 compared to Adr-treatment alone. Figures 3D and 3E show the regulation of the expression of Bax and Bcl-2 in MCF-7/A cells by the combinationtreatment, showing up-regulation of Bax and down-regulation of Bcl-2. Statistical analysis indicated that the ratio of Bax/Bcl-2 was obviously increased.

3.5. *Induction of mitochondrial membrane potential collapse and release of cytochrome c*

To investigate the mechanism of apoptosis, mitochondrial functions were examined by determining mitochondrial activity such as mitochondrial membrane potential and cytochrome c in the mitochondrion and cytosol of MCF-7/A cells. The change of mitochondrial membrane potential was analyzed using JC-1 which could selectively enter into the mitochondrion, and its color could change reversibly from red to green as membrane potential decreased (*12*). Cell apoptosis usually accompanies mitochondrial depolarization. JC-1 release in depolarized mitochondria causes the reduction of red fluorescence intensity. As shown in Figure 4A, the percentage of cells with depolarized mitochondria was significantly increased to 16.2% by DLJ14 and Adr combination treatment, which was 4.48% in the Adr alone treated group. Since mitochondrial membrane disruption is often associated with the release of mitochondrial proteins into cytosol,

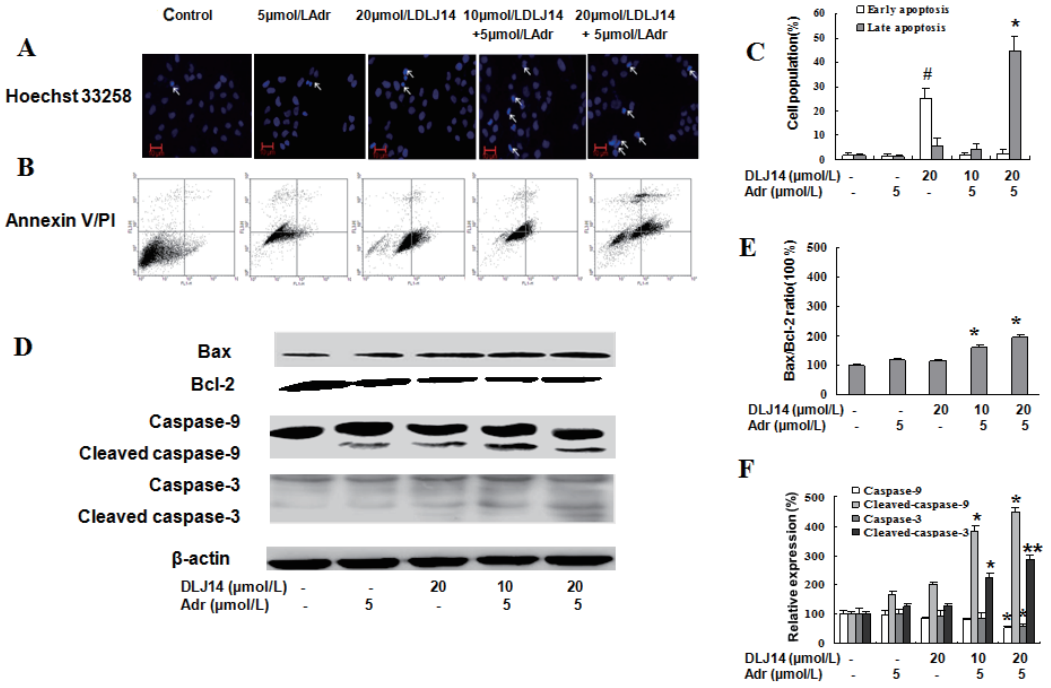

Figure 3. DLJ14 induces the apoptosis of MCF-7/A cells treated with Adr. MCF-7/A cells were treated with DLJ14, Adr, or the combination treatment of DLJ14 and Adr for 48 h, and then stained with DNA-binding fluorochrome Hoechst 33258 staining. Representative images of MCF-7/A cells are shown in **(A)**. Arrows indicate characteristic apoptotic features. Scale bar = 10 μm. Cell apoptosis was analyzed by flow cytometry following Annexin V/FITC and PI double-staining. The FL1 axis shows the Annexin V intensity, and the FL2 axis shows PI staining **(B)**. The percentages of apoptosis and necrosis cells were scored in three separate experiments and the results are shown in **(C)**. The expression of Bax, Bcl-2, caspase-9, cleaved caspase-9, caspase-3, and cleaved caspase-3 in MCF-7/A cells were detected by Western blotting method. Representative blots of three independent experiments are shown in **(D)**. Quantification of the protein levels were normalized to the expression of β-actin and are shown in (**E** and **F**). Data are expressed as mean ± SEM ($n = 3$). $^{\#}$ $p < 0.05$ *vs.* untreated cells, * $p < 0.05$, ** $p < 0.01$ *vs*. Adr treatment alone.

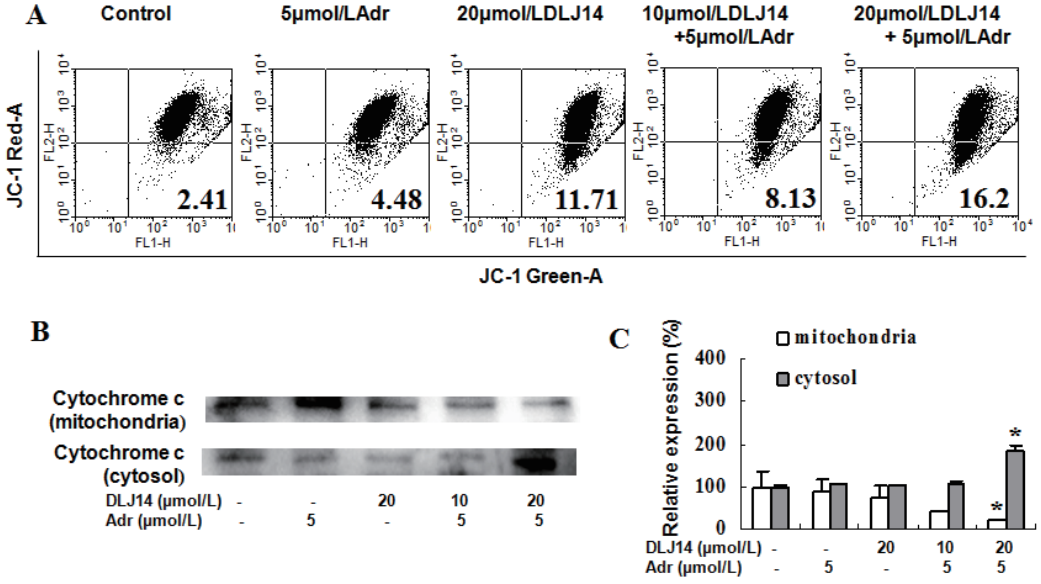

Figure 4. DLJ14 increases mitochondrial membrane potential collapse and the release of cytochrome c in MCF-7/A cells induced by Adr. MCF-7/A cells were treated with DLJ14, Adr, or the combination treatment of DLJ14 and Adr for 48 h, and then stained with JC-1 (the red passage: excitation 325 nm, emission 590 nm and the green passage: excitation 488 nm, emission 530 nm) to determine the depolarized cells by flow cytometry **(A)**. The expression of mitochondrial cytochrome c and cytosol cytochrome c in MCF-7/A cells were detected by Western blotting method. Representative blots of three independent experiments are shown in **(B)**. Quantification of the protein levels were normalized to the expression of β-actin and are shown in **(C)**. Data are expressed as mean ± SEM ($n = 3$). * $p < 0.05$, ** $p < 0.01$ *vs.* Adr treatment alone.

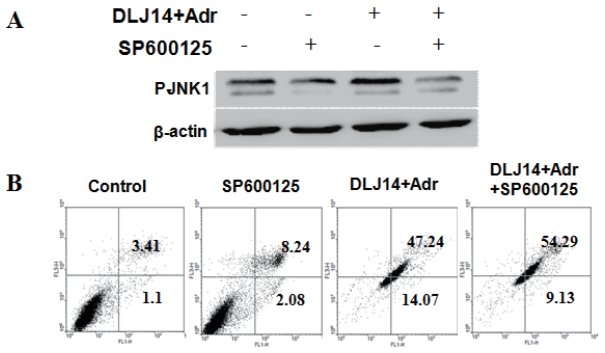

Figure 5. Effect of DLJ14 and Adr combination treatment on apoptosis induction in MCF-7/A cells. MCF-7/A cells were pretreated with or without specific JNK inhibitor (SP600125, 40μmol/l) for 30 min, and then exposed to the combination treatment of DLJ14 and Adr for 48 h. The expression of P-JNK1 was determined by Western blotting method. Representative blots of three independent experiments are shown in **(A)**. Cell apoptosis was analysed by flow cytometry following Annexin V/FITC and PI double-staining **(B)**.

the subcellular distribution of cytochrome c was then examined. Figures 4B and 4C showed that the level of cytochrome c in mitochondria was significantly decreased and the level of cytochrome c in cytosol was obviously increased in MCF-7/A cells by DLJ14 and Adr combination treatment compared to Adr treatment alone.

3.6. *Induction of apoptosis by DLJ14 and Adr combination treatment is not mainly due to the activation of the JNK1 signal pathway*

Our previous results showed that JNK1 phosphorylation was significantly elevated in MCF-7/A cells after DLJ14 treatment. In order to further explore the contribution of JNK signaling pathway to the enhanced therapeutic effect of DLJ14 and Adr combination treatment, SP600125, a small molecule JNK inhibitor, was used to block the JNK signaling pathway (*13*). We examined the regulation of JNK1 phosphorylation and the apoptosis induction of DLJ14 and Adr combinationtreatment. Results showed that the increase of phospho-JNK1 (p-JNK1) expression induced by the combination treatment was significantly blocked by SP600125 (Figure 5A). However, the apoptosis induction of MCF-7/A cells by the combination treatment was obviously not inhibited by SP600125, and the percentage of apoptotic cells was 61.31% or 63.42% in the DLJ14 and Adr combination treatment group without or with SP600125 (40 μM) (Figure 5B).

4. Discussion

TMP, one of active ingredients of the Chinese herb *Ligusticum chuanxiong* Hort, has been widely used for cerebrovascular and cardiovascular diseases in China (*14*). Moreover, TMP has been suggested to be useful as an adjuvant agent to reverse multidrug resistance of tumor cells (*15*). DLJ14, a novel TMP piperazine derivate, was synthesized for the improvement of low bioavailability and short half-life of TMP. In preliminary studies, DLJ14 enhanced therapeutic effects of Adr on resistant cancer cells K562/A02 or MCF-7/A *in vitro* or *in vivo*. In this study, we investigated the underlying molecular mechanism of DLJ14 and Adr combination treatment on Adr-resistant human breast cancer. In cancer cells, dysregulation of PI3K/Akt signaling pathways is associated with deficiency of apoptosis and the phenotype of multidrug resistance. PI3K/Akt is a major downstream signaling cascade of EGFR which is over-expressed in all subtypes of breast cancer. Thus, the effect of DLJ14 and Adr combination treatment on the EGFR/PI3K/Akt survival pathway in MCF-7/A cells was determined. Results showed that DLJ14 and Adr combination treatment effectively decreased the protein expression of EGFR, p-EGFR, PI3K, and p-Akt, but not total Akt, as compared to only Adr treatment. This indicated that DLJ14 and Adr combination treatment inhibited the survival pathway of EGFR/PI3K/Akt to enhance anti-tumor effects.

PI3K/Akt signaling pathway plays an important role in cancer cell growth. Activation of PI3K/Akt pathway not only promotes cell survival, but also exerts anti-apoptotic function *via* the mitochondrial pathway. It phosphorylates Bad for degradation and thus increases Bcl-2 activity (*16*). Moreover, PI3K/Akt pathway also blocks p53 *via* activation of Mdm2 (*17*). Therefore, inhibition of PI3K/Akt pathway could promote tumor cell apoptosis. Apoptosis has been accepted as a fundamental component in the pathogenesis of cancer. Apoptosis mediated by mitochondria can be triggered by MMP collapse, which is a decisive event in the process of cytochrome c release. The collapse of MMP can initiate the release of molecules from space between the outer and inner mitochondrial membranes into cytosol, and triggercaspase cascade and other apoptotic processes (*18*). Release of cytochrome c is also associated with decrease of Bcl-2 and increase of Bax followed by activating caspase-9 and caspase-3 (*19*). In this study, we observed that DLJ14 and Adr combination treatment increased Bax/Bcl-2 ratio and collapse of MMP, subsequently induced cytochrome c release from mitochondria to cytosol and caspase-9 and caspase-3 activation. These results indicated that DLJ14 and Adr combination treatment might induce apoptosis in MCF-7/A cells *via* activating the mitochondria-mediated intrinsic pathway.

Cell cycle is also a main regulatory mechanism of cell growth and many chemical compounds could trigger apoptosis in tumor cells accompanied by cell cycle arrest (*20,21*). Flow cytometry analysis showed that DLJ14 and Adr combinationtreatment induced G2/M phase arrest of MCF-7/A cells compared with Adr treatment alone. $p21^{Waf1/Cip1}$ is known as a cell cycle inhibitor involved in G2/M phase progression and its

up-regulation has been linked to cell cycle arrest at G1 or G2/M phase. Activated of p53 is able to regulate transcription of the cell cycle regulator gene $p21^{Waf1/Cip1}$ (*22*). Cyclin A is also related to cell cycle arrest at G2 phase through forming Cyclin A/Cdk2 complexes (*23*). This study, in parallel with flow cytometery analysis, showed that DLJ14 and Adr combination treatment could activate p53, upregulate the expression of $p21^{Waf1/Cip1}$ and downregulate the expression of Cyclin A.

JNK primarily contributes to pro-apoptotic cell death or tumor suppression in response to a variety of stress, inflammatory or oncogenic signals and a is major downstream signal pathway of EGFR (*24*). We used SP600125, a specific JNK inhibitor, to explore the role of JNK1 pathway in the enhanced therapeutic effects of the combination of DLJ14 and Adr treatment. Results showed that the apoptosis induction of MCF-7/A cells by the combination treatment was not inhibited significantly by SP600125. This result indicated that the JNK1 pathway might not play a crucial role in the apoptosis induction from DLJ14 and Adr combination treatment.

In summary, the combination treatment of DLJ14 and Adr enhanced anti-proliferation effects on MCF-7/A cells through inhibiting EGFR/PI3K/Akt survival pathway and induced cell apoptosis *via* the mitochondrial-mediated intrinsic pathway accompanied with G2/M cell cycle arrest. Moreover, JNK1 pathway activation induced by DLJ14 and Adr combination treatment might not be involved in apoptosis induction. DLJ14 therefore has the potential to be developed as a promising agent for treatment of cancers with Adr resistant cells.

Acknowledgements

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