Original Article
Dihydroartemisinin and cisplatinum synergistically induced apoptosis in human lung adenocarcinoma H1299 cells

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Abstract: Dihydroartemisinin (DHA), a kind of artemisinin derivatives, has been extensively investigated alone or together with other drugs for anti-cancer therapy in recent years. However, whether a synergistic effect between DHA and cisplatinum (Cis, an anti-cancer drug) existed in treatment of human lung adenocarcinoma is still unknown. This study was designed to explore the potential synergism of DHA and Cis using human lung adenocarcinoma H1299 cells. The cells were treated by DHA, Cis and DHA plus Cis respectively. Untreated cells were used as control. Cell inhibition and apoptosis were evaluated by MTT assays and flow cytometry. The apoptosis associated proteins were assessed by Western blotting and RT-qPCR. Flow cytometry revealed that both DHA and Cis induced significantly H1299 cell apoptosis compared to control group, while a higher apoptotic level was observed in DHA plus Cis treated group. Analysis of apoptotic proteins revealed that caspase-3, -8, -9 were significantly higher in DHA plus Cis treated cells than DHA or Cis alone. Meanwhile, The DHA plus Cis treatment also significantly enhanced caspase-8, -9, -3, -6, -7 and PARP activation compared with DHA or Cis alone, indicating that the caspase-8-related death receptor signaling pathway and the caspase-9-related mitochondrial signaling pathway may play important roles in the synergistic effect of DHA and Cis. In general, this study reveals the obvious synergistic action of the DHA and Cis in inducing apoptosis of H1299 cells via the death receptor and mitochondrial apoptosis pathway.

Keywords: Dihydroartemisinin, cisplatinum, apoptosis, synergistic action, caspase

Introduction
Lung cancer is the leading cause of cancer-related deaths worldwide [1, 2]. Non-small cell lung cancer (NSCLC) accounts for about 80% of lung cancer, among which the lung adenocarcinoma is the most common one. At present, the chemotherapy was still one of the most important treatment approaches for patients with lung adenocarcinoma. However, the clinical effectiveness of first line platinum-based chemotherapy is only ~30%. Thus, novel chemotherapy regimens to improve efficacy in patient are urgently needed.

Artemisinin and its derivatives have been proven to possess anticancer properties [3, 4] with little side effects [5, 6]. Dihydroartemisinin (DHA) is one of the artemisinin derivatives (ARTs), which have been demonstrated to have potent anticancer activity by inducing apoptosis without apparent side-effects and therefore have been used in the clinic for many years [7, 8]. Many investigations were targeted researching the possibility of utilizing the DHA for anti-cancer treatment. It was reported that DHA enhanced the human ovarian cancer cells sensitivity to carboplatin therapy [7], DHA plus histone deacetylase inhibitors expressed a significant inhibitory effect on liver cancer cells via strengthening apoptosis in vitro and in vivo [9]. Dihydroartemisinine enhances dictamine-induced apoptosis via a caspase dependent pathway in the invasion of lung adenocarcinoma A549 cells [10]. DHA showed prominent anticancer activity against pancreatic, leukaemic, osteosarcoma, and lung cancer cells [11].

“Resisting cell death” is the characteristic of malignant cancer cells [12]. Molecules in the
apoptosis pathway can be classified as regulators and effectors, and some may play key roles in the process of cancer development and evolution [13]. The activation of apoptosis process is very important for cancer treatment.

P53 gene, one of the tumor suppressor gene, plays a vital role in the process of tumorigenesis and p53 gene mutation occurs in 50%~70% of lung cancer patients [14]. p53 deletion are related to drug resistance of Cis [15]. In this study, H1299 cell line (a type of p53 deletion lung adenocarcinoma cell) was used to explore the potential synergistic effect of DHA and Cis in antitumor activity. We also sought to determine the underlying molecular mechanisms about DHA plus Cis to H1299 cell line.

Materials and methods

Cell culture

Human lung adenocarcinoma H1299 cells were kindly provided by Doctor. Ping Wang (General Hospital of PLA). Cells were grown in RPMI 1640 medium, supplemented with 10% Fetal Bovine Serum (FBS) plus penicillin (100 UI/mL) and streptomycin (100 μg/mL), at 37°C in a humidified atmosphere of 5% CO₂.

Drugs and reagents

The pro-apoptotic agent DHA was purchased from Holley-Cotec (Chongqing, China) and dissolved in dimethyl sulfoxide (DMSO) as a 100 mmol/L stock solution. Cis was obtained from Qilu Pharmaceutical General Factory, China. Penicillin, streptomycin, DMSO, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium-bromide (MTT), Hoechst 33258, Annexin-V, and propidium iodide (PI) were purchased from Sigma (St Louis, MO, USA). RPMI 1640 medium and FBS were purchased from Gibco (Grand Island, USA). Caspase-3, -8 and -9 inhibitors zDQMD-fmk, zIETD-fmk and zLEHD-fmk were from Sigma (St Louis, MO, USA). Antibodies against Caspase-3 Rabbit mAb 9665, Caspase-6 Antibody 9762, Caspase-7 Rabbit mAb 12827, Caspase-8 Mouse mAb 9746, Caspase-9 Mouse mAb 9508, PARP Antibody 9542, β-Actin Rabbit mAb 4970, Anti-rabbit IgG, HRP-linked Antibody 7074, Anti-mouse IgG, HRP-linked Antibody 7076, were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Evaluation of the anti-proliferative effects

We used the MTT assay to determine the level of cell proliferation. The numbers of cell per well were 3000 for H1299 cells in 96-well plates and incubated overnight. Serial dilution of DHA and Cis were used in the cell culture. After 24 h and 48 h of incubation, the cells were incubated with MTT for 4 h. And then, DMSO was added into each well to dissolve the resulted formazan and ODs were measured using a spectrometer at 490 nm. IC50 value was calculated using CalcuSyn software (BIOSOFT, Inc., Ferguson, MO, USA). To evaluate the anti-proliferative effects of the combined treatments, cells were treated with Cis and DHA for 24 h and 48 h. These serial doses were 1.56, 3.12, 6.25, 12.5, 25 and 50 μM for Cis and 2.5, 5, 10, 20, 40 and 80 μM for DHA to calculate the combination index (CI) value. The combination index (CI) equation is based on the multiple drug-effect equation of Chou-Talalay derived from enzyme kinetic models [16]. The equation is given by, CI = [(D1)/(Dx1) + (D2)/(Dx2)] + [(D1)/(Dx1)]/[[(D1) + (D2)]]; for mutually non-exclusive drugs that have totally independent modes of action. Drug1, (D1), and Drug2, (D2), (in the numerators) are the doses of drug1 and drug2 in combination inhibit x%. (Dx1) and (Dx2) (in the denominators) are the doses of drug1 and drug2 alone, respectively, inhibiting x%. CI < 1, = 1, and > 1 indicates synergism, additive effect, and antagonism, respectively. The CI values of the two drugs for anti-proliferative effects were calculated using CalcuSyn software (BIOSOFT, Inc., Ferguson, MO, USA).

The proliferation of treated cells was also detected by Ki-67 immunofluorescence staining. 5-7 random separate fields for each group were calculated for the percentage of Ki-67 positive cells and 5 samples were included in each group.

Apoptosis morphological assay

According to the reagent’s protocol, the morphological experiment for apoptosis was observed by Hoechst 333258 staining. The images that cell nucleus were stained by Hoechst 333258 were visualized using a Fluorescence Microscope (OLYMPUS, Japan).

Flow cytometry

H1299 cell line (3×10⁵/well) was implanted in 6-well plates overnight. The cells were exposed
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with the DHA, Cis or DHA combined with Cis for 24 h and 48 h in a humidified atmosphere of 5% CO₂. After that, the cells were harvested and washed with phosphate buffer. The cells were centrifuged at 200×g, 4°C for 5 minute and discarded the supernatant. The re-suspended cells in 100 ul Annexin V Binding Buffer were added with 5 ul Annexin V-FITC and 10 ul PI to each tubes. Then, the cells were incubated for 15 minute under dark condition at room temperature and added 400 ul Annexin V Binding Buffer. After staining, the cells were analyzed on the flow cytometer (FACS Caliber, BD Biosciences).

Western blotting analysis of apoptotic pathway protein expression

Western blotting was performed to evaluate the apoptosis-related proteins, which were extracted from each experimental group (DHA group, Cis group and combination group of DHA plus Cis) and controls group in the H1299 cell lines. Cells were collected and washed with PBS. RIPA lysis buffer was added for 10 min at 4°C and the reaction mixtures were centrifuged (13,000 rpm). Supernatants were boiled in 5×SDS-PAGE loading buffer and the protein contents were quantified using the Bicinchoninic acid (BCA) assay kit. Equal amounts of proteins and 5 μL of a marker were loaded on a 10% stacking gel for electrophoresis for 20 min at 80 V, followed with a further 50 min at 120 V electrophoresis in the 12% separating gel. After transferring to a PVDF membrane, the nonspecific protein targets were blocked by 5% skim milk powder in 1×TBST. Defined dilutions of specific primary antibodies were required for overnight incubation at 4°C: anti-β-actin Rabbit mAb 4970 at 1:1000, anti-Caspase-9 Mouse mAb 9508 at 1:1000, and anti-PARP Antibody 9542 at 1:1000. After that, PVDF membranes were then washed by 1×TBST and incubated with secondary antibodies for 1 h. The target protein bands were visualized on the film by enhanced chemiluminescence (ECL) (Applygen Technologies Inc.), quantified using a GS-710 Imaging Densitometer (Bio-Rad, Hercules, CA) and the band intensity was normalized.

Real-time qPCR

Total RNA of each group was extracted using TRIzol (Invitrogen, USA) reagent and cDNA was reversed transcribed using ReverTra Ace qPCR RT Kit (TOYOBO, Dalian, China) following the instruction of the manufacturer. Primers for real-time PCR were showed in Table 2. Put simply, 20 μL of total RT-qPCR reaction system was used including 2 μL DNA template, 0.8 μL forward primer (0.4 μM), 0.8 μL reverse primer (0.4 μM), 10 μL 1×SYBR Green Realtime PCR Master Mix (TOYOBO), and 6.4 μL distilled water. The reactions were incubated in a thermocycler at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 55°C for 10 s and 72°C for 15 s. The RT-PCR reaction was performed in Bio-Rad real-time PCR detection systems. (Bio-Rad, USA). We detected the threshold cycle (Ct) and calculated expression values of 2^ΔΔct, using β-actin as the internal reference for each sample [17, 18].

Statistical analysis

The data were collected and analyzed by SPSS software (version 22.0, USA). Data were presented as mean ± standard deviation. The percentage data was analyzed using the Kruskal-Wallis H nonparametric test. The rest of data were processed for one-sample Kolmogorov-Smirnov Test and homogeneity of variance test. Statistical comparisons were performed by one-way analysis of variance (ANOVA). P < 0.05 was considered to be statistically significant.

Results

DHA induces cytotoxicity in dose- and time dependent manner by MTT assay

DHA showed cytotoxic effects in H1299 cells. The H1299 cell line was incubated in different concentrations of DHA (2.5~80 μM) for 48 h or

Table 1. CI of different regimens of DHA in combination with Cis in H1299 cells (mean ± sd), n = 3

<table>
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<td>25</td>
<td>0.687±0.127</td>
</tr>
<tr>
<td>80</td>
<td>50</td>
<td>0.338±0.079</td>
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</table>

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20 μM of DHA for 0~72 h followed by MTT assay. The data demonstrated that inhibition ratio of DHA for different concentrations were 22.75%, 28.68%, 33.07%, 39.06%, 51.58% and 72.64% after treated for 48 h, indicating that DHA induced H1299 cell cytotoxicity in a dose-dependent manner (Figure 1A). Furthermore, inhibition ratios of DHA for different times were 27.41%, 39.06%, and 58.68% with 20 μM DHA, indicating that DHA induced a time-dependent cytotoxicity in H1299 cells (Figure 1B). Consistently, the Ki-67 results confirmed the time-dependent cytotoxicity of DHA in H1299 cells.

DHA induces cell apoptosis in a dose- and time-dependent manner by Hoechst staining

In order to identify whether the DHA induced cell death was associated with apoptosis, we stained H1299 cells with Hoechst 33258 to demonstrate the cell nuclear morphology by the fluorescent microscope. The control cells demonstrated homogeneous distribution of Hoechst 33258 (Figure 2A); however, after exposure to 20 μM DHA for 24 h, 48 h and 40 μM DHA for 24 h, the cells were observed with typical morphologic changes of apoptosis, such as chromatin condensation, nucleus shrinkage and fragmentation (Figure 2B-D).

To further determine if the DHA induced cell death was correlated with apoptosis, cells were stained with Annexin-V/PI kit and flow cytometry analysis was performed. Compared with control, DHA treatment induced an obvious increase in the percentage of cells with phosphatidyl-serine externalization from 9.09% (control) to 17.07% (24 h) and 24.31% (48 h) at 20 μM concentration, while from 9.09% (control) to 17.07% at 20 μM concentration and 22.5% at 40 μM concentration for 24 h (Figure 2E).

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DHA and Cis synergistic induce the pro-apoptosis of H1299 cells

As shown in Figure 3A, the inhibition ratio of A549 cells and H1299 cells were significantly different when the same doses of Cis were administrated, but DHA had similar effect on two cells (Figure 3B). The H1299 cell inhibition ratio by combined treatment was higher than single drug (Figure 3C). And the cellular proliferation was also confirmed by Ki-67 staining results (Figure 3D, 3E).

We used the MTT test to assess the suppression effect of DHA plus Cis on H1299 cells. Compared with single drugs therapy, the combined treatment significantly improved the inhibition ratio of H1299 cells (Figure 3C). The CI-value were 0.720, 0.675, 0.762, 0.840, 0.687 and 0.338 for 24 h, which were smaller than 1.0, implying that combination treatment has a obvious synergistic effect (Table 1).

Finally, we used the western blotting to evaluate the expression of apoptosis-associated protein caspase-3, -6, -7 and PARP. As shown in Figure 3F and 3G, following the different treatment (Cis, DHA, Cis+DHA and control), the protein levels of caspase-3, -6, -7, PARP and β-actin (as an internal control) were demonstrated. We found that the protein levels of cleave-caspase-3, -6, -7 and cleavage of PARP were significantly higher in single drug group than in control group, and the levels in combined treatment group were the highest.

DHA and Cis involved mitochondria and death receptor-related apoptotic signaling pathway

Caspase-9 plays a key role in the mitochondria apoptosis pathway, and upon release from mitochondria, cytochrome c binds to Apaf-1 and forms an activation complex with caspase-9. Caspase-9 was cleaved, producing p35 and p37 subunit to activate caspase-3, -6 and -7. Caspase-8 is also very important to death receptor apoptosis pathway and activated caspase-8 could stimulate apoptosis. As shown in Figure 4A, treatment with DHA, Cis or Cis in combination with DHA promoted casp-8, -9 cleavage into the active form, which resulted in the cleaved caspase-8 and -9 proteins expres-

<table>
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<th>Accession no.</th>
<th>Primers</th>
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<td>Caspase 8</td>
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<td>5’-CTGGGAGAGTAGCAGTAT-3’</td>
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<td>Caspase 9</td>
<td>NC_000001.11</td>
<td>5’-ATGTCTTTTATGCTGATGG-3’</td>
</tr>
<tr>
<td>β-actin</td>
<td>NC_000007.14</td>
<td>5’-GAG ACC TTC AAC ACC CGC C-3’</td>
</tr>
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</table>

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**Figure 1.** DHA induces dose- and time-dependent cytotoxicity in H1299 cell line tested. (A) DHA induced the dose-dependent cytotoxicity reflected by MTT assay. The H1299 cells were incubated in different concentrations of DHA (2.5~80 μM) for 48 h. **P < 0.01, compared with control; (B) DHA induced the time-dependent cytotoxicity reflected by MTT assay and (C) Ki-67 positive percentage. The H1299 cells was incubated in 20 μM DHA for 0, 24, 48 and 72 h. **P < 0.01, compared with control group (n = 5).

In recent years, the treatment of cancer using traditional Chinese medicines is being increasingly emphasized [19]. The anti-cancer activity of Dihydroartemisinin (DHA) was extensively studied in the past years. In the study, we demonstrated for the first time about the synergistic effect of DHA and Cis on inhibition of NSCLC H1299 cells, as well as its possible mechanisms.

Deletion of p53 is associated with resisting to chemotherapy in some tumor cells [20], this study showed the inhibitory rate of H1299 cells under the Cis treatment was lower than DHA treatment group. DHA can inhibit cell proliferation and induce cell death through a p53-independent mechanism [21]. In this study, the inhibition ratio of A549 cells and H1299 cells showed little change when different doses of combination of the two drugs for 24 h and 48 h, none-treated cells were used as control group. The qRT-PCR (Figure 5) demonstrated that the expression of Caspase-3, -8, -9 mRNA in H1299 cell were up-regulated when incubated with 20 μM DHA, 12.5 μM Cis and combination of the two drugs for 24 h and 48 h. Moreover, the results of Caspase-3, -8, -9 mRNA expressions after incubation with single drug and combination treatment were consistently with the western blotting results. In DHA group and combined treatment group, the expressions of Caspase-3, -8, -9 mRNA were significantly increased.

**Discussion**

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Figure 2. DHA induces dose- and time-dependent apoptosis in H1299 cells. (A) The control cells demonstrated homogeneous distribution of Hoechst 33258; (B-D) DHA induces cell apoptosis represented by Hoechst 33258 staining assay. The h1299 cells were incubated in 20 μM DHA for 24 h (B), 48 h (C) and 40 μM (D) DHA for 24 h. After that, fluorescent microscope was used to observe the changes of the cell nuclear morphology. Magnification 400. (E) Flow cytometry was used to detect the DHA induced cell apoptosis. H1299 cells were incubated in 20 μM DHA for 24 h, 48 h and 40 μM DHA for 24 h. The final step is to analyze result by flow cytometry after staining with Annexin V-FITC/PI. Apoptotic cells were stained with Annexin V+/PI- and Annexin V+/PI+ (n = 5).

DHA were used for the two kinds of cells at the same time. We speculated that cisplatin resistance in H1299 cell line was because of the lack expression of p53 protein, while combination treatment with DHA and Cis can highly improve the inhibition ratio of H1299 cells.

It is reported that DHA has better synergistic effect with many other anticancer drugs [10, 22, 23]. In present study, the inhibition ratio of H1299 cells by combination treatment was also higher than single drug. Furthermore, the calculated CI value by combination index (CI) equation showed that they were all less than 1.0 under the different concentrations for 48 h, providing further evidence that DHA combined with Cis has potent synergistic effect in human lung adenocarcinoma H1299 cells.
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Figure 3. DHA and Cis synergistic affect the H1299 cells pro-apoptosis. (A) MTT assay was used to appraise the inhibition ratio of Cis on A549 and H1299 cells. The two types of cells were incubated in different concentrations of Cis (1.56~50 μM) for 48 h. #P < 0.05, ##P < 0.01, compared with each other. (B) MTT assay was used to appraise the inhibition ratio of DHA on A549 and H1299 cells. The two types of cells were incubated in different concentrations of DHA (2.5~80 μM) for 48 h. $P > 0.05, compared with each other. (C) Synergistic effect of combined treatment
Previous study has shown that combined treatment with DHA and Cis inhibited the tumor vascularization by decreasing the protein expression levels of HIF-1α and VEGF [22]. In this article, the underlying apoptotic signal pathway of Caspase family under combination treatment has been further investigated. Caspase-3 is a critical executioner of apoptosis, as it is either partially or totally responsible for the cleavage of many key proteins [24]. Caspase-3 ultimately triggers the morphological changes of apoptosis, including DNA fragmentation and cell shrinkage. Caspase-6 and Caspase-7 have been identified as major contributors to the execution of apoptosis [25]. PARP appears to be involved in DNA repair in response to environmental stress [26]; cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis [27]. This protein is one of the main cleavage targets of caspase-3 in vivo [28, 29]. In our work, we found the apoptotic executioner protein levels of cleave-caspase-3, -6, -7 and PARP were significantly higher in combined treatment group than single drug using and control groups. The results indicated that combination treatment with DHA and Cis could induce the apoptosis of H1299 cell line. And, it is clear that the effect is obviously better than single drug.

Caspase-8 plays an important role in the TNFαR and DR3 promoted apoptosis via the adaptor proteins TRADD/FADD and the activation of caspase-8. The induced apoptosis through the CD95 receptor (Fas/APO-1) and tumor necrosis factor receptor 1 (TNFR1) activates caspase-8 and leads to the release of the caspase-8 active fragments [30-32]. Activated caspase-8 cleaves activates downstream effector caspasess such as caspase-3, -6, and -7. Caspase-9 is another important member of the caspase family [33, 34]. Upon apoptotic stimulation, cytochrome c released from mitochondria associates with the pro-caspase-9/ Apaf-1. Apaf-1 mediated activation of caspase-9 involves intrinsinc proteolytic process-

In summary, this study demonstrated the synergistic effect of the combination treatment with DHA and Cis in the induction of H1299 cell apoptosis. The caspase-8-participated death receptor signaling pathway and the caspase-
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9-participated mitochondria signaling pathway, following the crosstalk between the two apoptotic pathways, played important roles respectively in the synergistic effect of combination therapy in H1299 cells. This study will contribute to understand the mechanism of the combination treatment with DHA and Cis, and also provide more information for the future studies.

Figure 4. DHA and Cis combination treatment in H1299 cells involved mitochondria and death receptor apoptosis signaling pathway. A. Western blot analysis of the Mitochondria and death receptor apoptosis signaling pathway related proteins c-caspase8 and c-capase9 in H1299 cells treated with Cis, DHA and DHA in combination with Cis for 48 h, respectively. Combined treatment enhanced the c-caspase8 and c-capase9 expression and the relative quantitative analysis respectively. B. H1299 cells were pre-incubated with zDQMD-fmk, zIETD-fmk, zLEHD-fmk 1 h, and then Cis (12.5 μM) combined with DHA (20 μM) incubated cells for 24 h and the relative quantitative analysis respectively (n = 3).
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![Graphs showing mRNA expression level of Caspase-3, -8, -9 after 24 h and 48 h culture with or without medicine treatment (n = 3). *P < 0.05, **P < 0.01.]

of the combined treatment with DHA and Cis in patients with NSCLC.

Disclosure of conflict of interest

None.

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References


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