RESEARCH ARTICLE

Dihydroartemisinine Enhances Dictamnine-induced Apoptosis via a Caspase Dependent Pathway in Human Lung Adenocarcinoma A549 Cells

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Abstract

Dictamnine (Dic) has the ability to exert cytotoxicity in human cervix, colon, and oral carcinoma cells and dihydroartemisinin (DHA) also has potent anticancer activity on various tumour cell lines. This report explores the molecular mechanisms by which Dic treatment and combination treatment with DHA and Dic cause apoptosis in human lung adenocarcinoma A549 cells. Dic treatment induced concentration- and time-dependent cell death. FCM analysis showed that Dic induced S phase cell cycle arrest at low concentration and cell apoptosis at high concentration in which loss of mitochondrial membrane potential ($\Delta\Psi m$) was not involved. In addition, inhibition of caspase-3 using the specific inhibitor, z-DQMD-fmk, did not attenuate Dic-induced apoptosis, implying that Dic-induced caspase-3-independent apoptosis. Combination treatment with DHA and Dic dramatically increased the apoptotic cell death compared to Dic alone. Interestingly, pretreatment with z-DQMD-fmk significantly attenuated DHA and Dic co-induced apoptosis, implying that caspase-3 plays an important role in Dic and DHA co-induced cell apoptosis. Collectively, we found that Dic induced S phase cell cycle arrest at low concentration and cell apoptosis at high concentration in which mitochondria and caspase were not involved and DHA enhanced Dic induced A549 cell apoptosis via a caspase-dependent pathway.

Keywords: Dictamnine - dihydroartemisinin - apoptosis - caspase-3 - mitochondrial membrane potential

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Introduction

Dictamnine (Dic), a natural alkaloid, was isolated from the root of Dictamnus dasycarpus Turcz, and has been reported to possess anti-platelet aggregation, fungal activity and vascular-relaxing effects (Huang et al., 2005; Huang et al., 2007; Guo et al., 2008). Recently, it was reported that Dic could induce cytotoxicity in human cervix, colon, and oral carcinoma cells (Huang et al., 2005). In addition, derivatives from Dic have been exhibited anticancer activity (Huang et al., 2007). However, the characteristics and molecular mechanism of Dic-induced cell death are still poorly understood.

Apoptosis, one of the programmed cell death, plays an essential role in regulating growth, development and immune response by clearing redundant or abnormal cells in organisms (Fan et al., 2005). Apoptotic cells undergo various cellular morphological changes, including cell shrinkage, chromatin condensation, membrane blebbing, oligonucleosomal DNA cleavage and translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane and activation of a family of cysteine proteases called caspases (Elmore, 2007; Drakopanagiotakis et al., 2008). Typical apoptotic signaling pathway includes extrinsic (death receptor) and intrinsic (mitochondrial) pathway. The mitochondrial pathway was characterized by loss of mitochondrial membrane potential resulting in release of cytochrome c which can activate caspase-9 and then caspase-3, one of the major downstream caspase, leading to apoptosis in a wide variety of cancer cell types (Fan et al., 2005; Alvero et al., 2011).

Dihydroartemisinin (DHA), widely used as anti-malarial drugs, also have potent anticancer activity on various tumour cell lines (Gao et al., 2013). Many studies showed that DHA can inhibit proliferation and induce apoptosis via caspase-3-dependent mitochondrial pathway (Lu et al., 2009). However, it is not unclear whether Dic and DHA have synergistic effect on inducing cancer cell apoptosis. In the present study, we not only explored the role of mitochondria and caspase in Dic-induced apoptosis, but also investigated the co-effects of Dic and DHA on inducing human lung adenocarcinoma A549 cell apoptosis. In conclusion, we found that Dic induced

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S phase cell cycle arrest in low concentration and cell apoptosis in high concentration in which mitochondria and caspase were not involved and DHA enhanced Dic induced A549 cell apoptosis via caspase-dependent pathway.

Materials and Methods

Cell culture

A549 cell line was obtained from the Department of Medicine, Jinan University (Guangzhou, China), and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, USA) supplemented with 10% fetal calf serum. Cell cultures were maintained at 37°C in a humidified 5% CO_2 incubator.

Reagents

Dic was purchased from Herbpurify (Chengdu, China) and were prepared by dissolving the compound in ethyl alcohol before the experiments. The final concentration of ethyl alcohol was less than 2 % in all experiments. DHA was purchased from Bide Pharmaceutical Corporation (Guangzhou, China). z-DQMD-fmk (caspase-3 inhibitor) was purchased from Merck-Calbiochem (USA).

Cell viability and apoptosis assay

Cell viability was assessed by Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay according to manufacture's protocol. All experiments were performed in quadruple occasions.

Cell apoptosis observed by Hoechst 33258/PI staining. According to manufacture's protocol, the images of Hoechst 33258 were visualized under a Fluorescence Microscope (M4000, Leica, Germany).

In addition, cell apoptosis detection was performed by flow cytometry (FCM, FACSCantoII, BD, USA) analysis using Annexin V-FITC/PI apoptosis detection kit (BestBio, Shanghai, China) as previously described (Zhang et al., 2012), and for each FCM analysis 10,000 events were recorded. Apoptotic cells are those stained with Annexin V-FITC+/PI- (early apoptotic cells) and Annexin V-FITC+/PI+ (late apoptotic cells).

Measurement of mitochondrial membrane potential ($\Delta\Psi m$)

Rhodamine 123 (Rho 123, Sigma, St. Louis, USA) was used to analyze $\Delta\Psi$m by FCM as previously described (Zhang et al., 2012). Briefly, cells were harvested and stained with 10 μM Rho 123 for 30 min at 37 °C in the dark, and then washed with PBS twice and subsequently assayed by FCM. Results were expressed as the proportion of cells with low Rho123 fluorescence indicating the loss of $\Delta\Psi$m.

Cell cycle analysis

Cell cycle distribution was analyzed using FCM. Briefly, after cells were treated with 300 and 500 μM Dic for 24 h, the cells were harvested, washed twice with PBS, and fixed in 70 % ethanol at 4 °C for 1 h and centrifuged. The pellet was treated with RNase (20 μg/ml) at room temperature for 30 min and then incubated with PI (50 μg/ml) for 30 min.

Statistical analysis

Results were expressed as mean±SD of triplicates. Analysis of variance was used to compare the mean differences between samples using the statistical software SPSS version 10.0 (SPSS, Chicago). $P<0.05$ were considered to be statistically significant.

Results

Dic induces dose- and time-dependent cytotoxicity

To determine whether Dic has cytotoxicity, A549 cells were exposed to different concentrations (100, 300 and 500 μM) of Dic for 24 and 48 h followed by CCK-8 assay. Our data showed that Dic induced 13.5 %, 35.4 % and 66.3 % decrease of cell viability at 100, 300 and 500 μM at 24h indicating that Dic induced a dose-dependent A549 cells death (Figure 1A). Moreover, the results that A549 cells treated with 300 μM Dic for different times (8, 12, 18 and 24 h) demonstrated that Dic induced A549 cells death in a time-dependent manner (Figure 1B).

Low concentration of Dic induces cell cycle arrest in S phase

Dic-induced A549 cell viability decrease may via cell cycle arrest, apoptosis, necrosis or other cell death.

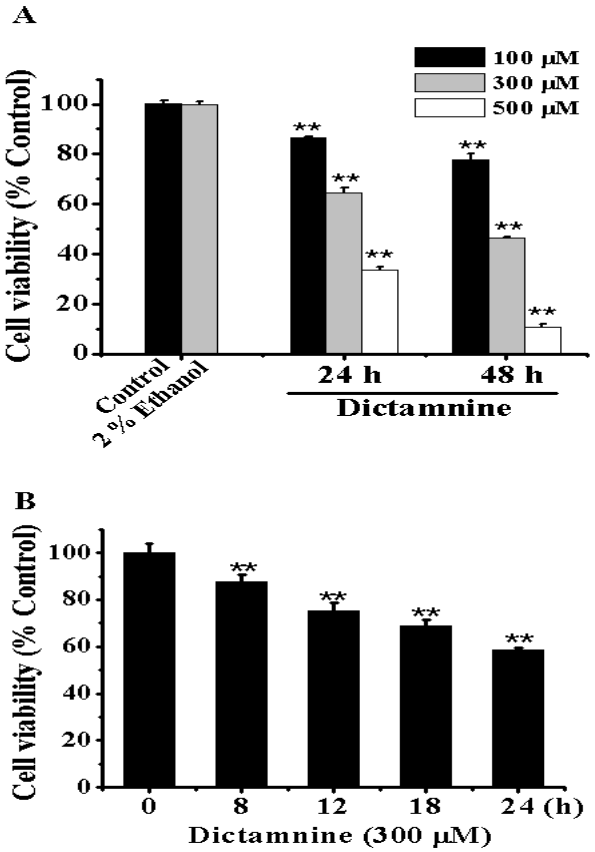

Figure 1. Inhibition of Dic on the Cell Viability Measured by CCK-8 Assay. (A) Dic -induced concentration-dependent reduction of cell viability. Cells were incubated with indicated concentrations (100, 300 and 500 μM) of Dic for 24 and 48 h. (B) Dic-induced time-dependent reduction of cell viability. Cells were incubated with 300 μM of Dic for the indicated times (0, 8, 12, 18 and 24 h). Data analyzed with SPSS10.0 software are expressed as mean ± standard deviations (SD) of triplicates. $^{**}P < 0.01$, compared with control

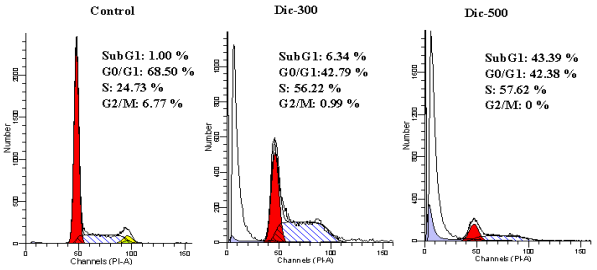

Figure 2. FCM Anslysis of Dic-induced Cell Cycle Arrest in S Phase. After treatment with indicated concentrations (300 and 500 μM) of Dic for 24 h, the cells stained with PI (10 μg/ml) for 30 min were analyzed for DNA content by FCM

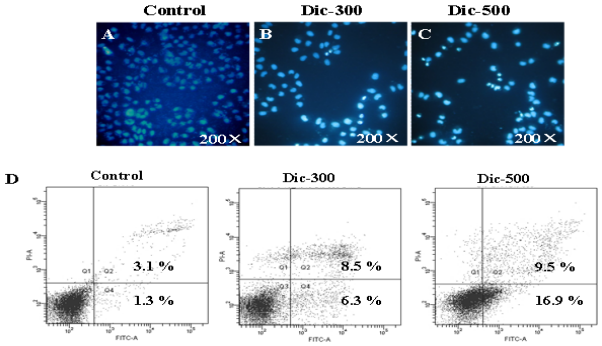

Figure 3. Dic Induces Apoptosis Assessed by Hoechst 33258 Staining and Annexin V-FITC /PI Analysis. (A) Nuclear morphology of control cells by Hoechst 33258 staining. Magnification is 200. (B) and (C) Nuclear morphology of Dic-treated cells by Hoechst 33258 staining. The cells were treated with Dic for 24 h before Hoechst 33258 staining. Magnification is 200. (D) FCM analysis of Dic-induced apoptosis by fluorescence-activated sorting analysis of annexinV-FITC/PI. Cells were treated with indicated concentrations (300 and 500 μM) of Dic for 24 h, respectively

To explore whether Dic induces cell cycle arrest, A549 cells were harvested at 24 h after Dic treatment and then stained with PI for FCM assay. The sub-G1 population indicated the chromatin degradation which is one of concomitant events that characterize the commitment of a cell to apoptosis. As the treatment concentration increased, the percentage of cells in sub-G1 significantly increased from 1.0 % (control) to 6.3 % (300 μM) and 43.4 % (500 μM) (Figure 2). In the non-apoptotic population, cells in S phase increased from 24.7 % (control) to 56.2 % (300 μM) and 57.6 % (500 μM) and cells in the G0/G1 phase decreased from 68.5 % (control) to 42.8 % (300 μM) and 42.4 % (500 μM) (Figure 2). These results suggested that Dic blocked A549 cell cycle from G0/G1 to S phase and arrested A549 cell cycle in S phase.

Dic induces dose-denpendent cell apoptosis

Cell cycle analysis assay have showed that Dic induced an increase of sub-G1 proportion (Figure 2). To verify Dic-induced cell death is associated with apoptosis, we stained A549 cells with Hoechst 33258 to show the cell nuclear morphology in the fluorescent microscope. As showed in Figure 3A, control cells showed homogeneous distribution of Hoechst 33258. However, after exposure to 300 and 500 μM Dic for 24 h, cells with typical morphologic changes of apoptosis that chromatin condensation and shrunken nucleus (Figure 3B and C) were increased.

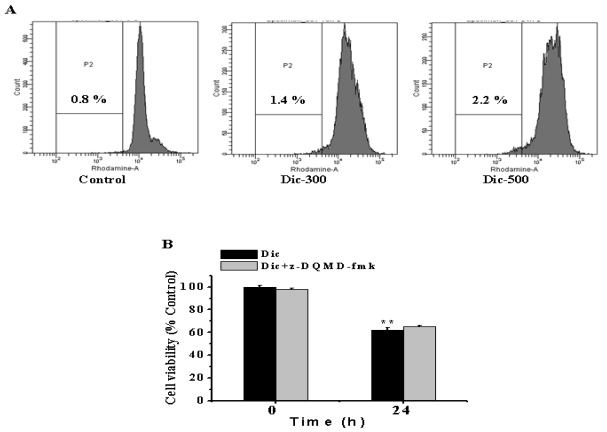

Figure 4. The Loss of Mitochondrial Membrane Potential (ΔΨm) and Activation of Caspase-3 Were not Involved in Dic Induces Apoptosis. (A) FCM analysis of RV-induced loss of mitochondrial membrane potential (ΔΨm). After treatment with 300 and 500μM of Dic for 24h, the cells were stained with 1 μM Rho123 for 30 min. The number in left quadrant represents the percentage of cells with reduction in ΔΨm. (B) Inhibition of z- DQMD-fmk on the Dic-induced apoptosis. $^{**}P < 0.01$, compared with control

To further confirm the apoptotic form of Dic induced cell death, we used Annexin V/PI staining assay to examine the integrality of cell membrane and the externalization of phosphatidyl-serine, the early apoptotic characteristic. Assessment of the cells exposed to Dic or not showed that the percentage of cells with phosphatidyl-serine externalization increased from 4.4 % (control) to 14.8 % (300 μM) and 26.4 % (500 μM) (Figure 3D). These demonstrated that Dic induced a dose-dependent A549 cell apoptosis.

Mitochondria and caspase-3 are not involved in Dic-induced cell apoptosis

Typical apoptosis pathway involves mitochondrial pathway and death receptor pathway. First, we measured the changes of ΔΨm using Rho 123 staining by FCM to determine the involvement of mitochondrial pathway in Dic-induced A549 cell apoptosis. Compared to Control, Dic treatment almost not induced the decrease of Rho 123 fluorescence intensity (Figure 4A), implying that mitochondria were not involved in the Dic-induced apoptosis. Moreover, we tested the effects of caspase-3 inhibitor, z-DQMD-fmk, on Dic-induced A549 cell apoptosis. Cells were pre-incubated with 10 μM z-DQMD-fmk for 1 h before 300 μM Dic treatment. As shown in Figure 4B, Dic-induced cytotoxicity was not attenuated by z-DQMD-fmk pretreatment, implying that caspase-3 was not involved in the Dic-induced apoptosis.

DHA enhances Dic induced cell cytotoxicity

To determine whether DHA enhances Dic induced cell death, cells were respectively co-treatment with 6 or 12 ug/ml DHA and 100 μM Dic (Figure 5A). CCK-8 assay showed that 100 μM Dic induced 23.3 % decrease of A549 cell viability and 22.3 % of 6 ug/ml DHA. However, co-treatment of 100 μM Dic and 6 ug/ml DHA induced 47.4 % decrease of A549 cell viability. In addition, co-treatment of 100 μM Dic and 12 ug/ml DHA induced

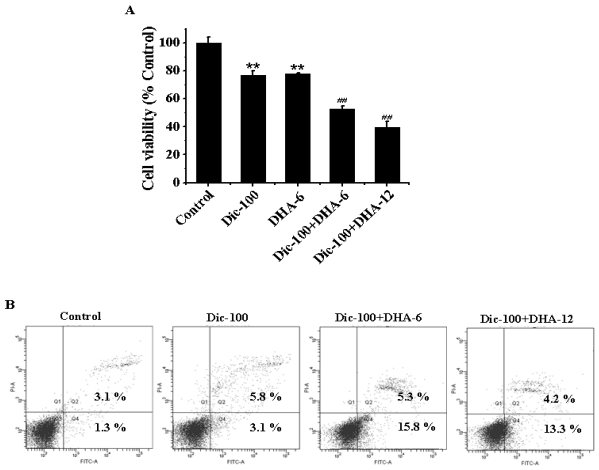

Figure 5. DHA Enhances Dic Induced Cell Apoptosis. (A) DHA enhances Dic -induced reduction of cell viability assessed by CCK-8. Cells were incubated with indicated concentrations of drugs (100 μM Dic, 6 μg/ml DHA, 100 μM Dic and 6 μg/ml DHA, 100 μM Dic and 12 μg/ml DHA) for 24h. (B) FCM analysis of the indicated concentrations of drugs (100 μM Dic, 100 μM Dic and 6 μg/ml DHA, 100μM Dic and 12 μg/ml DHA)-induced apoptosis by fluorescence-activated sorting analysis of annexinV-FITC/PI. Cells were treated with indicated concentrations of drugs for 24 h, respectively

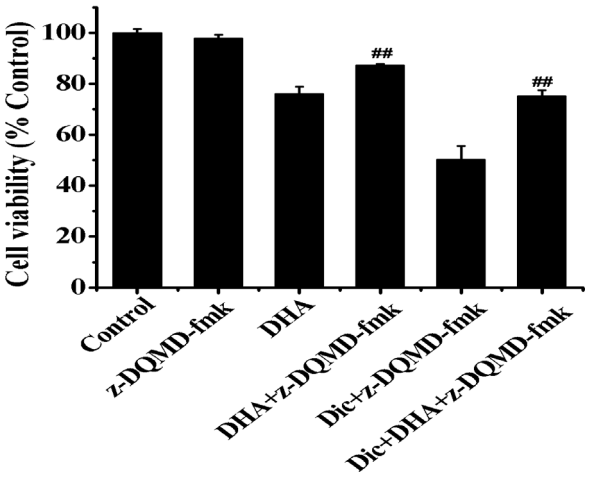

Figure 6. Combination Treatment with DHA and Dic Induce Caspase Dependent Cell Apoptosis. Inhibition of z- DQMD-fmk on the indicated concentrations of drug (6 μg/ml DHA; 100 μM Dic and 6 μg/ml DHA)-induced apoptosis. $^{\#\#}P < 0.01$, compared with DHA treatment or combination treatment with DHA and Dic

60.6 % decrease of A549 cell viability. These results suggested that DHA markedly increased Dic induced cell death. To define whether Dic and DHA co-induced cell death is associated with apoptosis, we used Annexin V/PI staining assay to examine the integrality of cell membrane and the externalization of PS. The results showed that the percentage of cells with PS externalization were increase from 8.9 % (treatment with Dic only) to 21.1 % (co-treatment with 6 ug/ml DHA and Dic) and 17.6 % (co-treatment with 12 ug/ml DHA and Dic) (Figure 5B), implying that DHA enhanced Dic-induced A549 cell apoptosis.

DHA enhances Dic-induced cell apoptosis via caspase-3 dependent pathway

It's known that DHA induces cancer cell apoptosis via caspase-3-dependent mitochondrial pathway (Lu et al., 2009) To determine whether caspase-3 was involved in DHA and Dic co-induced apoptosis, cells were pre-incubated with 10 μM z-DQMD-fmk for 1 h before drugs treatment. As shown in Figure 6, pretreatment with z-DQMD-fmk significantly attenuated not only DHA-induced apoptosis but also Dic and DHA co-induced apoptosis, implying that caspase-3 play an important role in Dic and DHA co-induced A549 cell apoptosis.

Discussion

In recent years, cancer prevention and treatment using traditional chinese medicines have attracted increasing interest (Caia et al., 2004). Here, we for the first time demonstrated that Dic dominantly induced S phase cell cycle arrest in low concentration and cell apoptosis in high concentration in which mitochondria and caspase-3 were not involved in A549 cell line. Moreover, we also found that DHA enhanced Dic-induced cell apoptosis via caspase-3-dependent pathway.

Cell viability assay showed that Dic induced dose- and time-dependent A549 cell apoptosis (Figure 1). Interestingly, our results showed that Dic induced S phase cell cycle arrest in low concentration (300 μM) (Figure 2A) and cell apoptosis in high concentration (500 μM) (Figure 2B). In response to genotoxic stress including etoposide and X-ray irradiation, cells may undergo cell cycle arrest and DNA repair or commit suicide to apoptosis when the damage is beyond repair (Jiang et al., 2005). It is also reported that C75, an inhibitor of fatty acid synthase (FAS), triggered apoptosis during S phase in human cancer cells (Zhou et al., 2003). Cyclin D1, cyclin A and cyclin B1 may be involved in the S phase cell cycle arrest (Joe et al., 2002). Therefore, we infer that Dic induces cell cycle arrest in low concentration and early stage of treatment, but induces cell apoptosis in high concentration and late stage of treatment when the damage can not be repaired.

The mitochondrial disruption and the activation of caspase-3 have been shown to play a critical role in apoptosis induced by arrange of stimuli in a wide variety of cell lines (Fan et al., 2005; Alvero et al., 2011). However, previous studies suggest the existence of mitochondria or/and caspases-independent cell death in programmed cell death (PCD). For instance, it has been variously reported that apoptotic pathway was activated by stimuli in a mitochondria-dependent and -independent (MacDonald et al., 1999) or caspase-independent (Kang et al., 2004; Li et al., 2004; Shrivastava et al., 2006; Zhang et al., 2011) or both mitochondria- and caspase-independent manner (Chauhan et al., 2004; Bhalla et al., 2009). Our results showed that Dic did not induced any mitochondrial depolarization and caspase-3 inhibition did not prevent Dic-induced A549 cell death indicating that Dic induced a mitochondria- and caspase-3-independent A549 cell apoptosis (Figure 4). It is well known that NF-κB induced the expression of antiapoptotic molecules such as Bcl-2 and Bcl-XL (Estrov et al., 2003). Bcl-2 overexpression

suppresses the loss of $\Delta\Psi m$, cytochrome c and apoptosis-inducing factor released from mitochondria, which prevents the activation of caspases (MacDonald et al., 1999). Other reports demonstrated that p53-mediated transcriptional repression is involved in the caspase-independent apoptotic pathway (Godefroy et al., 2004). Therefore, we infer that NF-κB and p53 may be the most possible involved in Dic-induced mitochondria and caspase-independent apoptosis. However, it remains unclear whether these molecules play a role in Dic-induced apoptosis.

DHA has been reported that have co-treatment effect with many other anti-tumor compounds (Caia et al., 2004; Zongo et al., 2007; Chen et al., 2013). After enrolled into cytosol, DHA can react with Fe^{2+} to generate a lot of reactive oxygen species which mediated caspase-9, -3 and/or caspase-8 activation resulting in cell apoptosis (Mercer et al., 2007; Mercer et al., 2011). In addition, chen et al (Chen et al., 2013) reported that caspase-8 and -3 were activated in the synergistic cytotoxicity of the combination treatment with DHA and X-ray irradiation. The anti-tumor pathway of DHA is much different from that of Dic. Therefore we explored their co-treatment effect on A549 cells. Importantly, we for the first time demonstrated that DHA markedly enhanced Dic-induced A549 cell death and apoptosis (Figure 5) in which caspase-3 played an important role (Figure 6). Cytochrome c was released from mitochondria after treatment with some of apoptotic stimuli, subsequently resulting activate caspase 9 and then caspase 3 (MacDonald et al., 1999). The enhancement of DHA on Dic-induced A549 cell apoptosis can be explained that Dic-induced S phase cell cycle arrest may change some proteins expression level which sensitize A549 cells to DHA treatment. However, the upstream molecular mechanism of the activation of caspase-3 induced by the combination treatment with DHA and Dic need to be further investigated.

In summary, our findings lead to the conclusion that as an anticancer drug, Dic induces A549 cell cycle arrest and apoptosis via mitochondria and caspase-independent apoptotic pathway. DHA enhances Dic-induced cell apoptosis via caspase-3-dependent apoptotic pathway. The detailed molecular mechanism on DHA how enhances Dic induced apoptosis of A549 cells reported here are valuable for further investigation.

Acknowledgements

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