Synergistic anti-cancer activity of the combination of dihydroartemisinin and doxorubicin in breast cancer cells

Guo-Sheng Wu, Jin-Jian Lu, Jia-Jie Guo, Ming-Qing Huang, Li Gan, Xiu-Ping Chen, Yi-Tao Wang

State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macao, China
College of Life Sciences, Zhejiang Chinese Medical University, Hangzhou, Zhejiang, 310053, China
College of Pharmacy, Fujian University of Traditional Chinese Medicine, Fuzhou, Fujian, 350008, China

Correspondence: Jin-Jian Lu, e-mail: jjian@lumac.com and Yi-Tao Wang, e-mail: ytwang@umac.mo

Abstract:
Background: Dihydroartemisinin (DHA) exhibits potent anti-malarial and anti-cancer activities. This study aimed to investigate the anti-proliferative effects of a combination of DHA and doxorubicin (DOX) on human breast cancer cells.

Methods: MTT assay and the combination index (CI) were used to show the anti-proliferative effects and calculate the synergism potential, respectively. Flow cytometry assay was used to detect apoptosis and the intracellular accumulation of DOX. JC-1 staining was used to determine the mitochondrial membrane potential. Western blot analysis was used to detect the protein expression of some apoptosis-related molecules.

Results: A synergistic anti-proliferative effect was found, and the enhanced anti-cancer activity was observed to be accompanied by the prompt onset of apoptosis in MCF-7 cells. The combinative treatment remarkably decreased the mitochondrial membrane potential and activated caspase cascades more than the mono-treatment. Pretreatment with DHA also did not influence the accumulation of DOX in MCF-7 cells.

Conclusion: This study presented a new opportunity to enhance the effectiveness of future treatment regimens of breast cancer using DOX.

Key words: dihydroartemisinin, doxorubicin, synergistic, anti-tumor, apoptosis; MCF-7

Introduction

Breast cancer is one of the most common cancers worldwide and the most common among women [8]. Doxorubicin (DOX), an anthracycline drug, is widely used as a chemotherapeutic agent for breast cancer. Despite the remarkable anti-cancer activity of DOX,
its practical therapeutic use is limited by toxicities such as cardiotoxicity [19]. Therefore, combined treatment with other drugs is desirable.

Traditional Chinese medicinal herbs have a long history and are widely viewed as new, attractive sources of therapeutic regimens without severe toxicity. The herb *Artemisia annua* L., which has been used in China for centuries to treat fever and chills, contains artemisinin as its active constituent. Artemisinin and its derivatives (ARTs) are extensively used as anti-malarial drugs without severe side effects and have been found to inhibit the growth of tumor cells [4, 11, 17, 18]. One of the main active metabolites of ARTs is dihydroartemisinin (DHA) (Fig. 1), which is one of the most effective anti-cancer compounds both *in vitro* and *in vivo*. DHA mediates cell cycle arrest, induces apoptosis, blocks angiogenesis, and inhibits metastasis [1, 7, 10–12, 17]. DHA also selectively kills cancer cells with little effect on normal cells [6, 13]. DHA enhances as well the anti-cancer potential of gemcitabine in human hepatoma cells and cooperates with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to induce apoptosis in human prostate cancer cells [5, 6]. Herein, we hypothesized that the combination of DHA and DOX in human breast cancer cells exerts a synergistic anti-cancer effect.

In this study, we found that the combination DHA and DOX exerted a synergistic effect on breast cancer cells. DHA pretreatment was found to enhance DOX-mediated apoptosis in MCF-7 cells significantly. This enhancement was accompanied by an increase in the cleavage of poly (ADP-ribose) polymerase (PARP) and the activation of caspase cascades. Our results revealed a new strategy for enhancing the effectiveness of future treatment regimens using DOX.

### Materials and Methods

#### Reagents

DHA was provided by Prof. Ying Li of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China). DOX was obtained from Sigma (St. Louis, MO, USA). 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyltetrazolium bromide (MTT) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyldi-carbo cyanine iodide (JC-1) were purchased from USB (OH, USA) and Molecular Probes (Eugene, OR, USA), respectively.

#### Cell lines and culture

MCF-7, MDA-MB-231, and T-47D human breast cancer cells were obtained from ATCC (Manassas, VA, USA). The cells were maintained at 37°C under an atmosphere of 5% CO₂ and 95% air in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin).

#### MTT assay

Exponentially growing MCF-7, MDA-MB-231, and T-47D cells were seeded into 96-well plates. The cells were treated with the indicated compounds, and cell viability was determined after 48 h of incubation by adding 20 µl of MTT (5 mg/ml). After slightly aspirating the MTT-containing medium after 4 h, 100 µl of dimethyl sulfoxide was added to solubilize the formazan followed by shaking 10 min in darkness. The absorbance at 570 nm was recorded using a Multilabel counter (PerkinElmer, Singapore).
**Observation of morphologic changes**

MCF-7 cells were seeded into six-well plates and treated with 10 µM DOX for 24 h with or without pretreatment of 20 µM DHA for 6 h. Cellular morphology was observed with an AxioCam HRc CCD camera (Carl Zeiss, Germany).

**Flow cytometry assay**

MCF-7 cells seeded into six-well plates were treated with 10 µM DOX for 24 h with or without pretreatment of 20 µM DHA for 6 h. The cells were harvested, fixed in 70% ethanol, and stored at 4°C overnight. Then, the cells were stained in phosphate-buffered saline (PBS) containing 5 µg/ml RNase and 20 µg/ml propidium iodide (PI) in the dark at room temperature for 30 min. Flow cytometry (Becton Dickinson FACSCanto™, Franklin Lakes, NJ, USA) was used to analyze the cells. At least 10,000 events were counted for each sample, and the sub-G1 was analyzed.

**Mitochondrial membrane potential (MMP) assay**

The MMP of intact cells was measured by a fluorescent inverted microscope with the lipophilic probe JC-1 [15]. MCF-7 cells were seeded into six-well plates and treated with 10 µM DOX for 24 h with or without pretreatment of 20 µM DHA for 6 h. JC-1 fluorescence was observed with a fluorescent microscope, and pictures were taken with an Axiovert 200 fluorescent inverted microscope (Carl Zeiss) and an AxioCam HRc CCD camera (Carl Zeiss).

**Western blot analysis**

After treatment under desired conditions, the cells were harvested and total proteins were extracted with radioimmunoprecipitation assay lysis buffer containing 1% phenylmethanesulfonyl fluoride and 1% protease inhibitor cocktail for 30 min. Equal amounts of total protein were separated by appropriate SDS-PAGE and transferred onto a poly (vinylidene fluoride) membrane. After blocking with 5% non-fat dried milk for 1 h, the membrane was incubated with the specific primary antibodies against PARP, caspase-7, caspase-9, and β-actin (Cell Signaling Technology, Beverly, MA, USA) followed by incubation with corresponding secondary antibodies at 37°C for 1 h. Specific protein bands were visualized with an ECL advanced western blot analysis detection kit.

**Intracellular accumulation of DOX**

MCF-7 cells were pre-cultured for 6 h with or without indicated concentrations of DHA and treated with different concentrations of DOX for 1 h. The cells were then harvested and resuspended in 1 ml of PBS. The cells were washed three times with PBS, and the mean fluorescence intensities of the cells were detected with a fluorescence-activated cell sorting (FACS)-Calibur cytometer (Becton Dickinson, San Jose, CA, USA).

**Statistical analyses**

The combination index (CI) is widely used to quantify drug synergism based on the multiple drug effect equation of Chou–Talalay [2, 3]. In our study, the CI values were determined for each concentration of DHA, DOX, and their combination in cell proliferation assays using CalcuSyn (Biosoft, Cambridge, UK). CI < 0.9 indicates synergism, CI = 0.9–1.10 indicates additive interaction, and CI > 1.10 indicates antagonism [20].

**Results**

**DHA synergistically increased the DOX-induced inhibition of cell proliferation**

We first examined the synergistic effects of DHA and DOX on the proliferation of MCF-7, MDA-MB-231, and T-47D human breast cancer cell lines. Figure 2A shows a schematic of the experimental protocol for the combined treatment. Cells in 96-well plates were pre-treated with serial concentrations of DHA for 6 h and further treated with or without indicated DOX for 48 h. The cell proliferation inhibition bars to DHA, DOX, and DHA combined with DOX from three independent experiments are shown in Figures 2B–D. DHA exhibited moderate cytotoxicity against MCF-7 and T-47D cancer cells but had little effect on MDA-MB-231 cells, consistent with our previous reports [11, 12]. After the combined treatment, markedly stronger anti-proliferation abilities were achieved.
Subsequently, CI values were calculated using CalcuSyn at fixed-ratio concentrations of DHA and DOX. DHA plus DOX exhibited synergy in the tested breast cancer cell lines except at the highest dose in MDA-MB-231 cells, in which an additive effect was observed (Fig. 2E).

**DHA sensitized DOX-triggered apoptosis**

We further investigated the effects of exposure to DHA, DOX, or their combination on the apoptosis-inducing abilities in MCF-7 cells. We first detected morphological changes after DHA, DOX, and DHA plus DOX treatment. A significantly higher number of round cells appeared in the combined-treatment group than in the other groups (Fig. 3A). We then used flow cytometry analysis with PI staining to detect the sub-G1 content, which is a characteristic of apoptosis. The cells treated with both DHA and DOX had the highest percentage in sub-G1. Given that mitochondria significantly influence DHA-induced apoptotic cell death [11] and mitochondrial changes including MMP collapse, the activation of caspases results in apoptosis. Therefore, we detected MMP changes after DHA, DOX, and DHA + DOX treatment. JC-1, which is a dual-emission fluorescent dye internalized and concentrated by respiring mitochondria, can reflect MMP changes in live cells [15]. In 24 h-treated MCF-7 cells, mitochondrial membrane depolarization as de-
Fig. 3. DOX + DHA triggers increased apoptosis in MCF-7 cells. MCF-7 cells seeded into six-well plates were treated with 20 µM DHA, 10 µM DOX, or their combination for 24 h; the morphological changes, sub-G1, MMP, and protein expression were detected. (A) Classical morphological changes. (B) Results of flow cytometry with PI staining. (C) JC1 mitochondrial probe for MMP test. (D) Protein extracts were immunoblotted with the specific antibodies for PARP, caspase-7, and caspase-9.

determined by JC-1 was moderately induced by DOX or DHA alone, whereas combined DOX and DHA resulted in a greater additive effect than the individual agents (Fig. 3C). We investigated further the effect of DHA, DOX, and their combination on PARP and caspases through western blot analysis. In MCF-7 cells, treatment with combined DOX (10 µM) and DHA (20 µM) for 24 h caused a much more significant cleavage of PARP and activation of caspase-7 than in mono-treated cells. This finding indicated the involvement of caspase-mediated apoptosis triggered by DHA + DOX, which favored the sensitizing effects of DHA on DOX-induced apoptosis (Fig. 3D). Besides, apoptosis induced by DHA + DOX was accompanied by the loss of MMP. This result prompted us to examine the involvement of caspase-9 in this process.

In MCF-7 cells, DOX combined with DHA activated caspase-9 to a much higher extent than the mono-treatments (Fig. 3D). Collectively, these data showed that DHA sensitized DOX in activating caspase cascades and triggering apoptosis.

**DHA pretreatment did not influence the intracellular concentration of DOX**

To determine whether DHA enhanced DOX activity by increasing the intracellular concentration of DOX, DOX was examined in the presence or absence of DHA. Given that DOX exhibited self-fluorescence, the intensity of intracellular fluorescence was adopted to reflect the intracellular concentration through FACS assay. MCF-7 cells were pretreated with DHA for 6 h and then with DOX for 1 h. We found that the fluo-
cence intensity in the DOX group was significantly higher than that in the blank and DHA-treated groups. However, the fluorescence intensity in the combined-treatment group was identical to that in the DOX group, indicating that DHA did not influence the intracellular concentration of DOX (Figs. 4A and B).

**Discussion**

The CI is a widely accepted qualitative measure of the extent of drug interaction. In this study, the CI was used to evaluate the combination effects of DOX and DHA on the proliferation of breast cancer MCF-7, MDA-MB-231 and T-47D cells. DHA potentiated DOX-imposed cytotoxicity as revealed by the CI values (Fig. 2E) and the obvious enhancement of proliferation inhibition in the combined-treatment group (Figs. 2B–D). Compared with MCF-7 and T-47D cells, MDA-MB-231 cells were much more susceptible to DOX. However, after being combined with DHA, DOX exhibited similar potent anti-proliferative capabilities especially at relatively high concentrations (Figs. 2B–D). More importantly, DHA did not enhance intracellular DOX accumulation in MCF-7 cells (Figs. 4A and B). Thus, the same anti-cancer effect may be preferred at low doses of combined DOX and DHA or high doses of DOX alone. DHA is also widely used as an anti-malarial drug with few side effects, and the DHA-resistant cancer cell line does not present a multidrug-resistant phenotype [9]. Thus, DHA + DOX has tremendous latent capacity for clinical treatment in the future. Artemisinin, the mother compound of ARTs, reportedly induces resistance to DOX in human HT29 colon cancer cells and MCF-7 breast cancer cells partially by inducing P-glycoprotein (P-gp) expression [14]. Thus, artemisinin pretreatment reduces the intracellular DOX because of P-gp expression. The discrepancies may be due to the different compounds used. Although DHA is one of the main metabolites of ARTs, the pharmacological activities of artemisinin and DHA differ [6]. However, the detailed mechanisms require further investigation.

The PI staining, JC-1 staining, and western blot analysis data indicated significant effects on apoptosis in the combined DOX and DHA groups compared with the mono-treated groups. This improvement partially explained the synergistic anti-proliferation ef-
fect of the DHA + DOX treatment. Actually, the enhancement of apoptosis is an effective way to improve the anti-proliferation potential in cancer cells [16]. However, how does DHA enhance DOX-induced apoptosis still warrants further study.

In summary, DHA + DOX significantly improved the anti-cancer activity of each component, as revealed by the synergistic inhibitory effects on cancer cell proliferation. The synergism was partially due to the sensitized execution of apoptosis. Therefore, the superior pharmacological activities of DOX + DHA enabled their use as a promising anti-cancer strategy, although further research is needed.

Acknowledgments:
This work was supported by Research Fund of Zhejiang Chinese Medicine University (No. 2006ZZ04), National Natural Science Foundation of China (No. 81001450), Research Fund of University of Macau (SRG0006-CMSY14-LUJ, UID1008-IV/CM(SYSTD/007/08)), and Science and Technology Development Fund of Macau Special Administrative Region (025/2007/Ad). We greatly thank Prof. Ying Li from Shanghai Institute of Materia Medica for providing DHA and Dr. Hong Zhu from Zhejiang University for the statistical analyses.

References:

Received: October 10, 2011; in the revised form: November 3, 2012; accepted: November 16, 2012.