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Original Contribution

DJ-1 mediates the resistance of cancer cells to dihydroartemisinin through reactive oxygen species removal

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ABSTRACT

Dihydroartemisinin (DHA), one of the main metabolites of artemisinin and its derivatives, presents anti-cancer potential in vitro and in vivo. To explore the mechanisms of resistance toward DHA, a DHA-resistant cell line, HeLa/DHA, was established with a resistance factor of 7.26 in vitro. Upon DHA treatment, apoptotic cells were significantly elicited in parental HeLa cells but minimally induced in HeLa/DHA cells. HeLa/DHA cells also displayed much less sensitivity to DHA-induced tumor suppression in cancer xenograft models than HeLa cells. Intriguingly, DHA-resistant cells did not display a multidrug-resistant phenotype. Based on a proteomic study employing LC–ESI–MS/MS together with pathway analysis, DJ-1 (*PARK7*) was found to be highly expressed in HeLa/DHA cells. Western blot and immunofluorescence assays confirmed the higher expression of DJ-1 in HeLa/DHA cells than in parental cells in both cell line and xenograft models. DJ-1 is translocated to the mitochondria of HeLa/DHA cells and oxidized, providing DJ-1 with stronger cytoprotection activity. Further study revealed that DJ-1 knockdown in HeLa/DHA cells abolished the observed resistance, whereas overexpression of DJ-1 endowed the parental HeLa cells with resistance toward DHA. Reactive oxygen species (ROS) were also significantly induced by either DHA or hydrogen peroxide in HeLa cells but not in resistant HeLa/DHA cells. When the cells were pretreated with *N*-acetyl-L-cysteine, the effect of DJ-1 knockdown on sensitizing HeLa/DHA cells to DHA was significantly attenuated. In summary, our study suggests that overexpression and mitochondrial translocation of DJ-1 provides HeLa/DHA cells with resistance to DHA-induced ROS and apoptosis.

Artemisinin, a natural product from the Chinese medicinal herb *Artemisia annua* L., and its derivatives (ARTs) are widely used for malaria treatment [1] and exhibit potent anti-cancer activities against a variety of cancer cells [2–10] and human tumor xenografts in nude mouse models, including colorectal carcinoma, hepatoma, pancreatic cancer, and ovarian cancer [3,6,10–12]. ARTs exert their anti-cancer effects through multiple mechanisms, including iron- or heme-mediated reactive oxygen species (ROS) generation and cytotoxicity [4,13], endoplasmic reticulum stress [9,14], and NF-κB inhibition [15].

Dihydroartemisinin (DHA), one of the main active metabolites of ARTs, has been extensively studied because of its potential anti-cancer capability in vitro and in vivo [3,5–9,12]. DHA presents synergistic anti-cancer potential when combined with cisplatin, carboplatin, cyclophosphamide, doxorubicin, gemcitabine, or tumor necrosis factor-related apoptosis-inducing ligands [3,6,16–18]. However, the ability of cancers to develop resistance to chemotherapeutic agents [19,20] indicates that the primal sensitive cancer cells may acquire resistance to DHA treatment. Therefore, further development of DHA (and other ARTs) as an anti-cancer agent necessitates a better understanding of the mechanisms by which cancer cells elude DHA treatment.

A DHA-resistant HCT116/R cell line with four times higher IC_{50} than the parental HCT116 cell line was previously established [21]. This cell line does not present a multidrug-resistant (MDR)

Abbreviations: ARTs, artemisinin and its derivatives; BCRP, breast cancer resistance protein; DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DHA, dihydroartemisinin; DHE, dihydroethidine; 5-FU, 5-fluorouracil; GCLC, glutamate–cysteine ligase catalytic subunit; MDR, multidrug-resistant; MDR1, multidrug-resistant protein 1; MRP1, MDR-associated protein 1; MS, mass spectrometry; Nrf2, nuclear factor erythroid 2-related factor; P-gp, P-glycoprotein; PI, propidium iodide; ROS, reactive oxygen species; shRNA, short hairpin RNA; siRNA, small interfering RNA; SRB, sulforhodamine B; TPT, topotecan; VP16, etoposide

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phenotype. Development of other DHA-resistant cancer cell lines would better illustrate whether cells with resistance to DHA present MDR phenotypes. In this study, a DHA-resistant HeLa/DHA subline was established. This subline is resistant to apoptosis caused by DHA and shows unaltered MDR-related gene expression. We also established, for the first time, a causal link between DJ-1 and resistance toward DHA by conducting a proteomic study and pathway analysis. DJ-1 was found to mediate the resistance of cancer cells to DHA through ROS removal.

Materials and methods

Chemicals and reagents

DHA was obtained from the National Institute for Food and Drug Control (Beijing, China). H_2O_2 and topotecan (TPT) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China) and Aosaikang Pharmaceutical Co. Ltd. (Nanjing, China), respectively. Taxol was obtained from Bristol–Myers Squibb (New York, NY, USA). Etoposide (VP16), 5-fluorouracil (5-FU), doxorubicin, propidium iodide (PI), and sulforhodamine B (SRB) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). MitoTracker red was purchased from Life Technologies (Carlsbad, CA, USA).

Cell lines and culture

Both human cervical cancer HeLa (ATCC) and HeLa/DHA cell lines were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and Hepes (10 mM), pH 7.4, in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Establishment of DHA-resistant HeLa/DHA subline

The DHA-resistant HeLa/DHA cell line was established as described previously, with minor modifications [8]. Briefly, HeLa cells were exposed to 5 μM DHA for 24 h and then cultured in fresh medium until the surviving cells had recovered and displayed normal exponential growth. Selection cycles were performed in the presence of gradually increasing DHA concentrations from 5 to 100 μM DHA for about 9 months (Supplementary Fig. S1). Surviving cells were harvested and propagated in drug-free medium for further studies. The newly established DHA-resistant HeLa subline was designated as HeLa/DHA. During HeLa/DHA cell line establishment, the parental HeLa cell line was always maintained in drug-free medium in parallel.

SRB assay

The effects of DHA, taxol, VP16, TPT, and 5-FU on cell proliferation inhibition were determined by SRB as described in a previous study [22]. Briefly, cells were seeded into 96-well plates, cultured overnight, and treated with the compounds for the indicated time. The cells were then fixed with 10% trichloroacetic acid and stained with SRB. SRB in the cells was dissolved in 10 mM Tris–HCl and measured at 515 nm using a multiwell spectrophotometer (Thermo Electron Corp., Marietta, OH, USA). The rate of inhibition of cell proliferation was calculated for each well as $[(A_{515\ \text{control cells}} - A_{515\ \text{treated cells}})/A_{515\ \text{control cells}}] \times 100\%$ (where A_{515} is the OD value at 515 nm).

Measurement of in vivo activity

Tumors were established by injection of HeLa or HeLa/DHA cells (5×10^6 cells per animal, subcutaneously into the armpit) into 5- to 6-week-old BALB/c female athymic mice (National Rodent Laboratory Animal Resource). Tumor volume (mm^3) was measured using calipers and calculated as $(W^2 \times L)/2$, where W is the width and L is the length. Athymic mice were administered vehicle or DHA (100 mg/kg, intraperitoneal injection) dissolved in 5% cremophor, 2% dimethyl sulfoxide, and 0.9% sterile sodium chloride solution five times per week. The tumor volume on day n was expressed as relative tumor volume (RTV) according to the following formula: $\text{RTV} = \text{TV}_n/\text{TV}_0$, where TV_n is the tumor volume on day n and TV_0 is the tumor volume on day 0. Therapeutic effects of treatment were expressed in terms of T/C %, using the calculation formula $T/C\% =$ (mean RTV of the treated group/mean RTV of the control group) × 100%. Animal care was in accordance with guidelines for the use of laboratory animals and approved by the Committee on the Ethics of Animal Experiments of Zhejiang University.

Annexin V–FITC assay

Cells seeded in six-well plates were exposed to 30 and 100 μM DHA for 48 h. Cells undergoing apoptosis were detected using an annexin V–FITC apoptosis detection kit (Invitrogen). Briefly, cells were resuspended in cold binding buffer and incubated for 15 min in the dark at room temperature; addition of 5 μl annexin V–FITC and 5 μl PI solutions followed. Flow cytometry analysis was performed using a FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA).

Doxorubicin intracellular accumulation

HeLa and HeLa/DHA cells were cultured for 2 h at the indicated doxorubicin concentrations. Cells were then harvested and resuspended in 1 ml of phosphate-buffered saline (PBS). The cells were washed twice with PBS and the mean fluorescence intensities of cells were detected using a FACSCalibur cytometer (BD Biosciences).

Mitochondrial fractionation

HeLa or HeLa/DHA cells were washed twice with cold PBS and fractionated using a mitochondria extraction kit (Beyotime, Haimen, China) according to the manufacturer's protocol.

Proteomic and pathway analyses

Experiments for proteomic analysis were performed by PTM Biolab, Inc. (Hangzhou, China). Briefly, the proteins of whole-cell lysates and mitochondrial fractions extracted from HeLa and HeLa/DHA cells were concentrated into gels. The gel slices were cut into small cubes of 1 mm^3 and then digested via a series of standard procedures. The peptides were separated and confirmed using liquid chromatography (LC)–electron spray ionization (ESI)–tandem mass spectrometry (MS/MS) analysis. Fragment spectra were searched against the human IPI version 3.87 database using the Mascot search engine (version 2.3.0, Matrix Science). The expression of proteins that differed between HeLa and HeLa/DHA cells was calculated (i.e., proteins extracted from whole-cell lysates of HeLa cells versus proteins extracted from whole-cell lysates of HeLa/DHA cells and proteins extracted from mitochondrial fractions of HeLa cells versus proteins extracted from mitochondrial fractions of HeLa/DHA cells). Proteins with twofold differences in expression between groups were selected, and pathway analysis