Dichloroacetate shifts the metabolism from glycolysis to glucose oxidation and exhibits synergistic growth inhibition with cisplatin in HeLa cells

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Abstract. The unique bioenergetic feature of cancer, aerobic glycolysis or the Warburg effect, is an attractive therapeutic target for cancer therapy. Reversing the glycolytic phenotype may trigger apoptosis in tumor cells. Recently, dichloroacetate (DCA) was proven to produce significant cytotoxic effects in certain tumor cells through this distinct mechanism. In this study, the effect of DCA on the metabolism of cervical cancer HeLa cells was explored and its synergistic growth inhibition with cisplatin was also evaluated. The intracellular changes in HeLa cells following DCA exposure were analyzed through cell viability, intracellular H_2O_2 and pH levels, mitochondrial membrane potential (MMP), expression of apoptotic proteins and Kv1.5 channel, and intracellular-free Ca^{2+} concentration ($[Ca^{2+}]_i$). For the evaluation of combination chemotherapy, HeLa cells were treated with a combination of DCA and cisplatin at various concentrations for 48 h. Cell viability was determined by CCK-8 assay and the synergy of the two agents was evaluated using the R index method. DCA shifted the metabolism of HeLa cells from aerobic glycolysis to glucose oxidation as shown by the increased intracellular H_2O_2 and pH levels. The change of the metabolism modality led to a drop in MMP and the increase of apoptotic proteins (caspase 3 and 9). The increased Kv1.5 expression and decreased $[Ca^{2+}]_i$ established a positive feedback loop that resulted in reduced tonic inhibition of caspases. Combination chemotherapy of DCA and cisplatin exhibited a significant synergy in inhibiting the proliferation of HeLa cells. The specific apoptotic mechanism of DCA as distinguished from the cisplatin may be partly responsible for the synergy and further *in vivo* study on combination chemotherapy of the two agents in cervical cancer xenografts in mice is warranted.

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Introduction

Cervical cancer is the second most common cancer among women, with an estimated worldwide burden of 493,000 new cases and 274,000 deaths each year (1). Nowadays, there are two inspiring landmarks in the battle against cervical cancer. The first is the development of the Papanicolaou smear in 1943 which made possible the screening and early detection of the disease (2). The second is the prevention of cervical dysplasia using the human papillomavirus (HPV) vaccine (Gardasil®) approved by FDA in 2006 (3,4).

Despite these advances, nearly one-third of patients with invasive cervical cancer die from recurrent or metastatic disease and the management of invasive cervical cancer still remains a clinical challenge. Systematic chemotherapy is the major treatment modality for invasive cervical cancer, although in most circumstances it is limited to palliative management. Until recently, ~60 cytotoxic agents have been tested in cervical cancer (5), among them cisplatin, a drug that can bind to and cause crosslinking of DNA and ultimately trigger apoptosis in tumor cells, has consistently proven to be the most effective single anticancer drug against the recurrent or metastatic cervical cancer (5-7). However, the response rate of cisplatin treatment is not satisfactory, and the response is usually accompanied by high rates of toxicity such as nephrotoxicity, neurotoxicity and severe nausea and vomiting. Moreover, the majority of cancer patients will eventually relapse with cisplatin-resistant disease. Thus more effective and less toxic chemotherapy strategies for this disease are urgently required.

Recently, the unique bioenergetic feature of cancer, aerobic glycolysis or the Warburg effect, has received great attention as a potential therapeutic target in cancer therapy (8,9). Most cancer cells exhibit increased glycolysis and utilize this metabolic pathway for ATP generation as their main source of their energy supply despite an available oxygen source. This glycolytic phenotype is closely associated with the state of apoptosis resistance, and reversing this

cytoplasm-based glycolysis to the mitochondria-based glucose oxidation may trigger apoptosis in tumor cells (10-14). The key regulator of cellular glucose oxidation metabolism is pyruvate dehydrogenase (PDH) which is inhibited by pyruvate dehydrogenase kinase (PDK) in most tumor cells. Bonnet *et al* firstly found that dichloroacetate (DCA), a small molecule and a well-characterized inhibitor of PDK that has been used for more than 30 years in the treatment of lactic acidosis, could significantly inhibit the PDK activity in tumor cells and promote apoptosis in lung, breast and glioblastoma cancer cells. DCA also inhibited the growth of subcutaneously implanted A549 tumors in athymic rats without observable toxicity (10). DCA could also sensitize both wild-type and overexpressing Bcl-2 human prostate cancer cells to radiation *in vitro* (15). A recent study by Michelakis *et al* proved that DCA could exhibit clinical antitumor efficacy in patients with glioblastoma and the dose-limiting toxicity was a dose-dependent, reversible peripheral neuropathy, and there was no hematologic, hepatic, renal, or cardiac toxicity (16).

Based on these inspiring results, and the fact that this distinctive metabolism has not been targeted by classical chemotherapeutics, we hypothesized that DCA may be cytotoxic to cervical cancer cells and exhibit synergistic effects with cisplatin. This might allow the use of lower concentration of cisplatin and might potentially decrease the cisplatin-associated side-effects in chemotherapy. In the present study, we determined the *in vitro* cytotoxicity of DCA and explored the underlying mechanisms it uses against cervical cancer HeLa cells, and assessed the potential synergism of DCA and cisplatin in inhibiting the growth of HeLa cells.

Materials and methods

Reagents. Dichloroacetate (sodium salt, DCA), cisplatin, rotenone, DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt), 4-aminopyridine (4-AP), t-butyl-H_2O_2 (tert-butyl hydro-peroxide), DAPI (4',6'-diamidino-2- phenylindole dihydrochloride), and cleaved caspase 3 antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amplex Red, SNARF-1/AM (seminaphtorhodafluor-1-acetoxymethylester), MitoTracker Red, Fluo-3/AM and Hoechst 33342 were obtained from Molecular Probes (Eugene, OR, USA). VIVIT (NFAT inhibitor) was from Calbiochem (San Diego, CA, USA). Goat anti-rabbit IgG-TR, goat anti-mouse IgG-FITC, Kv1.5 (H-120) and actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against caspase 3, caspase 9 and cleaved caspase 9 were obtained from Cell Signaling Technology (Danvers, MA, USA)

Cell culture. HeLa (cervix adenocarcinoma, CCL-2) and HUVEC (human umbilical vein endothelial cells, CRL-1730) cell lines were obtained from the American Type Culture Collection (Manassas, VA) and routinely maintained in our lab. HeLa cells and HUVEC were maintained in Dulbecco's modified Eagle's medium (DMEM) and DMEM/F1, respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U penicillin/ml and 100 μg streptomycin/ml in a humidified atmosphere of 5% CO_2 at 37˚C.

Cell viability assay. Cell viability was evaluated by Cell Counting kit-8 assay (CCK-8 assay, Dojindo Molecular Technologies, Gaithersburg, MD). In brief, $5x10^3$ HeLa cells per well were seeded into 96-well plates. After 24 h incubation, the cells were treated with increasing concentrations of DCA (2, 4, 8 and 16 mM) or cisplatin (0.5, 1, 2, 4, 8, 16 μM) single-drug exposure for 48 h. Then the CCK-8 agent was added and incubated for 2 h at 37˚C for color development. The absorbance values (A) at 450 nm were measured using a microplate reader (Model 550, Bio Bad). One column of cells without drug treatment was used as the control and another column without cells was used as the blank. The cell viability (%) was calculated using the following equation: cell viability (%) = $[(A_{sample}-A_{blank})/(A_{control}-A_{blank})]$ x 100%. We performed at least three experiments.

H_2O_2 assay. Production of H_2O_2 in live HeLa cells was measured by the AmplexRed assay (17). In brief, $5x10^4$ cells per well were cultured on sterile glass coverslips in 24-well plates. After 24 h incubation, cells were pre-incubated with DCA (8 mM) in the presence or absence of rotenone (0.5 μM) for 1 h. Then the cells were loaded with 50 μM AmplexRed reagent for 30 min at 37˚C. In the presence of peroxidase, the AmplexRed reagent reacts with H_2O_2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin. The fluorescent intensity represents the H_2O_2 level. Fluorescence was measured at 560 nm with excitation at 543 nm.

Intracellular pH assay. The SNARF-1/AM, a pH-sensitive fluoroprobe, was used to measure intracellular pH (10). In brief, HeLa cells were cultured on sterile glass coverslips in 24-well plates ($5x10^4$ cells/well). After 24 h incubation, the cells were treated with and without DCA (8 mM) for 48 h. Then the cells were loaded with 5 μM SNARF-1/AM for 45 min (37˚C) and washed with PBS for 30 min (37˚C). After that, Hoechst 33342 (1 μM) was used to stain nuclei for 10 min. Fluorescence was measured at 560 nm with excitation at 543 nm.

Mitochondrial membrane potential assessment. Mitochondrial membrane potential (MMP) was measured on the ArrayScan high content screening (HCS) system (Cellomics, Inc., Pittsburgh, PA, USA) (18). In brief, $5x10^3$ HeLa cells per well were seeded into 96-well plates. After 24 h incubation, the cells were treated with DCA (8 mM) at 37˚C for 48 h. As control, HeLa cells were pretreated with DIDS (0.5 mM), an inhibitor of the mitochondrial voltage-dependent anion channel (VDAC), for 10 min before exposure under DCA (19). Then 50 μl of the MitoTracker/Hoechst solution (Cellomics Inc.) was added and the cells were incubated for 30 min at 37˚C. Fixation solution (100 μl) was added directly to each well without removing the medium and the cells were incubated in a fume hood at room temperature for 10 min. The wells were aspirated and the plate was washed once with 200 μl of wash buffer-M. The plate was sealed and run on the ArrayScan HCS System. The intensity of red fluorescence represented the MMP of the cells. All MMP assessments were performed in triplicate.

Western blot analysis. HeLa cells were seeded into 6-well plates ($1.5x10^5$ cells per well). After 24 h incubation, cells were

treated with and without DCA (8 mM) for 48 h. The cells were lysed in RIPA buffer (10 mM Tris-HCl buffer, pH 7.5, containing 1% NP-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl and 1 mM EDTA-2Na). The lysates containing 20 μg proteins were separated by 12% SDS-PAGE and electrotransferred to PVDF membranes (Millipore, Bedford, MA, USA). After blockage with 5% non-fat milk in TBS containing 0.1% Tween-20, immunoblotting was performed by first incubating the membranes with primary antibodies against caspase 3 (1:500), cleaved caspase 3 (1:500), caspase 9 (1:500), cleaved caspase 9 (1:500), Kv1.5 (1:500) and actin (1:750) as control and then incubating with horseradish peroxidase-conjugated anti-rabbit or anti-rat IgG (1:5000, Zhongshan, Beijing, China) with respect to the corresponding first antibody at room temperature. The immunoblots were visualized with an enhanced chemiluminescence detection kit (Pierce Chemical, Rockford, IL, USA).

Kv 1.5 channel assay. The Kv1.5 channel expression was observed under the confocal laser scanning microscopy (CLSM, LSM-510, Carl Zeiss AG, Oberkochen, Germany). In brief, $5x10^4$ cells per well were cultured on sterile glass coverslips in 24-well plates. After 24 h incubation, the cells were treated with DCA (8 mM), t-butyryl-H_2O_2 (10 μM), or VIVIT (4 μM) for 48 h. Then Kv1.5 antibody (1:150) was used as per its manufacturer's instructions on fixed cells. Counterstain with DAPI (300 nM) was performed for 10 min at room temperature and washed with phosphate-buffered saline. Fluorescence was measured at 560 nm with excitation at 543 nm.

Intracellular Ca^{2+} measurement. Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was studied in live HeLa cells and measured according to a method described previously (20). In brief, $5x10^4$ cells per well were cultured on sterile glass coverslips in 24-well plates. After 24 h incubation, the cell was exposed to different treatments, and then were loaded with 5 μM Fluo-3/AM for 45 min (37˚C) and washed for 30 min in PBS (37˚C) to allow cleavage of the acetoxymethyl esters. Then Hoechst 33342 (1 μM) was used to stain nuclei for 10 min. Fluorescence of intracellular free Ca^{2+} was measured at 525 nm with excitation at 488 nm.

Combination chemotherapy analysis. In combination chemotherapy experiments, the cells were concurrently treated with various concentrations of cisplatin (0.5, 1, 2, 4, 8, 16 μM) and DCA (2, 4, 8 and 16 mM) for 48 h. Then CCK-8 agent was added and incubated for 2 h at 37˚C for color development. Cell viability was obtained using the method mentioned above. The nature of the interaction between DCA and cisplatin was determined by calculating the R index (RI) as described by Kern (21) and modified by Romanelli (22) The RI value is obtained as a ratio of the expected cell survival (S_{exp}, defined as the product of the survival observed with drug A alone and the survival observed with drug B alone) to the observed cell survival (S_{obs}) for the combination of A and B (RI= S_{exp}/S_{obs}). RI of 1 (additive effect) or lower indicates the absence of synergism. Synergism is then defined as RI of greater than unity. To further assess the statistical significance of the interactions, we performed one-sample t-test using the additive model based on the null hypothesis that there was no synergistic interaction between the two agents. Three-dimensional response surface graph of RI as a function of the concentrations of DCA and cisplatin were delineated using Statistica 6.0 software (StatSoft Inc., Tulsa, USA).

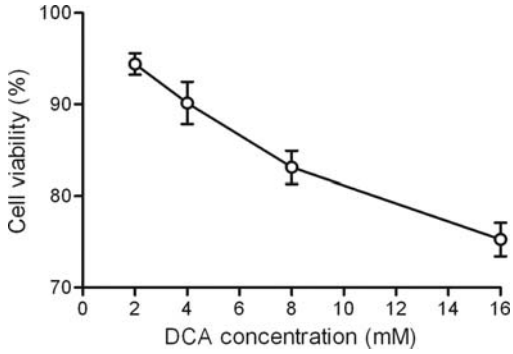

Figure 1. Effects of DCA on the viability of HeLa cells. CCK-8 assay was used to determine cell viability after treatment with increasing drug concentrations for 48 h. Values are expressed as mean ± SD, n=6.

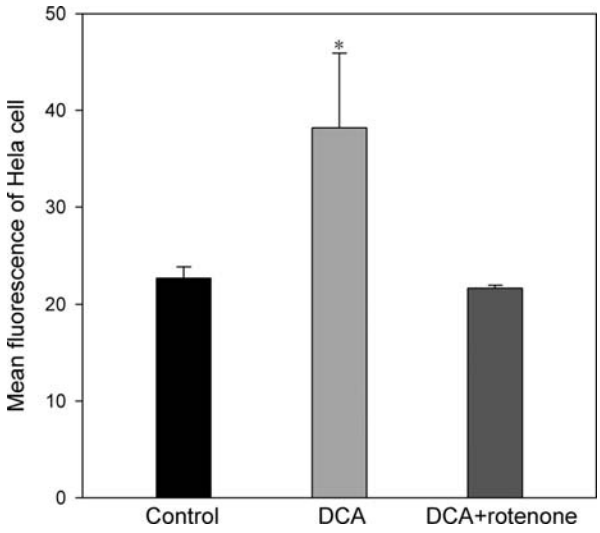

Figure 2. Quantitation of mean fluorescence intensity of HeLa cells after treatment with 8 mM DCA in the presence or absence of 0.5 μM rotenone for 1 h. AmplexRed reagent was used to measure H_2O_2 production in HeLa cells. The AmplexRed reagent reacted with H_2O_2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin. Fluorescence was measured at 560 nm with excitation at 543 nm. Values are expressed as mean ± SD, n=8. *P<0.01 vs control and DCA + rotenone.

Statistical analysis. The data are presented as the mean ± SD. Inter-group differences were assessed by Student's t-test or ANOVA with a follow-up LSD test using the SPSS 11.0 software. P<0.05 indicated a significant difference.

Results

Effects of DCA on the viability of HeLa cells. HeLa cells were exposed to increasing concentrations of DCA to determine the effect of the agent on cell viability. The results showed that DCA reduced the viability of HeLa cells in a dose-dependent manner after 48 h treatment and exerted significant cytotoxicity at a concentration of above 4 mM (Fig. 1).

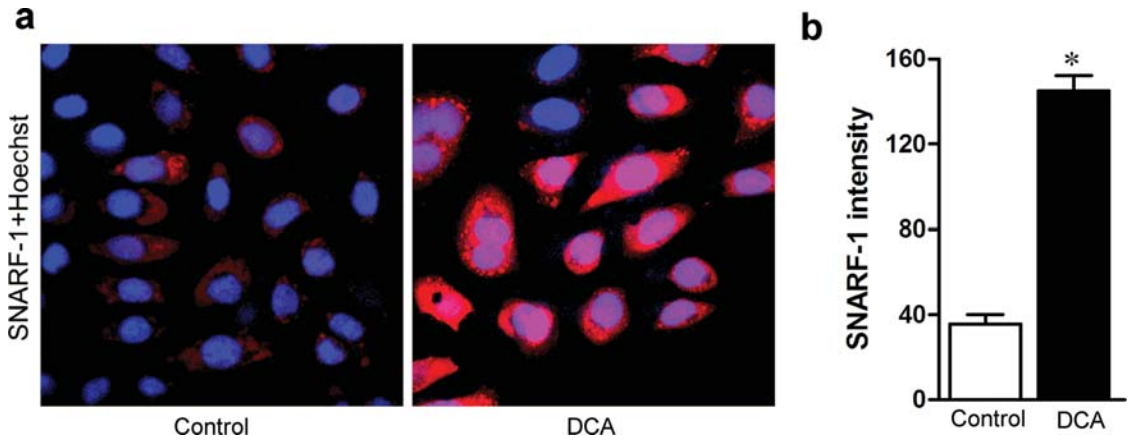

Figure 3. Effect of 8 mM DCA for 48 h on intracellular pH value in HeLa cells. (a) Cells were fluorescently stained with SNARF-1/AM dye, a sensitive pH indicator, to determine intracellular pH. The nuclei were stained with Hoechst 33342. (b) Quantitation of mean SNARF-1 fluorescence intensity in each HeLa cell. Values are expressed as mean ± SD, n=8. *P<0.01 vs control.

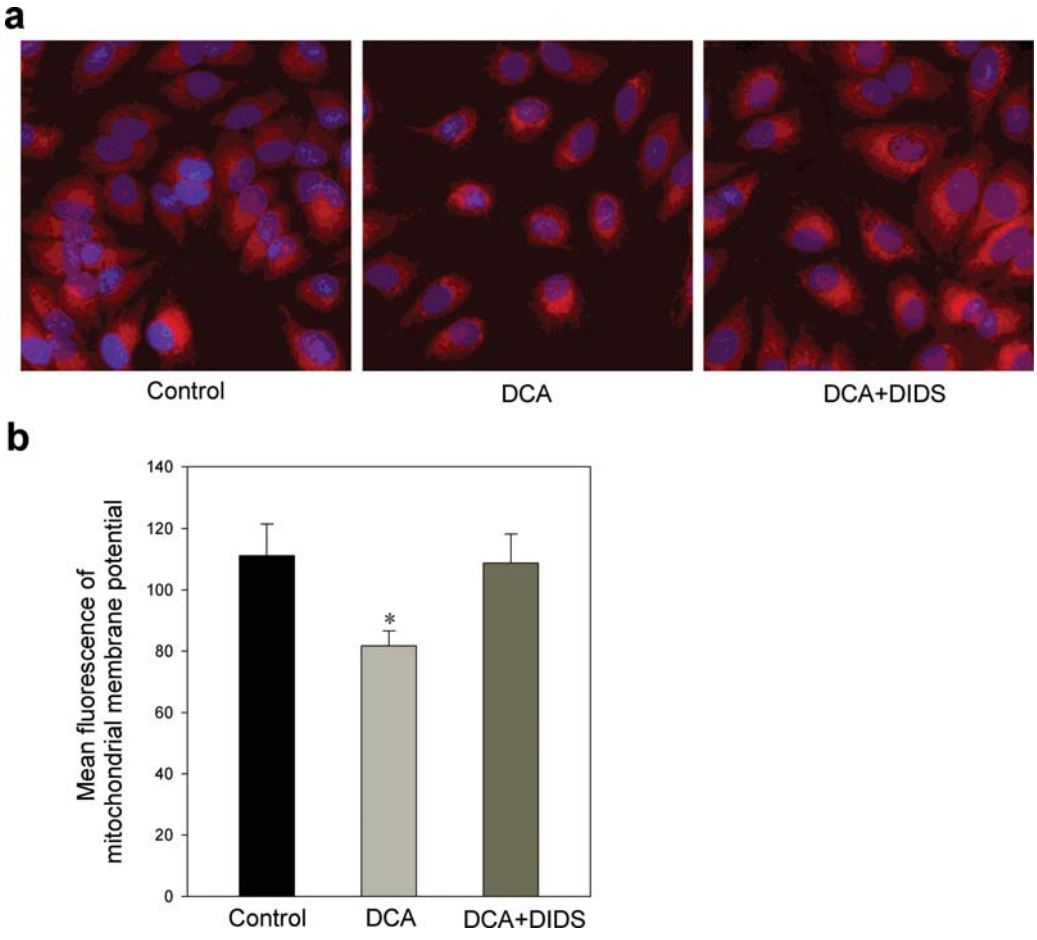

Figure 4. Effect of 8 mM DCA for 48 h on the mitochondrial membrane potential of HeLa cells in the presence or absence of DID. (a) The red fluorescence of MitoTracker red accumulated in mitochondria and was represented as a function of the cell MMP. The nuclei were stained with Hoechst 33342. (b) Quantitation of mean fluorescence intensity of MMP. Values are expressed as mean ± SD, n=8. *P<0.01 vs control and DCA + DIDS.

Influence of DCA on H_2O_2 production and pH in HeLa cells. After 1 h incubation, 8 mM DCA significantly increased H_2O_2 production in HeLa cells by 70% (P<0.01) and this increase could be completely inhibited by 0.5 μM rotenone (Fig. 2). Rotenone is an inhibitor of the mitochondrial electron transport chain (ETC) complex I, which is a critical component of oxidative phosphorylation. The results suggested that DCA-induced increase of H_2O_2 production in HeLa cells was achieved through promoting oxidative phosphorylation.

The intracellular fluorescence intensity of SNARF-1/AM represented the intracellular pH value. It showed that DCA (8 mM) treatment for 48 h increased the intracellular pH

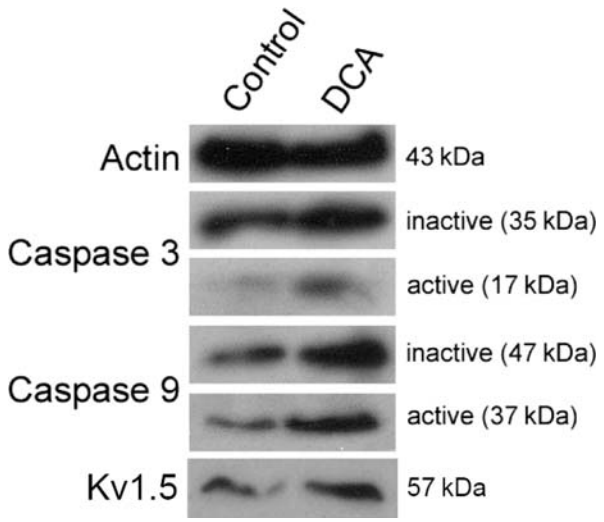

Figure 5. Effects of 8 mM DCA for 48 h on protein levels of caspases 3, 9 and Kv1.5 in HeLa cells. Results are representative of three independent experiments.

value, with a fluorescence intensity five times that of untreated HeLa cells ($P<0.01$) (Fig. 3). This phenomenon could be due to increased oxidative phosphorylation (Fig. 2) and decreased lactate production from aerobic glycolysis.

Effect of DCA on MMP and apoptosis-associated proteins in HeLa cells. MMP was determined by MitoTracker red staining and fluorescence assay on the ArrayScan HCS system. DCA (8 mM) treatment for 48 h significantly reduced the MMP of HeLa cells by 25% compared with the untreated cells ($P<0.01$) (Fig. 4), while it had no effect on the MMP of non-cancerous HUVEC (data not shown). The DCA-induced significant drop of MMP in HeLa cells could be completely inhibited by DIDS (Fig. 4), an inhibitor of the mitochondrial VDAC, which is a critical component of the mitochondrial transition pore (MTP).

DCA (8 mM) treatment for 48 h induced a significant increase in the expression of both caspase 3 and caspase 9, including both inactive and active sections, in HeLa cells (Fig. 5). These results proved that DCA induced apoptosis of HeLa cells through a mitochondria-regulated mechanism.

Influence of DCA on Kv1.5 expression and intracellular calcium levels in HeLa cells. The CLSM images showed that treatment with 8 mM DCA for 48 h induced higher expression of Kv1.5 in HeLa cells compared with the control cells (Fig. 6), which was consistent with the results of Western blot assays (Fig. 5). As expected, t-butyryl-H_2O_2, which mimicked the DCA-induced H_2O_2, and VIVIT, which is an inhibitor of NFAT that could translocate into the nucleus and decrease Kv1.5 expression, also increased Kv1.5 expression (Fig. 6).

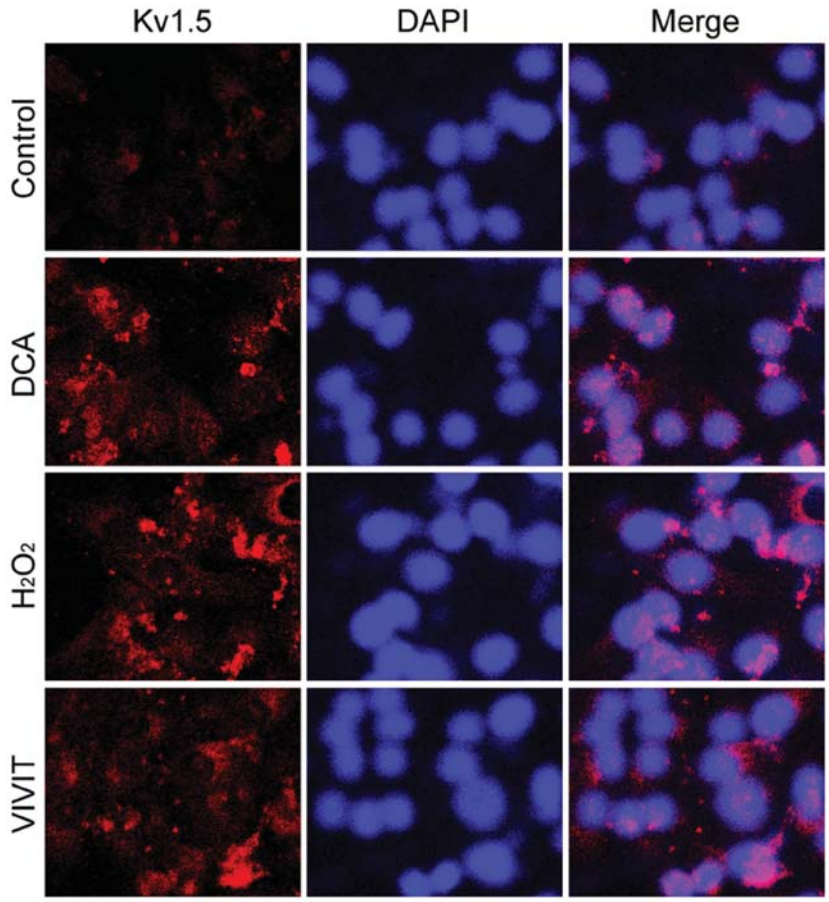

Figure 6. Effect of DCA (8 mM), t-butyl-H_2O_2 (10 μM) and VIVIT (4 μM) for 48 h on the expression of Kv1.5 in HeLa cells. Confocal imaging qualitatively showing the level of Kv1.5 expression (red). The nuclei (blue) were stained by DAPI.

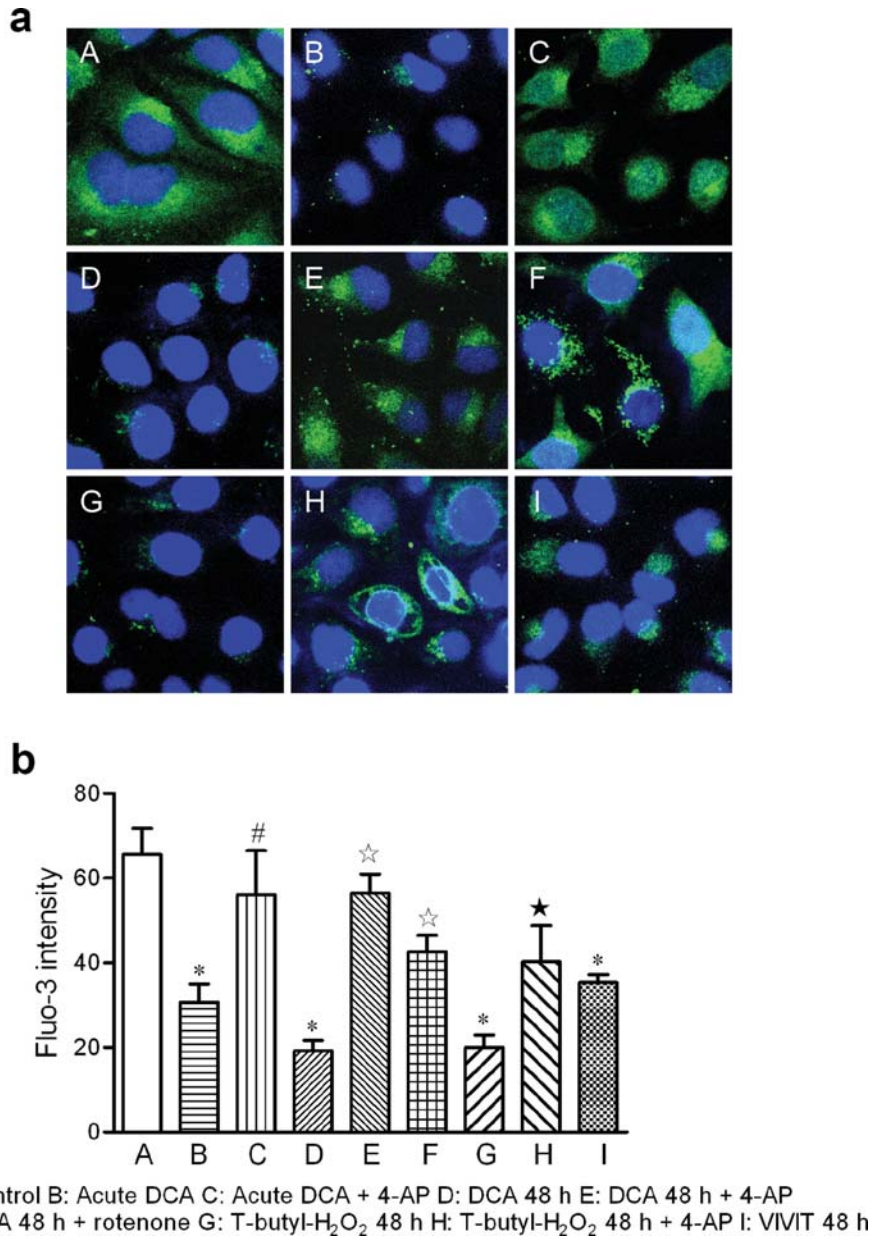

Figure 7. Effect of DCA on free cytosolic calcium concentration ($[Ca^{2+}]_i$) in HeLa cells. The cell treatments were carried out in nine groups. They were: (A) control without any treatment; (B) DCA (8 mM) for acute 5 min; (C) 4-AP (5 mM) pretreatment for 5 min and then DCA (8 mM) for 5 min; (D) DCA (8 mM) for 48 h; (E) 4-AP (5 mM) pretreatment for 5 min and then DCA (8 mM) for 48 h; (F) rotenone (0.5 μM) for 5 min and then DCA (8 mM) for 48 h; (G) t-butyl-H_2O_2 for 48 h; (H) 4-AP (5 mM) pretreatment for 5 min and then t-butyl-H_2O_2 for 48 h; (I) VIVIT (4 μM) for 48 h. (a) Fluo-3 was used to assay ($[Ca^{2+}]_i$) (green). (b) Quantitation of Fluo-3 fluorescence intensity. Values are expressed as mean ± SD, n=6. *P<0.01 vs A, $^{\#}$P<0.01 vs B, *P<0.01 vs D, *P<0.01 vs G.

The intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was detected using the Fluo-3/AM fluorescence probe (Fig. 7). The DCA-treated HeLa cells had lower $[Ca^{2+}]_i$ than the untreated cells. The decrease in $[Ca^{2+}]_i$ appeared within 5 min and was sustained after 48 h of DCA exposure. The effect of DCA on $[Ca^{2+}]_i$ could be inhibited by 4-AP (a specific Kv channel inhibitor) and rotenone, and this effect could be mimicked by t-butyryl-H_2O_2. All these results suggested that the decrease of $[Ca^{2+}]_i$ induced by DCA involved opening of Kv channels by the mitochondrial electron transport chain complex I-derived H_2O_2. By increasing the expression of Kv1.5 like DCA as shown in Fig. 8a, VIVIT also decreased the $[Ca^{2+}]_i$.

Effect of combination chemotherapy of DCA and cisplatin on the viability of HeLa cells. First, the effect of cisplatin alone on the viability of HeLa cells was examined. The results showed that cisplatin dose-dependently reduced cell viability in the concentration range from 0.5 to 16 μM after 48 h treatment and started to display obviously cytocidal efficacy at the concentration of 1 μM (Fig. 8a).

For the evaluation of combination chemotherapy, HeLa cells were treated in culture with a combination of DCA and cisplatin at various concentrations for 48 h. Cell viability was determined by CCK-8 assay and the synergy of the two agents was evaluated using the R index method. The combination of DCA and cisplatin at certain drug concentrations led to remarkable synergistic effect in decreasing the

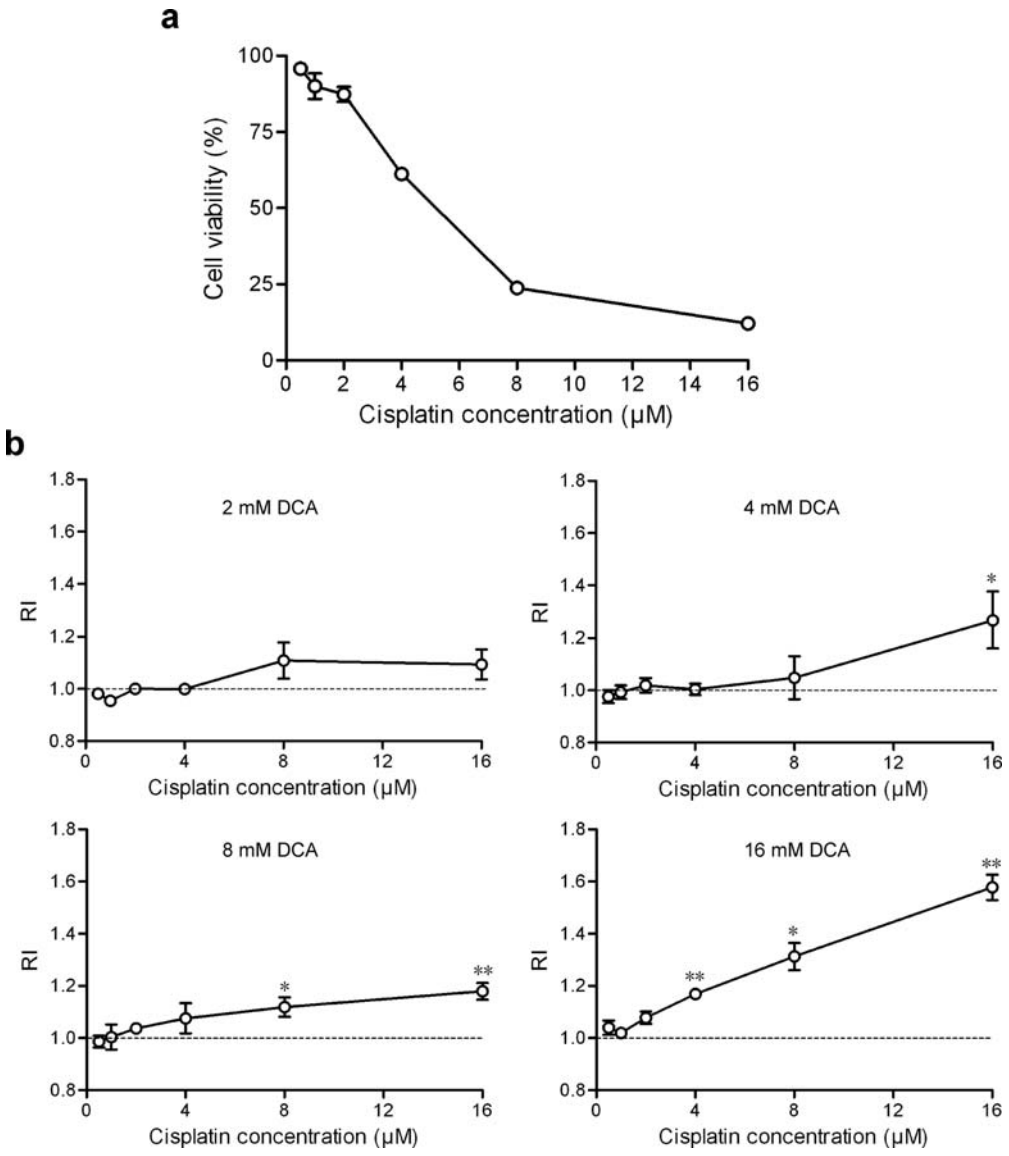

Figure 8. (a) Effects of cisplatin on the viability of HeLa cells. CCK-8 assay was used to determine cell viability after treatment with increasing drug concentrations for 48 h. Values are expressed as mean ± SD, n=6. (b) RI values calculated from the CCK-8 cell viability assay in response to combination chemotherapy of DCA and cisplatin to HeLa cells. Synergism is defined as RI>1. One-sample t-test using the additive model based on the null hypothesis that there was no synergistic interaction between the two agents. Values are expressed as mean ± SD, n=6. *P<0.05, **P<0.01.

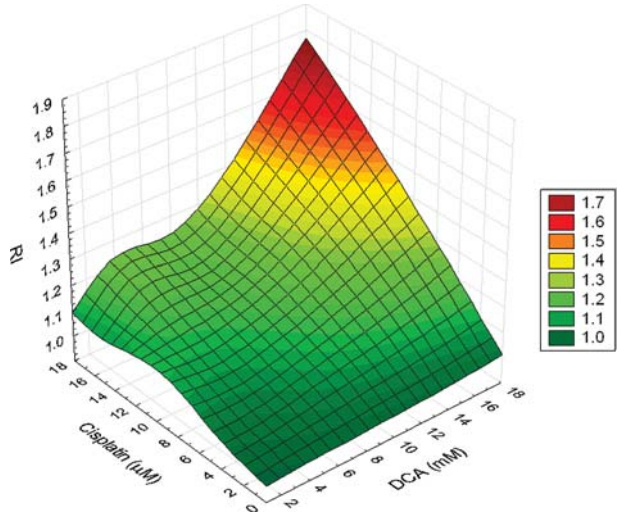

Figure 9. Three-dimensional response surface graphic representation of RI values as a function of the concentrations of DCA and cisplatin.

viability of HeLa cells, showing an RI of above 1 with statistical significance (Fig. 8b). At a DCA concentration of 16 mM, synergy was clearly observed at a cisplatin concentration of higher than 4 μM and the RI value reached 1.6 with cisplatin concentration of 16 μM (P<0.01). In contrast, no synergistic interaction was observed when the cells were treated with DCA at the low concentration of 2 mM (P>0.05). A three-dimensional response surface plot (Fig. 9) visually depicts the synergistic effect of DCA and cisplatin in decreasing the viability of HeLa cells, showing that the RI values increased with the concentration of either DCA or cisplatin.

Discussion

DCA dose-dependently reduced the viability of HeLa cells (Fig. 1) in the concentration range consistent with that of inhibiting endometrial cancer cells (12). Anderson *et al* also observed a decreased proliferation and viability of HeLa cells

exposed to DCA (23). In this study, we further found that intracellular H_2O_2 production was significantly increased after HeLa cells were treated by DCA for 1 h, and this was almost completely inhibited by rotenone, an inhibitor of mitochondrial electron transport chain complex I (Fig. 2). Meanwhile, 48 h of DCA significantly increased the intracellular pH of HeLa cells (Fig. 3). It is known that HeLa cells largely rely on aerobic glycolysis for their ATP budget (24). The results indicate that DCA may shift the metabolism of HeLa cells from glycolysis back to glucose oxidation.

The enhanced glucose oxidation led to sustained release of H_2O_2 (reactive oxygen species, ROS), which could inhibit the mitochondrial H^+ efflux and ultimately decrease the MMP (Fig. 4). The drop of MMP was almost completely inhibited by DIDS, an inhibitor of the VDAC, which is an important component of the mitochondrial transition pore (MTP). The opening of the MTP by DCA allowed the efflux of cytochrome c and apoptosis inducing factors, activated caspases 3 and 9 (Fig. 5), and decreased cell viability through apoptosis (Fig. 1).

Moreover, DCA treatment remarkably increased the expression of Kv1.5 which could be attributed to the increased H_2O_2 production as mimicked by t-butyryl-H_2O_2 (Fig. 6). The enhanced expression of Kv1.5 in the plasma membrane decreased the intracellular K^+ concentration ($[K^+]_i$) and thus led to reduced tonic inhibition of caspases (10,12). On the other hand, K^+ efflux hyperpolarized the cells and inhibited the voltage-dependent Ca^{2+} entry (Fig. 7). The decreased $[Ca^{2+}]_i$ inhibited the activation of NFAT and its translocation into the nucleus, thus further increasing Kv1.5 expression, establishing a positive feedback loop. Taken together, DCA shifted the metabolism of HeLa cells from aerobic glycolysis to glucose oxidation and induced cell apoptosis by depolarizing mitochondria and activating/up-regulating Kv1.5.

For recurrent or metastatic cervical cancer, intravenous infusion of 50 mg/m^2 cisplatin at 3-week intervals has been adopted by the oncology community as the standard regimen since 1986. But in subsequent trials, single-agent cisplatin used as the control arm showed a response rate of only 13-19%, a progression-free survival of 2.8-3.2 months, and an overall survival of only 6.5-8.8 months (5). The efficacy of single-agent cisplatin is still not satisfactory, though it is superior to 21 other known cytotoxic agents that have activity against cervical cancer. The combined-agent chemotherapy regimens based on cisplatin have been widely evaluated in clinical trials for improved efficacy. However, until recently, only the combination of cisplatin and topotecan has improved overall survival. In this study, DCA, a drug that can target the unique tumor metabolism Warburg effect, shift the aerobic glycolysis to glucose oxidation, and induce tumor cell apoptosis, was combined with cisplatin for cervical cancer cell chemotherapy for the first time.

A variety of *in vitro* assays and different mathematics models have been developed to investigate cytotoxic effects and to analyze the type of drug interactions in combination chemotherapy (25). In the present study, the improved sensitivity of HeLa cervical cancer cells to the combination chemotherapy was evaluated using the RI method. This method was adopted because treatment with DCA alone had a relatively low effect on cell viability compared with cisplatin (Fig. 1), so other methods, such as the median effect principle and isobologram methods, were not suitable (26,27). In this study, DCA exhibited significant synergistic interactions with cisplatin in decreasing the viability of HeLa cells with an increase of the drug concentration (Figs. 8 and 9). It should be noted that Dhar *et al* have developed a new compound, mitaplatin, which contains two DCA units and one platinum center (28). The cytotoxicity of mitaplatin is only comparable to that of cisplatin, although in a variety of cancer cell lines it equals or exceeds that of all known Pt(IV) compounds. We speculate that the fixed intracellular molar ratio of platinum and DCA (1:2) after the reduction of mitaplatin in cancer cells may not be the optimal combination of the two agents for cancer cell killing. When they were independently dosed as such in present study, the synergism of the two agents could be revealed and optimized. Cisplatin and DCA should have different potentials of penetrating cell plasma, thus the exact intracellular concentrations and molar ratio of the two agents in synergism remain to be determined.

Cisplatin is one of the most potent compounds and it has been used in cancer chemotherapy for more than 30 years as the first line drug in cervical cancer therapy, although its biochemical mechanism of action is still not fully elucidated. The accepted theory is that cisplatin induces its cytotoxic properties through binding to nuclear DNA and thereby interfering with normal transcription and/or DNA replication mechanisms (29,30). Several lines of evidence support that the cytotoxic effects induced by binding of cisplatin to non-DNA targets (especially proteins) may also contribute to its action (29,30). The sensitive cancer cells attacked by cisplatin finally die through apoptosis or necrosis, dependent on factors such as the availability of energy and the metabolic condition of the cell (30). We postulate that the specific apoptotic mechanism of DCA as distinguished from the cisplatin may be partly responsible for their synergistic cytocidal effect in HeLa cells. An *in vivo* study is now being undertaken in our laboratory to evaluate the synergistic effect in inhibiting the growth of tumor xenografts in mice, and we are also trying to identify potential predictive biomarkers with respect to the drug interaction.

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