RESEARCH PAPER

Cancer Biology & Therapy 16:5, 770–779; May 2015; © 2015 Taylor & Francis Group, LLC

p8 attenuates the apoptosis induced by dihydroartemisinin in cancer cells through promoting autophagy

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Keywords: autophagy, chloroquine, dihydroartemisinin, endoplasmic reticulum stress, p8

Abbreviations: DHA dihydroartemisinin; ART artemisinin; CQ chloroquine; AVOs acidic vesicular organelles; ATF4 activating transcription factor 4; CHOP C/EBP homologous protein; ERs endoplasmic reticulum stress; AO Acridine orange; MDC Dansylcadaverine

Dihydroartemisinin (DHA) exhibits anticancer activities in a variety of cancer cells, but DHA alone are not effective enough for cancer therapy. In this study we found the stress-regulated protein p8 was obviously increased after DHA treatment in several cancer cells, which further to induce autophagy by the upregulation of endoplasmic reticulum stress-related protein ATF4 and CHOP. Furthermore, when we silenced p8 by siRNA in cancer cells, the apoptosis induced by DHA were notably increased, whereas the overexpression of p8 in cancer cells leaded to the resistance to DHA-induced apoptosis. Moreover, we found the inhibition of autophagy with chloroquine (CQ) can enhance the anticancer effect of DHA both *in vitro* and *in vivo*. In conclusion, we found that p8-mediated autophagy attenuates DHA-induced apoptosis in cancer cells, which provides evidence to support the use p8 as a cancer therapeutic target, and suggests that the combination treatment with DHA and autophagy inhibitor might be an effective cancer therapeutic strategy.

Introduction

Dihydroartemisinin (DHA), a main active metabolite of artemisinin (ART), which is making a crucial contribution to the therapy of malaria,[1] has been demonstrated to exert preferentially cytotoxic effects toward various human cancers, including breast cancer,[2] ovarian cancer,[3] cervical cancer[4] and colorectal carcinoma.[5] Recent studies have shown that the anti-cancer activity of ART derivatives including DHA is mainly executed by iron-mediated cleavages of endoperoxide bridges.[5,6] Since most cancer cells have higher iron influx than normal cells, DHA exert selective toxicity toward cancer cells.[7,8] Additionally, clinical trials have shown that ART derivatives are well tolerated with insignificant side-effects in malaria therapy, indicating DHA have important potential in oncology.

p8, with a theoretical molecular mass of 8872.7Da, also known as nuclear protein 1(Nurp1) or candidate of metastasis-1 (Com-1), was first described as being preferentially upregulated in pancreatic acinar cells during the acute phase of pancreatitis.[9] P8 participates in a wide range of biological functions in tumorigenesis, including anti-inflammatory,[10] cell cycle regulation,[11] autophagy,[12] and apoptosis inhibition.[13] Accumulating studies have shown that p8 is overexpressed in various cancer cells including pancreatic cancer,[14] colorectal carcinoma[15] and brain tumors.[16] Moreover, it has been reported that p8 is involved in the resistance of cancer cells to gemcitabine and adriamycin.[14,17] Therefore, p8 might be a new drug-targetable gene in preventing the progression and metastasis of cancer.[18]

In this study, we discovered DHA upregulates p8 remarkably in several cancer cells, and we sought to find out what role p8 plays in the antiproliferative actions of DHA in cancer cells. It has been reported that endoplasmic reticulum stress (ERs)-associated molecule ATF4 (activating transcription factor 4) and CHOP (C/EBP homologous protein) are downstream targets of p8.[19] ER stress occurs when the accumulation of unfolded proteins in the ER is exceeded, and is caused by multiple stimuli including hypoxia, oxidants or reducing agents, glucose deprivation, protein inclusion bodies, etc.[20] The major pathway of ER stress is unfolded protein response (UPR), which is identified to recover ER homeostasis and promote cell survival.[21] In addition, ER stress has proved to be one of the inducers of autophagy.[22] Interestingly, DHA induced-ER stress has been observed in several tumor cells,[23,24] and our previous studies have demonstrated that DHA both induce apoptosis and autophagy in various cancer cells.[25,26] The Complex relationships among ER stress, autophagy and apoptosis remain unclear, but it's perhaps

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Submitted: 10/22/2014; Revised: 02/10/2015; Accepted: 03/01/2015
http://dx.doi.org/10.1080/15384047.2015.1026477

noteworthy that we found p8 is involved in all of these 3 biological functions induced by DHA.

As shown in our results, when silenced p8 in cancer cells, both ER stress and autophagy induced by DHA were reduced while the apoptosis rates were further increased. Furthermore, when inhibited autophagy in cancer cells through pretreating with chloroquine (CQ), which can arrest degradation of the autolysosome,[27] we found the anticancer effect of DHA was notably enhanced. Interestingly, CQ is also widely used as an antimalarial agent, and for the first time, we discovered the 2 classical antimalarial agents, DHA and CQ, have the synergistic effect in cancer suppression.

Results

Dihydroartemisinin upregulates p8, ATF4 and CHOP at transcription level and protein level in HeLa and HCT116 cells

We used human cervical carcinoma cell line HeLa and hunman colon carcinoma cell line HCT116 to investigate the expression changes of p8, ATF4 and CHOP after DHA treatment. As illustrated in **Figure 1A**, the time-dependent increases in p8, ATF4 and CHOP were observed in both 2 cell lines that were treated with 20 μM DHA. The up-regulation of p8 was observed as early as at 3 h, and the increase of ATF4 was observed at 6 h, whereas the increase of CHOP was observed at least after 9 h treatment under our experimental condition. Likewise, by using real-time quantitative PCR, we confirmed that DHA upregulates the mRNA levels of p8, ATF4 and CHOP both in HeLa and HCT116 cells (**Fig. 1B** and **C**).

To investigate whether ATF4 and CHOP are regulated by p8, which are involved in the response to endoplasmic reticulum stress (ERS), we transfected cells with p8 siRNA, and then we measured the expression levels of ATF4 and CHOP. The transfection efficiency was 84.0%, which detected by real-time quantitative PCR (**Fig. 1D**). We measured ATF4 and CHOP levels after treatment with DHA for 24 h by western blot. To compare with the negative-knockdown cells, the level of ATF4 and CHOP significantly decreased after DHA exposure in p8-knockdown cells (**Fig. 1E**). The identical results were obtained in mRNA levels (**Fig. 1F** and **G**). Conversely, when cells were transfected with p8-overexpress plasmid, the ATF4 and CHOP express levels further increased than the negative control cells after DHA treatment (**Fig. 1D**). Taken together, these data demonstrated that DHA-induced increases of ATF4 and CHOP were mediated by p8 upregulation. In addition, the CHOP expression was reduced when ATF4 was knockdown in both HeLa and HCT116 cells (**Fig. 1H**), indicating that the expression of CHOP is regulated by ATF4 after DHA treatment. The analogous results were validated in human hepatoma HepG2 cells and ovarian carcinoma SKOV3 cells (**Fig. S1A** and **Fig. S2A**).

P8 upregulation decreases the sensitivity of tumor cells to dihydroartemisinin

We next investigated the role of p8 in DHA-induced tumor suppression. Cells were transfected with p8 siRNA or p8 overexpressed plasmid and treated with a series of concentration of DHA for 48 h, and after that we figured out the survival functions by SRB assays. As shown in **Figure 2A** and **Figure S1B–C**, the survival functions decreased when p8 was silenced by siRNA, whereas the survival functions increased in p8 overexpressed cells compared with the negative control cells.

We further observed the apoptosis after treatment with DHA in p8-knockdown or p8 overexpressed cells. Since activations of caspases are key players in apoptosis execution,[28] we investigated the effect of DHA on the protein levels of PARP(a major substrate of caspase3), caspase3 and cleaved-caspase3 in HeLa and HCT116 cells. Compared with in negative-knockdown cells, DHA caused an enhanced cleavage of caspase3 and PARP in p8-knockdown cells (**Fig. 2B** and **Fig. S1A**). But in p8 overexpressed cells, DHA caused less cleavage of caspase3 and PARP than in negative control cells (Fig. 2B). The same results were observed when we detected apoptosis rate by flow cytometry analysis. As presented in **Figure 2C** and **D**, the apoptosis rate remarkably increased in p8-knockdown HeLa cells treated with DHA (20 μM, 24 h), while the percentage of apoptotic cells caused by DHA was much lower in p8 overexpressed HeLa cells than in negative control HeLa cells (**Fig. 2E** and **F**). These results confirmed that the higher expression of p8, the lower effect of DHA performs in tumor inhibition.

ATF4 or CHOP depletion enhances the sensitivity of tumor cells to dihydroartemisinin

In this study we have confirmed ATF4 and CHOP are regulated by p8 after DHA treatment, to test the correlation between the expression levels of ATF4 or CHOP and sensitivity to DHA treatment, we silenced ATF4 or CHOP by transfecting specific siRNAs into HeLa and HCT116 cells before DHA treatment and Annexin V-FITC/PI assays. Notably, ATF4-knockdown increased DHA-driven apoptosis to a greater extent in HeLa cells treated with 20 μM DHA (**Fig. 3A** and **C**), and the apoptosis rate of CHOP-knockdown cells rise significantly comparing to negative control cells (**Fig. 3B** and **C**). The similar result was also observed in the effect of DHA on the apoptosis execution protein levels. Whether ATF4 depletion or CHOP depletion, the PARP cleavage and caspase3 cleavage driven by DHA were notably increased in both HeLa and HCT116 cells (**Fig. 3D**), further suggesting that the silencing of ATF4 or CHOP enhances the sensitivity of tumor cells to DHA-driven apoptosis *in vitro*. The similar results were obtained in HepG2 cells and SKOV3 cells (**Fig. S2A-D**).

p8-ATF4-CHOP axis is involved in dihydroartemisinin -induced autophagy in HeLa and HCT116 cells

In our early study we have demonstrated that DHA stimulates the induction of autophagy in several cancer cell lines including HeLa and HCT116 cells.[26] In this study we found that p8-ATF4-CHOP axis is closely related to DHA-induced autophagy. We determined the effect of DHA on formation of acidic vesicular organelles (AVOs) by acridine orange (AO) staining and MDC staining, to visualize autophagosome formation in the cells. In negative-knockdown HeLa cells, treatment with 20 μM

DHA resulted in the formation of red fluorescent AVOs, whereas the DHA-induced AVOs were reduced in p8-knockdown HeLa cells (**Fig. 4A**). Likewise, when performed by DMC staining after DHA treatment, the fluorescent density was decreased in p8-knockdown HeLa cells compared to the negative-knockdown cells (**Fig. 4A**), indicating p8 knockdown reverses DHA induced autophagy.

The conversion of LC3-I to LC3-II is a widely accepted autophagy marker.[29] As showed in **Figure 4B** and **Figure S1A**, the LC3-II accumulations induced by DHA were reduced when p8 was silenced by siRNA. Conversely, the LC3-II accumulations were further increased after DHA treatment when p8 was overexpressed. We discovered similar results when the ATF4 or CHOP was silenced by siRNAs (**Fig. 4C** and **Fig. S2A-B**), indicating the p8-ATF4-CHOP axis plays a critical role in DHA induced autophagy in tumor cells.

Inhibition of autophagy increases apoptosis in dihydroartemisinin-treated tumor cells

Autophagy is a lysosomal degradation pathway for the breakdown of damaged or superfluous proteins and organelles, and is a double-edged sword in cancer.[30] However, autophagy has a more prominent role in prolonging survival in tumor cells with defects in apoptosis.[31] To determine whether autophagy promotes or attenuates apoptosis in DHA-treated tumor cells, we pretreated with 1 mM 3-MA (an inhibitor of an early stage of autophagy) or 10 μM CQ (an inhibitor of which block the downstream steps of autophagy) and then detected the apoptosis rate in HeLa cells that were treated with 20 μM DHA. 3-

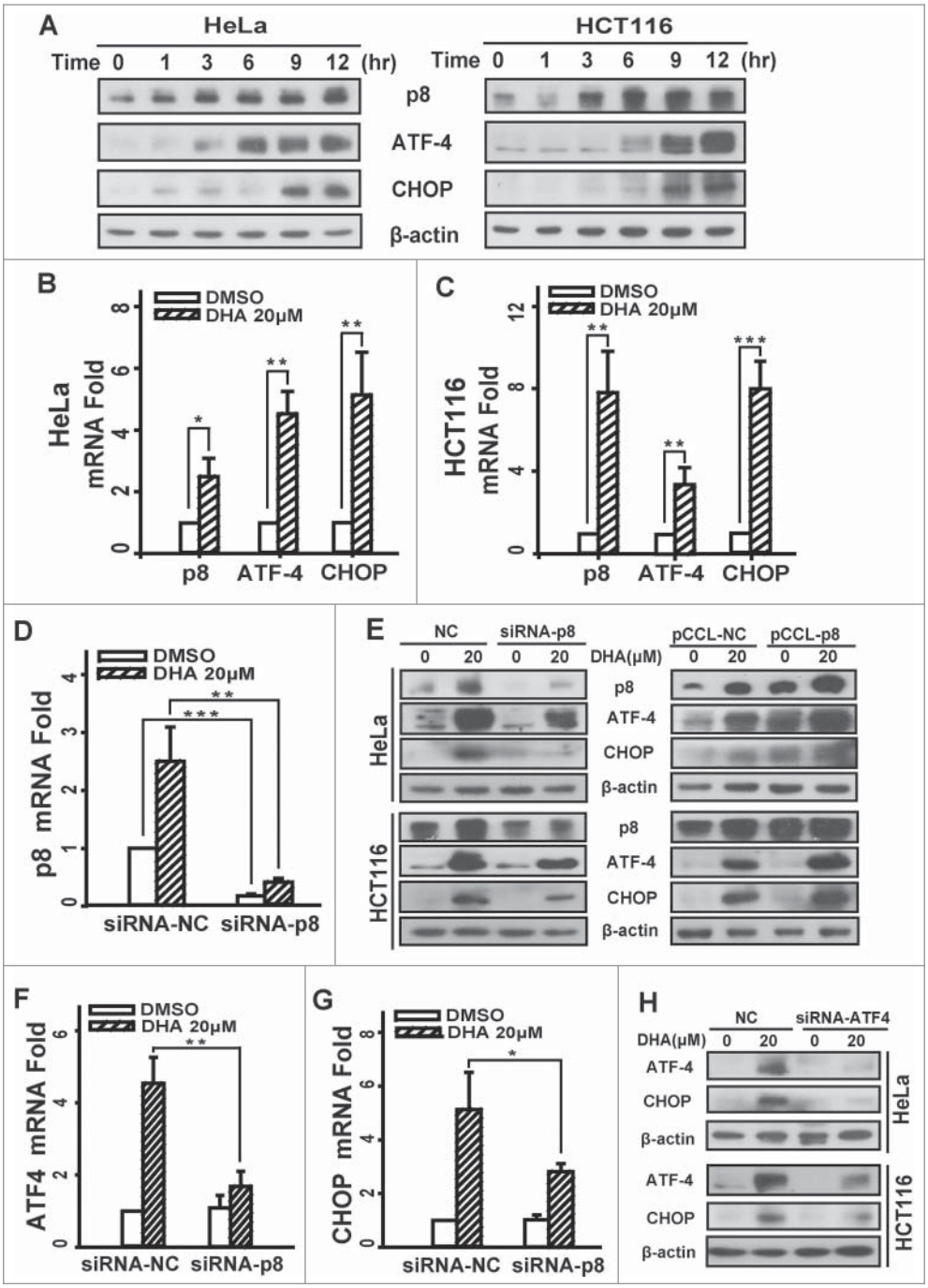

Figure 1. Dihydroartemisinin upregulates p8, ATF4 and CHOP at transcription level and protein level in HeLa and HCT116 cells. (**A**). HeLa and HCT116 cells were treated with 20 μM DHA for the indicated times, and total cell extracts were probed with antibodies against p8, ATF4, CHOP and β-actin. (**B and C**).HeLa and HCT116 cells treated with 20 μM DHA for 9 h, then total RNA was extracted and p8, ATF4 and CHOP mRNA expression was analyzed by RT-PCR, using GAPDH as a control gene. Three independent experiments were performed and the values were expressed as the mean ± SD. (E and H). After transfection with pCCL^{-} p8 plasmid or specifically targeted siRNA (p8 or ATF4) for 24 h, HeLa and HCT116 cells were treated with DHA (20 μM) for 24 h, after which p8, ATF4 and CHOP protein levels were measured by protein gel blot analysis. β-actin was measured as the loading control. (**D, F-G**) After transfection with specifically targeted p8 siRNA for 24 h, HeLa cells treated with DHA (20 μM) for 9 h, after which total RNA was extracted and p8, ATF4 and CHOP mRNA expression was analyzed by RT-PCR, using GAPDH as a control gene. Three independent experiments were performed and the values were expressed as the mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

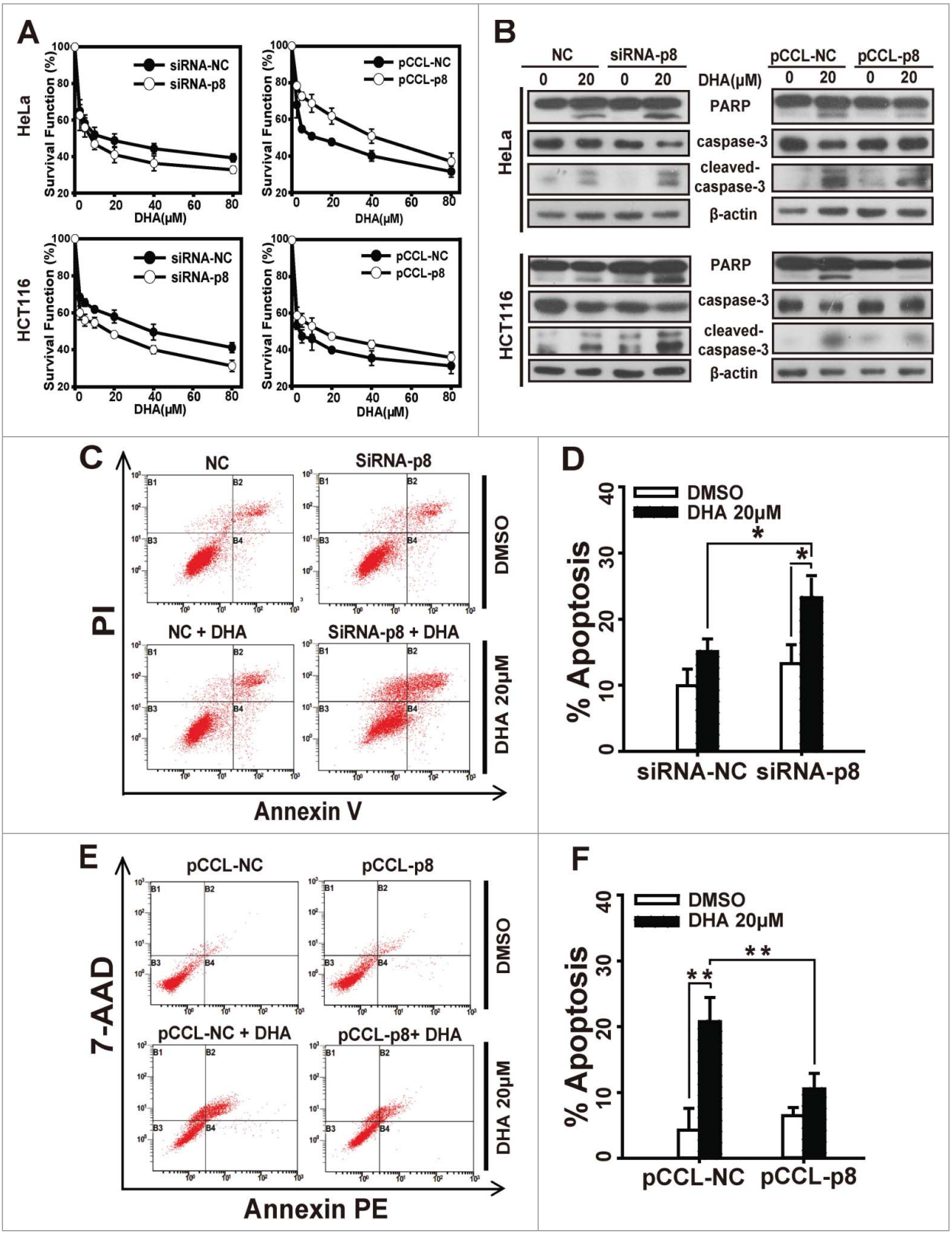

Figure 2. p8 upregulation decreases the sensitivity of tumor cells to dihydroartemisinin. After transfection with pCCL^{-} p8 plasmid or specifically targeted p8 siRNA for 24 h, (**A**) HeLa and HCT116 cells were treated with serial concentrations of DHA for 48 h, and then cell survival was detected by SRB assay. (**B**) HeLa and HCT116 were treated with 20 μM DHA for 24 h, after which PARP, caspase-3 and cleaved-caspase-3 protein levels were measured by western blot analysis. (**C-F**) siRNA or plasmid-transfected HeLa cells treated with 20 μM DHA for 24 h, and quantification of apoptosis was performed using Annexin V/PI or Annexin V-PE/7-AAD followed by FACS analysis. Three independent experiments were performed and the values were expressed as the mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

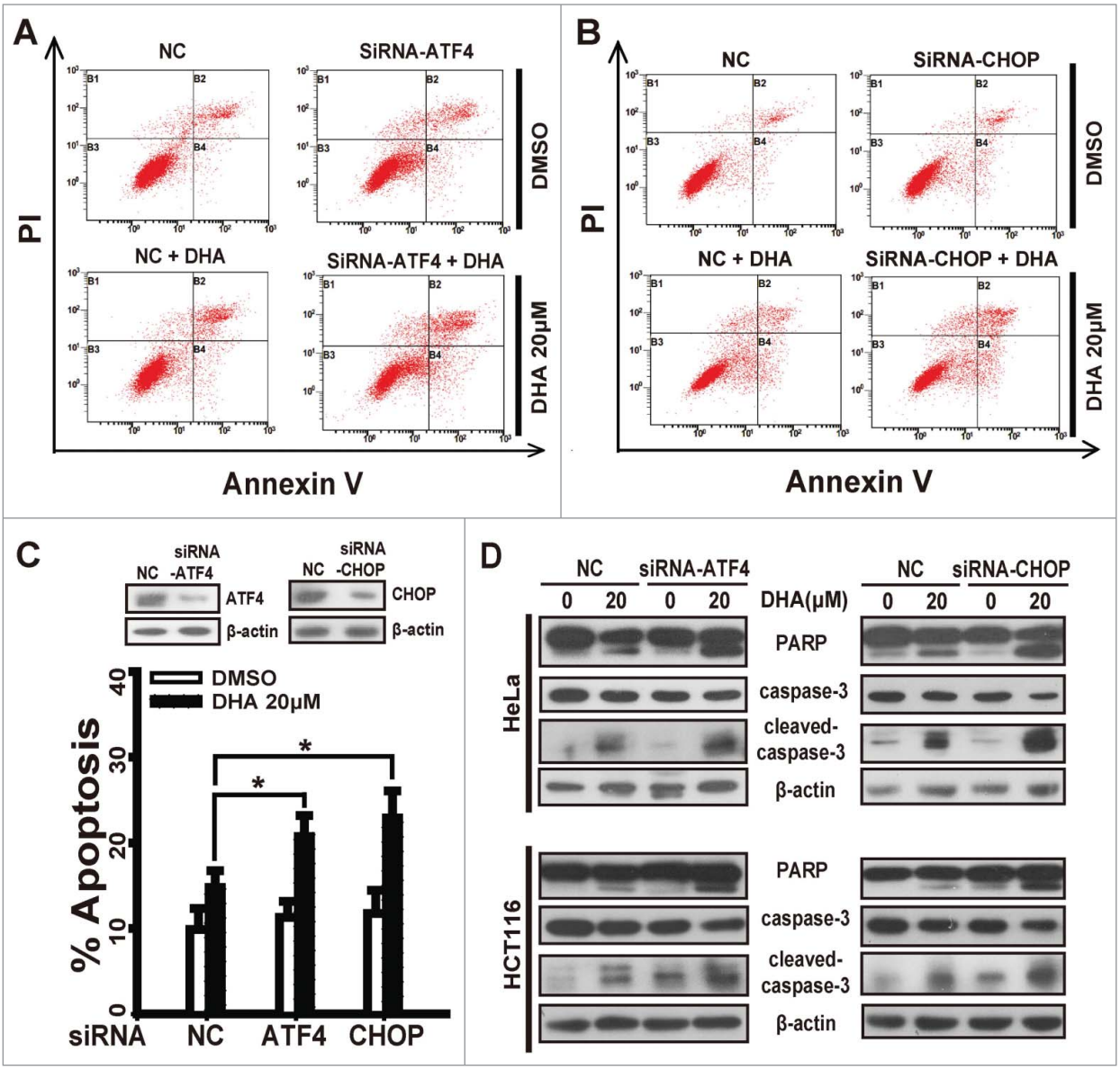

Figure 3. ATF4 or CHOP depletion enhances the sensitivity of tumor cells to dihydroartemisinin. After transfection with specifically targeted siRNA (ATF4 or CHOP) for 24 h, (**A-C**) HeLa cells treated with 20 μM DHA for 24 h, and quantification of apoptosis was performed using Annexin V/PtdIns followed by FACS analysis. Three independent experiments were performed and the values were expressed as the mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (**D**) HeLa and HCT116 were treated with 20 μM DHA for 24 h, after which PARP, caspase-3 and cleaved-caspase-3 protein levels were measured by protein gel blot analysis.

MA or CQ alone induced low percentages of cells undergoing apoptosis, which were close to the control group. DHA alone induced 20.6% of cells undergoing apoptosis, while combined with 3-MA or CQ, the apoptosis rates rose significantly (**Fig. 5A and B**). Furthermore, we knockdown autophagy related protein 5(Atg5) to block autophagy in Hela cells, and found that DHA treatment induced higher apoptosis rate in Atg5-knockdown cells than in negative-knockdown cells (**Fig. 5C**). Taken together, pharmacological inhibition of autophagy at different steps or selective knockdown of Atg5 both strongly increased DHA-induced apoptosis.

The combination of dihydroartemisinin and chloroquine exerted synergistic anti-tumor efficacy both *in vitro* and *in vivo*

In present study we have observed that CQ, as an inhibitor of autophagy, enhances the apoptosis rate in DHA-treated HeLa cells, so next we sought to further demonstrate the synergistic effects of DHA and CQ on anti-tumor efficacy. First, we employed SRB assay to figure out the survival function and combination index (CI values) of DHA/CQ combination treatment in HeLa and HCT116 cells. Survival fraction curves were displayed in **Figure 6A** and **B**, compared with mono-treatment, the combination of DHA and CQ notably decreased the survival

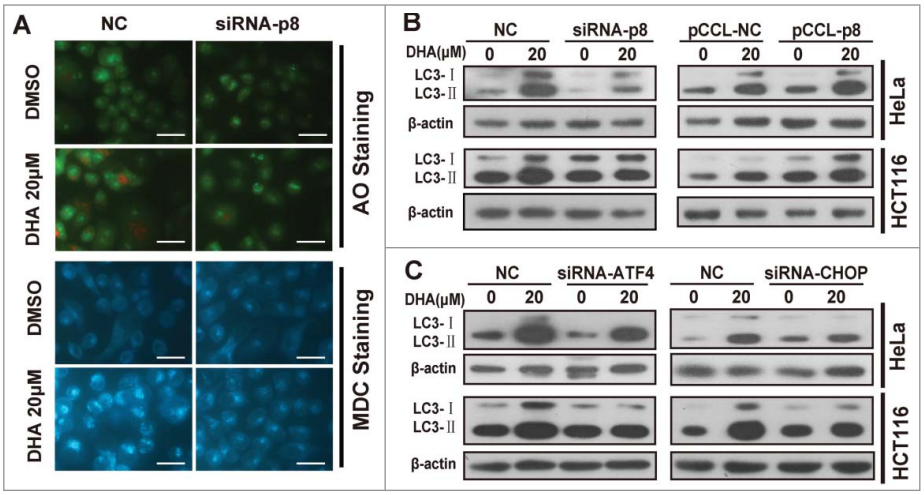

Figure 4. p8-ATF4-CHOP axis is involved in dihydroartemisinin -induced autophagy in HeLa and HCT116 cells. (**A**) After transfection with specifically targeted p8 siRNA for 24 h, HeLa cells treated with 20 μM DHA for 24 h, and then Acridine orange (AO) staining, MDC staining were performed. Bar, 50 μM. (**B**) After transfection with pCCL^{-} p8 plasmid or specifically targeted siRNA (p8, ATF4 and CHOP) for 24 h, HeLa and HCT116 were treated with 20 μM DHA for 24 h, after which LC3 protein levels were measured by western blot analysis.

function in both HeLa and HCT116 cells. Subsequently, CI values were calculated using Calcucyn at the fixed-ratio concentrations of DHA and CQ. As shown in **Table 1**, most groups of DHA plus CQ exerted synergy (CI < 0.70) or strong synergy (CI < 0.30) in the tested cancer cells lines. Next, we performed protein gel blot assays. Treatment with DHA (20 μM) combined with CQ (10 μM) for 24 h caused much more significant cleavage of PARP, caspase3 compared with the single agent-treated cells, indicating the involvement of caspases-mediated apoptosis triggered by DHA/CQ combined treatment (**Fig. 6C**), and further favoring the synergistic efficacy of CQ on DHA-induced apoptosis.

To test whether this synergistic effect could be reproduced *in vivo*, the combination therapy of DHA and CQ was tested against HeLa xenografts in nude mice. The i.g. administration of DHA at the dose of 100 mg/kg daily or i.p. administration of CQ at dose of 50 mg/kg daily for 13 days only modestly reduced tumor weight; and the combination therapy with DHA and CQ significantly decreased the tumor weight (**Table 2**). As illustrated in **Figure 6D**, similar tumor growth inhibitory effect of DHA and CQ were observed on HeLa xenografts models. Both of single agent treatment groups resulted in a moderate therapeutic effect, as the T/C value of DHA-treated group was 74.7%, and T/C value of CQ-treated group was 87.0%. As expected, the combination therapy exhibited distinct tumor growth inhibition (the T/C value 51.2%), with a significant greater extent than DHA-, CQ- or vehicle-treated mice (mean RTV of combination group vs mean RTV of DHA-group: $P < 0.05$; mean RTV of combination group vs

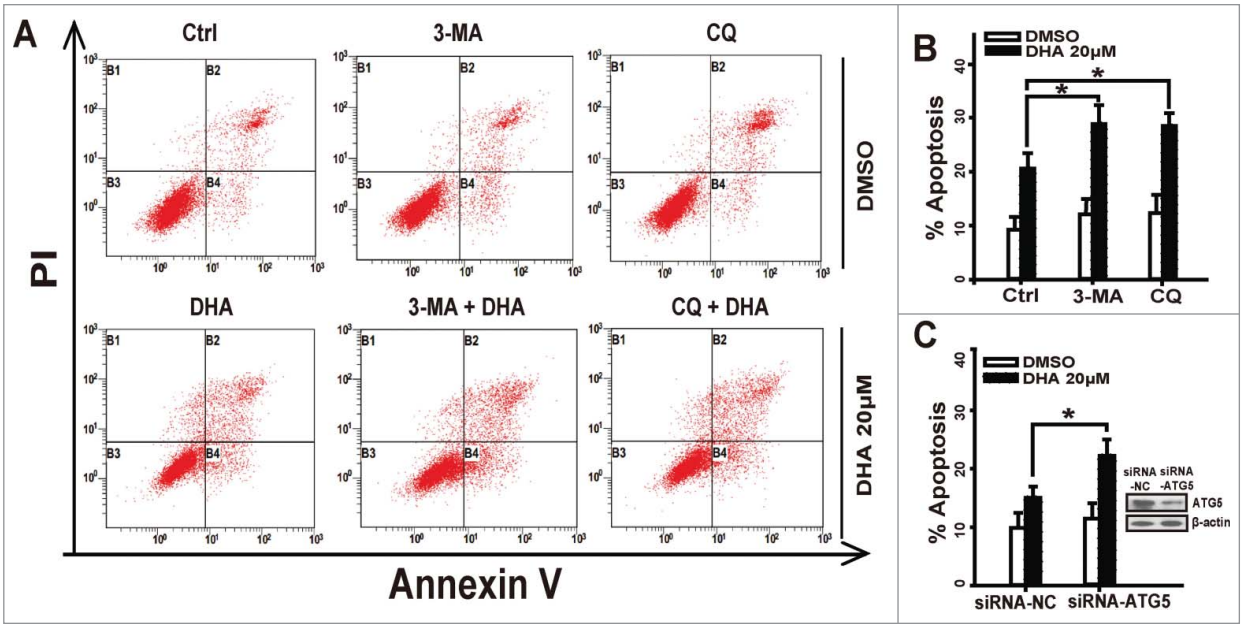

Figure 5. Inhibition of Autophagy increases apoptosis in dihydroartemisinin-treated tumor cells. (**A-B**) HeLa cells were pretreated with 3-MA(1 mM) or CQ(10 μM)for 1 h, then cotreated with DHA(20 μM) for 24 h and were stained with Annexin V/PI and detected by FACS analysis. (**C**) After transfection with specifically targeted Atg-5 siRNA for 24 h, HeLa cells were treated with 20 μM DHA and were stained with Annexin V/PtdIns and detected by FACS analysis. Three independent experiments were performed and the values were expressed as the mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

mean RTV of CQ-group: $P < 0.05$; mean RTV of combination group vs mean RTV of vehicle-group: $P < 0.001$), but caused no extended body weight loss to the animals. Taken together, these data revealed that the combination of DHA and CQ produced much more potent tumor growth inhibitory effects, without significant toxicities to the animals.

Discussion

The cytotoxicity of dihydroartemisinin in multiple kinds of cancer cells have been demonstrated by numerous of researches. Moreover, some clinical trials have also been reported, which provides evidence on the good tolerability of DHA and shows orally administered DHA improved clinical symptoms of advanced cervix carcinoma.[32] However, since it has been reported that DHA alone is not effective enough for cancer therapy,[33] new effective chemotherapy strategies such as combination of other compounds with DHA are urgently needed. In the present study, we find upregulation of p8 in DHA-treated cancer cells induced autophagy to decrease the anticancer effect, thus we speculate that combination of DHA with p8 inhibitor or autophagy inhibitor may have the synergistic anti-tumor effect. Since we have not found any kinds of p8 inhibitor, we only investigated the combination treatment of DHA and chloroquine (CQ), which is well known as an autophagy inhibitor. Coincidentally, like DHA, CQ is another classical antimalarial agent, and several clinical trials have shown CQ exerts well anticancer effects.[34] CQ exerts its effects mainly through blunting the activivty of lysosomal enzymes,[35] to arrest degradation of the autolysosome, the latter step of autophagy,

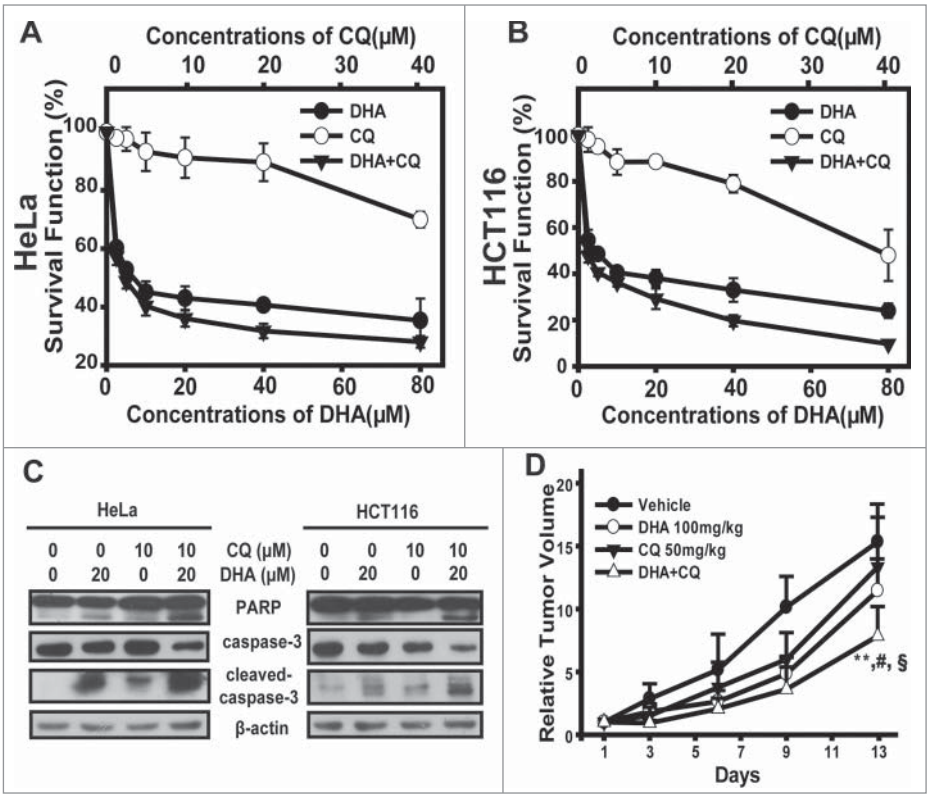

Figure 6. The combination of dihydroartemisinin and chloroquine exerted synergistic anti-tumor efficacy both *in vitro* and *in vivo*. (**A–B**) SRB assays were used to examine the cell proliferation inhibitory activities in HeLa cells (**A**) and HCT116 cells (B). Cells in 96-well plates were exposed to serial concentrations of DHA, CQ or the constant-ratio mixture of these 2 for 48 h. Dose–response curves of 2 cell lines to DHA, CQ and the combination were presented. Each condition had triplicates, and standard deviation was represented as error bars. The CI values were demonstrated in **Table 1**. (**C**) HeLa and HCT116 cells were pretreated with CQ(10 μM)for 1 h, then cotreated with DHA(20 μM) for 48 h and after which PARP, caspase-3 and cleaved-caspase-3 protein levels were measured by protein gel blot analysis. (**D**) The mice transplanted with HeLa human xenografts were randomly divided into 4 groups and given administration of DHA (100 mg/kg, i.g.), CQ (50 mg/kg, i.p.), the combination or vehicle daily for a period of 13 days. Relative tumor volume are expressed as mean ± SD (n = 8 per group). ** Relative to Vehicle group, $P < 0.01$; # Relative to DHA group, $P < 0.05$;§Relative to CQ group, $P < 0.05$. Tumor weight, inhibition rate, and T/C value (RTV of treatment/RTV of control) were shown in **Table 2.**

Table 1. CI values of DHA at concentrations applied in combination with CQ in Hela and HCT116 cells

Hela			HCT116		
DHA (μM)	**CQ (μM)**	**Combination index**	**DHA (μM)**	**CQ (μM)**	**Combination index**
2.5	1.25	0.899	2.5	1.25	0.487
5	2.5	0.538	5	2.5	0.446
10	5	0.315	10	5	0.544
20	10	0.335	20	10	0.485
40	20	0.351	40	20	0.289
80	40	0.396	80	40	0.126

A CI less than 0.90 indicates synergism; 0.10, very strong synergism; 0.10–0.30, strong synergism; 0.30–0.70, synergism; 0.70–0.85, moderate synergism; 0.85–0.9, slight synergism; 0.90–1.10, additive; and more than 1.10, antagonism.

Table 2. *In vivo* efficacy of DHA in combination with CQ against human HeLa xenografts

	No. of animals		Body Weight				
	start	end	start	end	Tomur weight (g)	Inhibition (%)	T/C (%)
Vehicle	8	8	19.3	20.8	0.28 ± 0.17	/	/
DHA(100 mg/kg)	8	8	20.1	22.1	0.21 ± 0.08	26.9	74.7
CQ(50 mg/kg)	8	8	19.1	20.9	0.24 ± 0.08	16.6	87.0
DHA + CQ	8	8	18.9	20.7	0.14 ± 0.05 *,#, §§	51.5	51.2

which break the energy supply through the autophagy pathway.[27] As our expectation, the combination treatment of DHA and CQ reveals notable synergistic anticancer effect both *in vitro* and *in vivo*. The mechanism underlying the synergistic effect may be that CQ broke the DHA-induced autophay to enhance apoptosis level in cancer cells. However, it needs to be further investigated because both of 2 agents have complex cellular functions.

During p8 upregulation and autophagy induced by DHA, endoplasmic reticulum stress is also triggered in this process. ER stress is initiated by an imbalance between ER protein folding load and capacity in the ER lumen, and its signal transduction pathway is known as unfolded protein response (UPR). Since ER stress can be caused by oxidative stress, the iron-mediated endoperoxide cleavages of DHA result in the unbalance of redox to induce ER stress.[5] To response to ER stress, the translation of ATF4 mRNAs selectively increases, which induces the expression of genes involved in restoring ER homeostasis.[36] Among the ATF4 target genes, CHOP is one of the most induced, which is common considered as a mediator of apoptosis. But recent studies have identified that CHOP promotes pro-survival autophagic process through the cooperation with ATF4.[37] In this study, we demonstrated that p8 acts upstream of ATF4 and CHOP in DHA-induced ER stress response in tumor cells. Although the exact p8 precise role in these mechanisms has still to be further clarified, the pro-survival autophagy induced by p8-ATF4-CHOP axis should not be ignored in cancer therapies with DHA.

Several studies have shown that ER stress can activate autophay, and even suggested that at least part of autophagosomal membrane originates from enlarged ER membrane.[22,38] Like as autophagy, ER stress can activate both prosurvival programs as well as death mechanisms. Carracedo's study identified that ER stress induced by cannabinoid promoted autophagy-mediated cell death via TRB3-dependent inhibition of mTORC1 axis in human glioma cells.[12] But another study demonstrated that ER stress induced by salinomycin promoted pro-survival autophagy via inhibiting AkT1 and mTORC1 in human non-small cell lung cancer.[39] Therefore, the paradoxical ability of ER stress and autophagy to promote cell survival or death seems to depend on the type of cancer and cytotoxic agents used. In our study, by using RNA interference and autophagy inhibitors, we confirmed that the p8-ATF4-CHOP axis-mediated autophagy contributes to promote cell survival in DHA-treated cancer cells.

In summary, for the first time, we found the stress-inducible protein p8 is upregulated rapidly by DHA treating in tumor cells. Furthermore, we demonstrated that the upregulation of p8 induces ATF4 and CHOP expression, and consequently leads to autophagy, which was confirmed to attenuate apoptosis in DHA treatment. In addition, the combination therapy of DHA and CQ showed well synergistic antitumor effect in both *vitro* and *in vivo*. These results provide evidence to support the use p8 as a cancer therapeutic target, and suggest that the combination treatment of DHA and CQ might be an effective cancer therapeutic strategy.

Materials and Methods

Reagents

Dihydroartemisinin (DHA) was kindly provided by Engineer Liuxu of Guiling Pharmaceutical Co. Sulforhodamine B (SRB), Acridine orange (AO), Dansylcadaverine (MDC), Chloroquine diphosphate salt (CQ), and 3-Methyladenine (3-MA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against ATF4, CHOP, LC3B, Caspase-3, cleaved-caspase3 were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Antibodies against PARP and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against p8 was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

HeLa, HCT116, HepG2 and SKOV3 cell lines were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and were cultured in RPMI-1640 (HeLa and SKOV3) or DMEM (HCT116 and HepG2), supplemented with 10% fetal serum in a humidified atmosphere of 5%CO_2 at 37°.

SRB assay

The effects of DHA and CQ on cell proliferation inhibition were determined by the sulforhodamine B (SRB) assay. 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 $[(A_{515\ \text{control cells}}\text{-}A_{515\ \text{treated cells}})/A_{515\ \text{control cells}}] \times 100\%$ (whereA_{515} is the OD value at 515 nm).

siRNA transfection

The siRNA sequence was duplexes produced by Genepharma, Co. (Shanghai, China). The sequences of siRNAs used were as follows (sense): si-p8#1 (for HeLa) 5′-GGAUGAAUCUGACCUCUAUTT-3′, si-p8#2 (for HCT116,SKOV3 and HepG2) 5′-GACUCCUGCCUUGGUUACATT-3′, si-ATF4#1 (for HeLa, SKOV3 and HepG2) 5′-TCCCTCAGTGCATAAAGGATT-3′, si-ATF4#2 (for HCT116) 5′-GCCTAGGTCTCTTAGATGATT-3′, si-CHOP 5′-GAGCUCUGAUUGACCG-AAUTT-3′, siAtg5 5′-GGAAUAUCCUGCAGAAGAAUU-3′, negative control siRNA 5′-UUCUCCGAACGUGUCACGUTT-3. The transfection was performed using Opti-MEM (Invitrogen), siRNA and Oligofectamine (Invitrogen) according to the manufacturer's recommendations, 24 h prior to the drug treatment.

Quantitative real-time reverse transcription PCR assay

Total RNA was prepared using Trizol reagent (Sangon Biotech Co., Ltd), precipitated by isopropyl alcohol, and rinsed with 70% ethanol. Single-stranded cDNA was prepared from the purified RNA using an oligo(dT) primer (Thermoscript RT kit; Invitrogen) followed by SYBR(Takara) green real-time PCR. The primers were as follows(forward primer and reverse primer): p8, 5′-GAAGAGAGGCAGGGAAGACA-3′ and 5′-CTGCCGTGCGTGTCTATTTA-3′; ATF4, 5′-GGCCACCATGGCGTATTA-3′ and 5′-TGCTGAATGCCGTGAGAA-3; CHOP, 5′-GCACCTCCCAGAGCCCTCACTCTCC-3′ and 5′-GTCTACTCCAAGCCTTCCCCCTGCG-3′; GAPDH, 5′-TGCACCACCAACTGCTTAGC-3′ and 5′-GGCATGGACTGTGGTCATGAG-3′. Relative expression levels of the target genes were normalized with the control gene, GAPDH.

AO staining and MDC staining

Acridine orange (AO) and Dansylcadaverine (MDC) were used to evaluate the abundance of autophagic vacuoles in the cells. Following treatment, the cells were stained with AO (1 μg/ml) or MDC (50 μM) for 15 min at 37° and subsequently examined by fluorescence microscopy.

Annexin V-FITC/PtdIns and Annexin V-PE/7-AAD assay

Cells undergoing apoptosis were detected using the Annexin V-FITC/PI apoptosis detection kit (Invitrogen) or Annexin V-PE/7-AAD apoptosis detection kit (MultiSciences Biotech Co, Ltd).Briefly, cells were resuspended in cold binding buffer and incubated for 15 min in dark at room temperature; addition of Annexin V–FITC(or Annexin V-PE) and PtdIns (or 7-AAD) solutions following the manufacturer's instructions. Flow cytometry analysis was performed using a FACSCalibur cytometer (Beckman Coulter, FC500MCL).

Western blotting

The cells were collected and lysed in 2× SDS gelloading buffer [24 mM Tris-HCl (pH 6.8), 0.02% mercaptoethanol, 4% SDS, 0.4% bromphenol blue, 20% glycerol] and then boiled for 10–15 minutes. Equal volumes of cell lysates were resolved on 8%–15% SDS-PAGE gels, and the proteins were transferred to PVDF membranes (Pierce Chemical). The blots were incubated with the indicated primary antibodies and then the appropriate horseradish peroxidase-conjugated secondary antibodies. The signals were visualized by the ECL Plus western blotting detection system (Abbkine, Inc., Redlands, CA, USA).

Measurement of *in vivo* activity

Human cervical cancer HeLa xenografts were established by 5×10^6 cells subcutaneously into the armpit of the nude mice. When the tumor reached a mean group size of50 mm^3, the mice were randomized to vehicle and treated groups, and received vehicle (0.1%CMC-Na in physiological saline solution, i.g. administration and physiological saline solution i.p. administration) daily, DHA (100 mg/kg, i.g. administration) daily, CQ (50 mg/kg, i.p. administration) daily, and combination group (DHA, 100 mg/kg, i.g. administration and CQ, 50 mg/kg, i.p. administration) daily for 13 days. Tumor volume was calculated as $V = (\text{length} \times \text{width}^2)/2$. The individual relative tumor volume (RTV) was calculated according to the following formula: $RTV = V_n/V_0$, where V_n is the tumor volume on day n and V_0 is the tumor volume on day of initial treatment. The T/C% was determined by calculating relative tumor volume (RTV) as T/C% = 100 × (mean RTV of treated group)/(mean RTV of vehicle group). The tumor growth inhibition rate was calculated by using the following formula: inhibition rate (%) = (1−TWt/TWv) × 100, where TWt and TWv are the mean tumor weight of the treatment and vehicle groups, respectively. The animal study was approved by the Animal Research Committee at Zhejiang University (log number Zju2011101065 and Zju2012101016), and animal care was provided in accordance with institutional guidelines.

Statistical analysis

For all parameters measured, the values for all samples in the different experimental conditions were averaged, and the standard error or standard deviation of the mean was calculated. Analysis of variance followed by Student's t-test was used for the statistical analyses. Significance was defined as $P < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

This study was supported by research grants from the National Natural Science Foundation of China (No. 81072659), the Zhejiang Provincial Science and Technology Program (No. 2008C23067) and the Health Bureau of Zhejiang Province, China (No. 2008W10923).

Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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