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ORIGINAL ARTICLE

Quercetin greatly improved therapeutic index of doxorubicin against 4T1 breast cancer by its opposing effects on HIF-1α in tumor and normal cells

Gangjun Du · Haihong Lin · Mei Wang · Shuo Zhang · Xianchuang Wu · Linlin Lu · Liyan Ji · Lijuan Yu

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Abstract
Purpose The anthracycline antibiotic doxorubicin (DOX) has been used successfully for treating various types of cancers. However, the therapeutic efficacy of DOX was greatly restricted by its cumulative dose-related cardiotoxicity and common side effects such as bone marrow and immune suppression. Quercetin had better cardioprotective and hepatoprotective activities. The present study was to observe whether quercetin could improve therapeutic index of DOX and explore its mechanisms.
Methods Effects of quercetin on doxorubicin (DOX)-induced cytotoxicity were investigated in 4T1 cells and murine spleen cells by methylthiazoletetrazolium assay, flow cytometry and single cell gel electrophoresis. Influences of quercetin on therapeutic efficacy and systemic toxicity of DOX were evaluated in BALB/c mice with 4T1 breast cancer. Hypoxia-inducible factor-1 alpha (HIF-1α) in tumor and normal cells was examined to explore mechanisms of quercetin by Western blot and enzyme-linked immunosorbent assay.
Results In vitro, quercetin at dose less than 100 μM had only slight effects on cell viability and DOX-induced cytotoxicity in 4T1 cells under normoxia, but it could reverse 4T1 cell resistance to DOX under hypoxia and protect spleen cells against DOX-induced cytotoxicity. In vivo, quercetin suppressed tumor growth and prolonged survival in BALB/c mice bearing 4T1 breast cancer. Importantly, quercetin enhanced therapeutic efficacy of DOX and simultaneously reduced DOX-induced toxic side effects. Further study showed that quercetin suppressed intratumoral HIF-1α in a hypoxia-dependent way but increased its accumulation in normal cells. HIF-1α siRNA abolished effects of quercetin on both tumor and normal cells.
Conclusions These results suggested that quercetin could improve therapeutic index of DOX by its opposing effects on HIF-1α in tumor and normal cells, and was a promising candidate as anticancer agents.

Keywords Quercetin · Doxorubicin · Therapeutic index · HIF-1α · Breast cancer

Introduction

Breast cancer is the most common cancer and the second major cause of cancer deaths in women [1]. Despite advances in early detection and therapeutic approaches in the past few years, breast cancer, especially advanced breast cancer, remains the poor prognosis [2]. The high mortality rate of breast cancer is mostly due to the low therapeutic selectivity of antitumor agents and the high rate of metastasis and recurrence [3]. The anthracycline antibiotic DOX is one of the most effective anticancer drugs for treating various types of cancers. In most advanced breast cancer, DOX is a main option and can improve both the quantity and quality of the patients' lives [4]. However, the therapeutic effectiveness of DOX is greatly limited by conventional toxicities and dose-dependent cardiotoxicity,

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especially when administered as a combination of more than two drugs [5]. Therefore, the new combination treatment of DOX and other agents with increased efficacy and low toxicity is necessary.

Flavonoids have recently received much attention as potential chemopreventive and chemotherapeutic agents [6]. Quercetin is one of the most frequently studied dietary flavonoids which is ubiquitous in various vegetables, fruits, seeds, nuts, tea and red wine [7]. Due to very promising for cancer prevention and treatment in preclinical models and clinical trials [8, 9], quercetin has become a prospective anticancer drug candidate. Several studies showed that quercetin was especially relevant for gynecological cancers such as breast cancer [10, 11]. This was well in agreement with experimental facts that quercetin had potent inhibitory action on human breast cancer cells with selective cytotoxicity [12]. Taking potential cardioprotective and hepatoprotective effects of quercetin into account [13, 14], together with the antioxidant capacity in human lymphocytes and pro-oxidant activities in many cancer cell lines [15, 16], we hypothesis that quercetin might synergistically potentiate the antitumor effects of DOX and simultaneously protect normal cells against DOX-induced damages. To verify this hypothesis, we assessed the biological activities of quercetin alone and in combination with DOX in the mouse 4T1 cell line in vitro and in BALB/c mice bearing 4T1 breast cancer. Further, we exploited the mechanisms that quercetin suppressed tumor cells and simultaneously protected normal cells.

Materials and methods

Materials

Quercetin, yellow powder, purity 95% (HPLC), was from Shanxi Huike Botanical Development Co. (China), dissolved to 50 mmol/l in dimethyl sulfoxide and diluted with the medium. DOX was provided by Haizheng Corp. (Zhenjiang, China). RPMI1640 medium was from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS), penicillin, and streptomycin were from Gibco BRL (Rockville, MD). Apoptosis detection kit was from BD Pharmingen (San Diego, CA). Concanavalin A (Con A), methylthiazoletetrazolium (MTT) and anti-actin were from Sigma Chemical (St. Louis, MO, USA). Anti-HIF-1α was from BD Biosciences Co. (San Jose, CA). HIF-1α enzyme-linked immunosorbent assay (ELISA) kit was from Wuhan USCN Sciences Co. Ltd. (China). A secondary antibody (sheep anti-mouse IgG horseradish peroxidase-linked antibody) was from Sino-American Biotechnology Co. Ltd. (China). Enhanced chemiluminescence substrate was from ECL Plus (Germany). Vascular endothelial growth factor (VEGF), interferon-gamma (IFN-γ) and interleukin-2, -4, -10 (IL-2, IL-4, IL-10) ELISA kits were from R&D Systems (Minneapolis, MN). siRNA specific to HIF-1α was designed and synthesized by GenePharma Company (Shanghai, China). All other reagents were of analytical grade from commercial sources.

Animal

Pathogen-free female BALB/c mice weighing 18–22 g were from Beijing Weitonglihua Animal Co. The mice were maintained in a pathogen-free animal facility for at least 1 week before each experiment. The animal use committee of Pharmacy College of Henan University approved all animal study protocols described in this publication, and experiments were conducted in compliance with the guide for the care and use of laboratory animals.

Cell culture

The murine breast cancer (4T1) cells were purchased from American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured in RPMI1640 medium supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/l glutamine. All cells were incubated at 37°C with 5% CO_2. Hypoxia treatment was performed by placing cells in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) flushed with a mixture of gas consisting of 1% O_2, 5% CO_2, and 94% N_2.

Cell proliferation assay

4T1 cells (5×10^3 cells/well) were plated in 96-well plates and allowed to attach overnight. Cells were then treated with quercetin or DOX at various concentrations or the vehicle (DMSO, final concentration 0.2%) and incubated under normoxia or hypoxia at 37°C for 72 h. Cell proliferation was examined using MTT assay as previously described [17], and the IC50, defined as the concentration of drug required to block 50% cell viability, was determined using SPSS software (version 10.0, SPSS Inc., Chicago, IL).

Preparation and culture of splenocyte

Spleen was aseptically taken from female BALB/c mice, crushed gently and separated into single cells by squeezing in 10 ml D-Hank's solution. After lysis of the erythrocytes with 10 ml of sterile 0.01 mol/l Tris–0.83% NH_4Cl, single cell suspension was washed twice with D-Hank's and was resuspended in RPMI1640 medium supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml

streptomycin. Cell viability was evaluated by trypan blue exclusion and only cell preparations with more than 95% viability were used. Cells were plated in 96-well plates at a density of 2×10^5 cells/well and treated for 72 h with quercetin or DOX at various concentrations in the presence of 5 μg/ml Con A. Cell proliferation was determined by MTT assay as previously described [17].

Transfer of HIF-1α siRNA to cells

4T1 (2×10^5 cells/well) and spleen cells (2×10^7 cells/well) were seeded into six-well plates the day before transfection. After 24 h (30–40% confluence), the media were replaced with media without serum or antibiotics. Cells were then transfected with the specific siRNA to HIF-1α using Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's instructions. After 5 h, the transfection media were replaced with regular complete medium. After 72 h, cells were harvested or treated as needed for further experiments.

Single cell gel electrophoresis

DNA strand break formations in the murine spleen cells were determined using the comet assay as previously described with minor modifications [18]. Murine spleen cells were cultured in complete RPMI1640 medium containing 5 μg/ml of Con A and were treated with DOX 2 μM or indicated concentrations of quercetin for 24 h under normoxia and hypoxia. Slides previously covered with 100 μl 1% NMP (normal melting point) agarose in PBS buffer were covered with a second layer consisting of 80 μl 0.75% LMP (low melting point) agarose and 20 μl cell suspension (2×10^4 cells). After storage for 30 min at 4°C to allow solidification of the second layer, slides were immersed in lysis buffer (2.5 mmol/l NaCl, 100 mmol/l EDTA, 10 mmol/l Tris–base, and 1% Triton X-100, pH 10) for 1 h at 4°C. Then the slides were transferred to an electrophoresis box containing an alkaline solution at pH 13 (300 mmol/l NaOH, 1 mmol/l Na_2EDTA) for 40 min unwinding time at 4°C. Electrophoresis was conducted at 300 mA and 25 V for 15 min. Following three times neutralization using neutralization buffer (0.4 mmol/l Tris–HCl, pH 7.5), slides were stained with 20 μg/ml PI. Analysis of comet appearance was performed on an Olympus BX60 fluorescence microscope at 200× magnification. For each individual experiment, at least two times 100 cells were analyzed randomly per treatment, using a comet image analysis software program (Comet 5.0, Kinetic Imaging Ltd., Liverpool, UK). DNA damage was defined as the product of tail length and the fraction of total DNA in the comet tail.

Apoptosis assay

Apoptotic and/or necrotic cells were evaluated by Annexin V binding and 7-AAD uptake using an Annexin V-FITC/7-AAD kit as described by the manufacturer. Briefly, 4T1 cells were plated at a density of 5×10^5 cells/well into six-well plates for 24 h. The cells were treated with DOX 0.5 μmol/l or quercetin 50 μmol/l for 72 h under hypoxia. The cells were washed with cold PBS and resuspended in Annexin V binding buffer. The cells were stained with Annexin V-FITC for 15 min, washed, and then stained with 7-AAD. The samples were analyzed by flow cytometer with CellQuest Pro software.

Western blot analysis

Whole-cell extracts were prepared from quercetin-treated or -untreated cells cultured in six-well plates. After incubation, cells were harvested and resuspended in lysis buffer, washed with ice-cold PBS and lysed in extraction buffer (40 mM Tris–HCl, pH 7.5, 150 mM KCl, 1 mM EDTA, 1% Triton X-100, 100 mM $NaVO_3$, 1 mM PMSF) supplemented with the protease inhibitor cocktail. The protein (50 μg) was separated on 8% SDS-PAGE and transferred onto a PVDF membrane (Roth, Karlsruhe, Germany). The membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS) at 4°C, and then incubated with mouse anti-HIF-1α monoclonal antibody (1:500), or mouse anti-β-actin in TBS containing 5% non-fat milk for 1 h at room temperature. Horseradish peroxidase-linked anti-mouse IgG (1:1,000) was used as a secondary antibody (in TBS containing 5% non-fat milk for 30 min at room temperature), and antigen–antibody complexes were detected using an enhanced chemiluminescence kit (ECL Plus, Amersham, Freiburg, Germany). The relative density of the band was calculated by comparing the ratio of intensity of HIF-1α and β-actin.

In vivo tumor models

4T1 cells (1×10^5 cells suspended in 0.1 ml PBS) were injected into the second mammary fat pad of 7–8-week-old BALB/c mice. The mice were kept in a pathogen-free environment and checked twice weekly. When the tumors reached a size of 200–400 mm^3 (2 weeks after tumor implantation), mice were randomized into one of four experimental groups (15 per group) and treatment was initiated according to the indicated schedule/dose. Quercetin (100 mg/kg) or vehicle, formulated in 0.5% CMC-Na, was administered daily by oral gavage for 3 weeks; DOX (5 mg/kg), formulated in 0.9% NaCl, was injected intravenously on days 1, 8 and 15 after grouping. Tumors were measured with calipers twice weekly, and tumor volume was calculated using the following formula: tumor volume

(mm^3) = [(width)^2 × length]/2. Treatment-related toxicity was determined by mouse weights weekly.

Five mice in each group were killed at day 22 after treatment. Tumors were dissected and weighed. Regional lymph nodes and lungs were collected and evaluated for metastases. Serum cytokines including IFN-γ, IL-2, IL-4, IL-10 were determined by ELISA according to manufacturer's instructions. Spleens were removed and examined for cell apoptosis and HIF-1α level. Hearts were removed and prepared sections for cardiac toxic assay. VEGF and HIF-1α in tissues were measured by ELISA as described by the manufacturer. The total number of hematopoietic cells in bone marrow and leukocytes from blood was counted using a Zeiss microscope.

The remaining mice in each group were used for survival analysis. Kaplan–Meier plots were constructed to show the percentage of animals remaining in the study as a function of time following treatment. Statistical significance between the treated versus control groups was evaluated by log-rank analysis using SPSS software.

The tissue samples of tumor and spleen were prepared as described previously [19]. Briefly, fresh tissue was frozen in liquid nitrogen and chopped. 0.2 g tissue was resuspended in 1 ml lysis buffer (10 mM HEPES, pH 7.6, 2 mM DTT, 1 mM Na_2VO_4, 100 mM NaF, 0.4 mM PMSF, 0.1 mM EGTA, 10 mM $Na_4P_2O_7$, 1× protease inhibitor cocktail) for 15 min on ice. After centrifugation at 1,500 rpm for 5 min, supernatants were used as tissue extracts. The pellet was resuspended in 300 µl lysis buffer (20 mM HEPES, pH 7.5, 400 mM NaCl, 10 mM NaF, 10 mM PNPP, 1 mM Na_2VO_4, 0.1 mM EDTA, 10 µM Na_2MoO_4, 10 mM β-glycerophosphate, 20% glycerol, 1 mM DTT, and 1× protease inhibitor cocktail) for 30 min at 4°C, then centrifuged at 40,000 rpm for 1 h, the supernatants were used as nuclear extracts.

Statistical analysis

Data are mean ± SD, the differences in the mean values among different groups were carried out by a one-way analysis of variance (ANOVA) followed with a post hoc test (Dunnett's multiple comparison test). The differences of the variables between groups were performed with the Student's t test using SPSS 10.0 program, $p < 0.05$ was considered as statistically significant.

Results

Quercetin exhibits preferential activity against 4T1 cells in a hypoxia-dependent manner in vitro

We first investigated the effects of quercetin alone and in combination with DOX on 4T1 cell viability using MTT assay. As shown in Fig. 1a, unlike DOX displaying a potent anti-proliferative activity against 4T1 cells, quercetin at dose less than 100 µM had only slight effects on cell viability and DOX-induced cytotoxicity in 4T1 cells under normoxia. Interestingly, quercetin showed an anti-proliferative effect on 4T1 cells in a dose-dependent manner under hypoxia, its IC50 was 51.42 µM (Fig. 1b). Importantly, quercetin at low dose that had a little effect on tumor cells significantly enhanced cytotoxicity of DOX and reversed the hypoxia-conferred tumor resistance to DOX in 4T1 cells under hypoxia (Fig. 1c). IC50 of DOX was 0.29 µM under normoxia versus 1.58 µM under hypoxia in 4T1 cells, whereas IC50 of DOX in combination with quercetin 10 or 25 µM was 0.40 and 0.31 µM, respectively, under hypoxia. Consistently, quercetin 50 µM synergistically promoted DOX-induced apoptosis in 4T1 cells under hypoxia (Fig. 1d, e). Surprisedly, under normoxia, quercetin significantly decreased the cytotoxicity of DOX compared with DOX alone in spleen cells, IC50 of DOX in combination with quercetin 10 or 25 µM was increased by three- and fourfold, respectively, compared with that of DOX alone (Fig. 2a, b). We further confirmed the protective effect of quercetin 10 or 50 µM on spleen cells against DOX-induced cytotoxicity by single cell gel electrophoresis (Fig. 2c, d).

Antitumor effects of quercetin alone and in combination with DOX in vivo

Based on the preferential activity of quercetin shown in 4T1 cells in vitro, we explored the antitumor activity of quercetin alone and in combination with DOX in vivo using an implant model of 4T1 breast cancer. As shown in Fig. 3, mice treated with quercetin 100 mg/kg day or DOX 5 mg/kg had statistically significantly smaller tumors than untreated mice at days 7, 14, and 21 following initiation of treatment, combination treatment with quercetin and DOX significantly resulted in a greater antitumor effect compared with either agent alone (Fig. 3a). The median survival times for quercetin-treated mice were 55 days (95% CI 50–60 days, $p < 0.01$, Kaplan–Meier curves, log-rank test) versus 38 days (95% CI 36–40 days) for untreated mice (Fig. 3b), whereas it was 65 days (95% CI 45–85 days, $p < 0.01$) for combination-treated mice. In contrast, treatment with DOX alone did not significantly increase the median survival times ($p > 0.05$).

The 4T1 breast cancer can spontaneously metastasize from the primary tumor in the mammary gland to multiple distant sites, especially lung. To extend our observations in this model, we also explored the effects of quercetin alone and in combination with DOX on lung metastasis. As shown in Fig. 3c, quercetin treatment significantly resulted in less metastatic nodes in lung than untreated control,

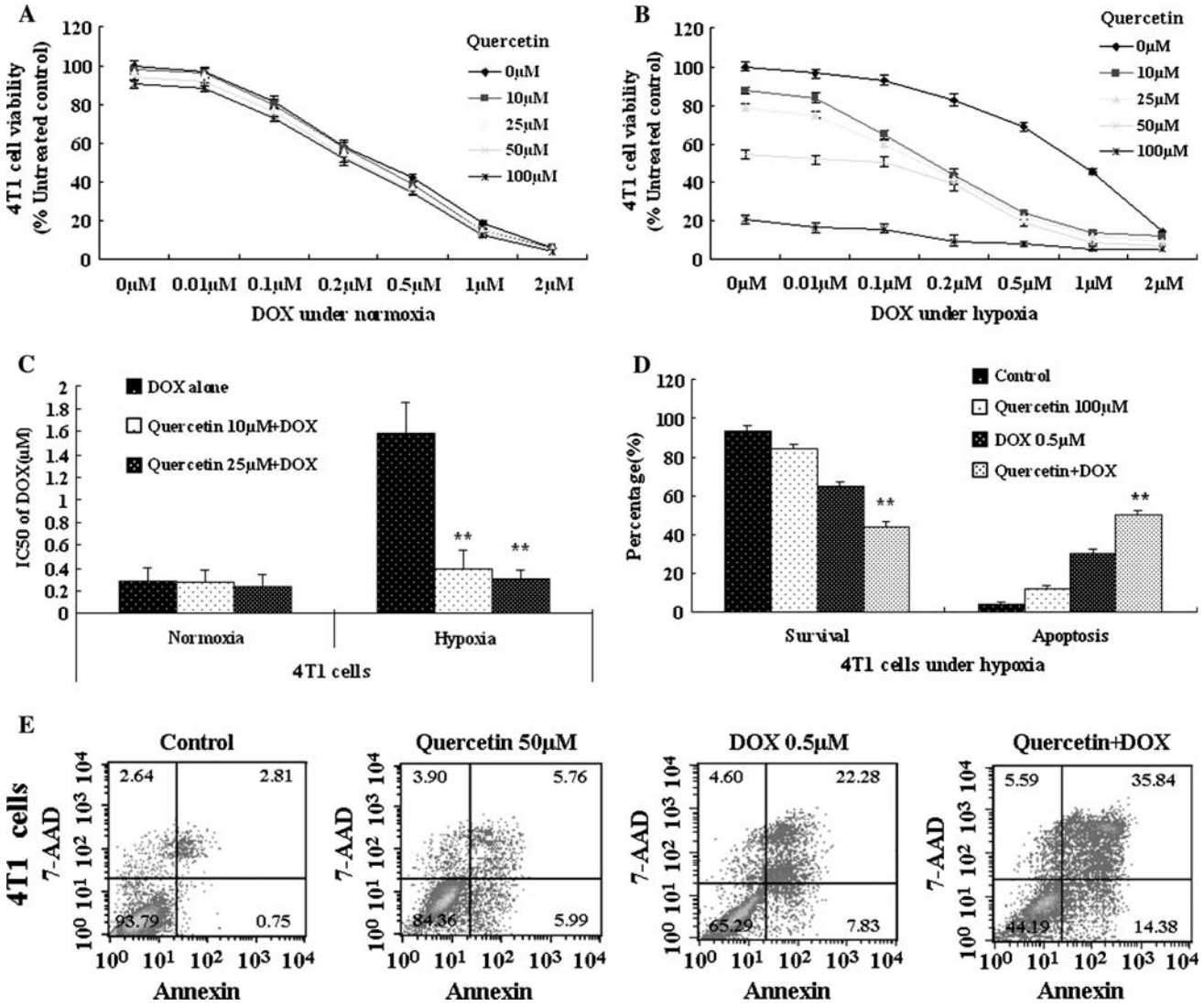

Fig. 1 Effects of quercetin on cell viability and DOX-induced cytotoxicity in 4T1 cells, cells were treated for 72 h. Quercetin had only slight effects on cell viability and DOX-induced cytotoxicity under normoxia (**a**) but showed an anti-proliferative effect in a dose-dependent manner and enhanced DOX-induced cytotoxicity under hypoxia (**b**) examined by MTT assay. Quercetin reduced IC50 of DOX under hypoxia but not under normoxia (**c**). Quercetin synergistically promoted DOX-induced apoptosis under hypoxia (**d**, **e**). Cells' apoptosis was examined by double staining with Annexin V-FITC and 7-AAD, earlier apoptotic cells are only stained by Annexin and later apoptotic cells are double stained by Annexin and 7-AAD, apoptotic cells include earlier apoptotic cells and later apoptotic cells. Results represent mean values of three independent experiments. Compared with DOX-treated cells alone, $^{**}p < 0.01$

combination treatment was significantly more active. Unexpectedly, mice treated with DOX alone developed similar lung metastasis to untreated mice, likely due to systemic toxicity. This was also confirmed by metastatic tumor weight in lung calculated by subtracting mean weight of lung without tumor from tumor-bearing lung (Fig. 3d).

Effects of quercetin on systemic toxicity of DOX

Due to protective effect of quercetin on spleen cells against DOX-induced cytotoxicity in vitro, we intended to test whether quercetin could protect normal cells against DOX-induced systemic toxicity. Hematopoietic system was investigated by blood leukocytes and bone marrow cells. Immune system was examined by spleen cell apoptosis and serum cytokines including the type 1 cytokines IFN-γ and IL-2, and the type 2 cytokines IL-4 and IL-10. As shown in Fig. 4a–d, normally, DOX alone resulted in severe systemic toxicity including body weight lose, hematopoietic damage, immune suppression and cardiotoxicity. Quercetin alone had little effects on above system apart from serum cytokines, whereas administering quercetin in combination with DOX abrogated the above systemic toxicity. In addition, quercetin also reduced DOX-induced spleen cell apoptosis in vivo (Fig. 4d, e). Representatively, DOX alone caused severe histopathologic lesions, the muscle fibers showed

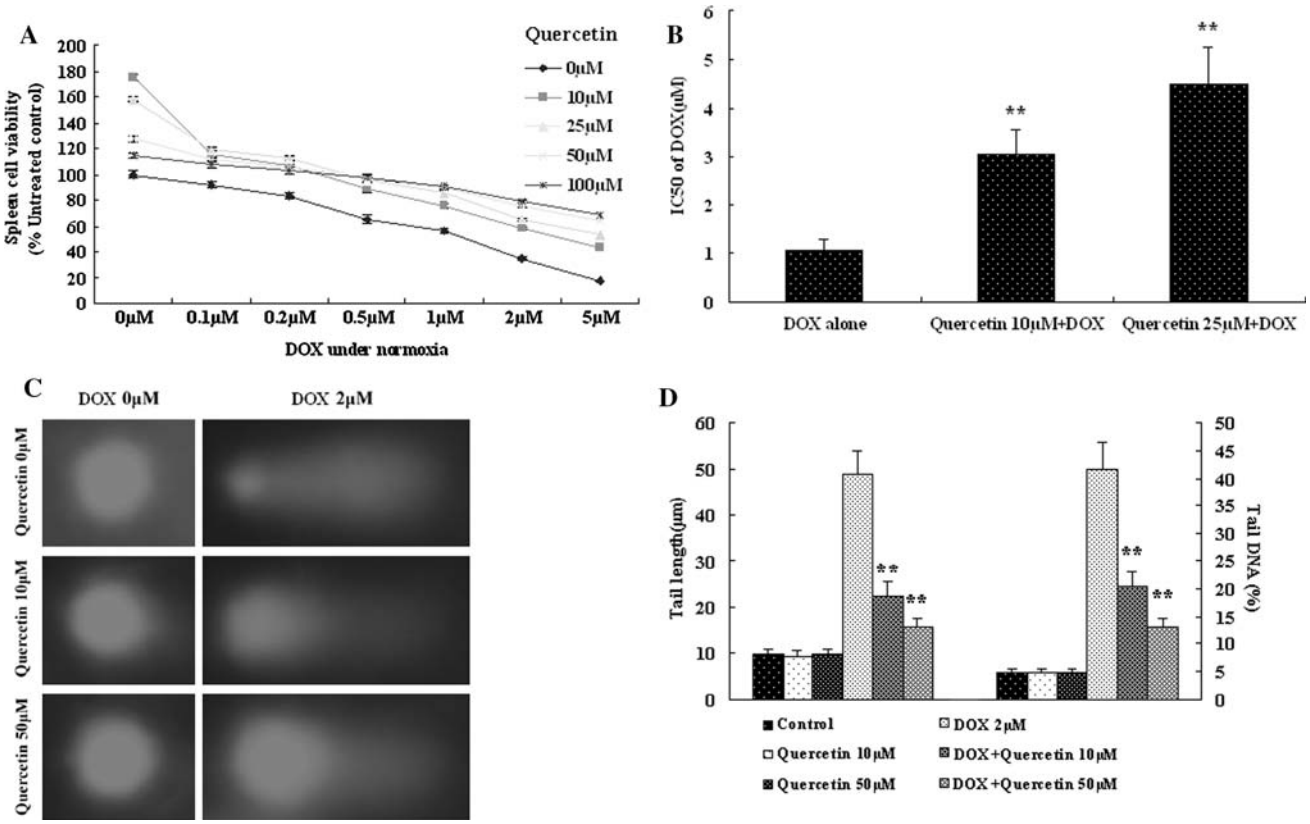

Fig. 2 Effects of quercetin on cell viability and DOX-induced cytotoxicity in spleen cells, cells were treated for 72 h. Quercetin significantly decreased DOX-induced cytotoxicity (**a**) and increased IC50 (**b**) compared with DOX alone under normoxia examined by MTT assay. Quercetin protected spleen cells against DOX-induced cytotoxicity under normoxia examined by single cell gel electrophoresis (bright-field microscopy ×40) (**c**, **d**). Results represent mean values of three independent experiments. Compared with DOX-treated cells alone, $*p < 0.05$ and $**p < 0.01$

varying degrees of damage ranging from loss of striation to complete fragmentation, whereas quercetin alone or in combination with DOX showed no signs of toxicity to muscle fibers in heart sections, as shown in Fig. 4f.

The mechanisms that quercetin suppressed tumor cells and simultaneously protected normal cells

Based on hypoxia-dependent activity of quercetin on 4T1 cells in vitro, we investigated effect of quercetin on HIF-1α level by Western blot. As expected, treatment with quercetin 10 μM significantly induced HIF-1α degradation in 4T1 cells under hypoxia but not under normoxia. By contrast, quercetin 10 μM promoted HIF-1α accumulation in spleen cells under normoxia (Fig. 5a). The similar results were obtained using other dose of quercetin (data not shown). To confirm similar effect of quercetin on HIF-1α level in vivo, we examined tissue HIF-1α level by ELISA. As shown in Fig. 5b and c, quercetin reduced cytoplasm and nuclear HIF-1α level in tumor, but increased them in spleen. This was further confirmed by tissue VEGF which is an activated product of downstream signals in the HIF-1 system (Fig. 5d). These results suggested that the mechanisms that quercetin suppressed tumor cells and simultaneously protected normal cells were likely due to opposing regulation of HIF-1α. To verify this precise mechanism, we transfected HIF-1α siRNAs into 4T1 and spleen cells and observed effects of quercetin on cell viability again in 4T1 and spleen cells. As shown in Fig. 6a–d, HIF-1α siRNA downregulated HIF-1α expression and abrogated efficacy of quercetin, including activities of quercetin alone (Fig. 6c) and effect on DOX-induced cytotoxicity (Fig. 6d) in 4T1 and spleen cells.

Discussion

Breast cancer treatment requires the joint efforts of a multidisciplinary team. Currently, neoadjuvant systemic therapy is an important tool in not only assessing tumor response to an agent but also studying the mechanisms of action of the agent and its effects at the cellular level [20]. The more effective and less toxic treatment should be based on knowledge of the benefits and potential acute and late toxic

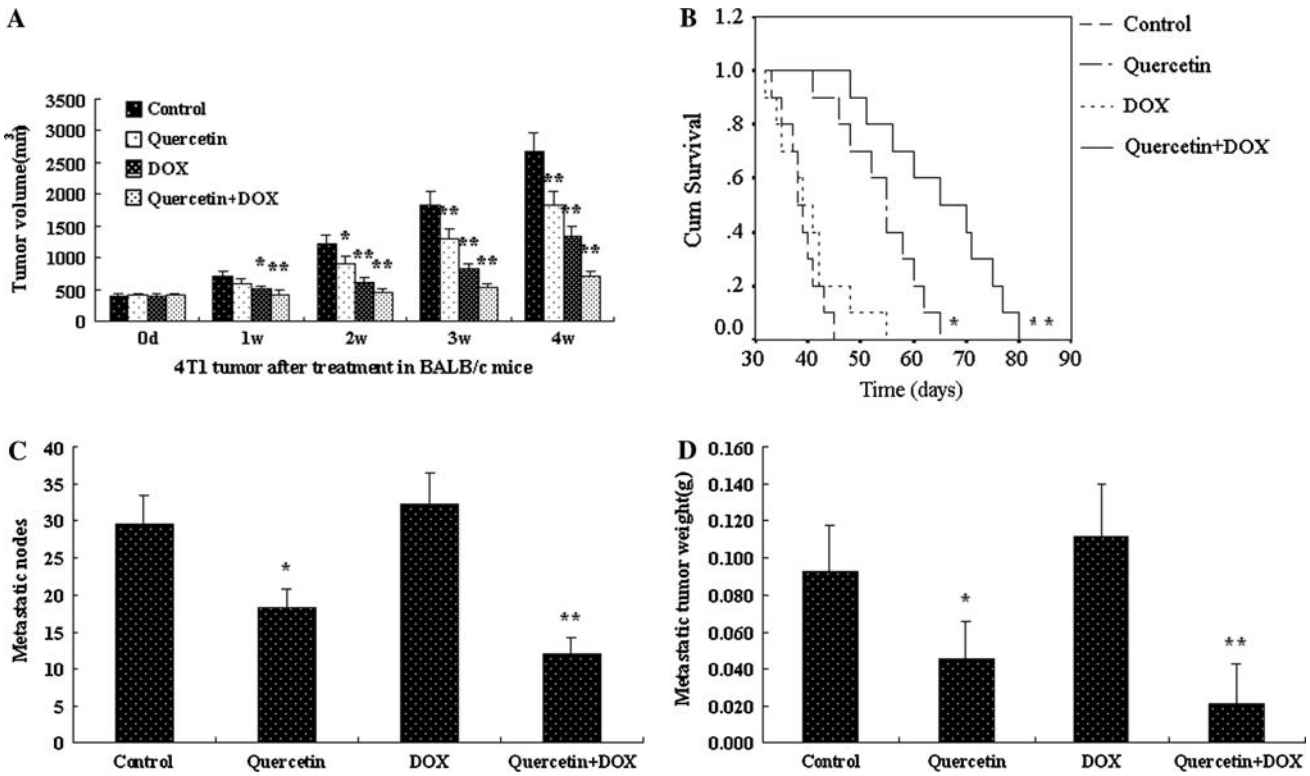

Fig. 3 Effects of quercetin on orthotopic growth and lung metastasis of 4T1 breast cancer. Quercetin inhibited orthotopic growth of 4T1 breast cancer (**a**) and prolonged life span (**b**) in BALB/c mice bearing tumor, combination treatment with quercetin and DOX significantly resulted in a greater antitumor efficacy compared with either agent alone ($n = 10$ per group). DOX alone did not reduced metastatic nodes (**c**) and metastatic tumor weight (**d**) in lung, combination treatment also resulted in a greater antimetastatic efficacy compared with either agent alone ($n = 5$ per group). Compared with DOX-treated mice alone, $^{*}p < 0.05$ and $^{**}p < 0.01$

effects of each of the therapy regimens. In this article, we reported a new strategy for breast cancer therapy by combination with quercetin and DOX.

Despite the anti-proliferative effects in many cancer cell lines, the role of quercetin as single agent for cancer treatment appears to be limited. In this study, we showed that quercetin had a more poor effect against 4T1 breast cancer under normoxia and exhibited an antitumor activity under hypoxia in vitro. Interestingly, quercetin in combination with DOX had preferential activity against 4T1 cells in vitro and in vivo. In order to show the greater antitumor effect of combination treatment compared with DOX alone, the concentration of quercetin in the in vitro studies was chosen through the significant downregulation of HIF-1α and the less growth inhibition in cancer cells under hypoxia, the dose of quercetin in the in vivo studies was based on the optimal dose that resulted in the greatest antitumor effect as single agent (date not shown), simultaneously, we selected the maximum tolerated dose of DOX in vivo. In our studies, quercetin could promote cytotoxicity of DOX in tumor cells and simultaneously protected normal cells against DOX-induced damages in vitro and in vivo, therefore, enhanced the therapeutic index of DOX.

In previous studies, quercetin could inhibit HIF-α protein accumulation and induce DNA strand breaks in cancer cells [21]. This effect was the possibility that quercetin could also lead to relatively toxicity in normal cells. However, under the same in vitro and in vivo experimental conditions, quercetin had selective even opposing effects in tumor cells and normal cells. To explore this mechanism, we examined the effect of quercetin on HIF-α in cancer and normal cells. Surprisedly, quercetin promoted HIF-α degradation in cancer cells but increased its accumulation in normal cells, whereas HIF-α siRNA could abrogate efficacy of quercetin. Therefore, the different effects of quercetin on cancer and normal cells were due to the opposing regulation of HIF-α, likely to the biological differences between normal and cancer cells, it was not simply explained by previous mechanism that quercetin increased the intracellular level of HIF-1α by inhibiting HIF-prolyl hydroxylase and chelating cellular iron ions [22, 23]. In addition, in this study, it should be noted that the best protective effect of quercetin on spleen cells was related to used dose and cell number, the optimal dose was 25 μM in MTT assay and 50 μM in single cell gel electrophoresis although quercetin inhibited tumor cells in a dose- and hypoxia-dependent

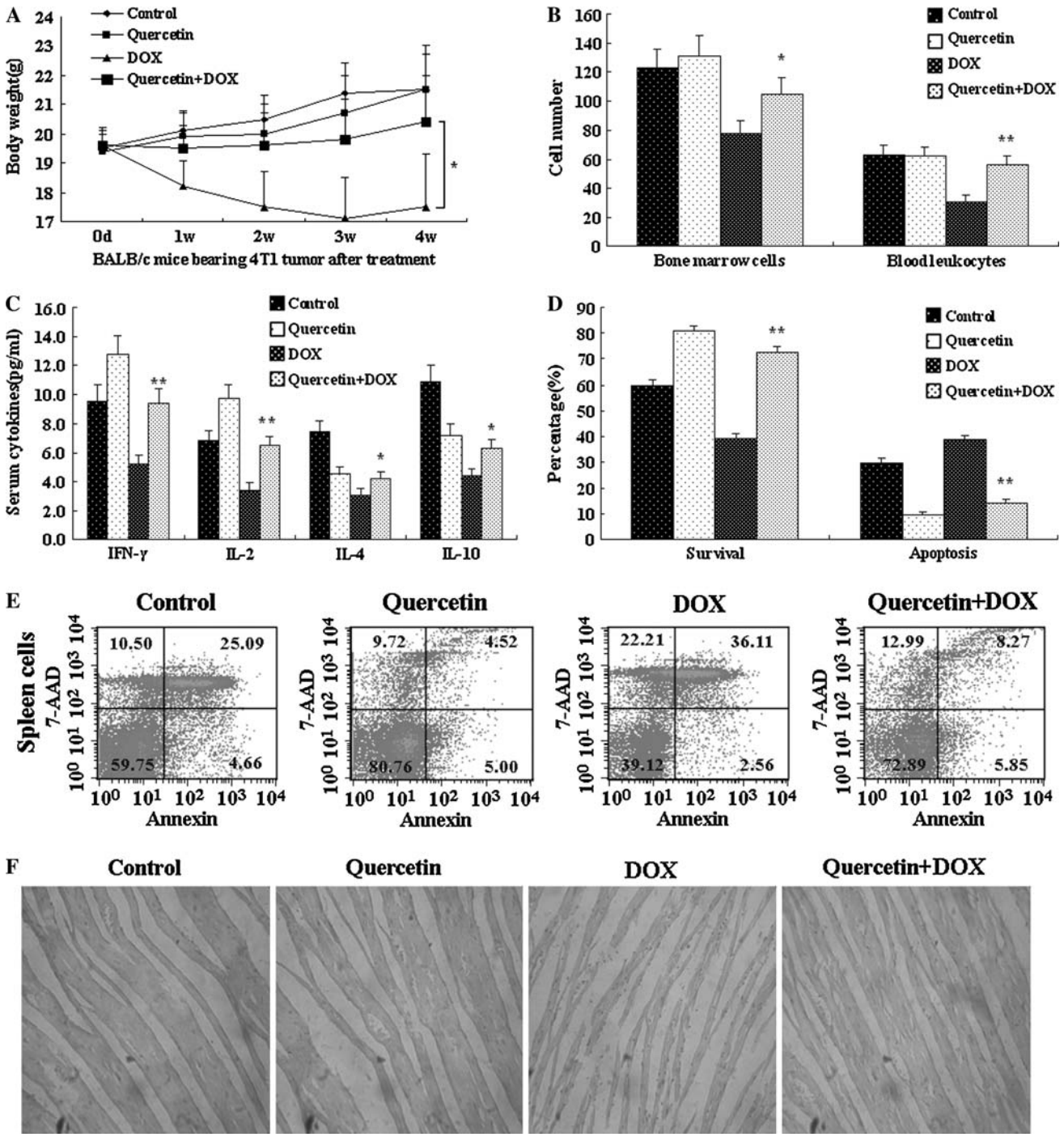

Fig. 4 Effects of quercetin on DOX-induced systemic toxicity in BALB/c mice. Quercetin prevented DOX-induced reduction in body weight ($n = 10$ per group) (**a**), the number of bone marrow cells and blood leukocytes ($n = 5$ per group) (**b**), serum cytokines including serum cytokines including the type 1 cytokines IFN-γ and IL-2, and the type 2 cytokines IL-4 and IL-10 ($n = 5$ per group) (**c**). Bone marrow cells ($\times 10^6$ ml^{-1}) and blood leukocytes ($\times 10^5$ ml^{-1}) were counted using a Zeiss microscope. Serum cytokines were examined by ELISA. Quercetin also prevented DOX-induced spleen cell apoptosis (**d**) and cardiac tissue toxicity examined by H&E staining (bright-field microscopy $\times 10$) (**e**). Compared with DOX-treated mice alone, $^*p < 0.05$ and $^{**}p < 0.01$

manner, this implied that quercetin had more complicated mechanisms.

Several previous reports have proposed that quercetin without metabolic activation displayed mutagenic activity in the Ames test and mammalian cells such as hamster embryo cells, L5178Y mouse cells and Chinese hamster lung cells [24]. Therefore, the issue of carcinogenicity of quercetin has received considerable attention. However,

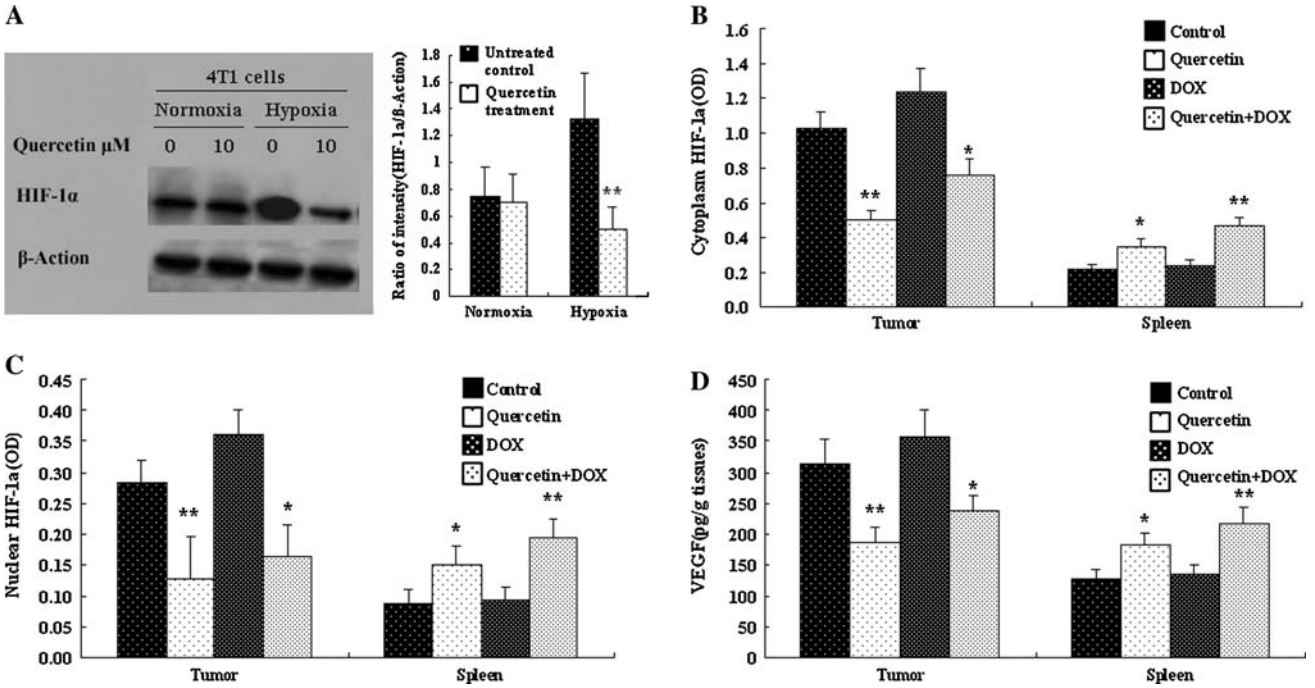

Fig. 5 Effects of quercetin on HIF-1α in vitro and in vivo. Quercetin induced HIF-1α degradation in 4T1 cells under hypoxia but not under normoxia and promoted HIF-1α accumulation in spleen cells under normoxia in vitro examined by Western blot ($n = 3$ per group) (**a**). Quercetin reduced cytoplasm and nuclear HIF-1α level in tumor but increased them in spleen in mice with 4T1 breast cancer examined by ELISA ($n = 5$ per group) (**b**, **c**). Quercetin had similar effects on tissue VEGF levels examined by ELISA ($n = 5$ per group) to HIF-1α (**d**). Compared with DOX-treated mice alone, $^{*}p < 0.05$ and $^{**}p < 0.01$

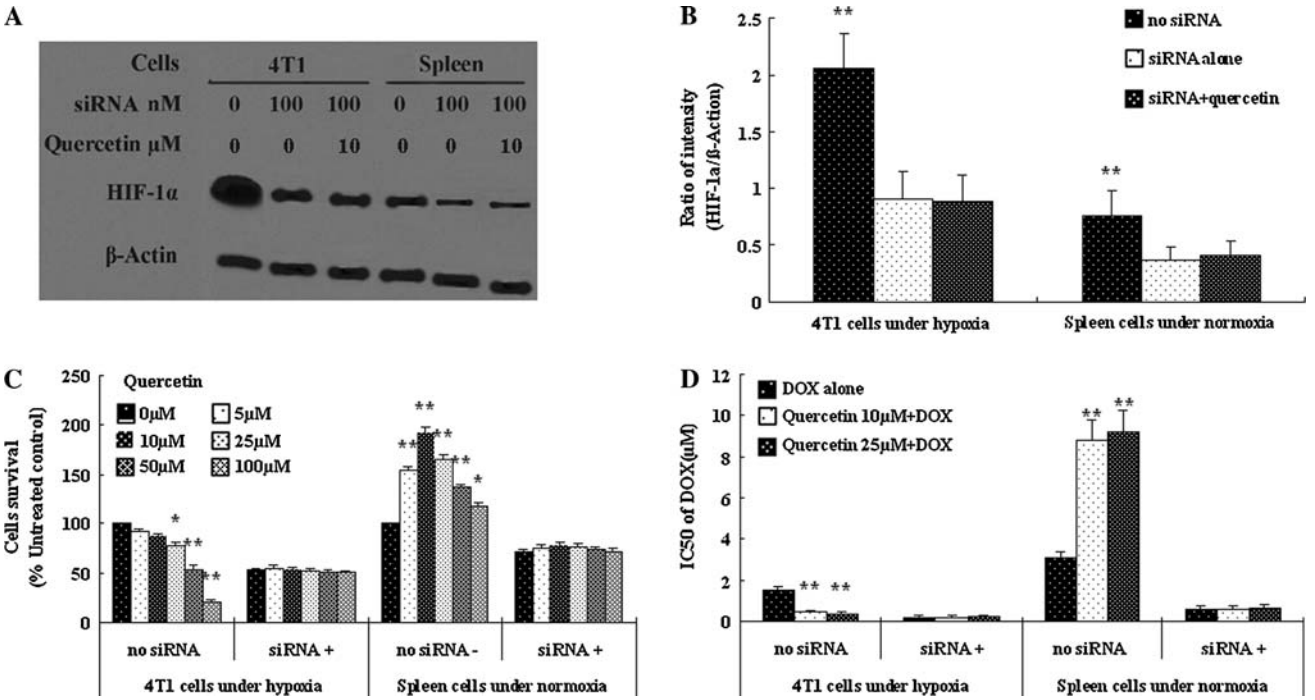

Fig. 6 Effects of HIF-1α siRNA on quercetin activities in vitro. HIF-1α siRNA downregulated HIF-1α expression (**a**, **b**) and abrogated efficacy of quercetin, including activities of quercetin alone (**c**) and effect on DOX-induced cytotoxicity (**d**) in 4T1 and spleen cells. Results represent mean values of three independent experiments. Compared with siRNA-treated cells alone or quercetin-untreated cells, $^{*}p < 0.05$ and $^{**}p < 0.01$

most results published to date have been negative in vivo in spite of some positive results in vitro [24]. Inversely, quercetin has been reported to protect mice against DMBA-, BP-, *N*-methyl-*N*-nitrosourea-, and BP-7,8-dihydrodiol-9 IQ-epoxide-induced skin tumorigenesis [24]. Mice fed during 28 days at doses of 3,000 mg/kg body weight/day of quercetin did not show any alterations in biochemical parameters, clinical signs or organ weight [25]. The recent data related to the genotoxicity of quercetin also supported the safety of quercetin in addition to food [26, 27].

In conclusion, our data suggest that the combination treatment of quercetin and DOX provides a novel therapeutic regimen to treat breast cancer. Previous finding that quercetin potentiated the effect of doxorubicin in a multidrug-resistant MCF-7 human breast cancer cell line extended the possible therapeutic application of quercetin as a promising anticancer agents either alone or in combination with other drugs in multidrug-resistant breast cancer [28]. Although the low oral bioavailability of quercetin in previous study [29], daily supplementation of healthy humans with graded concentrations of quercetin for 2-week dose dependently increased plasma quercetin concentrations but did not affect antioxidant status, oxidized LDL, inflammation, or metabolism [30]. A limitation of our study is that the antitumoral effect of quercetin was studied only in established tumor cell lines. It is important to note that extensive pharmacokinetic and pharmacodynamic testing and simulation are warranted to optimize the dose regimen before the application of this strategy in patients with clinical breast cancer.

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References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ (2008) Cancer statistics, 2008. CA Cancer J Clin 58:71–96
2. Gonzalez-Angulo AM, Morales-Vasquez F, Hortobagyi GN (2007) Overview of resistance to systemic therapy in patients with breast cancer. Adv Exp Med Biol 608:1–22
3. Morse R, Rodgers J, Verrill M, Kendell K (2003) Neuropsychological functioning following systemic treatment in women treated for breast cancer: a review. Eur J Cancer 39:2288–2297
4. Gianni L (2009) Anthracyclines and early breast cancer: the end of an era? J Clin Oncol 27:1155–1157
5. Gianni L, Salvatorelli E, Minotti G (2007) Anthracycline cardiotoxicity in breast cancer patients: synergism with trastuzumab and taxanes. Cardiovasc Toxicol 7(2):67–71
6. Benavente-García O, Castillo J, Alcaraz M, Vicente V, Del Río JA, Ortuño A (2007) Beneficial action of citrus flavonoids on multiple cancer-related biological pathways. Curr Cancer Drug Targets 7:795–809
7. Formica JV, Regelson W (1995) Review of the biology of quercetin and related bioflavonoids. Food Chem Toxicol 33:1061–1080
8. Hirpara KV, Aggarwal P, Mukherjee AJ, Joshi N, Burman AC (2009) Quercetin and its derivatives: synthesis, pharmacological uses with special emphasis on anti-tumor properties and prodrug with enhanced bio-availability. Anticancer Agents Med Chem 9:138–161
9. Mulholland PJ, Ferry DR, Anderson D, Hussain SA, Young AM, Cook JE, Hodgkin E, Seymour LW, Kerr DJ (2001) Pre-clinical and clinical study of QC12, a water-soluble, pro-drug of quercetin. Ann Oncol 12:245–248
10. Damianaki A, Bakogeorgou E, Kampa M, Notas G, Hatzoglou A, Panagiotou S, Gemetzi C, Kouroumalis E, Martin PM, Castanas E (2000) Potent inhibitory action of red wine polyphenols on human breast cancer cells. J Cell Biochem 78:429–441
11. Conklin CM, Bechberger JF, MacFabe D, Guthrie N, Kurowska EM, Naus CC (2007) Genistein and quercetin increase connexin43 and suppress growth of breast cancer cells. Carcinogenesis 28:93–100
12. Hakimuddin F, Paliyath G, Meckling K (2004) Selective cytotoxicity of a red grape wine flavonoid fraction against MCF-7 cells. Breast Cancer Res Treat 85:65–79
13. Verma N, Behera BC, Makhija U (2008) Antioxidant and hepatoprotective activity of a lichen *Usnea ghattensis* in vitro. Appl Biochem Biotechnol 151:167–181
14. Mojzisová G, Mirossay L, Kucerová D, Kyselovic J, Mirossay A, Mojzis J (2006) Protective effect of selected flavonoids on in vitro daunorubicin-induced cardiotoxicity. Phytother Res 20:110–114
15. Duthie SJ, Collins AR, Duthie GG, Dobson VL (1997) Quercetin and myricetin protect against hydrogen peroxide-induced DNA damage (strand breaks and oxidised pyrimidines) in human lymphocytes. Mutat Res 393:223–231
16. Granado-Serrano AB, Martín MA, Bravo L, Goya L, Ramos S (2006) Quercetin induces apoptosis via caspase activation, regulation of Bcl-2, and inhibition of PI-3-kinase/Akt and ERK pathways in a human hepatoma cell line (HepG2). J Nutr 136:2715–2721
17. Tang N, Du GJ, Wang N, Liu CC, Hang HY, Liang W (2007) Improving penetration in tumors with nanoassemblies of phospholipids and doxorubicin. J Natl Cancer Inst 99:1004–1015
18. Collins AR (2004) The comet assay for DNA damage and repair: principles, applications, and limitations. Mol Biotechnol 26:249–261
19. Choi HJ, Eun JS, Kim BG, Kim SY, Jeon H, Soh Y (2006) Vitexin, an HIF-1α inhibitor, has anti-metastatic potential in PC12 cells. Mol Cells 22:291–299
20. Early Breast Cancer Trialists' Collaborative Group (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 365:1687–1717
21. Lee DH, Lee YJ (2008) Quercetin suppresses hypoxia-induced accumulation of hypoxia-inducible factor-1alpha (HIF-1alpha) through inhibiting protein synthesis. J Cell Biochem 105:546–553
22. Jeon H, Kim H, Choi D, Kim D, Park SY, Kim YJ, Kim YM, Jung Y (2007) Quercetin activates an angiogenic pathway, hypoxia inducible factor (HIF)-1-vascular endothelial growth factor, by inhibiting HIF-prolyl hydroxylase: a structural analysis of quercetin for inhibiting HIF prolyl hydroxylase. Mol Pharmacol 71:1676–1684
23. Triantafyllou A, Liakos P, Tsakalof A, Chachami G, Paraskeva E, Molyvdas PA, Georgatsou E, Simos G, Bonanou S (2007) The flavonoid quercetin induces hypoxia-inducible factor-1alpha (HIF-1alpha) and inhibits cell proliferation by depleting intracellular iron. Free Radic Res 41:342–356
24. Middleton E Jr, Kandaswami C, Theoharides TC (2000) The effects of plant flavonoids on mammalian cells: implications for

inflammation, heart disease, and cancer. Pharmacol Rev 52:673–751
25. Ruiz MJ, Fernández M, Picó Y, Mañes J, Asensi M, Carda C, Asensio G, Estrela JM (2009) Dietary administration of high doses of pterostilbene and quercetin to mice is not toxic. J Agric Food Chem 57:3180–3186
26. Harwood M, Danielewska-Nikiel B, Borzelleca JF, Flamm GW, Williams GM, Lines TC (2007) A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties. Food Chem Toxicol 45:2179–2205
27. Utesch D, Feige K, Dasenbrock J, Broschard TH, Harwood M, Danielewska-Nikiel B, Lines TC (2008) Evaluation of the potential in vivo genotoxicity of quercetin. Mutat Res 654:38–44
28. Scambia G, Ranelletti FO, Panici PB, DeVincenzo R, Bonanno G, Ferrandina G, Piantelli M, Bussa S, Rumi C, Cianfriglia M, Mancuso S (1994) Quercetin potentiates the effect of adriamycin in a multidrug-resistant MCF-7 human breast cancer cell line: P-glycoprotein as a possible target. Cancer Chemother Pharmacol 34:459–464
29. Boots AW, Li H, Schins RP, Duffin R, Heemskerk JW, Bast A, Haenen GR (2007) The quercetin paradox. Toxicol Appl Pharmacol 222:89–96
30. Egert S, Wolffram S, Bosy-Westphal A, Boesch-Saadatmandi C, Wagner AE, Frank J, Rimbach G, Mueller MJ (2008) Daily quercetin supplementation dose-dependently increases plasma quercetin concentrations in healthy humans. J Nutr 138:1615–1621