Pharmaceutical Research, Vol. 26, No. 4, April 2009 (© 2008)
DOI: 10.1007/s11095-008-9793-y

Research Paper

Naringenin Enhances the Anti-Tumor Effect of Doxorubicin Through Selectively Inhibiting the Activity of Multidrug Resistance-Associated Proteins but not P-glycoprotein

Fa Yun Zhang,[1] Gang Jun Du,[1,2] Ling Zhang,[1] Chun Ling Zhang,[1] Wan Liang Lu,[3] and Wei Liang[1,4,5]

Received July 12, 2008; accepted November 7, 2008; published online December 10, 2008

Purpose. Naringenin has shown paradoxical results to modulate the function of multidrug resistance-associated proteins (MRPs). The aim of this study is to interpret whether naringenin can reverse intrinsic and/or acquired resistance of cancer cells to chemotherapeutic agents.
Methods. The effects of naringenin on the uptake, retention and cytotoxicity of doxorubicin were investigated in A549, MCF-7, HepG2 and MCF-7/DOX cells. Cellular efflux pathways modulated by naringenin were assessed with their specific substrates and inhibitors. The improved antitumor activity of doxorubicin in combination with naringenin was also investigated *in vivo*.
Results. The IC_{50} values of doxorubicin in combination with naringenin in A549 and MCF-7 cells were approximately 2-fold lower than that of doxorubicin alone. The increased sensitivity to doxorubicin by naringenin in HepG2 and MCF-7/DOX cells was not observed. Naringenin increased the cellular doxorubicin accumulation through inhibiting doxorubicin efflux in the cells expressing MRPs but not P-gp. In contrast to doxorubicin alone, doxorubicin in combination with naringenin enhanced antitumor activity *in vivo* with low systemic toxicity.
Conclusion. Naringenin enhances antitumor effect of doxorubicin by selective modulating drug efflux pathways. Naringenin will be a useful adjunct to improve the effectiveness of chemotherapeutic agents in treatment of human cancers.

KEY WORDS: doxorubicin; drug efflux pumps; multidrug resistance; naringenin.

INTRODUCTION

Chemotherapeutic strategies for cancer commonly require the combination of agents. However, intrinsic and acquired resistance to chemotherapeutic agents and toxicity to normal cells are the major causes of treatment failure in most solid tumors (1). Tumor cells overexpressing multidrug export pumps, which are members of the family of ATP-binding cassette (ABC) transport proteins, such as P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRP1 and MRP2) and the breast cancer resistance protein (BCRP), may contribute to their resistance to chemotherapeutic agents (2–6). Many efforts have been made to search for modulators to reverse P-gp and MRPs related multidrug resistance (7–10). However, tumor cells often response poorly to anticancer drugs at the beginning of treatment, thus exhibiting the intrinsic resistant phenotype (11–14). Non-small cell lung cancer (NSCLC) overexpressing the MRP1 and MRP3 proteins tends to be intrinsically resistant to chemotherapy (11). The expression of MDR proteins in malignant glioma patients has been investigated. The data suggest that glioma chemoresistance is mostly intrinsic, while no information is strictly affordable about acquired resistance after drug exposure (12,13). MRP2 and MRP3 but not MRP1 were found to contribute to the intrinsic multidrug resistance in the frequent primary cancer of the liver (14). Therefore, it is necessary to investigate the mechanism of the intrinsic drug resistance and to develop the agents for reversing intrinsic drug resistance for cancer therapy.

Doxorubicin is one of the most frequently used anticancer agents in the treatment of human malignancies. However, the therapeutic use of doxorubicin is limited by its severe cumulative dose-related cardiotoxicity and resistance (15,16).

[1] Protein and Peptide Pharmaceutical Laboratory, National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China.
[2] Department of Pharmacology, Pharmaceutical College of Henan University, Kaifeng 475001, China.
[3] State Kay Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing 100083, China.
[4] National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Beijing 100101, China.
[5] To whom correspondence should be addressed. (e-mail: weixx@sun5.ibp.ac.cn)

ABBREVIATIONS: DMSO, Dimethyl sulfoxide; DOX, Doxorubicin; FITC, Fluorescein isothiocyanate; MRPs, Multidrug resistance-associated proteins; MTT, Methylthiazoletetrazolium; Nar, Naringenin; PBS, Phosphate-buffered saline; P-gp, P-glycoprotein; PI, Propidium iodide; Rho123, Rhodamine 123; Vrp, Verapamil; 5-CFDA, 5-Carboxyflurescein diacetate.

To overcome such problems, strategies must be devised for its better cancer cell killing and reduction of systemic toxicity.

Flavonoids have recently been found to have the inhibitory function of ABC transporters, including P-gp (17,18), MRPs and BCRP (19,20). Collected data demonstrate that the combinations of doxorubicin with flavonoids can enhance the sensitivity of tumor cells to doxorubicin (21) and reduce its cardiac toxicity (22,23). Naringenin (4′,5,7-trihydroxyflavanone),as one of the natural predominant flavonoids in citrus fruits, has been shown to inhibit the efflux of mitoxantrone, which is a substrate of BCRP (24). Other studies have suggested that naringenin has no inhibitory effect on P-gp-mediated efflux (25) and only low MRP1 and MRP2 inhibitory activity (20) due to its lack of 2,3-double bond in the C ring. Therefore, the mechanism of naringenin enhancing chemosensitivity remains unclear. Since distribution of transporters varies widely among cancer types, there is an effort to try to understand whether naringenin can reverse intrinsic and/or acquired resistance to chemotherapeutic drugs. Here we demonstrate that naringenin increases doxorubicin uptake and retention in the tumor cell lines expressing MRPs but not P-gp. These data indicate that naringenin may be useful to prevent or to reduce the development of the chemoresistance phenotype in non-expressing P-gp cancer cells by inhibiting MRPs activity.

MATERIALS AND METHODS

Chemical and Reagents

Naringenin (Nar) was purchased from Shanxi Huike Botanical Development Co. (China). 5-carboxyflurescein diacetate (5-CFDA), Rhodamine 123 (Rho123) and verapamil (Vrp) were obtained from Sigma-Aldrich Co. MK571 was from Calbiochem (USA). Doxorubicin (DOX) was provided by HaiZheng Co. (China). Fluorescein isothiocyanate (FITC)-conjugated human anti-P-gp (17F9) was obtained from BD Biosciences. Naringenin and 5-CFDA were stored as a stock solution in dimethyl sulfoxide (DMSO) and used after diluting with medium for each assay.

Cell Lines

A549 (human non-small cell lung carcinoma, NSCLC) and HepG2 (human hepatocellular carcinoma, HCC) cells were purchased from American Type Culture Collection (ATCC; Manassas, VA). MCF-7 (human breast carcinoma) and MCF-7/DOX cells were provided by the Academy of Military Medical Sciences. MCF-7 cells, expressing *MRP1*, *MRP2* but not *MDR1*, were treated with increasing doses of doxorubicin, and they became chemoresistant in association with *MDR1* expression induction. Compared to MCF-7 cells, multidrug resistance-1 gene (*MDR1*)/P-gp was overexpressed in MCF-7/DOX cells. The accumulation of doxorubicin in MCF-7/DOX cells was only 16.7% of that in wild type MCF-7 cells. Cell culture medium and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). Culture flasks and dishes were from Corning (Corning, NY). Cells were cultured in RPMI 1640 or DMEM medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 U/ml).

Cell Growth Inhibition Assay

A549, HepG2, MCF-7 and MCF-7/DOX cells were plated at a density of 5×10^3 cells per well in 100 μl RPMI 1640 or DMEM medium in 96-well plates and grown for 24 h, respectively. Cells were exposed to a series of concentrations of doxorubicin combined with naringenin or with verapamil for 48 h, and the viability of cells was measured using the methylthiazoletetrazolium (MTT) method, as previously described (26). Briefly, 100 μl methylthiazoletetrazolium solution (0.5 mg/ml in phosphate-buffered saline [PBS]) was added to each well. The plates were incubated for 4 h at 37°C. After incubation, 100 μl of DMSO (Sigma) was added to each well for 10 min at room temperature. Absorbance was measured at 570 nm using a plate reader (Thermo, Erlangen, Germany). The mean percentage of cell survival relative to that of untreated cells was estimated from data of three individual experiments. The concentration of DOX at which cell killing was 50% was calculated by curve fitting using SPSS software (version 12.0, SPSS, Chicago, IL).

Apoptosis Evaluation

Annexin V-PI staining and DNA ladder analysis were used to examine the apoptosis of A549 cells. 2×10^5 cells were plated in 6-well plates and exposed to 100 μM naringenin, 0.52 μM DOX or DOX combined with naringenin for 48 h. Propidium iodide (PI) and FITC-conjugated Annexin V double-stained kit (Jingmei Biotech, China) were used to stain the cells according to the manufacture's instructions and analyzed by flow cytometry. We defined the cells with Annexin V-FITC positive and PI negative as the early apoptotic cells and the cells with both Annexin V-FITC and PI positive as the apoptotic cells. Total apoptotic cells were the sum of the early apoptotic cells and the apoptotic cells. The cells treated as described above, were also collected to analyze DNA fragments, and the DNA ladder was visualized as described previously (27).

Quantification of Intracellular Doxorubicin

To measure the intracellular amount of doxorubicin quantitatively, A549, HepG2, MCF-7 and MCF-7/DOX cells were cultured on 6-well plates for 24 h to achieve approximately 80% confluence. DOX alone, DOX combined with naringenin or verapamil or MK571 (DOX concentration: 6.9 μM; Naringenin concentration: 100 μM; verapamil concentration: 10 μM; MK571 concentration: 50 μM) were then added to designated wells. After incubation for 2 h, the culture medium containing DOX was discarded and the cells were washed with PBS. Fresh medium with 0.1% DMSO (as the control), naringenin or verapamil was then added to the wells. After incubation for another 2 h, the cells were collected for measurement of doxorubicin fluorescence.

To further clarify whether naringenin can affect the uptake and efflux of doxorubicin, A549 cells were cultured on 6-well plates for 24 h to achieve approximately 80% confluence. For detection of DOX uptake, DOX (1.7 μM) or DOX combined with 100 μM naringenin were added to cells. After incubation for different times (0, 0.5, 1, 2, 4, 8 and 12 h), the culture medium was discarded, and then cells were

collected for detection of DOX fluorescence. For detection of DOX efflux, DOX (6.9 μM) was added to designated wells. After incubation for 2 h, the culture medium containing DOX was discarded and the cells were washed with PBS. Fresh medium with 100 μM naringenin was then added to the wells. After incubation for different times (0, 1, 2, 4, 8 h), the cells were collected for measurement of DOX fluorescence using flow cytometry (FACSCalibur, BD, San Jose, CA), excitation was with the 488 nm line of an argon laser, and emission fluorescence between 564 and 606 nm was measured. For all experiments in which the intracellular DOX was quantified using flow cytometry, at least 10,000 cells were measured from each sample.

Reverse Transcription-PCR

To compare the mRNA expression of the multidrug resistant genes in different types of cancer cells, total RNAs were isolated from A549, HepG2, MCF-7 and MCF-7/DOX cells using TRIzol (Invitrogen, USA). To address the effect of naringenin or verapamil on the mRNA expression of the multi-drug resistant genes, RNAs were also isolated from the four types of cells treated with naringenin (100 and 200 μM) or verapamil (10 and 20 μM), respectively. The mRNAs were reversely transcribed to cDNAs by M-MLV reverse transcriptase (Invitrogen). Primers for human *MDR1*: 5′-AAAGCTGTC AAGGAAGCCAA-3′ and 5′-TGACTC CATCATCGAAACCA-3′, *MRP1*: 5′-ATGTCACGTG GAATACCAGC-3′ and 5′-GAAGACTGAACTCCC TTCCT-3′, *MRP2*: 5′-ACAGAGGCTGGTGGCAACC-3′ and 5′-ACCATTACCTTGTCACTGTCCATGA-3′, *MRP3*: 5′-GGACCCTGCGCATGAACCTG-3′ and 5′-AGGCA AGTCCAGCATCTCTGG-3′, *MRP4*: 5′-GGATCCAAGAA CTGATGAGTTAAT-3′ and 5′-TCACAGTGCTGTCTCG AAAATAG-3′, *MRP5*: 5′-GCTGTTCAGTGGCACTGT CAG-3′ and 5′-TCAGCCCTTGACAGCGACCTT-3′, *MRP6*: 5′-CACTGCGCTCCAGGATCAGC-3′ and 5′-CAGACCAGGCCTGACTCCTG-3′ were used. The primers for β-Actin were sense primer 5′-CATGTACGTTGC TATCCAGGC-3′ and antisense primer 5′-CTCCTTAATG TCACGCACGAT-3′. After initial denaturation at 95°C for 5 min, PCR was performed for 30 cycles (30 s at 94°C, 30 s at annealing temperature and 40 s at 72°C) using Taq polymerase (TaKaRa, Japan). Reaction products (20 μl per lane) were electrophoresed in 1% agarose, stained with ethidium bromide and photographed.

P-gp Immunoassay

P-gp expression in the four types of cancer cells was examined by flow cytometry. Briefly, 1×10^6 A549, HepG2, MCF-7 and MCF-7/DOX cells were treated with trypsin, respectively, and then washed and resuspended in 100 μl staining buffer (2% fetal calf serum and 0.1% NaN3 in PBS, pH7.4). 20 μl FITC conjugated mouse anti-human monoclonal antibody 17F9, which recognizes an external epitope of P-gp, was added and incubated for 30 min in the dark at 4°C. Cells were washed and immediately analyzed using flow cytometry with an excitation of 488 nm and emission collected through a 530/515 band pass filter for FITC (FL1-H). Analysis was performed using the Cellquest (Becton Dickinson Immunocytometry Systems) computer programs.

Activities of P-gp and MRPs

To detect P-gp and MRPs like transport functions, 5-CFDA, a fluorescent substrate for MRPs (28), and Rho123, a fluorescent substrate for P-gp (29), were used to treat the cells with or without 100 μM naringenin or 10 μM verapamil or 50 μM MK571. The four types of cells were cultured in 6-well plates for 24 h to achieve approximately 80% confluence, and then 5-CFDA or Rho123 were added to designated wells at final concentration of 4 or 0.6 μM, respectively. After incubation for 30 min, the cells were washed with PBS. Fresh medium containing 0.1% DMSO as the control, naringenin or verapamil or MK571 containing medium was then added to the wells at final concentration of 100 or 10 or 50 μM, respectively. After 1 h incubation, the cells were collected, and then the intracellular fluorescence intensity derived from 5-CFDA or Rho123 was measured by flow cytometry.

Transfection of siRNAs

Double-stranded siRNAs specific to MRP1 and MDR1 were designed and synthesized by GenePharma Company (Shanghai, China) according to literatures (30,31), mock nontargeting siRNA (sense strand 5′-GAUACGAAUUGA-CACCGUA) was as negative control. Transfection with Lipofectamine2000 (Invitrogen) was carried out as directed by the manufacturer guideline. MCF-7 and MCF-7/DOX cells (3×10^5/well) were plated in 6-well plates. They were transfected at 30–40% confluence. The final concentration of siRNA was 100 nM. The MRP1-targeted siRNA were transfected into MCF-7 cells to modulate MRP1 expression and MDR1-targeted siRNA was transfected into MCF-7/DOX cells to modulate P-gp expression. Transfection was performed in DMEM without serum or antibiotics in a final volume of 2 ml. After 5 h incubation at 37°C, removed the old medium, and the completed DMEM with FBS and antibiotics were added. The cells were incubated for 48 h to detect their mRNA expression and for 72 h to measure the drug efflux.

In vivo Tumor Study

C57Bl/6 female mice (6–8 weeks old) were from the Institute of Materia Medica, Chinese Academy of Medical Sciences. All animal procedures were performed following the protocol approved by the Institutional Animal Care and Use Committee at the Institute of Biophysics, Chinese Academy of Sciences. The mice were injected subcutaneously in the right flank with 0.2 ml of cell suspension containing 3×10^5 Lewis lung carcinoma (LLC) cells. Mice (ten in each group) received 5 mg/kg of doxorubicin for three times at day 7, day 14, and day 21, or received a combination of 5 mg/kg of doxorubicin for three times with orally administrated naringenin at a dose of 50 mg/kg daily for 3 weeks following injection of doxorubicin, or received only orally administrated naringenin at a dose of 50 mg/kg daily for 3 weeks, when tumor volume reaches about 40 mm^3 7 days after tumor cells inoculation. The control mice were injected intravenously with 10 ml/kg of saline buffer. The body weight, tumor length and

width were measured trice weekly in a blinded manner, and tumor volume was calculated as the length×width^2×0.5.

Statistics

Data and results are reported as means ±SE unless noted otherwise. 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 11.0 program. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Naringenin Enhances Selectively Doxorubicin-Induced Cancer Cell Growth Inhibition

To test whether naringenin enhances selectively cytotoxicity of doxorubicin in different types of cancer cells, four types of cancer cells from lung, liver, or breast both wild type and acquired resistance to doxorubicin were treated with doxorubicin alone or doxorubicin in combination with naringenin (100 μM) or verapamil (10 μM). Verapamil, a well known substrate of P-gp, was used here as a positive control for inhibiting activity of P-gp. The cell viability was determined by MTT assay, and the effects of doxorubicin and doxorubicin combined with naringenin or verapamil on the growth of different types of cancer cells were shown in Fig. 1A-D. The proliferation of all four types of cells was not affected by treatment with 100 μM naringenin for 48 h. However, this concentration of naringenin offered the improved sensitivity of A549 and MCF-7 cells to doxorubicin. For A549 cells treated with doxorubicin alone or in combination of naringenin, the IC_{50} values were 0.65 and 0.29 μM, respectively. For MCF-7 cells, a similar result to that of A549 cells was observed; the IC_{50} values of doxorubicin and its combination with naringenin were 1.06 and 0.53 μM, respectively. The improved sensitivity of HepG2 and MCF-7/DOX cells to doxorubicin by naringenin was not observed (Fig. 1B and D). However, compared with doxorubicin alone (IC_{50} values for HepG2 and MCF-7/DOX cells were 0.80 and 2.03 μM, respectively), the growth of cells treated with

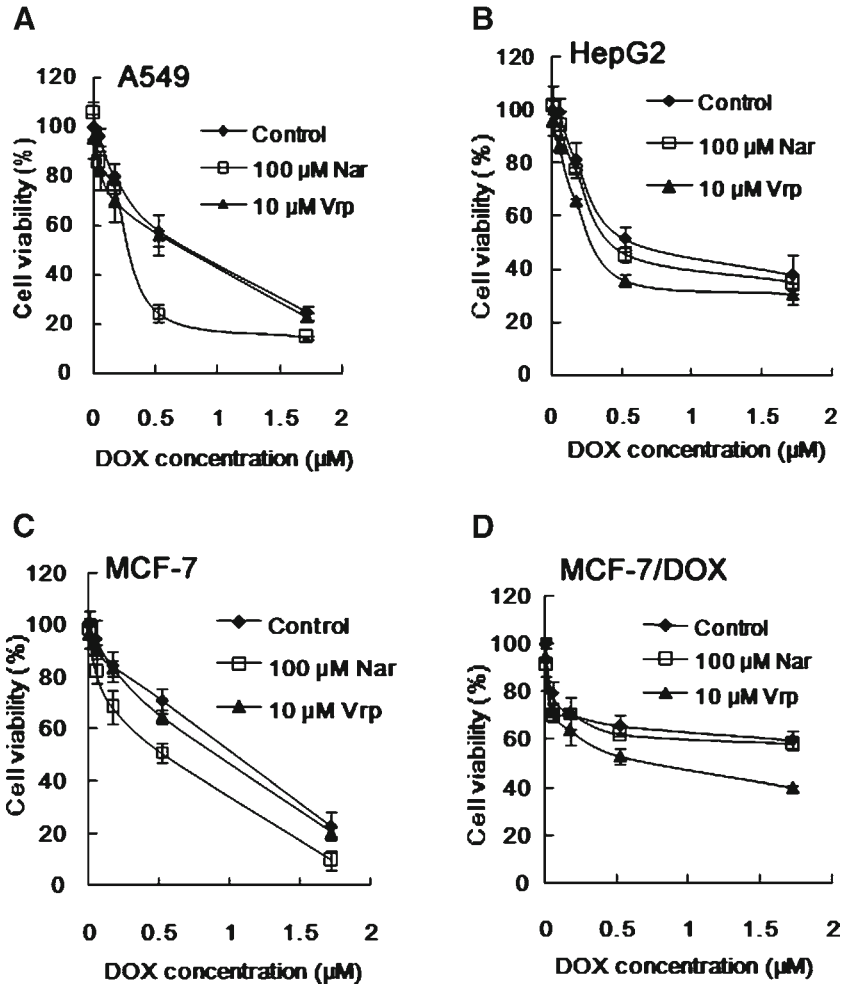

Fig. 1. Naringenin enhances the cytotoxicity induced by doxorubicin *in vitro*. The effect of naringenin (100 μM Nar) on the cytotoxicity of doxorubicin in A549 (**A**), HepG2 (**B**), MCF-7 (**C**) and MCF-7/DOX (**D**) cells, verapamil (10 μM Vrp) as positive control. The percentage of viable cells was quantified using the methylthiazoletetrazolium method. Each concentration was replicated three times. Points, mean $n=3$; bars, SE.

doxorubicin combined with verapamil was significantly inhibited (IC_{50} values for HepG2 and MCF-7/DOX cells were 0.41 and 0.67 μM, respectively). A549 and MCF-7 cells were also treated with doxorubicin in combination with verapamil, each IC_{50} value was to be the same as the cells treated with doxorubicin alone (Fig. 1A and C). These results indicate that naringenin has a selectivity to enhance cancer cell growth inhibition induced by doxorubicin, which is cancer cell type-dependent. Naringenin offers the improved sensitivity of cancer cells to doxorubicin that is very different from verapamil.

To examine whether naringenin enhanced cytotoxicity of doxorubicin to cancer cells was mediated through increased apoptosis, annexin V/PI staining and DNA ladder analysis were used to detect the apoptosis in A549 cells. From the statistical analysis of cytometry data, we found that doxorubicin combined with 100 μM naringenin induced more apoptotic cells (32.74±1.56%, $p<0.01$) than doxorubicin alone (19.65±1.25%, Fig. 2A and B). In addition, a similar result was observed by DNA ladder analysis (Fig. 2C).

Naringenin Inhibits Efflux of Doxorubicin and Enhances Uptake of Doxorubicin

To address whether the improved sensitivity of cancer cells to doxorubicin by naringenin might be associated with the accumulation of drug in the cells, the cellular accumulation of doxorubicin was evaluated in the presence or absence of naringenin by flow cytometric analysis. Intracellular accumulation of doxorubicin was increased in the presence of naringenin in A549 and MCF-7 cells but not in HepG2 and MCF-7/DOX cells, compared with that in the absence of naringenin. In A549 and MCF-7 cells, the presence of naringenin increased doxorubicin accumulation by 69% and 65%. In contrast, cells treated with doxorubicin in combination with verapamil showed that the intracellular accumulation of doxorubicin was increased in HepG2 and MCF-7/DOX cells but not in A549 and MCF-7 cells compared with doxorubicin alone (Fig. 3A and B). The results indicate that the improved sensitivity of cancer cells to doxorubicin can be achieved by increasing its cellular level in the different cancer cell types.

To further address whether the increased cellular accumulation of doxorubicin by naringenin might be mediated by inhibiting drug efflux, the cellular accumulation of doxorubicin was measured by flow cytometric analysis in two separated experiments. One is that A549 cells continuously treated with doxorubicin in the presence or absence of naringenin for different time intervals. Second is that A549 cells exposed to doxorubicin for 2 h prior to treatment with naringenin for different time intervals, seeing methods for detail. As shown in Fig. 3C, compared to the treatment with doxorubicin alone**,** the uptake of doxorubicin was increased in a time-dependent manner when cells treated with doxorubicin in combination with naringenin. Fig. 3D showed that the efflux of doxorubicin from the cells was less in the presence of naringenin than in the absence of naringenin. Taken together, these results suggest that naringenin increases the cellular accumulation of doxorubicin through inhibiting doxorubicin efflux.

Naringenin Has no Effect on Expression of mdr1 and mrps

In the present study, we found that naringenin had a selectivity to enhance cancer cell growth inhibition induced by doxorubicin in a pattern of cancer cell type-dependent. Naringenin has shown to inhibit the efflux of mitoxantrone (24) and to have no effect on P-gp mediated efflux (25). We

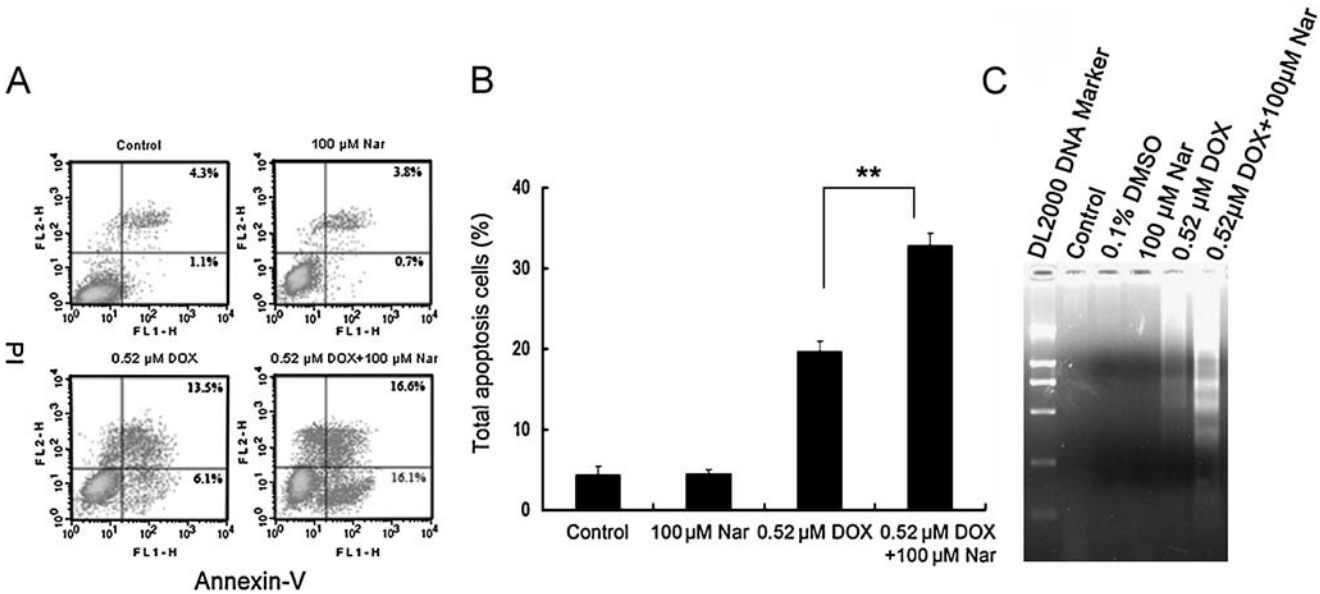

Fig. 2. Naringenin enhances the apoptosis induced by doxorubicin in A549 cells. **A** doxorubicin-induced apoptosis in the presence or absence of naringenin was determined by flow cytometry in A549 cells after 48 h treatment. **B** apoptosis estimated by flow cytometry assay in A549 cells after 48 h treatment with naringenin (100 μM Nar), doxorubicin (0.52 μM DOX), or their combination (0.52 μM DOX+100 μM Nar) ($n=4$). Columns, mean; *Bars*, SE. Combination induces a significant increase of apoptosis compared with other treatments (**$P<0.01$). **C** DNA ladder analysis of DNA fragments from A549 cells after 48 h treatment with doxorubicin, naringenin or their combination.

first examined the mRNA expression of *MDR1*, *MRP1*, *MRP2*,*MRP3*,*MRP4*, *MRP5* and *MRP6* using RT-PCR. The amount of mRNA for each targeted gene was normalized to the mRNA level of β-actin gene. The mRNA levels of *MRP1*, *MRP2* were higher than other *MRPs* and had no difference in the four types of cancer cells. However, a high mRNA expression of *MDR1*/P-gp was detected in HepG2 and MCF-7/DOX cells, the mRNA expression of *MDR1*/P-gp in A549 and MCF-7 cells was not detected (Fig. 4A). To further confirm the expression of P-gp, the cells were stained with anti-P-gp antibody. The expression level of P-gp was very low in A549 and MCF-7 cells, which even could not been stained by FITC-P-gp antibody. However, the FITC fluorescence intensity of P-gp in HepG2 and MCF-7/DOX cells was 3-fold and 8-fold higher, respectively, than their basic fluorescence intensity of cells (Fig. 4B and C).

Next, we examined whether naringenin affected the expression of *MDR1*, *MRP1* and *MRP2*. A549, MCF-7, HepG2 and MCF-7/DOX cells were treated with naringenin (100 and 200 μM), verapamil (10 and 20 μM) as a control. The mRNA expressions of *MDR1*, *MRP1* and *MRP2* were analyzed by RT-PCR. As shown in Fig. 4D, the effect of naringenin on the mRNA expression of *MDR1*, *MRP1* and *MRP2* in all four cell lines was not observed, and similar

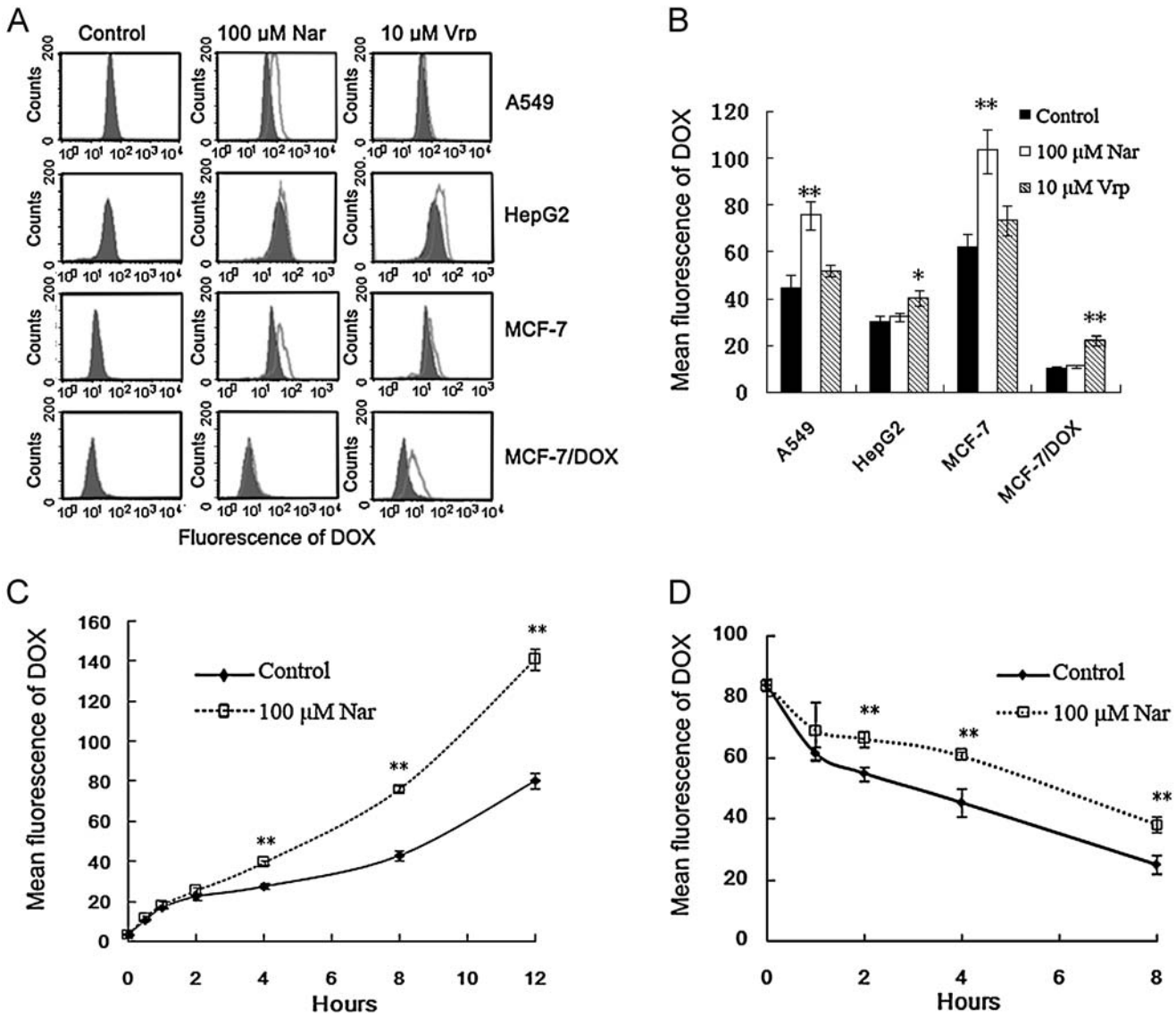

Fig. 3. Naringenin increases the intracellular doxorubicin fluorescence in A549 and MCF-7 cells but not in HepG2 and MCF-7/DOX cells. **A** flow cytometry analysis of the intracellular doxorubicin fluorescence in A549, MCF-7, HepG2 and MCF-7/DOX cells after 2 h treatment of doxorubicin in combination with naringenin or verapamil. **B** intracellular doxorubicin fluorescence estimated by flow cytometry assay in A549, MCF-7, HepG2 and MCF-7/DOX cells after 2 h treatment of doxorubicin in combination with naringenin or verapamil (n=4). Columns, mean; *Bars*, SE. Values significantly different from control cells were marked (*P<0.05; **P<0.01). **C** intracellular doxorubicin fluorescence estimated by flow cytometry assay in A549 cells after different hours (0, 0.5, 1, 2, 4, 8, 12 h) treatment of 1.7 μM doxorubicin in combination with 100 μM naringenin (n=4). Points, mean; *Bars*, SE. Combination induces a significant increase in intracellular fluorescence compared with doxorubicin alone (**P<0.01). **D** intracellular doxorubicin fluorescence estimated by flow cytometry assay in A549 cells. Cells were treated with 6.9 μM doxorubicin for 2 h and then removed drug containing medium, the cells were incubated in the naringenin-containing fresh medium for different times (n=4). Points, mean; *Bars*, SE. Naringenin increases significantly the retention of intracellular fluorescence (**P<0.01).

result was found for verapamil treatment. Previous studies have shown that verapamil can prevent the drug pump of P-gp by inhibiting its activity (32,33). Our results suggest that naringenin enhances the cellular accumulation of doxorubicin through modulating the function of MRPs but not their expression levels.

Naringenin Modulates the Activity of MRPs but not P-gp

We determined the effect of naringenin on cellular uptake of two fluorescent substrates: Rho123 for P-gp and 5-CFDA for MRPs. Compared to the untreated and verapamil treated cells, naringenin significantly enhanced the uptake of 5-CFDA in all four cell lines (Fig. 5A and C). Naringenin had no effect on Rho123 uptake in all four cell lines, but verapamil significantly increased the uptake of Rho123 in HepG2 and MCF-7/DOX cells, which expressed the high level of P-gp. Interestingly, verapamil stimulated a nonsignificant decrease in the uptake of Rho123 in A549 and MCF-7 cells (Fig. 5B and D), which expressed very low level of P-gp (Fig. 4A–C). We used MK571, an inhibitor of MRP1, to make another comparison with naringenin (34). MK571 showed a similar effect on modulation of drug efflux to naringenin. MK571 increased the intracellular accumulation of doxorubicin only in A549 and MCF-7 cells and the intracellular retention of 5-CFDA in all four cell lines, while, MK571 did not show any effect on the intracellular retention of Rho123, indicating that naringenin may play the same role like MK571 (Fig. 6). These results indicate that naringenin selectively interacts with the MRP transporters rather than P-gp.

Naringenin Increases Antitumor Effect of Doxorubicin *in vivo*

To test the efficiency of doxorubicin combined with naringenin on tumor growth *in vivo*, LLC subcutaneous model

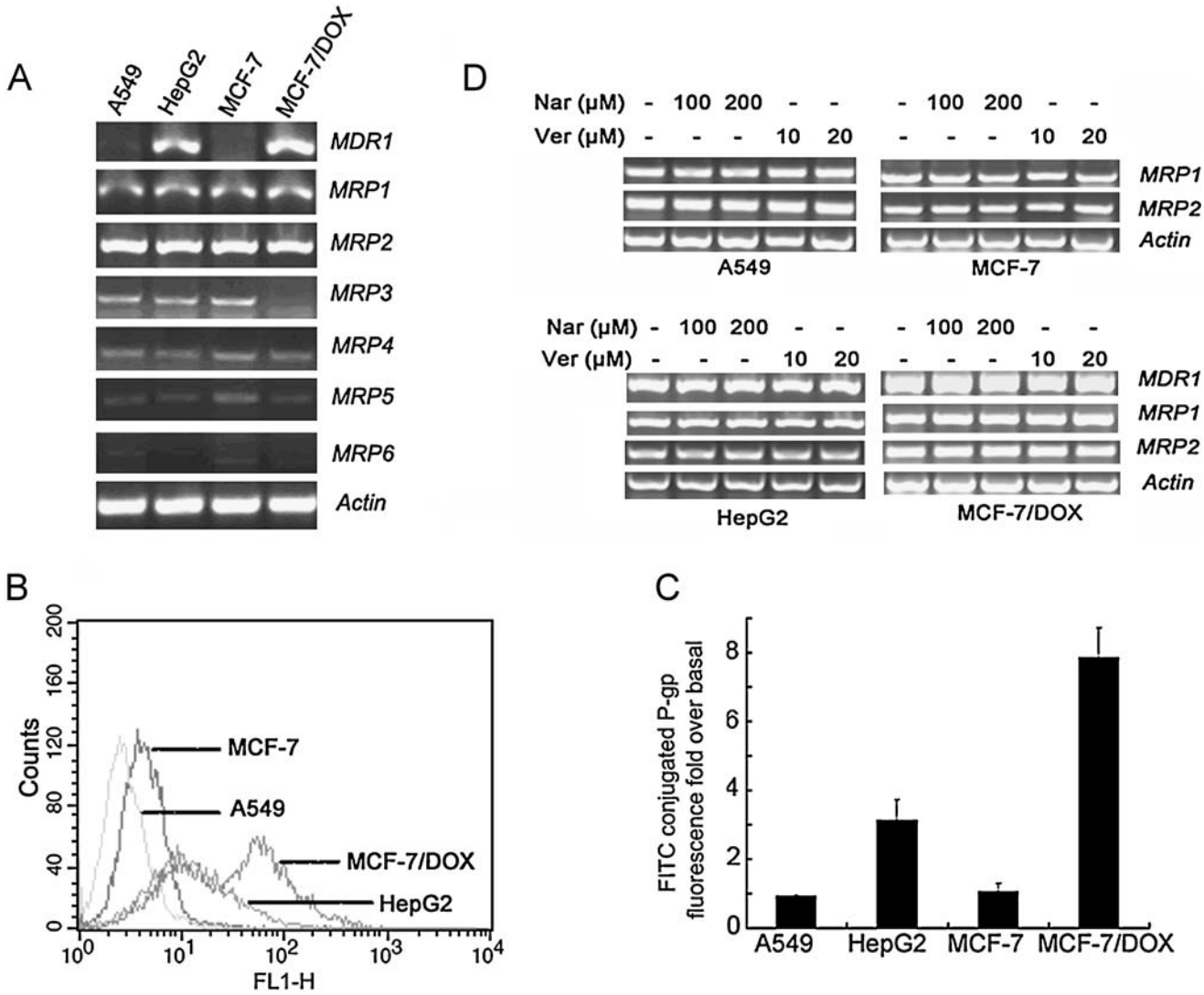

Fig. 4. Naringenin does not affect the expression of *MDR1*, *MRP1* and *MRP2*. **A** reverse transcription-PCR analyses of mRNA levels of *MDR1*, *MRP1*, *MRP2*, *MRP3*, *MRP4*, *MRP5* and *MRP6* in A549, MCF-7, HepG2 and MCF-7/DOX cells. **B** flow cytometry detection of P-gp expression in A549, MCF-7, HepG2 and MCF-7/DOX cells. **C** P-gp expression estimated by flow cytometry assay in A549, MCF-7, HepG2 and MCF-7/DOX cells ($n=3$). *Columns*, mean; *Bars*, SE. **D** reverse transcription-PCR analyses of mRNA levels of *MDR1*, *MRP1* and *MRP2* in A549, MCF-7, HepG2 and MCF-7/DOX cells after 12 h treatment with naringenin or verapamil.

was used. As shown in Fig. 7 A, tumor growth in mice treated with doxorubicin in combination with naringenin was dramatically decreased after oral administration of naringenin for 3 weeks, compared to mice treated doxorubicin alone. Treatment with naringenin alone had no difference in delaying tumor growth compared with untreated control mice. Moreover, as Fig. 7B shown, the mice that were given doxorubicin alone showed a decrease in body weight. In contrast, mice treated with the same dose of doxorubicin combined with naringenin began to gain weight at the start of treatment and attained body weight equal to that of control on day 35 ($P > 0.05$). The results demonstrate that naringenin might synergistically enhance the antitumor effect of doxorubicin.

DISCUSSION

To our knowledge, this is the first time to demonstrate that naringenin enhances the sensitivity of cancer cells to doxorubicin *via* selectively inhibiting the activity of MRPs and in turn increasing the accumulation of drug in the tumor cells, which express MRPs but not P-gp. Naringenin has a little or no effect on the tumor cells with intrinsic and/or acquired resistance to chemotherapeutic drugs by overexpressing P-gp. This is in agreement with the previous report (35). Naringenin did not increase the uptake of Rho123 in all four tumor cell lines that indicates that naringenin has no influence on the transports of Rho123. However, naringenin strikingly increased the accumulation of 5-CFDA in all four tumor cell lines that implies some transporters of 5-CFDA, presumably MRPs, that are known to be expressed in these cells, were inhibited by naringenin (Fig. 5A and C). A low MRP1 and MRP2 inhibitory activity of naringenin has been reported (20). This difference from our finding might be explained by that the cell lines and the naringenin concentration used in our study were different from theirs. Verapamil only increased the uptake of Rho123 in the cells overexpressing P-gp, but did not effect on the uptake of

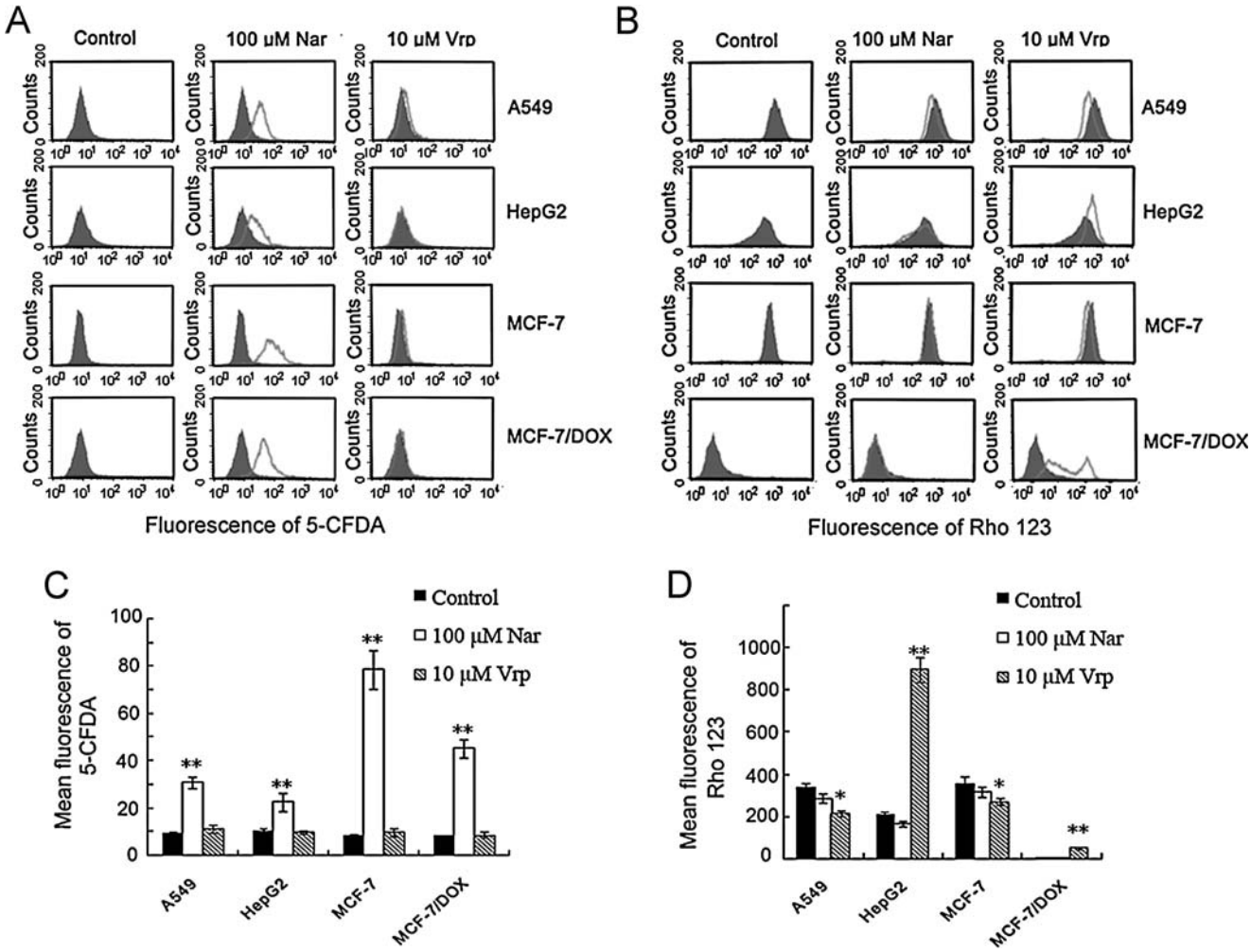

Fig. 5. Naringenin increases the retention of intracellular 5-CFDA. **A** flow cytometry determination of 5-CFDA uptake in A549, MCF-7, HepG2 and MCF-7/DOX cells after 1 h treatment with 100 μM naringenin or 10 μM verapamil. **B** flow cytometry determination of Rho123 retention in A549, MCF-7, HepG2 and MCF-7/DOX cells after 1 h treatment with 100 μM naringenin or 10 μM verapamil. **C** intracellular 5-CFDA fluorescence estimated by flow cytometry assay in A549, MCF-7, HepG2 and MCF-7/DOX cells after 1 h treatment with 100 μM naringenin or 10 μM verapamil ($n=4$). *Columns*, mean; *Bars*, SE. **D** intracellular Rho123 fluorescence estimated by flow cytometry assay in A549, MCF-7, HepG2 and MCF-7/DOX cells after 1 h treatment with 100 μM naringenin or 10 μM verapamil ($n=4$). *Columns*, mean; *Bars*, SE. Values significantly different from control cells were marked (*$P<0.05$; **$P<0.01$).

Rho123 in the cells non-expressing P-gp. Verapamil did not effect on the uptake of 5-CFDA in all four tumor cell lines. Thus, verapamil definitely modulates P-gp's function rather than MRPs' function. Naringenin and verapamil selectively inhibit each substrate transport indicate that they modulate separate the activity of multidrug resistance proteins.

To exclude the effect of naringenin on P-gp (MDR1), MCF-7/Dox cells treated with siRNA of MDR1 (targeting sequence: sense, 5′-GAAACCAACUGUCAGUGUA, 31) to downregulate the expression of MDR1 gene and to investigate efflux of doxorubicin mediated by this gene. The expression of MDR1 mRNA was markedly reduced in the cells treated with siRNA of MDR1 compared with untreated cells or the cells treated with mock siRNA (sense 5′-GAUACGAAUUGACACCGUA). The downregulation of MDR1 significantly increased the uptake of doxorubicin. The increased uptake of doxorubicin in the combination of siRNA with naringenin was much more than that of siRNA alone. Furthermore, MCF-7 cells treated with siRNA of MRP1 (targeting sequence: sense, 5′-GAUGACACCUCUCAA CAAAUU, 30) to downregulate the expression of MRP1 gene and to investigate efflux of doxorubicin mediated by the gene. The expression of MRP1 mRNA was dramatically decreased in the cells treated with siRNA of MRP1 compared with untreated cells or the cells treated with mock siRNA. The uptake of doxorubicin was significantly increased by downregulating MRP1. The combination of siRNA with naringenin did not enhance the uptake of doxorubicin compared with siRNA alone (Fig. 8). These results suggest that naringenin enhances the intracellular accumulation of

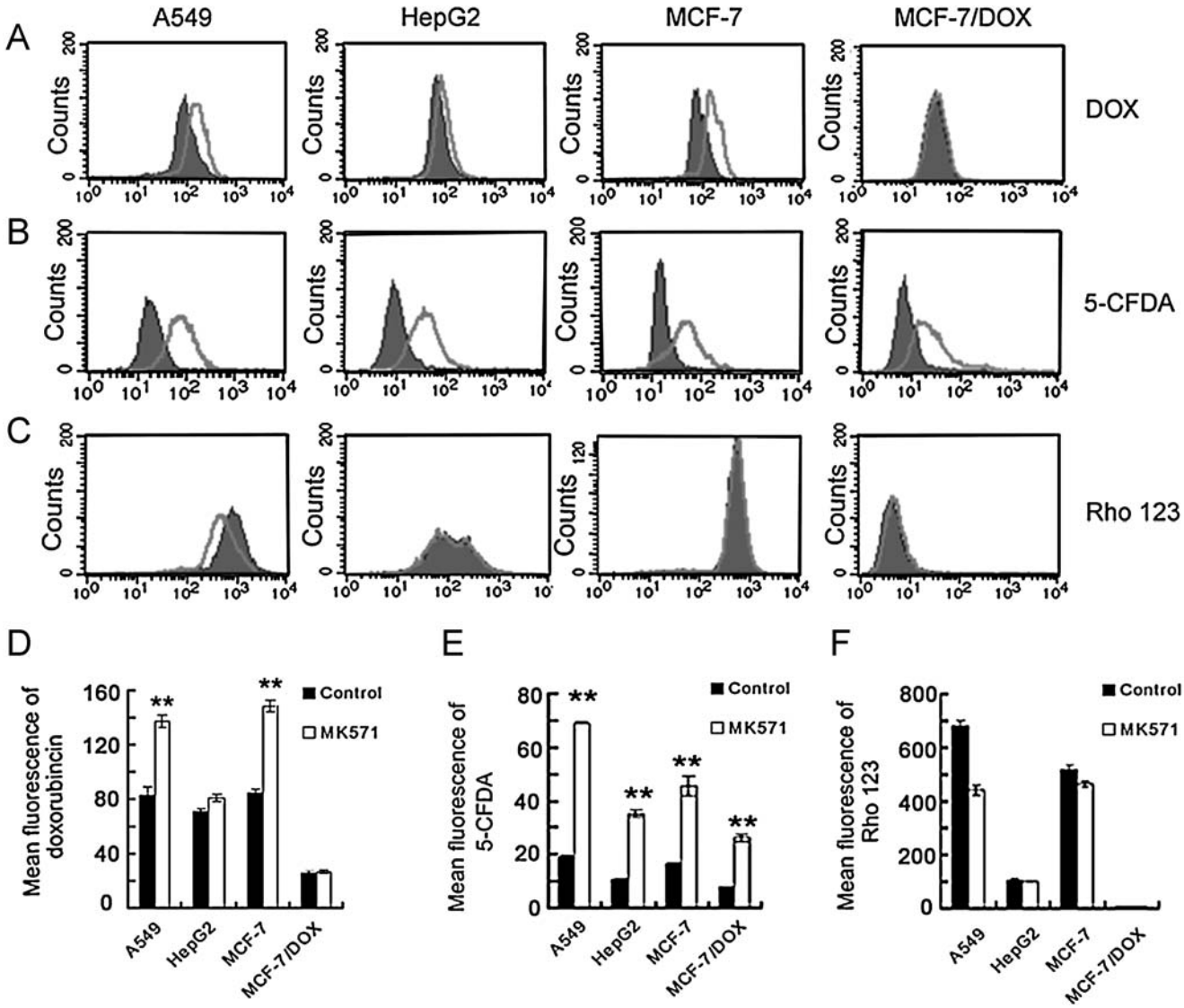

Fig. 6. MK571 increases the retention of intracellular 5-CFDA and intracellular doxorubicin fluorescence. **A** flow cytometry analysis of the intracellular doxorubicin fluorescence in A549, MCF-7, HepG2 and MCF-7/DOX cells after 2 h treatment of doxorubicin in combination with 50 μM MK571. **B** flow cytometry determination of 5-CFDA retention in A549, MCF-7, HepG2 and MCF-7/DOX cells after 1 h treatment with 50 μM MK571. **C** flow cytometry determination of Rho123 retention in A549, MCF-7, HepG2 and MCF-7/DOX cells after 1 h treatment with 50 μM MK571. **D** intracellular doxorubicin fluorescence estimated by flow cytometry assay (n=3). **E** intracellular 5-CFDA fluorescence estimated by flow cytometry assay (n=3). **F** intracellular Rho123 fluorescence estimated by flow cytometry assay (n=3). *Columns*, mean; *Bars*, SE. Values significantly different from control cells were marked (**P<0.01).

doxorubicin by modulating MRPs (MRP1) but not P-gp (MDR1).

Naringenin, at a non-cytotoxic concentration, in combination with doxorubicin increased the cytotoxicity of doxorubicin to A549 and MCF-7 cells. The enhanced cellular accumulation of doxorubicin by naringenin is in line with its increased cytotoxicity. Moreover, verapamil also showed the increased cytotoxicity of doxorubicin to HepG2 and MCF-7/DOX cells by raising its cellular concentration. These results suggest the high intracellular concentration of doxorubicin attributes to the added cytotoxicity. Annexin V/PI staining and DNA fragment analysis further confirmed that naringenin increased the sensitivity of A549 cells to doxorubicin-induced apoptosis. This enhancement of cytotoxicity by naringenin was not restrict to doxorubicin, but also observed in other cytotoxic drugs including vinorelbine and daunorubicin (data not shown).

Verapamil, a well known inhibitor of the P-glycoprotein-induced MDR phenotype, has been used to modulate the activity of P-gp and to reverse the multidrug resistance (32,33). However, our study showed that verapamil was ineffective in increasing cytotoxicity of doxorubicin in the non-expressing P-glycoprotein cells such as A549 and MCF-7 cells (Fig. 1). Some cancer cell lines overexpressing MRPs and/or breast cancer and lung cancer resistance proteins contribute to the multidrug resistance phenotype (19,36,37). The modulation of ABC protein-related transporters as the part of initial therapy may prevent subsequent emergence of drug resistance (38,39). Our findings showed that naringenin modulated the function of MRPs and enhanced the cellular accumulation of doxorubicin, thus in turn increased its cytotoxicity to cancer cells. Naringenin may be useful to prevent or to reduce the development of the chemoresistance phenotype in the non-expressing P-gp cancer cells by inhibiting MRPs activity. In addition, we found that naringenin induced tumor cell apoptosis and had an antiangiogenic effect (40). These may be useful for clinical cancer therapy (41).

The combination of doxorubicin with naringenin had increased antitumor activity in subcutaneous LLC tumor model compared with doxorubicin alone (Fig. 7A). Moreover, mice treated with this combination showed fewer signs of toxicity than that treated with doxorubicin only (Fig. 7B). These results indicate that naringenin might synergistically enhance the antitumor activity of doxorubicin, through modulating the activity of MRPs, inducing the apoptosis and inhibiting the angiogenesis. It may contribute to reducing the systemic toxicity of doxorubicin that naringenin can prevent heart from doxorubicin-induced cardiac toxicity (42). Our unpublished data also showed that naringenin could effectively inhibit tumor growth by regulating the microenvironment in tumors. Therefore, naringenin could be potentially useful in combination with chemotherapeutic agents for the treatment of cancers in the clinic.

CONCLUSIONS

Our results clearly showed that naringenin enhanced the sensitivities of cancer cells to doxorubicin both *in vitro* and *in vivo*. The combination of naringenin with chemotherapeutic agents may serve as a new approach for the treatment of human cancers, particularly in cancers having intrinsic drug resistance modulated by MRPs. However, further in-depth studies including clinical trials are needed to fully evaluate the value of naringenin in combination with chemotherapeutic agents for the treatment of human cancers.

ACKNOWLEDGMENTS

This work was supported by grants from the State Key Development Plan Project (2006CB705706 and 2007CB935801), National Natural Science Foundation of China (90606019, 90713024) and the Chinese Academy of Sciences (KSCX2-YW-R-21 and KJCX2-YW-M02).

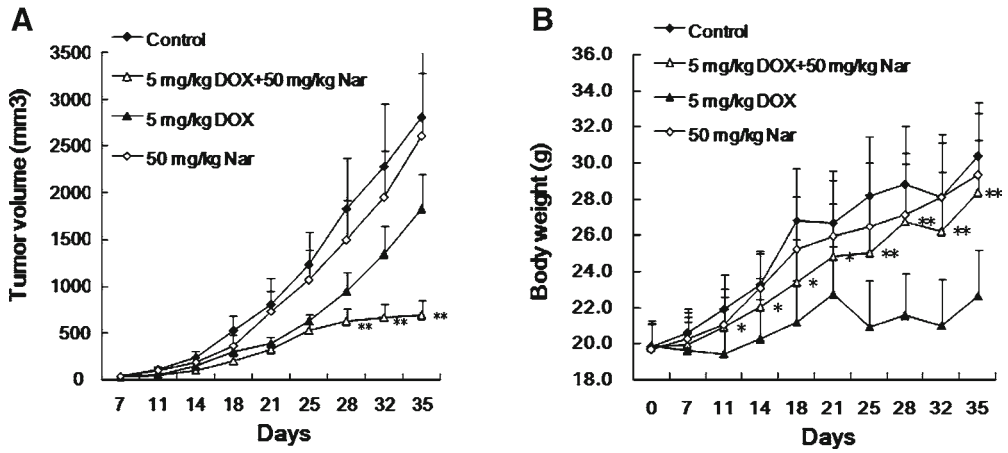

Fig. 7. The antitumor effect of doxorubicin combined with naringenin *in vivo*. **A** tumor growth kinetics; the mean calculated tumor volume was plotted with error bars for clarity. $N=10$ mice per group. Mice received 50 mg/kg naringenin daily for 3 weeks (*hollow diamonds*), 5 mg/kg doxorubicin at day 7, day 14 and day 21 (*filled triangles*), a combination of 50 mg/kg naringenin daily for 3 weeks and 5 mg/kg doxorubicin three times (*hollow triangles*), or saline buffer as the control (*filled diamonds*). **B** mean body weight of mice treated with above regiments. $N=10$ mice per group; *Bars*, SE. Values significantly different from doxorubicin treated group were marked ($^*P<0.05$; $^{**}P<0.01$).

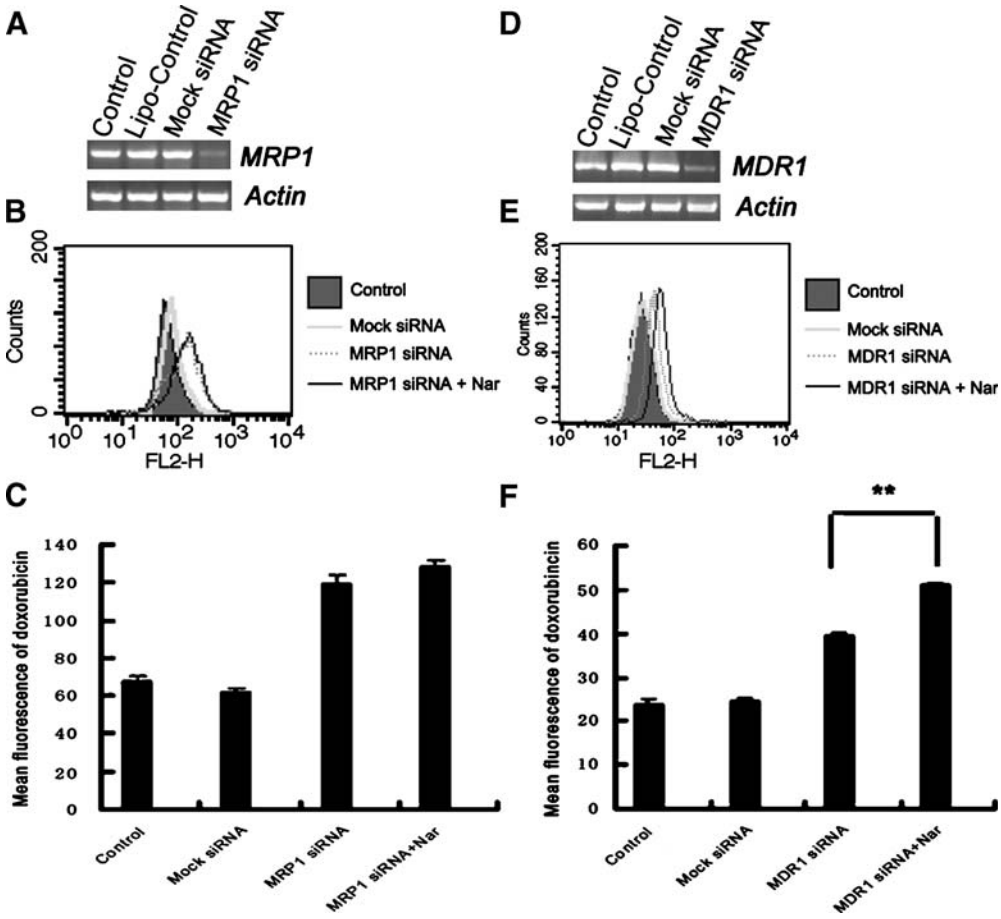

Fig. 8. Combination of naringenin with siRNA of *MDR1* increases the intracellular doxorubicin fluorescence. **A** reverse transcription-PCR analyses of mRNA levels of *MRP1* in MCF-7 cells after 48 h treatment with 100 nM siRNA of *MRP1*. **B** flow cytometry analysis of the intracellular doxorubicin fluorescence in MCF-7 cells after 72 h treatment with 100 μM naringenin and 100 nM siRNA of *MRP1*. **C** intracellular doxorubicin fluorescence estimated by flow cytometry assay in MCF-7 cells ($n=3$). *Columns*, mean; *Bars*, SE. **D** reverse transcription-PCR analyses of mRNA levels of *MDR1* in MCF-7/DOX cells after 48 h treatment with 100 nM siRNA of *MDR1*. **E** flow cytometry analysis of the intracellular doxorubicin fluorescence in MCF-7/DOX cells after 72 h treatment with 100 μM naringenin and 100 nM siRNA of *MDR1*. **F** intracellular doxorubicin fluorescence estimated by flow cytometry assay in MCF-7/DOX cells ($n=3$). *Columns*, mean; *Bars*, SE. Significantly different values were marked (**$P<0.01$).

REFERENCES

1. M. M. Gottesman, T. Fojo, and S. E. Bates. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat. Rev. Cancer.* **2**:48–58 (2002). doi:10.1038/nrc706.
2. M. Bredel. Anticancer drug resistance in primary human brain tumors. *Brain Res. Brain Res. Rev.* **35**:161–204 (2001). doi:10.1016/S0165-0173(01)00045-5.
3. M. M. Gottesman, and I. Pastan. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.* **62**:385–427 (1993). doi:10.1146/annurev.bi.62.070193.002125.
4. P. Borst, R. Evers, M. Kool, and J. Wijnholds. A family of drug transporters: the multidrug resistance-associated proteins. *J. Natl. Cancer Inst.* **92**:1295–1302 (2000). doi:10.1093/jnci/92.16.1295.
5. S. P. Cole, G. Bhardwaj, J. H. Gerlach, J. E. Mackie, C. E. Grant, K. C. Almquist, A. J. Stewart, E. U. Kurz, A. M. V. Duncan, and R. G. Deeley. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science.* **258**:1650–1654 (1992). doi:10.1126/science.1360704.
6. L. A. Doyle, W. Yang, L. V. Abruzzo, T. Krogmann, Y. Gao, A. K. Rishi, and D. D. Ross. A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc. Natl. Acad. Sci. USA.* **95**:15665–15670 (1998). doi:10.1073/pnas.95.26.15665.
7. E. K. Rowinsky, L. Smith, Y. M. Wang, P. Chaturvedi, M. Villalona, E. Campbell, C. Aylesworth, S. G. Eckhardt, L. Hammond, M. Kraynak, R. Drengler, J. Stephenson Jr., M. W. Harding, and D. D. Von Hoff. Phase I and pharmacokinetic study of paclitaxel in combination with biricodar, a novel agent that reverses multidrug resistance conferred by overexpression of both MDR1 and MRP. *J. Clin. Oncol.* **16**:2964–2976 (1998).
8. R. A. Peck, J. Hewett, M. W. Harding, Y. M. Wang, P. R. Chaturvedi, A. Bhatnagar, H. Ziessman, F. Atkins, and M. J. Hawkins. Phase I and pharmacokinetic study of the novel MDR1 and MRP1 inhibitor biricodar administered alone and in combination with doxorubicin. *J. Clin. Oncol.* **19**:3130–3141 (2001).
9. R. V. Kondratov, P. G. Komarov, Y. Becker, A. Ewenson, and A. V. Gudkov. Small molecules that dramatically alter multidrug resistance phenotype by modulating the substrate specificity of P-glycoprotein. *Proc. Natl. Acad. Sci. USA.* **98**:14078–14083 (2001). doi:10.1073/pnas.241314798.
10. H. Minderman, K. L. O'Loughlin, L. Pendyala, and M. R. Baer. VX-710 (biricodar) increases drug retention and enhances chemosensitivity in resistant cells overexpressing P-glycoprotein, multidrug resistance protein, and breast cancer resistance

protein. *Clin. Cancer Res*. **10**:1826–1834 (2004). doi:10.1158/1078-0432.CCR-0914-3.
11. L. C. Young, B. G. Campling, S. P. Cole, R. G. Deeley, and J. H. Gerlach. Multidrug resistance proteins MRP3, MRP1, and MRP2 in lung cancer: correlation of protein levels with drug response and messenger RNA levels. *Clin. Cancer Res*. **7**:1798–1804 (2001).
12. T. Abe, S. Hasegawa, K. Taniguchi, A. Yokomizo, T. Kuwano, M. Ono, T. Mori, S. Hori, K. Kohno, and M. Kuwano. Possible involvement of multidrug-resistance-associated protein (MRP) gene expression in spontaneous drug resistance to vincristine, etoposide and adriamycin in human glioma cells. *Int. J. Cancer*. **58**:860–864 (1994). doi:10.1002/ijc.2910580619.
13. C. Calatozzolo, M. Gelati, E. Ciusani, F. L. Sciacca, B. Pollo, L. Cajola, C. Marras, A. Silvani, L. Vitellaro-Zuccarello, D. Croci, A. Boiardi, and A. Salmaggi. Expression of drug resistance proteins Pgp, MRP1, MRP3, MRP5 and GST-pi in human glioma. *J. Neurooncol*. **74**:113–121 (2005). doi:10.1007/s11060-004-6152-7.
14. A. T. Nies, J. König, M. Pfannschmidt, E. Klar, W. J. Hofmann, and D. Keppler. Expression of the multidrug resistance proteins MRP2 and MRP3 in human hepatocellular carcinoma. *Int. J. Cancer*. **94**:492–499 (2001). doi:10.1002/ijc.1498.
15. E. H. Rubin, D. P. de Alwis, I. Pouliquen, L. Green, P. Marder, Y. Lin, R. Musanti, S. L. Grospe, S. L. Smith, D. L. Toppmeyer, J. Much, M. Kane, A. Chaudhary, C. Jordan, M. Burgess, and C. A. Slapak. A phase I trial of a potent P-glycoprotein inhibitor, Zosuquidar.3HCl trihydrochloride (LY335979), administered orally in combination with doxorubicin in patients with advanced malignancies. *Clin. Cancer Res*. **8**:3710–3717 (2002).
16. L. Gandhi, M. W. Harding, M. Neubauer, C. J. Langer, M. Moore, H. J. Ross, B. E. Johnson, and T. J. Lynch. A phase II study of the safety and efficacy of the multidrug resistance inhibitor VX-710 combined with doxorubicin and vincristine in patients with recurrent small cell lung cancer. *Cancer*. **109**:924–932 (2007). doi:10.1002/cncr.22492.
17. S. Zhang, and M. E. Morris. Effects of the flavonoids biochanin A, morin, phloretin and silymarin on P-glycoprotein-mediated transport. *J. Pharmacol. Exp. Ther*. **304**:1258–1267 (2003). doi:10.1124/jpet.102.044412.
18. M. Hadjeri, M. Barbier, X. Ronot, A. M. Mariotte, A. Boumendjel, and J. Boutonnat. Modulation of P-glycoprotein-mediated multidrug resistance by flavonoid derivatives and analogues. *J. Med. Chem*. **46**:2125–2131 (2003). doi:10.1021/jm021099i.
19. S. Zhang, X. Yang, and M. E. Morris. Combined effects of multiple flavonoids on breast cancer resistance protein (ABCG2)-mediated transport. *Pharm. Res*. **21**:1263–1273 (2004). doi:10.1023/B:PHAM.0000033015.84146.4c.
20. J. J. van Zanden, H. M. Wortelboer, S. Bijlsma, A. Punt, M. Usta, P. J. Bladeren, I. M. Rietjens, and N. H. Cnubben. Quantitative structure activity relationship studies on the flavonoid mediated inhibition of multidrug resistance proteins 1 and 2. *Biochem. Pharmacol*. **69**:699–708 (2005). doi:10.1016/j.bcp.2004.11.002.
21. C. Zanini, G. Giribaldi, G. Mandili, F. Carta, N. Crescenzio, B. Bisaro, A. Doria, L. Foglia, L. C. di Montezemolo, F. Timeus, and F. Turrini. Inhibition of heat shock proteins (HSP) expression by quercetin and differential doxorubicin sensitization in neuroblastoma and Ewing's sarcoma cell lines. *J. Neurochem*. **103**:1344–1354 (2007). doi:10.1111/j.1471-4159.2007.04835.x.
22. A. Bast, H. Kaiserová, G. J. den Hartog, G. R. Haenen, and W. J. van der Vijgh. Protectors against doxorubicin-induced cardiotoxicity: flavonoids. *Cell Biol. Toxicol*. **23**:39–47 (2007). doi:10.1007/s10565-006-0139-4.
23. H. Kaiserová, T. Simůnek, W. J. van der Vijgh, A. Bast, and E. Kvasnicková. Flavonoids as protectors against doxorubicin cardiotoxicity: role of iron chelation, antioxidant activity and inhibition of carbonyl reductase. *Biochim. Biophys. Acta*. **1772**:1065–1074 (2007).
24. S. Zhang, X. Yang, and M. E. Morris. Flavonoids are inhibitors of breast cancer resistance protein (ABCG2)-mediated transport. *Mol. Pharmacol*. **65**:1208–1216 (2004). doi:10.1124/mol.65.5.1208.
25. S. Kitagawa. Inhibitory effects of polyphenols on P-glycoprotein-mediated transport. *Biol. Pharm. Bull*. **29**:1–6 (2006). doi:10.1248/bpb.29.1.
26. T. Mosmann. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*. **65**:55–63 (1983). doi:10.1016/0022-1759(83)90303-4.
27. Y. Li, and F. H. Sarkar. Inhibition of nuclear factor kappaB activation in PC3 cells by genistein is mediated via Akt signaling pathway. *Clin. Cancer Res*. **8**:2369–2377 (2002).
28. V. Jekerle, W. Klinkhammer, D. A. Scollard, K. Breitbach, R. M. Reilly, M. Piquette-Miller, and M. Wiese. *In vitro* and *in vivo* evaluation of WK-X-34, a novel inhibitor of P-glycoprotein and BCRP, using radio imaging techniques. *Int. J. Cancer*. **119**:414–422 (2006). doi:10.1002/ijc.21827.
29. C. Kimchi-Sarfaty, J. M. Oh, I. W. Kim, Z. E. Sauna, A. M. Calcagno, S. V. Ambudkar, and M. M. Gottesman. A "silent" polymorphism in the MDR1 gene changes substrate specificity. *Science*. **315**:525–528 (2007). doi:10.1126/science.1135308.
30. C. L. Hammond, R. Marchan, S. M. Krance, and N. Ballatori. Glutathione export during apoptosis requires functional multidrug resistance-associated proteins. *J. Biol. Chem*. **282**:14337–14347 (2007). doi:10.1074/jbc.M611019200.
31. V. Stierlé, A. Laigle, and B. Jollès. Modulation of MDR1 gene expression in multidrug resistant MCF7 cells by low concentrations of small interfering RNAs. *Biochem. Pharmacol*. **70**:1424–1430 (2005).
32. M. Biscardi, E. Teodori, R. Caporale, R. Budriesi, F. Balestri, B. Scappini, S. Gavazzi, and A. Grossi. Multidrug reverting activity toward leukemia cells in a group of new verapamil analogues with low cardiovascular activity. *Leuk. Res*. **30**:1–9 (2006).
33. E. Teodori, S. Dei, A. Garnier-Suillerot, F. Gualtieri, D. Manetti, C. Martelli, M. N. Romanelli, S. Scapecchi, P. Sudwan, and M. Salerno. Exploratory chemistry toward the identification of a new class of multidrug resistance reverters inspired by pervilleine and verapamil models. *J. Med. Chem*. **48**:7426–7436 (2005). doi:10.1021/jm050542x.
34. P. Mitra, C. A. Oskeritzian, S. G. Payne, M. A. Beaven, S. Milstien, and S. Spiegel. Role of ABCC1 in export of sphingosine-1-phosphate from mast cells. *Proc. Natl. Acad. Sci. USA*. **103**:16394–16399 (2006). doi:10.1073/pnas.0603734103.
35. S. Kitagawa, T. Nabekura, T. Takahashi, Y. Nakamura, H. Sakamoto, H. Tano, M. Hirai, and G. Tsukahara. Structure-activity relationships of the inhibitory effects of flavonoids on P-glycoprotein-mediated transport in KB-C2 cells. *Biol. Pharm. Bull*. **28**:2274–2278 (2005). doi:10.1248/bpb.28.2274.
36. G. A. Thomas, M. A. Barrand, S. Stewart, P. H. Rabbitts, E. D. Williams, and P. R. Twentyman. Expression of the multidrug resistance-associated protein (MRP) gene in human lung tumours and normal tissue as determined by *in situ* hybridisation. *Eur. J. Cancer*. **30**:1705–1709 (1994). doi:10.1016/0959-8049(94)00290-L.
37. C. M. Laurençot, G. L. Scheffer, R. J. Scheper, and R. H. Shoemaker. Increased LRP mRNA expression is associated with the MDR phenotype in intrinsically resistant human cancer cell lines. *Int. J. Cancer*. **72**:1021–1026 (1997). doi:10.1002/(SICI)1097-0215(19970917)72:6<1021::AID-IJC17>3.0.CO;2-7.
38. L. Beketic-Oreskovic, G. E. Durán, G. Chen, C. Dumontet, and B. I. Sikic. Decreased mutation rate for cellular resistance to doxorubicin and suppression of mdr1 gene activation by the cyclosporin PSC 833. *J. Natl. Cancer Inst*. **87**:1593–1602 (1995). doi:10.1093/jnci/87.21.1593.
39. X. F. Hu, A. Slater, P. Kantharidis, D. Rischin, S. Juneja, R. Rossi, G. Lee, J. D. Parkin, and J. R. Zalcberg. Altered multidrug resistance phenotype caused by anthracycline analogues and cytosine arabinoside in myeloid leukemia. *Blood*. **93**:4086–4095 (1999).
40. F. Zhang, G. Du, and W. Liang. Naringenin exerts its anti-tumor effect via antiangiogenesis. 8th world congress for microcirculation. Milwaukee, Wisconsin, USA, 2007, pp. 159–163.
41. F. Tosetti, N. Ferrari, S. De Flora, and A. Albini. Angioprevention: angiogenesis is a common and key target for cancer chemopreventive agents. *FASEB J*. **16**:2–14 (2002). doi:10.1096/fj.01-0300rev.
42. H. M. Arafa, M. F. Abd-Ellah, and H. F. Hafez. Abatement by naringenin of doxorubicin-induced cardiac toxicity in rats. *J. Egypt Natl. Canc. Inst*. **17**:291–300 (2005).