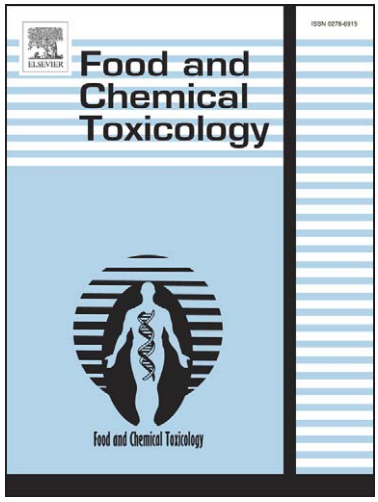

Naringenin-7-O-glucoside protects against doxorubicin-induced toxicity in H9c2 cardiomyocytes by induction of endogenous antioxidant enzymes

Xiuzhen Han, Jihong Pan, Dongmei Ren, Yanna Cheng, Peihong Fan, Hongxiang Lou

PII: S0278-6915(08)00378-5
DOI: 10.1016/j.fct.2008.06.086
Reference: FCT 4495

To appear in: *Food and Chemical Toxicology*

Received Date: 2 March 2008
Revised Date: 17 June 2008
Accepted Date: 27 June 2008

Please cite this article as: Han, X., Pan, J., Ren, D., Cheng, Y., Fan, P., Lou, H., Naringenin-7-O-glucoside protects against doxorubicin-induced toxicity in H9c2 cardiomyocytes by induction of endogenous antioxidant enzymes, *Food and Chemical Toxicology* (2008), doi: 10.1016/j.fct.2008.06.086

Naringenin-7-O-glucoside protects against doxorubicin-induced toxicity in H9c2 cardiomyocytes by induction of endogenous antioxidant enzymes

Xiuzhen Han[1], Jihong Pan[2], Dongmei Ren[1], Yanna Cheng[3], Peihong Fan[1], Hongxiang Lou[1]*

[1]*Department of Natural Product Chemistry, School of Pharmaceutical Sciences, Shandong University, 44 West Wenhua Road, Jinan, 250012, China*

[2]*Shandong Medicinal and Biotechnology Center, Shandong Academy of Medical Sciences, 89 Jingshi Road, Jinan, 250062, China*

[3]*Department of Pharmacology, School of Pharmaceutical Sciences, Shandong University, 44 West Wenhua Road, Jinan, 250012, China*

***Corresponding author:**

Name Hongxiang Lou, Ph.D.

Address Department of Natural Product Chemistry, School of Pharmaceutical Sciences, Shandong University, 44 West Wenhua Road, Jinan, 250012, China

Tel +86-531-88382012

Fax +86-531-88382019

E-mail louhongxiang@sdu.edu.cn.

Running title: NARG protects against doxorubicin toxicity

Abbreviations: ARE, Antioxidant response element; CAT, Catalase; DAB; 3, 3'-Diaminobenzidine tetrahydrochloride; DMEM, Dulbecco's modified Eagle's medium; DMSO, Dimethylsulfoxide; DTT, Dithiotreitol; EDTA, Ethylenediamine tetraacetic acid; ERK, Extracellular signal-regulated kinase; GCL, Glutamate cysteine ligase; GCLC, Glutamate-cysteine ligase catalytic subunit; GCLM, Glutamate-cysteine ligase modifier subunit; GSH, Reduced glutathione; HO-1, Heme oxygenase 1; MDA, Malondialdehyde; MnSOD, Manganese-dependent superoxide dismutase; NQO1, NAD (P) H: quinone oxidoreductase 1; NARG, Naringenin-7-O-glucoside; Nrf2, Nuclear factor-erythroid 2 p45-related factor 2; PMSF, Phenylmethylsulfonyl fluoride; ROS, Reactive oxygen species.

Abstract

Doxorubicin, a widely used chemotherapeutic agent, can give rise to severe cardiotoxicity that limits its clinical use by generation of reactive oxygen species (ROS) and apoptosis. Protection or alleviation of doxorubicin cardiotoxicity can be achieved by administration of natural phenolic compounds via activating endogenous defense systems and antiapoptosis. Naringenin-7-O-glucoside (NARG), isolated from *Dracocephalum rupestre* Hance, has been demonstrated to protect against cardiomyocyte apoptosis. In the present study, we investigated the effects of NARG on endogenous antioxidant enzymes against doxorubicin toxicity and the potential role of extracellular signal-regulated kinase (ERK) in regulation of NARG-induced Nrf2-dependent gene expression in H9c2 cardiomyocytes. The mRNA expression of glutamate-cysteine ligase modifier subunit (GCLM) and glutamate-cysteine ligase catalytic subunit (GCLC) was upregulated by NARG as detected by RT-PCR. NARG (10, 20, and 40 μM) pretreatment increased NAD (P) H: quinone oxidoreductase (NQO1), ERK, and Nrf2 protein levels in cardiomyocytes as detected by Western blotting. These results suggest that NARG could prevent cardiomyocytes from doxorubicin-induced toxicity by induction of endogenous antioxidant enzymes via phosphorylation of ERK1/2 and nuclear translocation of Nrf2.

Keywords

Naringenin-7-O-glucoside (NARG); NAD (P) H: quinone oxidoreductase(NQO1); glutamate-cysteine ligase(GCL); extracellular signal-regulated kinase (ERK); Nrf2;

doxorubicin

1. Introduction

Doxorubicin is an anthracycline antibiotic that is widely used to treat different types of human neoplastic diseases (Young et al., 1981). However, its clinical use is limited by its severe cumulative dose-related cardiotoxicity (Keefe, 2001). Many studies have shown that the major molecular mechanism involved in doxorubicin-induced cardiactoxicity is the generation of reactive oxygen species (ROS) due to the catalytic quinone moiety of doxorubicin and inducing cardiomyocyte apoptosis (Keizer et al., 1990; Takemura and Fujiwara, 2007). Treatment with antioxidants or natural phenolic compounds has been found to protect against doxorubicin-induced cardiotoxicity (Bast et al., 2007).

Flavonoids, a group of polyphenols, possess potent antioxidant activity and cardioprotective efficacy (Chlopcíková et al., 2004; Kaiserová et al., 2007). It has been reported that flavonoids can exhibit protective effects against doxorubicin-induced cardiactoxicity by activating the cellular antioxidative system and modulating the expression of some endogenous antioxidant enzymes (Bast et al., 2007; Du et al., 2007). Overexpression of manganese-dependent superoxide dismutase (MnSOD) or catalase (CAT) can suppress doxorubicin-induced cardiotoxicity (Yen et al., 1996; Kang et al., 1996). The protective phase 2 detoxifying enzymes such as NAD(P)H: quinine oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), and glutamylcysteine ligase (GCL) play an important role in the defense

system by enhancing cellular antioxidant capacity. Regulation of both basal and inducible expression of these protective enzymes is mediated mainly by nuclear factor E2 P45-related factor 2 (Nrf2) (Kang et al., 2007). Naturally occurring flavonoids or polyphenols have been reported to upregulate HO-1 expression by activating Nrf2 to bind with the antioxidant response element (ARE) in the ho-1 gene promoter region (Yao et al., 2007). Extensive studies in recent years have indicated that activation process of Nrf2 is modulated by phosphorylation cascade and extracellular signal-regulated kinase (ERK) has been implicated in the process of Nrf2 activation (Manandhar et al., 2007; Cullinan et al., 2003).

The Chinese traditional medicine *Dracocephalum rupestre* Hance, a wild perennial herb found throughout western China, contains high contents of flavonoids (Wu and Li, 1977), and has offered a therapeutic potential for cardiovascular diseases. In our continuous search for cardioprotective substances from natural products, NARG, a major active flavonoid isolated from *D. rupestre*, has been demonstrated that NARG was able to upregulate the expression of HO-1 and attenuate doxorubicin-induced H9c2 cell apoptosis (Han et al., 2008).

In the present study, the protective effects and possible mechanisms of NARG on cardiomyocyte injury induced by doxorubicin were evaluated by detecting endogenous antioxidant enzymes; the results demonstrated that NARG could prevent cardiomyocytes from doxorubicin-induced toxicity by induction of endogenous antioxidant enzymes via phosphorylation of ERK1/2 and nuclear translocation of Nrf2, having an effect comparable to that of quercetin.

2. Materials and methods

2.1. Chemicals and materials

By Silica gel chromarography, Sephadex LH-20 chromatography and Chiral HPLC, thirty-eight compounds were isolated from the ethanol extract of *D. rupestre* in our laboratory (Ren et al., 2007; Ren et al., 2008). NARG was a major active component and its purity was more than 98% detected by HPLC. The chromatographic conditions were phenomenex C_{18} column, methanol-acetonitrile-water (32:8:60) was used as the mobile phase, flow rate was 1.0 ml/min, and detected at a wavelength of 284 nm (Ren et al., 2003; Ren et al., 2005). In the present study, NARG was dissolved in dimethylsulfoxide (DMSO) for the in vitro assay.

Dulbecco's modified Eagle's medium (DMEM) was from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum was from Tianjin TBD Biotechnology Development Center (Tianjing, China). Pepstatin A, phenylmethylsulfonyl fluoride (PMSF), and ethylenediamine tetraacetic acid (EDTA) were from Sigma. SOD, reduced glutathine (GSH), malonaldehyde (MDA), and CAT assay kits were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Dithiotreitol (DTT) was from Merck. Acrylamide, RNase inhibitor, and leupeptin were from Amresco. 3, 3'-Diaminobenzidine tetrahydrochloride substrate kit (DAB) was from Zhongshan Goldenbridge Biotechnology LTD (Beijing, China). Polyvinylidene difluoride was from Millipot. RNA PCR kit was from TaKaRa. Bradford protein assay kit was from Beyotime Institute of Biotechnology (Beijing, China). Antibodies against NQO1 and

Nrf2 were from ABCAM. Antibody against ERK1/2 was from Cell Signalling Technology, Inc. Antibodies against beta-actin and horseradish peroxidase-conjugated secondary antibody were from Boster Biological Technology LTD (Wuhan, China).

2.2. Cell culture

Rat cardiac H9c2 cells (ATCC, Rockville, MD, USA) were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U ml^{-1} of penicillin, and 100 μg ml^{-1} of streptomycin in tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2. The cells were fed every two–three days and subcultured once they reached 70–80% confluence. Cells were plated at an appropriate density according to each experimental design.

2.3. MTT assay

MTT assay was used to determine cell viability (Han et al., 2008). The H9c2 cells were incubated with chemicals (10, 20, 40, and 80 μM) in DMEM supplemented with 0.5% fetal bovine serum at 37°C for 12, 24, 48, 72 h respectively, followed by incubation with doxorubicin (10 μM) for another 24 h, and then treated with MTT solution (final concentration, 0.5 mg/ml) for 4 h at 37°C in 96-well plates. The supernatants were removed carefully, followed by the addition of 100 μl DMSO to each well to dissolve the precipitate. Then, the absorbance was measured at 570 nm in a microplate reader (Synergy HT).

2.4. Cells treatment with NARG

H9c2 cells were incubated with NARG (10, 20 and 40 μM) for 24 h followed by incubation with doxorubicin (10 μM) for another 24 h. After this incubation, cells were collected by centrifugation (1500 rpm×5min) and parameters were measured as described in materials and methods.

2.5. Preparation of cell extracts for measurement of endogenous antioxidant enzymes

Treated cells (1×10^7 cells) were collected, washed with ice-cold PBS and resuspended in RIPA buffer [50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 60 mM DTT, 1 mM PMSF, 2.0 μg ml^{-1} leupeptin, 5 μg ml^{-1} pepstatin A]. Cell lysis was carried out at 4°C by vortexing for 15 s, and the cell suspension was then stored for 10 min in ice. After centrifugation at 13,000×g for 10 min, the supernatants were separated and stored at -70°C until use.

2.6. Measurement of cellular GSH content, MDA levels and activities of cellular antioxidant enzymes

The supernatants separated were used for measurement of cellular GSH content, MDA levels and cellular antioxidant enzymes such as SOD and CAT using the commercially available colorimetric assay kits respectively. The protein concentration was determined by using the Bradford protein assay kit.

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from H9c2 cells using Trizol (Invitrogen) as described by the manufacturer. First-strand cDNAs were generated by reverse transcription using oligo (dT) from RNA samples. Primer sequences (Sbsgene, Shanghai, China) are shown below. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 470bp), LP: 5‘-GAGGGGCCATCCACAGTCTTCTG-3’, RP: 5‘-CCCTTCATTGACCTCAACTACATGGT-3’; GAPDH (286bp), LP: 5'-TGC TGAGTATGTCGTGGAGTC-3', RP: 5'-CTTCTGAGTGGCAGTGATGG-3'; GCLC, LP: 5‘-GATGATAGAACACGGGAGG-3’, RP: 5‘-CATTGGTCGGAACTCTACTC-3’; GCLM, LP: 5‘-ACATTGAAGCCCAGGAGT-3’, RP: 5‘-CATTGCCAAACCA CCACA-3’; NQO1, LP: 5‘-CATTCCAGCCGACAACCAGA-3’, RP: 5‘-ATTCCCTCCTGCCCTAAACC-3’.

After cDNA synthesis, PCR was performed, using the following conditions: 95°C for 3 min; 94°C for 30 s; 58 or 60°C for 30 s (depending on the sequences of the primers); and 72°C for 1 min, 72°C for 5 min for 30 cycles. PCR products were electrophoresed in 2% agarose gel and visualized with ethidium bromide. The relative expression was quantified densitometrically using the Alphalmager ^{TM} 2200 System (Alpha Innotech Corporation) and calculated according to the reference bands of GAPDH.

2.8. Cytosolic and nuclear protein extraction

Treated cells (1×10^7 cells) were collected and washed with ice-cold PBS. Then

cytosolic and nuclear proteins were extracted as described previously (Dignam et al., 1983). Briefly, collected cells were suspended in 100 μl hypotonic buffer (1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 10 mM Hepes, pH 7.9) containing protease inhibitor cocktail and their outer membranes were disrupted by homogenization. The supernatant was then collected as cytosolic fraction by centrifuge at 10,000×g for 20 min. The resultant nuclear pellet was resuspended, homogenized and incubated in 50 μl nuclear extraction buffer [1.5 mM $MgCl_2$, 0.42M NaCl, 0.2 mM EDTA, 1 mM DTT, 20 mM Hepes, 25% (v/v) glycerol] containing the protease inhibitor cocktail. Finally, the nuclear suspension was centrifuged at 20,000×g for 15 min to collect supernatant as nuclear fraction.

2.9. Western blot analysis

After addition of sample loading buffer, protein samples were electrophoresed on a 15% SDS–polyacrylamide gel. Proteins (25 μg) were transferred to polyvinylidene difluoride blots at 150 mA for 2 h. The blots were blocked for 2 h at room temperature in PBST (PBS and 0.1% Tween 20, pH 7.4, containing 5% non-fat dried milk). Sheets were incubated with goat anti-NQO1 antibody (1:250), rabbit anti-phospho-ERK1/2(1:300), rabbit anti-Nrf2 (1:300), and rabbit anti-beta-actin antibody (1:200) in PBS with 5% non-fat dried milk. Following three washes with PBST, the blots were incubated with horseradish peroxidase-conjugated secondary antibody (1:2000) for beta-actin, NQO1, ERK1/2 and Nrf2 in PBST with 5% non-fat dried milk for 2 h at room temperature. Then sheets were washed again three times in

PBST buffer, and transferred proteins were incubated and visualized with DAB substrate solution for 10 min according to the manufacturer's instructions.

3.0. Statistical analysis

All data are expressed as mean ± S.D. from at least four independent experiments. Differences between mean values of multiple groups were analyzed by Student's t-test. Statistical significance was considered at $P<0.05$.

3. Results

3.1. Effects of NARG on doxorubicin-mediated cytotoxicity in H9c2 cells

To analyze the protective effects of NARG on doxorubicin-induced cytotoxicity in H9c2 cells, the rates of cell growth inhibition were evaluated based on the OD value as estimated with the MTT assay. As shown in Fig. 1, NARG (10, 20, 40 μM) pretreatment had a significant protective effects against doxorubicin-mediated cytotoxicity. The protective effects decreased at doses higher than 80 μM. Furthermore, the protective effects against doxorubicin cardiotoxicity in 24h were better than other term, therefore pretreatment with NARG (10, 20, 40 μM) for 24h was chosen in the following experiments.

3.2. Effects of NARG on cellular GSH content, MDA level, and activities of cellular antioxidant enzymes

GSH is an important protective antioxidant against oxidative stress. SOD and CAT are two key enzymes in the detoxification of ROS. They have been suggested to be protective against various forms of oxidative cardiovascular injuries (Rohde et al., 2005). We therefore measured cellular GSH levels and the activities of SOD and CAT in cardiomyocytes. As shown in Table.1, compared with the control, doxorubicin treated cells possessed significantly less GSH levels and activities of SOD and CAT, whereas NARG (10, 20 and 40 μM) pretreatment could effectively prevent doxorubicin-induced these reduction. The intracellular GSH level was significantly increased after treatment with NARG compared to the doxorubicin group. Incubation

of H9C2 cells with NARG resulted in significant upregulation in SOD and CAT activities when compared to the doxorubicin group. Furthermore, pretreatment with NARG (10, 20 and 40 μM) could significantly decrease the level of MDA compared to the doxorubicin group.

3.3. Effects of NARG on the mRNA expression of GCLC and GCLM

To explore the cellular mechanism responsible for the NARG-induced increase in cellular GSH levels, we measured the effect of NARG in the mRNA expression of GCL, the rate-limiting enzyme involved in the de novo GSH synthesis. As shown in Fig.2A and Fig.2B, the addition of doxorubicin to the cells induced a drop in the mRNA expression of GCLC and GCLM. NARG pretreatment (10, 20, and 40 μM) significantly increased the mRNA expression of GCLC and GCLM.

3.4. Effects of NARG on the expression of NQO1 in mRNA and protein level

Although SOD and CAT could provide a first line of defense and convert ROS into less noxious compounds, other endogenous enzymes such as HO-1 and NQO1 (Andreadi et al., 2006; Siegel et al., 2004) are still necessary to limit ROS-mediated damages to biological macromolecules. As shown in Fig.2C and Fig.3A, the addition of doxorubicin to the cells induced a drop in the mRNA expression of NQO1. NARG pretreatment (10, 20, and 40 μM) significantly increased the mRNA expression of NQO1. Western blot analysis showed that NARG (10, 20, and 40 μM) pretreatment significantly upregulated the protein level of NQO1.

3.5. Effects of NARG on the protein level of ERK1/2 and Nrf2

In order to investigate the cellular mechanism responsible for the NARG-induced increase in endogenous antioxidant enzymes, we measured the effects of NARG on Nrf2 nuclear translocation and levels of phosphorylated ERK1/2 by using Western blot analysis. As shown in Fig.3B and Fig.3C, NARG (10, 20, and 40 μM) pretreatment significantly enhanced Nrf2 nuclear translocation and upregulated the protein level of phosphorylated ERK1/2.

4. Discussion

Doxorubicin is a widely used chemotherapeutic agent for treatment of various cancers. However, the clinical use of doxorubicin has been limited due to the severe cardiomyopathy or congestive heart failure which has been shown to be correlated with cumulative doses of the drug when administered (Singal and Iliskovic, 1998; Jones et al., 2006). Although doxorubicin-induced injury appears to be multifactorial, one of the possible mechanisms is cellular damage mediated by generation of ROS and apoptosis (Keefe, 2001), which has been suggested to play an important role in the development of cardiomyopathy and congestive heart failure (Outomuro et al., 2007). Due to the successful action of doxorubicin as a chemotherapic agent, several strategies have been tried to prevent/attenuate the side effects of doxorubicin. Treatment with antioxidants or natural phenolic compounds such as flavonoids has been found to protect against doxorubicin-induced cardiotoxicity via radical-scavenging (Sadzuka et al., 1997), iron-chelating (van Acker et al., 1998) and activating endogenous defense systems (Bast et al., 2007; Crespo et al., 2008). Flavonoids can perform metal chelating because of the presence of hydroxyl groups attached to the ring structure such as 3', 4'-dihydroxy group, 4-carbonyl group, and 5-hydroxy arrangements (Cheng and Breen, 2000; Heim et al., 2002). Interestingly, other study has demonstrated that the ability of flavonoids to inhibit lipid peroxidation does not seem to correlate with hydroxyl radical scavenging potency (Kaiserová et al., 2007). Furthermore, no direct correlation was found between the iron-chelating and/or antioxidant effect and the cardioprotective effects (Kaiserová et al., 2007). This

suggests that cardioprotective effects of flavonoids against doxorubicin-induced toxicity probably are mediated via different mechanisms and modulation of antioxidant enzymes and activating endogenous defense systems may be also important in their protective effects.

NARG, a major active flavonoid from *D. rupestre*, had been demonstrated that it did not cause any apparent cytotoxicity at each of low doses alone. NARG (10, 20, and 40 μM) pretreatment had a significant protective effect against doxorubicin-induced decrease in cell viability and apoptosis in a dose-dependent manner at low doses (Han et al., 2008). Furthermore, the protective effects against doxorubicin cardiotoxicity in 24h were better than other term as shown in Fig.1.

GSH is the most abundant cellular thiol and an important protective antioxidant against oxidative stress that plays a role in numerous detoxifications, bioreduction, and conjugation reactions (Anderson, 1998). SOD and CAT are two key enzymes in the detoxification of ROS, which could convert ROS into less noxious compounds. They have been suggested to be protective against various forms of oxidative cardiovascular injuries (Rohde et al., 2005). The results of this study demonstrated that pretreatment with NARG could elevate the activities of SOD and CAT and decrease the level of MDA. The intracellular GSH level was significantly increased after treatment with NARG compared to the doxorubicin group. GSH is a tripeptide that is synthesized by sequential reactions. Glutamate and cysteine are first ligated by GCL to form c-glutamylcysteine in the rate-limiting step (Fraser et al., 2003). C-glutamylcysteine is then joined to glycine by glutathione synthetase to form

GSH. GCL holoenzyme is a heterodimer consisting of GCLC and GCLM. Whereas GCLC itself is capable of synthesizing c-glutamylcysteine, interaction with GCLM improves its catalytic properties (Zheng et al., 2007). In this report, the expression of both GCLC and GCLM mRNA was induced by NARG, which might increase the activity of GCL and stimulate the de novo synthesis of GSH, ultimately leading to the attenuation of oxidative stress.

SOD and CAT can provide a first line of defense against superoxide and hydrogen peroxides. They are of enormous importance in limiting ROS-mediated damages to biological macromolecules, but they are not able to be 100% effective because certain compounds generated by the interaction of ROS with macromolecules are highly reactive. It is then mandatory to detoxify these secondary products in order to prevent further intracellular damage, degradation of cell components and eventual cell death. This second line of defense against ROS is provided by phase 2 metabolizing enzymes such as HO-1 and NQO1, which play a major role in the cellular detoxification of oxidative damaging (Andreadi et al., 2006; Siegel et al., 2004). HO-1 is the rate-limiting enzyme in the conversion of heme into biliverdin, releasing free iron and carbon monoxide. Experimental evidence suggests that induction of the HO-1 is an important endogenous mechanism for cytoprotection and is widely recognized as an effective cellular strategy to counteract a variety of stressful events (Kirkby and Adin, 2006). HO-1 expression by pharmacological modulator may represent a novel target for therapeutic intervention. Particularly, the identification of a non-cytotoxic inducer of HO-1 may maximize the intrinsic

antioxidant potential of cells. Several antioxidants from plant origins have been reported to induce HO-1 expression in a variety of cells (Andreadi et al., 2006; Park et al., 2007). NQO1 catalyzes the two-electron reduction of electrophilic quinone compounds, thus limiting the formation of semiquinone radicals through one-electron reduction and the subsequent generation of ROS (Li et al., 2006; Ross et al., 2000). Moreover, NQO1 has been suggested to be able to maintain the cellular levels of biological antioxidants involved in the detoxification of ROS (Siegel et al., 2004). Therefore, the coordinated actions of the above cellular antioxidants and phase 2 enzymes ensure effective detoxification of ROS and electrophilic species. Our experiments showed that pretreatment with NARG, levels of mRNA and protein of HO-1 (Han et al., 2008) and NQO1 were found to be greatly elevated in cardiomyocytes. The induction of HO-1 and NQO1 may contribute to their protective effects against the cardiotoxicity of doxorubicin. In this context, simultaneous induction of a panel of antioxidants and phase 2 enzymes by NARG can inhibit lipid peroxidation and protect against doxorubicin-induced cardiotoxicity.

The transcription factor Nrf2, a member of the Cap'n'collar family of basic leucine transcription factors, plays an essential role in the antioxidant response element (ARE) mediated expression of phase II detoxifying enzymes and stress inducible genes. Nrf2 can regulate the basal and inducible expression of numerous detoxifying and antioxidant genes via its activation and nuclear translocation (Cavin et al., 2008). Cytosolic Nrf2 are known to be maintained in low levels due to the function of the Keap1-proteasomal system (Motohashi and Yamamoto, 2004).

Furthermore, many studies have suggested that ERK is a central pathway involved in Nrf2 activation and translocation for highly specialized protein synthesis including HO-1 and GSH (Andreadi et al., 2006, Calabrese et al., 2005; Jeong et al., 2006; Burdo et al., 2008). The ERK cascade appeared to facilitate nuclear translocation of Nrf2, leading to protection of this protein from cytosolic degradation. The ERK1/2 pathway is known to contribute to cell survival acting as an anti-apoptotic factor in response to oxidative stress (Sugden and Clerk, 2006). To investigate NARG mediated the effect on Nrf2 through the ERK signaling pathway, we determined the effects of NARG on Nrf2 nuclear translocation and levels of phosphorylated ERK1/2 following treatment of NARG. The levels of phosphorylated ERK1/2 were found to elevate following treatment with NARG (Fig. 3B). Phosphorylation cascade via ERK1/2 is required for nuclear accumulation of Nrf2 and subsequent transactivation of its target genes following treatment with NAGR in H9C2 cells. We have demonstrated that NAGR could protect against doxorubicin-induced apoptosis partly by induction of HO-1 (Han et al., 2008). Taken together, it is possible to speculate that NARG from *D. rupestre* Hance might be useful for protecting against the cardiotoxicity of doxorubicin by the induction endogenous antioxidant enzymes and antiapoptopic properties.

Partly due to their radical-scavenging and iron-chelating properties, flavonoids can be considered as potential protectors against chronic cardiotoxicity caused by doxorubicin. Flavonoids may vary significantly in their antioxidant activities depending on slight variations in their structural features. It has been reported that the

chelation property of flavonoids for Fe and Cu ions are derived primarily from the presence of 3-hydroxypyran-4-one and secondarily from the presence of 5-hydroxy and 4-carbonyl groups in the C ring, rather than the 3', 4'-dihydroxy group in the B ring (Paganga et al., 1996). NARG, containing 5-hydroxy and 4-carbonyl groups in the C ring, decreased the level of MDA and may interact with Cu and Fe ions. The Fe-chelating and direct antioxidant effects of NARG are the next target of our scientific effort.

In conclusion, this study demonstrated for the first time that a number of endogenous antioxidant enzymes in cultured cardiomyocytes can be simultaneously induced by low micromolar concentrations of NARG and the upregulation of cellular defenses is accompanied by a markedly increased resistance to cardiomyocyte injury elicited by doxorubicin. As mentioned above, efficient detoxification of ROS requires the coordinate actions of various cellular antioxidants enzymes. Accordingly, simultaneous induction of a scope of key cellular antioxidant enzymes by NARG in cardiomyocytes appears to be a promising strategy for protecting against oxidative injury and may be an important mechanism underlying the protective effects of NARG observed in doxorubicin-induced cardiotoxicity. Furthermore, our present study indicates that ERK1/2 is the common signal transducing pathway in response to NARG to enhance nuclear translocation of Nrf2, leading to an elevation of expression of endogenous antioxidant enzymes.

Acknowledgements

This work was supported by the National Natural Science Foundation (No. 30472072) and the Natural Science Foundation of Shandong Province (No.Y2007C098) of P.R.China. The Authors wish to thank Prof. Xueqin Gao (Shandong Medicinal and Biotechnology Center, Shandong Academy of Medical Sciences, China) for her skillful technical help.

References

Anderson, M.E., 1998. Glutathione: an overview of biosynthesis and modulation. Chem. Biol. Interact. 111-112, 1-14.

Andreadi, C.K., Howells, L.M., Atherfold, P.A., Manson, M.M., 2006. Involvement of Nrf2, p38, B-Raf, and nuclear factor-kappaB, but not phosphatidylinositol 3-kinase, in induction of hemeoxygenase-1 by dietary polyphenols. Mol. Pharmacol. 69, 1033-1040.

Bast, A., Kaiserová, H., den Hartog, G.J., Haenen, G.R., van der Vijgh, W.J., 2007. Protectors against doxorubicin-induced cardiotoxicity: flavonoids. Cell Biol. Toxicol. 23, 39-47.

Burdo, J., Schubert, D., Maher, P., 2008. Glutathione production is regulated via distinct pathways in stressed and non-stressed cortical neurons. Brain Res. 1189, 12-22.

Calabrese, V., Ravagna, A., Colombrita, C., Scapagnini, G., Guagliano, E., Calvani, M., Butterfield, D.A., Giuffrida Stella, A.M., 2005. Acetylcarnitine induces heme oxygenase in rat astrocytes and protects against oxidative stress: involvement of the transcription factor Nrf2. J. Neurosci, Res. 79, 509-521.

Cavin, C., Marin-Kuan, M., Langouët, S., Bezençon, C., Guignard, G., Verguet, C., Piguet, D., Holzhäuser, D., Cornaz, R., Schilter, B., 2008. Induction of Nrf2-mediated cellular defenses and alteration of phase I activities as mechanisms of chemoprotective effects of coffee in the liver. Food Chem.Toxicol. 46, 1239-1248.

Cheng, I.F., Breen, K., 2000. On the ability of four flavonoids, baicilein, luteolin, naringenin, and quercetin, to suppress the Fenton reaction of the iron-ATP complex, Biometals. 13, 77-83.

Chlopcíková, S., Psotová, J., Miketová, P., Simánek, V., 2004. Chemoprotective effect of plant phenolics against anthracycline-induced toxicity on rat cardiomyocytes. Part I. Silymarin and its flavonolignans. Phytother. Res. 18, 107-110.

Crespo, I., García-Mediavilla, M.V., Almar, M., González, P., Tuñón, M.J., Sánchez-Campos, S., González-Gallego, J., 2008. Differential effects of dietary flavonoids on reactive oxygen and nitrogen species generation and changes in antioxidant enzyme expression induced by proinflammatory cytokines in Chang Liver cells. Food Chem.Toxicol. 46, 1555-1569.

Cullinan, S.B., Zhang, D., Hannink, M., Arvisais, E., Kaufman, R.J., Diehl, J.A., 2003. Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. Mol. Cell Biol. 23, 7198-7209.

Dignam, J.D., Lebovitz, R.M., Roeder, R.G., 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic. Acids Res.11, 1475-1489.

Du, Y., Guo, H., Lou, H., 2007. Grape seed polyphenols protect cardiac cells from apoptosis via induction of endogenous antioxidant enzymes. J. Agric. Food Chem. 55, 1695-1701.

Fraser, J.A., Kansagra, P., Kotecki, C., Saunders, R.D., McLellan, L.I., 2003. The modifier subunit of Drosophila glutamate-cysteine ligase regulates catalytic

activity by covalent and noncovalent interactions and influences glutathione homeostasis in vivo. J. Biol. Chem. 278, 46369-46377.

Han, X., Ren, D., Fan, P., Shen, T., Lou, H., 2008. Protective effects of naringenin-7-O-glucoside on doxorubicin-induced apoptosis in H9C2 cells. Eur. J. Pharmacol. 581, 47-53.

Heim, K.E., Tagliaferro, A.R., Bobilya, D.J., 2002. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. J.Nutr.Biochem.13, 572-584.

Jeong, W.S., Jun, M., Kong, A.N., 2006. Nrf2: a potential molecular target for cancer chemoprevention by natural compounds. Antioxid. Redox Signal. 8, 99-106.

Jones, R.L., Swanton, C., Ewer, M.S., 2006. Anthracycline cardiotoxicity. Expert. Opin. Drug Saf. 5, 791-809.

Kaiserová, H., Simůnek, T., van der Vijgh, W.J., Bast, A., Kvasnicková, E., 2007. Flavonoids as protectors against doxorubicin cardiotoxicity: Role of iron chelation, antioxidant activity and inhibition of carbonyl reductase. Biochim. Biophys. Acta 1772, 1065-1074.

Kang, K.A., Lee, K.H., Park, J.W., Lee, N.H., Na, H.K, Surh, Y.J., You, H.J., Chung, M.H., Hyun, J.W., 2007. Triphlorethol-A induces heme oxygenase-1 via activation of ERK and NF-E2 related factor 2 transcription factor. FEBS Lett. 581, 2000-2008.

Kang, Y.J., Chen, Y., Epstein, P.N., 1996. Suppression of doxorubicin cardiotoxicity by overexpression of catalase in the heart of transgenic mice. J. Biol. Chem. 271, 12610-12616.

Keefe, D.L., 2001.Anthracycline-induced cardiomyopathy. Semin. Oncol. 4, 2-7.

Keizer, H.G., Pinedo, H.M., Schuurhuis, G.J., Joenje, H., 1990. Doxorubicin (adriamycin): a critical review of free radical-dependent mechanisms of cytotoxicity. Pharmacol. Ther. 47, 219-231.

Kirkby, K.A., Adin, C.A., 2006. Products of heme oxygenase and their potential therapeutic applications. Am. J. Physiol. Renal Physiol. 290, F563-F571.

Li, Y., Cao, Z., Zhu, H., 2006. Upregulation of endogenous antioxidants and phase 2 enzymes by the red wine polyphenol, resveratrol in cultured aortic smooth muscle cells leads to cytoprotection against oxidative and electrophilic stress. Pharmacol. Res. 53, 6-15.

Lu, S.C., 1999. Regulation of hepatic glutathione synthesis: current concepts and controversies. FASEB J. 13, 1169-1183.

Manandhar, S., Cho, J.M., Kim, J.A., Kensler, T.W., Kwak, M.K., 2007. Induction of Nrf2-regulated genes by 3H-1, 2-dithiole-3-thione through the ERK signaling pathway in murine keratinocytes. Eur. J. Pharmacol. 577, 17-27.

Motohashi, H., Yamamoto, M., 2004. Nrf2-Keap1 defines a physiologically important stress response mechanism. Trends Mol. Med. 10, 549-557.

Outomuro, D., Grana, D.R., Azzato, F., Milei, J., 2007. Adriamycin-induced myocardial toxicity: new solutions for an old problem? Int. J. Cardiol. 117, 6-15.

Paganga, G., Al-Hashim, H., Khodr, H., Scott, B.C., Aruoma, O.I., Hider, R.C., Halliwell, B., Rice-Evans, C.A., 1996. Mechanisms of antioxidant activities of quercetin and catechin. Redox. Rep. 2, 359-364.

Park, S.H., Jang, J.H., Li, M.H., Na, H.K., Cha, Y.N., Surh, Y.J., 2007. Nrf2-mediated heme oxygenase-1 induction confers adaptive survival response to tetrahydropapaveroline-induced oxidative PC12 cell death. Antioxid. Redox Signal. 9, 2075-2086.

Ren, D.M., Guo, H.F., Wang, S.Q., Lou, H.X., 2007. Separation and structure determination of two diastereomeric pairs of enantiomers from Dracocephalum rupestre by high-performance liquid chromatography with circular dichroism detection. J. Chromatogr. A. 1161, 334-337.

Ren, D.M., Guo, H.F., Yu, W.T., Wang, S.Q., Ji, M., Lou, H.X., 2008. Stereochemistry of flavonoidal alkaloids from Dracocephalum rupestre. Phytochemistry. 69, 1425-1433.

Ren, D., Lou, H., Ji, M., 2005. Studies on constituents of Dracocephalum rupestra. Chin. Pharm. J. 40, 1695-1696.

Ren, D.M., Lou, H.X., Ma, B., Ji, M., 2003. Determination of naringenin-7-O-glucoside and eriodictyol-7-O-glucoside in Dracocephalum rupestra and its different parts by HPLC. Nat. Prod. Res. Dev. 15, 229-231.

Rohde, L.E., Belló-Klein, A., Pereira, R.P., Mazzotti, N.G., Geib, G., Weber, C., Silva, L.F., Clausell, N., 2005. Superoxide dismutase activity in adriamycin-induced cardiotoxicity in humans: a potential novel tool for risk stratification. J. Card. Fail 11, 220-226.

Sadzuka, Y., Sugiyama, T., Shimoi, K., Kinae, N., Hirota, S., 1997. Protective effect of flavonoids on doxorubicininduced cardiotoxicity. Toxicol. Lett. 92, 1-7.

Siegel, D., Gustafson, D.L., Dehn, D.L., Han, J.Y., Boonchoong, P., Berliner, L.J., Ross, D., 2004. NAD(P)H:quinone oxidoreductase 1: role as a superoxide scavenger. Mol. Pharmacol. 65, 1238-1247.

Singal, P.K., Iliskovic, N., 1998. Doxorubicin-induced cardiomyopathy. N. Engl. J. Med. 339, 900-905.

Sugden, P.H., Clerk, A., 2006. Oxidative stress and growth-regulating intracellular signaling pathways in cardiac myocytes. Antioxid. Redox Signal. 8, 2111-2124.

Takemura, G., Fujiwara, H., 2007. Doxorubicin-induced cardiomyopathy from the cardiotoxic mechanisms to management. Prog. Cardiovasc. Dis. 49, 330-352.

van Acker, S.A., van Balen, G.P., van den Berg, D.J., Bast, A., van der Vijgh, W.J., 1998. Influence of iron chelation on the antioxidant activity of flavonoids. Biochem. Pharmacol. 56, 935-943.

Wu, Z.Y., Li, X.W., 1977. Flora Reipularis Sinicase (Zhongguo Zhiwu Zhi). Science Press Beijing 65, 378-380.

Yao, P., Nussler, A., Liu, L., Hao, L., Song, F., Schirmeier, A., Nussler, N., 2007. Quercetin protects human hepatocytes from ethanol-derived oxidative stress by inducing heme oxygenase-1 via the MAPK/Nrf2 pathways. J. Hepatol. 47, 253-261.

Yen, H.C., Oberley, T.D., Vichitbandha, S., Ho, Y.S., St Clair, D.K., 1996. The protective role of manganese superoxide dismutase against adriamycin-induced acute cardiac toxicity in transgenic mice. J. Clin. Invest. 98, 1253-1260.

Young, R.C., Ozols, R.F., Myers, C.E., 1981.The anthracycline antineoplastic drugs.

N. Engl. J. Med. 305,139-153.

Zheng, S., Yumei, F., Chen, A., 2007. De novo synthesis of glutathione is a prerequisite for curcumin to inhibit hepatic stellate cell (HSC) activation. Free Radic. Biol. Med. 43, 444-453.

Legends for figures

Fig. 1. Effects of NARG on doxorubicin-mediated cytotoxicity in H9c2 cells. Cells were incubated with NARG (10, 20, 40, 80 μM) and quercetin (20 μM) for 12, 24, 48, 72 h, followed by incubation with doxorubicin (10 μM) for another 24 h. After this incubation, cell viability was determined with the MTT assay. Nor: normal cells; Dox: doxorubicin; Que: quercetin. Values represented means ±S.D. (n=6).

Fig. 2. Effects of NARG on GCLC, GCLM, and NQO1 mRNA expression in H9c2 cells. After twenty-four hours of treatment without (normal) or with NARG (10, 20, and 40 μM) or quercetin (20 μM), cardiomyocytes were incubated with doxorubicin (10 μM) for 24 h. Normalization relative to GAPDH was performed. Nor: normal cells; Dox: doxorubicin; Mar: the molecular weight markers; Que: quercetin. Results presented in bar graphs are the mean ±S.D. of four separate experiments. $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with normal and $^{*}P<0.05$, $^{**}P<0.01$ compared with doxorubicin.

Fig. 3. Effects of NARG on the protein level of NQO1, ERK1/2 and Nrf2 in H9c2 cells. After twenty-four hours of treatment without (normal) or with NARG (10, 20, and 40 μM) or quercetin (20 μM), cardiomyocytes were incubated with doxorubicin (10 μM) for 24 h. Cell lysates were probed with antibodies against NQO1, ERK1/2, and Nrf2 respectively. Immunoblots shown were representative of four experiments. Nor: normal cells; Dox: doxorubicin; Que: quercetin.

Table 1. Effects of NARG on cellular GSH content, MDA level, and activities of cellular antioxidant enzymes (mean±S.D., n=4)

Group	Final concentration (μM)	GSH(mg/mg protein)	SOD(U/mg protein)	CAT(U/mg protein)	MDA(nmol/mg protein)
Normal	equal volume	3.49±0.36	89.58±8.62	82.59±5.79	1.15±0.18
Doxorubicin	10	1.52±0.23$^{\#\#}$	54.35±7.03$^{\#\#}$	40.07±7.34$^{\#\#}$	2.23±0.14$^{\#\#}$
NARG	10	2.48±0.47*	68.02±7.48*	58.37±7.30*	1.85±0.09*
NARG	20	3.09±0.38**	81.64±6.18**	71.47±6.51**	1.52±0.11**
NARG	40	2.79±0.48**	71.95±8.33*	67.54±5.76**	1.68±0.17*
Quercetin	20	2.63±0.59*	72.39±5.46**	66.22±5.97**	1.52±0.07*

$^{\#\#}$P<0.01 vs. normal, *P<0.05, **P<0.01 vs. doxorubicin.

Fig.1

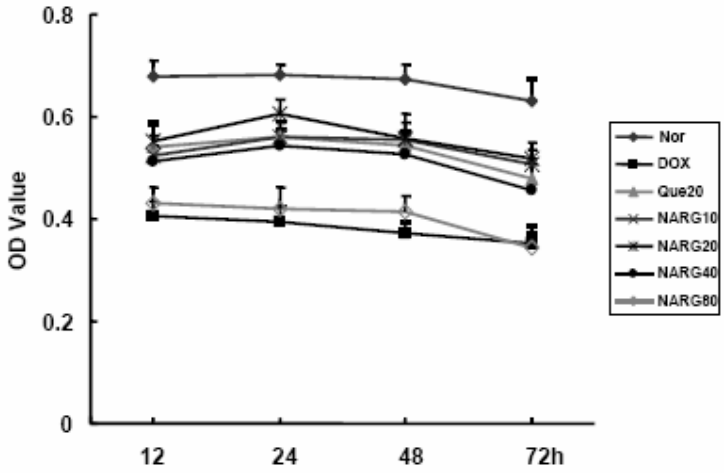

Fig.2A

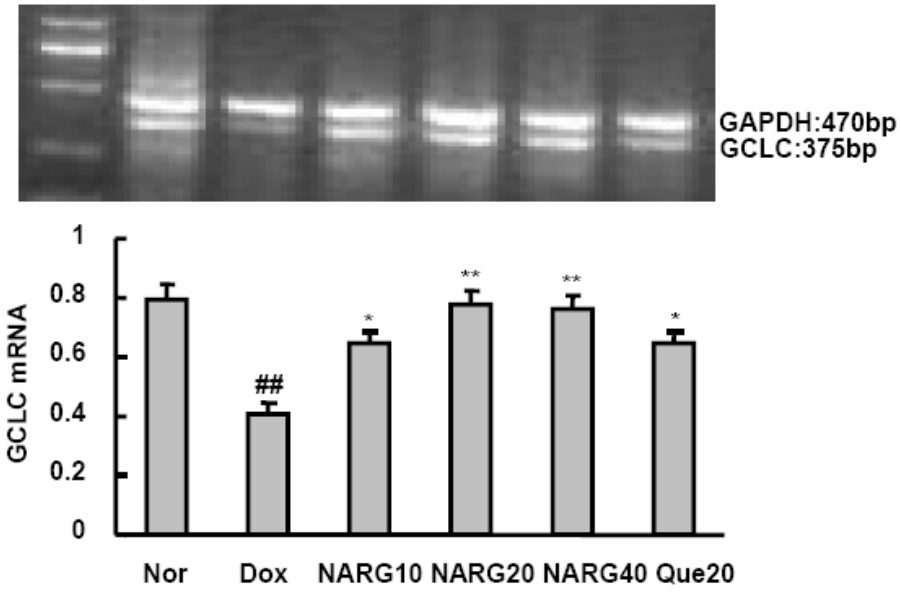

Fig.2B

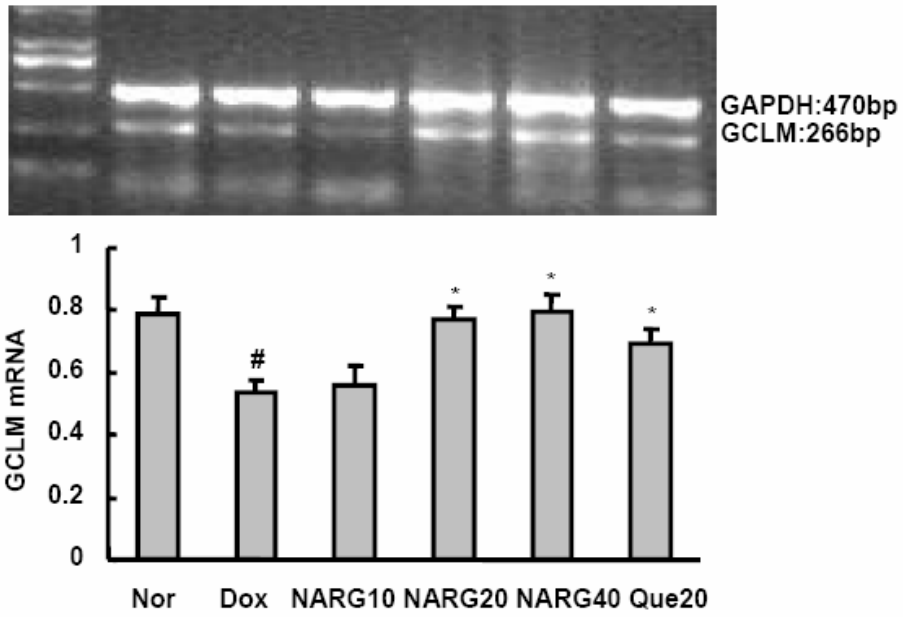

Fig.2C

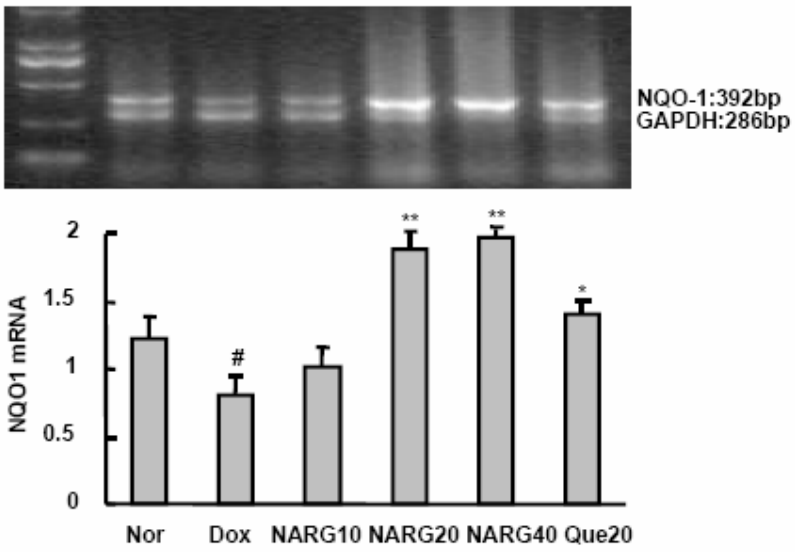

Fig.3A

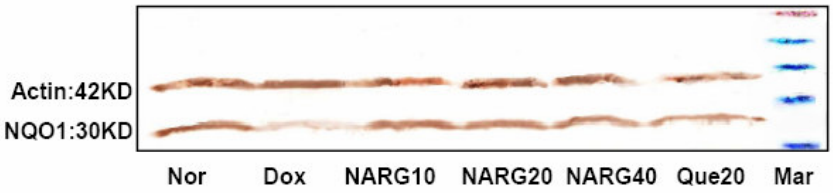

Fig.3B

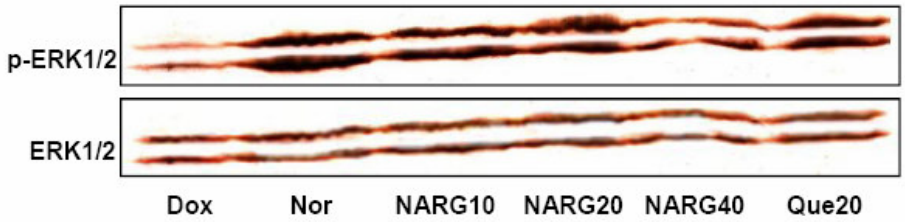

Fig.3C