Geranylgeranylacetone Blocks Doxorubicin-Induced Cardiac Toxicity and Reduces Cancer Cell Growth and Invasion through RHO Pathway Inhibition


Abstract

Doxorubicin is a widely used chemotherapy for solid tumors and hematologic malignancies, but its use is limited due to cardiotoxicity. Geranylgeranylacetone (GGA), an antiulcer agent used in Japan for 30 years, has no significant adverse effects, and unexpectedly reduces ovarian cancer progression in mice. Because GGA reduces oxidative stress in brain and heart, we hypothesized that GGA would prevent oxidative stress of doxorubicin cardiac toxicity and improve doxorubicin’s chemotherapeutic effects. Nude mice implanted with MDA-MB-231 breast cancer cells were studied after chronic treatment with doxorubicin, doxorubicin/GGA, GGA, or saline. Transthoracic echocardiography was used to monitor systolic heart function and xenografts evaluated. Mice were euthanized and cardiac tissue evaluated for reactive oxygen species generation, TUNEL assay, and RHO/ROCK pathway analysis. Tumor metastases were evaluated in lung sections. In vitro studies using Boyden chambers were performed to evaluate GGA effects on RHO pathway activator lysophosphatidic acid (LPA)–induced motility and invasion. We found that GGA reduced doxorubicin cardiac toxicity, preserved cardiac function, prevented TUNEL-positive cardiac cell death, and reduced doxorubicin-induced oxidant production in a nitric oxide synthase–dependent and independent manner. GGA also reduced heart doxorubicin-induced ROCK1 cleavage. Remarkably, in xenograft-implanted mice, combined GGA/doxorubicin treatment decreased tumor growth more effectively than doxorubicin treatment alone. As evidence of antitumor effect, GGA inhibited LPA-induced motility and invasion by MDA-MB-231 cells. These anti-invasive effects of GGA were suppressed by geranylgeraniol suggesting GGA inhibits RHO pathway through blocking geranylation. Thus, GGA protects the heart from doxorubicin chemotherapy-induced injury and improves anticancer efficacy of doxorubicin in breast cancer.

Introduction

Doxorubicin (Adriamycin) is one of the most widely used anticancer agents and it is currently a first-choice chemotherapeutic drug for the treatment of primary, recurrent, and metastatic breast cancer (1–4). Unfortunately, the use of doxorubicin in breast cancer chemotherapy is frequently limited due to its severe cumulative cardiac toxicity (5). Clinical signs of cardiac toxicity may occur during weeks, months, or even years after chemotherapy. Strategies that reduce cardiac toxicity could potentially allow higher dosages of doxorubicin to be used with an improved long-term survival in patients with improved quality of life.

A prevention strategy that reduces oxidative stress, a common mechanism of doxorubicin cardiac toxicity (5), led us to consider potential benefits of geranylgeranacetone (GGA), an acyclic polyisoprenoid (Selbex), which has been used since 1984 as an antiulcer drug in Japan with no adverse reactions (6, 7). In multiple animal models of ischemia and reperfusion, GGA prevents oxidative stress in liver, heart, brain, kidney, and retina (8–14). A second mechanism linked to GGA’s mechanism of action is its ability to inhibit the activation of the RHO family of GTPases through reduction of protein geranylgeranylation, a posttranslational modification required for RHO family membrane targeting and signaling (15, 16). Targeting the RHO/ROCK pathway with specific inhibitors is beneficial in a number of cardiovascular diseases, including hypertension, angina, ischemia-reperfusion injury, cardiac hypertrophy, chronic heart failure (17–23), all conditions with some level of oxidative stress.
Interestingly, this molecular activity of GGA might also make it useful as a cancer therapeutic because RHO GTPases, and their downstream target, RHO-associated kinases (ROCK), are implicated in a variety of physiologic functions associated with cancer-related changes in the actin cytoskeletal assembly, such as cell adhesion, motility, and migration (24).

Because GGA may reduce a variety of molecular functions important for cancer cell growth and migration, and at the same time, may reduce oxidative stress in the heart, we undertook an investigation to determine whether GGA can inhibit the cardiac adverse effects of doxorubicin while simultaneously inhibiting cancer cell growth. Specifically, we developed a breast cancer mouse model of chronic doxorubicin injury to the heart and investigated cellular and molecular responses to various treatments involving GGA and doxorubicin.

Materials and Methods

Reagents and materials
GGA (Lot # 17022802) was provided by Eisai Co Ltd.. GGA was dissolved in 100% ethanol for in vitro studies. 1-Oleoyl LPA (18:1 LPA; Cat. # 857130) was obtained from Avanti Polar Lipids. Doxorubicin (Cat. # NDC 55390-238-01) was from Bedford Laboratories. Matrigel (Cat. # 354234) and cell culture inserts (Cat. # 353182) for invasion and motility assay were obtained from BD Biosciences. RHO-associated coiled-coil protein kinase 1 (ROCK1) primary antibody (Cat. # A300-457A) was from Bethyl Laboratories. RPMI 1640 (Cat. # 11835-030) was from Gibco (Life Technologies). FBS (Cat. # SH30088.03) was from HyClone (Thermo Fisher Scientific). penicillin–streptomycin solution (Cat. # 30-001-CI) was from Cellgro.

Cell line
The human breast cancer cell line MDA-MB-231 (ATCC) was obtained in 2010, and was used both for xenograft in vivo studies and in cell culture for in vitro experiments. Cells were grown in RPMI medium 1640, supplemented with 10% (v/v) FBS, penicillin (10 U/mL)–streptomycin (10 U/mL) at 37°C in humidified 5% CO₂ atmosphere.

Animal studies
Five- to six-week-old female athymic nude-Foxn1nu mice (Harlan Laboratories) were exposed to 500 cGy of radiation. The next day the mice were anesthetized and an incision was made near the right flank to expose the mammary fat pad. A total of 1 × 10⁶ cells (MDA-MB-231 breast cancer cells) were injected using a Hamilton syringe into the mammary fat pad. Tumor development was followed and when xenografts averaged 4 mm in each dimension (length, width, and height, approximately 3 weeks after the implantation), mice with comparable sized tumors were randomly divided between the four treatment groups: DOX 9 mg/kg, DOX 9 mg/kg and GGA, GGA, and saline. Doxorubicin has been reported to induce cardiotoxicity in a wide range of dosages (4 to 25 mg/kg; refs. 25–29); we selected an intermediate dosage, which would allow for gradual cardiotoxicity development with multiple doxorubicin injections (25, 26). Doxorubicin was administered via tail vein injection every 2 weeks for a total of 4 injections. GGA treatment (1 mg/g body weight) was given 48 hour before doxorubicin per os method (pipette). GGA was previously shown to elicit a protective response when given 24 to 48 hours before stressor (30–35) in dosages from 200 to 1,000 mg/kg (orally or intraperitoneally; refs. 34–36).

Tumor progression was evaluated by palpation and tumor size measurements with calipers. Tumor volumes were calculated by the following formula: (L × W × H; ref. 37), in which L is the length, W is the width, and H is the height. All mice were housed under a 12-hour light-dark cycle with free access to food and water. This study was performed in accordance with the “Guide for the Care and Use of Laboratory Animals” (2011) of the NIH. The protocol was approved by the Animal Care and Use Committee of the Johns Hopkins Medical Institutions (Baltimore, MD; Animal Welfare Assurance # A-3273-01).

Echocardiography
Transthoracic echocardiography was performed on conscious mice using Acuson Sequoia C256 ultrasound machine (Siemens Corps) equipped with the 15-MHz linear array transducer. The mouse heart was imaged in a two-dimensional mode followed by M-mode using the parasternal short axis view at a sweep speed of 200 mm/sec. Measurements were acquired using the leading-edge method, according to the American Echocardiography Society guidelines (38). Left ventricle wall thickness and left ventricle chamber dimensions were acquired during the end diastolic and end systolic phase, including interventricular septum (IVSD), left ventricular posterior wall thickness (PWTED), left ventricular end diastolic dimension (LVEDD), and left ventricular end systolic dimension (LVESD). Three to five values for each measurement were acquired and averaged for evaluation. The LVEDD and LVESD were used to derive fractional shortening (FS) to measure left ventricular performance by the following equation: FS (%) = [(LVEDD – LVESD)/LVEDD] × 100.

Necropsy
Mice were euthanized (CO₂) and received postmortem examination and weighed. The hearts were immediately excised, rinsed in cold PBS, weighed, and sectioned at the level of attachment of papillary muscles. Sections of left ventricle, right ventricle, and septum were frozen in liquid nitrogen for molecular studies. The remainder of the heart was fixed in 10% formalin for histopathology.

Western blot
The tissue was homogenized in radioimmunoprecipitation assay buffer [25 mmol/L Tris-HCl; 150 mmol/L NaCl; 1% NP-40; 1% sodium deoxycholate; 0.1% SDS; 1×
proteinase inhibitor (Roche; cat.\#11697498001); 1 × phosphatase inhibitors (Sigma; cat.\#P5726 and P0044) and centrifuged at 12,000 \( \times g \) at 4 °C for 15 minutes. Protein measurements were performed using a Bio-Rad protein assay (BioRad); equal amounts of total protein (40 \( \mu g \)) were used per lane. Proteins were denatured in SDS gel-loading buffer (0.125 mol/L Tris, pH 6.8, 20% glycerol, 5% mercaptoethanol, 4% SDS, and 0.002% bromophenol blue) at 100 °C for 5 minutes and separated on a 4% to 12% SDS-PAGE gradient gel using an XCell SureLock Mini-Cell Electrophoresis System with Kaleidoscope-pre-stained molecular weight standards (Bio-Rad). After electrophoresis, the proteins were transferred to a poly-vinylidene difluoride membrane. Blots were blocked with 5% nonfat milk in Tris Buffered Saline with 0.1% Tween 20 (TBST; 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, and 0.1% Tween 20) and probed with primary antibody (diluted in 5% nonfat milk-TBST). Immunoblots were processed with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G; bound antibodies were detected using a Western blot chemiluminescence reagent kit (Pierce).

**Histology**

Histopathology was assessed in each treatment group as described previously (39). The hearts were fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned at a thickness of 5 \( \mu m \), and stained with hematoxylin–eosin (H&E). Lungs were inflated with 10% formalin after euthanasia, allowed to fix for 48 hours, embedded in paraffin, sectioned at a thickness of 5 \( \mu m \), and stained with H&E. Lung sections with 5 \( \mu m \) lobs were scanned by Aperio and evaluated for the presence of tumor metastases.

**Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling**

Staining was performed using DeadEnd Fluorometric TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) system (Promega) according to the manufacturer’s instructions, as described previously (40). Counterstaining with 4’,6-diamidino-2-phenylindole (DAPI) aided in the morphologic evaluation of normal and apoptotic nuclei, in which normal nuclei were stained as blue and apoptotic nuclei as green. The number of TUNEL-positive cells within a 2.5-mm\(^2\) field in left ventricle free wall was counted, and eight randomly selected fields per slide and five sections per hearts were averaged for statistical analysis.

**Measurement of myocardial superoxide generation**

Fresh-frozen left ventricular myocardium was homogenized on ice and sonicated. After centrifugation (30 seconds, 4,000 RPM), the supernatant was added to a lucigenin (5 \( \mu mol/L \)) solution containing NADPH (100 \( \mu mol/L \)). Superoxide generation was measured using lucigenin-enhanced chemiluminescence (Beckman LS6000IC) and corrected for the baseline value. Superoxide generation was expressed as counts per minute (cpm)/mg tissue. Nitric oxide synthase (NOS)–dependent superoxide generation was measured by adding L-NAME (100 \( \mu mol/L \)) to the solution (41).

**Invasion and motility assay**

Invasion studies were performed as described previously (16). MDA-MB-231 cells were plated in serum-free medium (2 × 10\(^4\)/well) onto 12-well Transwell plates containing Matrigel. Complete medium (10% FBS) was added into the bottom well and the cells were incubated at 37°C. Lysophosphatidic acid (LPA; 25 \( \mu mol/L \)) was applied to stimulate the invasion of the cancer cells through Matrigel and LPA-induced invasiveness was compared with the basal level of cell invasiveness. Seventy-two hours later, the number of cells in the bottom well was counted. Motility assays were performed similar to invasion assay, but no Matrigel was used.

**Cell viability/proliferation quantification with MTT assay**

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on the MTT conversion into formazan crystals by live cells, proportionally to mitochondrial activity, thus allowing to estimate the number of viable cells. MDA-MB-231 cells were plated in 96-well plates (0.3 × 10\(^4\)/well) and treated with specified concentrations of doxorubicin, GGA, or both drugs for 24 hours. MTT stock solution was prepared [5 mg of MTT (Sigma M-5655) to 1 mL of the growth media], further diluted 1:10 with growth media, and the cells were incubated in MTT solution for 2 to 3 hours, until visible formation of blue formazan crystals was confirmed under light microscope. After that, the formazan crystals were solubilized with MTT solubilization solution (10% Triton X-100, 0.1 N HCL in 200 mL isopropanol) and absorbance was read at 570 nm.

**Statistical methods**

GraphPad Prism software (GraphPad) was used to perform statistical analysis. After determining mean and SDs, the unpaired Student \( t \) test or one-way ANOVA were performed to compare two or three or more unrelated groups as appropriate, with a \( P \) value of <0.05 deemed significant.

**Results**

**GGA prevents cardiac dysfunction induced by doxorubicin**

To our knowledge, GGA has not been used previously in the setting of doxorubicin therapy. However, because GGA is protective in models of oxidative stress (8–14), we questioned whether GGA would be cardioprotective in mice with oxidative stress in the heart induced by doxorubicin. To evaluate cardiac function, echocardiography was performed in conscious mice from four treatment groups: saline control, GGA alone, doxorubicin (9 mg/kg) alone, and doxorubicin...
combined with GGA. We chose to use the dosing schedule, in which GGA treatment is initiated 48 hours before the oxidative stress, because a 48-hour pretreatment likely induces the induction of gene expression, and pretreatment is required for maximal protective effect (33, 34). Echocardiography of animals at 6 weeks after initiation of doxorubicin treatment demonstrated significantly decreased systolic function, as evidenced by lower FS percentage (Fig. 1A and B). Remarkably, however, pretreatment with GGA significantly protected animals from the doxorubicin-induced decrease in cardiac function. This protective effect of GGA was unlikely due to a direct stimulatory effect on cardiac function, because GGA treatment alone had no effect on systolic function compared with saline treatment. GGA did not have a significant effect on the weight loss seen with doxorubicin treatment (Fig. 1C).

GGA blocks doxorubicin-induced cell death in the heart

Extending these findings of GGA-induced protection of cardiac function to a cellular level, we investigated whether GGA can reduce doxorubicin-induced cell death in the heart, using TUNEL staining to evaluate heart sections. The percentage of TUNEL-positive nuclei in left ventricles was compared among four treatment groups: saline control,
GGA alone, doxorubicin (9 mg/kg) alone, and doxorubicin combined with GGA. We found that doxorubicin treatment resulted in significant increase in cell death in the hearts, whereas GGA pretreatment significantly reduced the doxorubicin-induced cell death (Fig. 2A and B), consistent with the protective effects seen with measurements of cell death in the hearts of the animals treated with doxorubicin was significantly higher than in controls \( (P = 0.0491) \). In the group that received combinatorial therapy, cell death was significantly lower than in the doxorubicin group \( (P = 0.0124) \). Cell death in the GGA group was not significantly different from the control group. Data, mean ± SD; \( n = 3 \) to 8 mice per group. \( * \), \( P < 0.05 \). C, representative H&E-stained heart sections from mice treated with saline, GGA, DOX, or combination of DOX and GGA. DOX induces fine vacuolization with cardiomyocytes cytoplasm and GGA/DOX combination hearts have fewer vacuoles. The presence of cytoplasmic vacuoles is used in human and animal studies to assess degree of cardiac toxicity \((65–67)\). D, Western blot of ROCK1. ROCK1 is cleaved in the hearts of doxorubicin-treated mice, but not in the hearts of mice treated both with doxorubicin and GGA. ROCK1 cleavage is further reduced in the hearts of GGA–treated mouse. ROCK1 full length = 160 kDa, cleaved ROCK1 = 130 kDa. E, densitometry of cleaved ROCK1 expressed as a fold change from the cleaved ROCK1 levels in saline–treated mouse hearts. Doxorubicin treatment significantly increased cleaved ROCK1 levels \( (P = 0.0054) \), whereas addition of GGA to doxorubicin therapy reduced ROCK1 cleavage \( (P = 0.0002) \). GGA treatment did not affect cleaved ROCK1 levels. Data, mean ± SD, \( n = 3 \) mice per group. \( ** \), \( P < 0.01 \); \( *** \), \( P < 0.001 \).
systolic function (Fig. 1) in corresponding treatment groups. H&E sections of heart from each treatment group reveal that GGA blocks the characteristic cytoplasmic vacuolization induced by doxorubicin (Fig. 2C).

**GGA blocks doxorubicin-induced ROCK1 cleavage in the heart**

GGA is known to inhibit RHO activation in cancer cells (15, 16), although the role of GGA on RHO inactivation and ROCK1 cleavage in the heart has not been previously addressed. Normally, ROCK1 is folded and auto-inhibited, but RHO–GTP binding prevents that folding and auto-inhibition, thus RHO activates ROCK1. ROCK1 cleavage occurs during apoptosis and is mediated by activated Caspase-3 (42). To explore the effects of GGA on the downstream effectors of RHO signaling, we evaluated the ROCK1 cleavage in all four treatment groups by Western blotting. The cleaved product was reported previously to be absent in normal hearts, whereas it is present in myocardium from heart failure patients (42). ROCK1 cleavage has been induced by doxorubicin in cell culture, yet it is not known if ROCK1 cleavage product is present in the myocardium after doxorubicin treatment in vivo. A representative blot shows that cleaved ROCK1 (130 kDa) levels were increased in the doxorubicin treatment group, accompanied by decreased full-length ROCK1 (top band; 160 kDa), compared with the saline group. The combination of GGA with doxorubicin treatment significantly reduced the level of ROCK1 cleavage comparable with saline treatment. Hearts of mice treated with GGA alone showed further decrease of cleaved ROCK1 (Fig. 2D and E).

**GGA reduces doxorubicin-induced oxidative stress in the heart**

One of the described mechanisms of doxorubicin cardiac toxicity is the generation of highly reactive oxygen species (ROS), such as superoxide radical (5, 43). GGA treatment is known to reduce oxidative stress in various animal models (8–14), yet GGA has never been used in the setting of doxorubicin toxicity. To evaluate the contribution of ROS generation in doxorubicin-induced cardiac toxicity and whether GGA pretreatment would reduce oxidative stress, lucigenin chemiluminescence assay was performed to measure superoxide in freshly frozen mouse cardiac tissue (41). In doxorubicin-treated mice, superoxide production from the heart was significantly increased, as compared with saline controls. GGA treatment reduced the basal level of superoxide production and when given before doxorubicin, significantly abolished the doxorubicin-induced superoxide production (Fig. 3).

Doxorubicin induces uncoupling of NOS enzymes so that doxorubicin undergoes redox activation by the enzyme to form a doxorubicin semiquinone and superoxide (44). Endothelial NOS (eNOS)–deficient mice are less susceptible to doxorubicin-induced cardiac toxicity, suggesting a role of eNOS in the mechanism of oxidant generation and cell damage (43). To elucidate the role of GGA in inhibiting NOS and doxorubicin-induced superoxide production, we used L-NAME, a potent inhibitor of NO synthases. L-NAME did not have a significant effect on basal superoxide generation in controls or in GGA-only–treated animals, but significantly reduced production of superoxide in doxorubicin-treated animals. L-NAME did not cause further significant change in superoxide production in doxorubicin–GGA-treated hearts, suggesting that other sources of superoxide production exist in doxorubicin-treated hearts not related to NOS (Fig. 3).

**GGA enhanced doxorubicin-induced chemotherapy effects in breast cancer xenografts**

In light of the cardioprotective effects of GGA during doxorubicin therapy, we next asked whether GGA would also attenuate or enhance the antineoplastic effects of doxorubicin. Previously, inactivation of the RHO pathway by GGA was found to inhibit ovarian tumor cell growth and invasion (15, 16), leading us to hypothesize that GGA might actually contribute to antineoplastic activity during doxorubicin chemotherapy, while at the same time have a cardioprotective effect. To test this hypothesis, MDA-MB-231 breast cancer cells (45) (with a high level of RHO expression) were implanted in fat pad of athymic nude female mice. When xenograft tumors became palpable, tumors were measured and mice were randomly assigned to treatment groups. After treatment, compared with saline controls, GGA control mice did not have a significant difference in tumor volumes. But
remarkably, GGA given with doxorubicin significantly enhanced the antitumor effect of doxorubicin compared with doxorubicin treatment alone (Fig. 4A).

Additionally, we compared metastatic lung tumors occurrence in all treatment groups, using H&E-stained lung sections evaluation, evaluating the average number of tumor nodules per lung section (Fig. 4B). Representative lung sections are provided (Fig. 4C). GGA alone did not affect tumor volumes, but significantly enhanced antitumor effect of doxorubicin ($P = 0.0012$). Data, mean ± SD; $n = 5$ to 7 mice per group. **, $P < 0.01$, B and C, lung metastases were evaluated using H&E-stained lung sections, comparing the average number of tumor nodules (black arrows) per lung section. GGA treatment (alone or in combination with doxorubicin) did not significantly change the number of metastatic nodules. Data, mean ± SD; $n = 9$ to 13 per group.

Figure 4. Effects of GGA on xenograft tumor volumes in doxorubicin-treated mice. A, effects of doxorubicin and GGA treatment on the average xenograft tumor volume. The mice were injected with MDA-MB-231 breast cancer cells into the mammary fat pad. Tumor development was followed and the mice were divided into four treatment groups: DOX 9 mg/kg, DOX 9 mg/kg and GGA, GGA, and saline. Doxorubicin (9 mg/kg) was given once every 2 weeks for 4 cycles. GGA administration was given 48 hours before doxorubicin. Mice were euthanized at 6 weeks after the initial injection of doxorubicin. GGA alone did not affect tumor volumes, but significantly enhanced antitumor effect of doxorubicin ($P = 0.0012$). Data, mean ± SD; $n = 5$ to 7 mice per group. **, $P < 0.01$, B and C, lung metastases were evaluated using H&E-stained lung sections, comparing the average number of tumor nodules (black arrows) per lung section. GGA treatment (alone or in combination with doxorubicin) did not significantly change the number of metastatic nodules. Data, mean ± SD; $n = 9$ to 13 per group.

GGA inhibited LPA-induced tumor cell motility and invasion in breast cancer cells

To explore possible cellular mechanisms for the anticancer effects of GGA, we examined effects of GGA on tumor cell migration and invasiveness using LPA RHO signaling stimulus. LPA exerts its biologic effects through the LPA receptors in cancer cells (46) and is associated with breast cancer cell migration and invasiveness (47, 48). LPA as an inducer of RHO signaling and tumor invasion is used in *in vitro* systems to study cancer cells invasion (49, 50). GGA reduced invasion of ovarian cancer cells *in vitro* (16), although its role in motility and invasion in breast cancer cells has not been evaluated. Therefore, we performed *in vitro* experiments to assess GGA effects on motility and invasiveness of MDA-MB-231 breast cancer cells that express a high level of RHOA (45). In motility
experiments, GGA reduced cell motility in the presence of LPA (Fig. 5A). GGA treatment alone reduced the basal level of invasion, whereas GGA in combination with LPA further reduced invasion of MDA-MB-231 cells (Fig. 5B).

Because GGA inhibits the RHO pathway in ovarian cancer, we hypothesized that GGA would inhibit RHO activation in breast cancer cells by inhibiting the RHO family member activation through reduced geranylgeranylation. To confirm the role of RHO family geranylgeranylation in GGA-induced inhibition of invasion of MDA-MB-231 cells, we applied GGOH (geranylgeranyl) to a subset of experiments testing the mechanism of GGA’s inhibition on LPA-induced migration. GGOH, added to the cells before migration, is metabolized to geranylgeranylpyrophosphate, which subsequently aids RHO geranylgeranylation, to rescue the inhibitory effects of GGA (16). In our experiments, GGOH reduced GGA anti-invasive effect, providing evidence that GGA inhibits RHO family geranylgeranylation in MDA-MB-231 cells suggesting a mechanism for the role of GGA in reducing tumor cell invasion (Fig. 5B). GGOH also reduced but did not completely eliminate GGA anti-invasive effect in LPA-treated cancer cells (similarly to the findings in ovarian cancer cells), suggesting additional mechanisms in LPA-induced invasiveness.

GGA decreases cell viability/proliferation in breast cancer cells

To evaluate the effects of GGA alone or in combination with doxorubicin on cancer cells viability in vitro, we performed MTT assay with various concentrations of GGA and doxorubicin. GGA alone significantly reduced MTT absorbance in MDA-MB-231 cells treated for 24 hours, whereas GGA in combination with doxorubicin did not result in a significant change compared with doxorubicin alone (Fig. 5C).

Discussion

The optimal cardiac toxicity prevention strategy for doxorubicin would include an agent that improves the efficacy of the doxorubicin-based cancer therapy and prevents cardiac toxicity. In our experiments, pretreatment with GGA blocked doxorubicin cardiac toxicity by

Figure 5. Effects of GGA on invasiveness of MDA-MB-231 breast cancer cells in vitro. Invasion studies were performed with MDA-MB-231 cells. LPA was applied to stimulate the motility or invasion of the cancer cells, and LPA-induced motility and invasiveness were compared with the basal level of cell motility and invasiveness. GGOH treatment was used in invasion studies to counteract the GGA inhibitory effect on cancer cells invasion. A, GGA does not affect basal motility of MDA-MB-231 breast cancer cells, but reduces motility in LPA presence ($P = 0.0195$). Data, mean ± SD; $n = 3$ wells per group. B, GGA treatment decreases invasiveness of MDA-MB-231 cells in vitro. GGA treatment reduced invasion ($P = 0.0423$), and GGA in combination with LPA further reduced invasion of MDA-MB-231 cells ($P = 0.0121$). GGOH abolishes GGA anti-invasive effect ($P = 0.0550$). Bar graph represents invasion of MDA-MB-231 cells as a total number of cells used in the assay. Data, mean ± SD; $n = 3$ wells per group. **, $P < 0.01$, versus basal. C, GGA decreases MDA-MB-231 cells viability in vitro, while not significantly altering doxorubicin effects in MTT assay. Data, mean ± SD; $n = 7$ wells per group. ****, $P < 0.0001$. ***, $P < 0.01$, versus basal.
maintaining systolic function and decreasing cell death in the heart. Most remarkably, GGA also contributed to doxorubicin’s chemotherapy efficacy in MDA-MB-231 xenografts in parallel with protecting the heart. GGA’s antineoplastic effect is likely due to its inhibition of RHO family proteins in both the heart and cancer cells, and we selected MDA-MB-231 for these experiments because of the endogenous high RHO activity in these cells. Because GGA has been used in Japan since 1984 to prevent stomach ulcers and has a long history of safety and lack of adverse effects, we suggest that this novel approach to prevention of doxorubicin toxicity should be further investigated.

By comparison, dexrazoxane, an iron chelator used to reduce superoxide formation in doxorubicin toxicity (51), currently has limited use due to European Medicines Agency and Food and Drug Administration restrictions. In 2011, dexrazoxane was restricted for use only in patients with breast cancer when doxorubicin dose exceeds 300 mg/m² or epirubicin exceeds 540 mg/m². This restriction was based on clinical trials that reported cases of acute myeloid leukemia and myelodysplastic syndrome in children receiving dexrazoxane (52, 53). Furthermore, there are anecdotal reports of reduced anticancer therapeutic efficacy for dexrazoxane, GGA, by contrast, offers an attractive alternative to dexrazoxane, with molecular activity that both prevents cardiac toxicity and increases effectiveness of doxorubicin chemotherapy.

The efficacy of cardiac protection by GGA may be related to multiple mechanisms. First, GGA induced a reduction in NOS-dependent superoxide production, with overall reduction in oxidative stress in the heart, an important mechanism of doxorubicin-induced cardiomyocyte cell death and toxicity. Second, GGA also prevented doxorubicin-induced ROCK1 cleavage, thus blocking the proapoptotic RHO/ROCK1 pathway in the heart, resulting in less TUNEL-positive cells in the myocardium. In the heart, the RHO/ROCK pathway is currently emerging as a potential target for inhibition in cardiovascular disease (17–23). In multiple settings of cardiac disease (17), specific RHO/ROCK inhibitors have shown promise in disease prevention yet RHO/ROCK inhibitors have never been used to prevent doxorubicin cardiac toxicity, although ROCK1 was found to be activated with doxorubicin treatment in vitro (42). This finding suggested to us that GGA treatment may be beneficial to induce RHO/ROCK inhibition in the heart and thus reduce cardiac toxicity. We hypothesized that ROCK activation occurs in hearts in mice treated with doxorubicin. In the current study, we demonstrate that ROCK activation does occur in the heart of mice treated with doxorubicin and this provides rationale to use GGA as a cardioprotective agent. In total, our study demonstrates that GGA can inhibit doxorubicin-induced RHO activation in the heart, oxidative stress, and cardiac toxicity.

The connection between oxidative stress and ROCK1 activation has recently been studied in erythroid cells (54), and we observed that GGA reduced both in the current study. Doxorubicin treatment induced an elevation of ROCK1 cleavage product, a downstream effect or protein indicative of RHO pathway activation. In addition, doxorubicin induced an increase of superoxide formation. The L-NAME experiments demonstrated that a portion of superoxide induced by doxorubicin treatment was NOS-dependent. We suggest that the non–NOS-dependent superoxide is related to RHO activation in the doxorubicin-treated mice, because GGA further inhibited the extent of superoxide production when comparing doxorubicin versus doxorubicin and GGA treatment groups. GGA may also inhibit uncoupled eNOS-dependent superoxide production, because L-NAME did not decrease superoxide production neither in the GGA-only treatment group nor in the doxorubicin+GGA treatment group. This mechanism may be possible due to positive GGA effects on HSP90 expression (55). RHOA-RHO kinase is also a well-documented inducer of oxidative stress, and oxidative stress is known to induce RHO family activation (56–58).

Inhibiting the RHO family of proteins has a different role in cancer cells, which might explain how GGA could have divergent effects on cardiac cells (nonmotile, nondividing cells) and cancer cells. In ovarian cancer cells, GGA was shown to inhibit both RHO and RAS activation, possibly through inhibiting geranylation (15, 16). Multiple types of human cancers (including breast cancer) have a high activity of RHO family proteins, which likely contributes to the invasive malignant phenotype and tumor metastasis (59, 60). Because the RHO family is responsible for this phenotype, this subgroup of cancers will be more susceptible to GGA-induced RHO pathway inhibition. We selected MDA-MB-231 due to high RHO activity and found that in xenograft-implanted nude mice, GGA+doxorubicin treatment also significantly reduced tumor mass versus doxorubicin treatment alone. The numbers of metastases in the lung were reduced in GGA versus control groups, although these differences did not reach statistical significance. The numbers of metastases were significantly reduced in both doxorubicin groups with and without GGA. Due to the high effectiveness of doxorubicin (a high dose needed to produce cardiac toxicity) in this xenograft model, any added effect of GGA was masked. However, in our in vitro studies, GGA inhibited LPA-induced Matrigel invasion by MDA-MB-231 cells, suggesting that RHO pathway inhibition is an important target of GGA in breast cancer cell motility and invasion. GGA anti-invasive effect was suppressed by GGOH, demonstrating that RHO geranylation is inhibited by GGA.

Our finding of GGA enhancing doxorubicin antitumor effect in vitro can be attributed to GGA-negative effect on cancer cells viability, as shown in our in vitro study and in previously published studies (61). We did not see an additive effect in combined GGA–doxorubicin treatment groups in vitro; however, in vivo multiple factors contribute to the tumor progression, which may not be accounted for with in vitro assays on isolated
cancer cells, but which may be affected by GGA treatment. For example, angiogenesis, an important factor which regulates tumor development, may be affected by GGA. It was shown that inhibition of geranylgeranylation interferes with angiogenesis, evaluated by tube formation by human dermal microvascular endothelial cells (62). RHO GTPases and ROCK inhibition are also reported to affect angiogenesis (63). In addition, the RHO/ROCK1 pathway is involved in cancer cells survival and propagation through various functions, including cell adhesion, motility, and migration (24). ROCK1 is found in MDA-MB-231 cells, although in low quantities, and GGA-negative effects on the RHO/ROCK1 pathway may also contribute to overall GGA effects on cancer progression, observed in our study (64).

Our studies demonstrate that GGA has both important cardioprotective properties and antineoplastic properties, which together can be therapeutically effective when GGA is used in combination with doxorubicin. Because GGA has been used for 30 years without adverse effects, we suggest that this novel approach to prevention of doxorubicin cardiac toxicity should be considered for clinical testing in patients undertaking doxorubicin treatment for cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: P. Sysa-Shah, K.L. Gabrielson
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