Geranylgeranylacetone blocks doxorubicin-induced cardiac toxicity and reduces cancer cell growth and invasion through RHO pathway inhibition


1 Johns Hopkins Medical Institutions, Department of Molecular and Comparative Pathobiology, Baltimore, MD, USA
2 New York Medical College, Department of Physiology, Valhalla, NY, USA
3 Johns Hopkins Medical Institutions, Department of Cardiology, Baltimore, MD, USA
4 Johns Hopkins University, Department of Radiology, Baltimore, MD, USA
5 Johns Hopkins University School of Medicine, Department of Pathology, Baltimore, MD, USA

*Current address: Hajime Orita, Juntendo Shizuoka Hospital, Department of Surgery, Izunokuni, Shizuoka, Japan

To whom correspondence should be addressed: Dr. Kathleen Gabrielson, Department of Molecular and Comparative Pathobiology, Johns Hopkins Medical Institutions, MRB 807, 733 N. Broadway, Baltimore, MD, USA, 21205. Tel.: 410 955 4584; Fax: 443-287-2954; E-mail: kgabriel@jhmi.edu

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Abstract

Doxorubicin is a widely used chemotherapy for solid tumors and hematological malignancies, but its use is limited due to cardiotoxicity. Geranylgeranylacetone (GGA), an anti-ulcer agent used in Japan for thirty years, has no significant adverse effects, and unexpectedly reduces ovarian cancer progression in mice. Since 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. Trans-thoracic 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 NOS-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 anti-tumor 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 anti-cancer 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 geranylgeranylacetone (GGA), an acyclic polyisoprenoid (Selbex®), which has been used since 1984 as an anti-ulcer 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 post-translational 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 since RHO GTPases, and their downstream target, RHO-associated kinases (ROCKs), are implicated in a variety of physiological functions associated with cancer-related changes in the actin cytoskeletal assembly, such as cell adhesion, motility, and migration (24).
Since 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. (Tokyo, Japan). GGA was dissolved in 100% ethanol for *in vitro* studies. 1-Oleoyl LPA (18:1 LPA) (Cat. # 857130) was obtained from Avanti Polar Lipids (Alabaster, AL). Doxorubicin (Cat. # NDC 55390-238-01) was from Bedford Laboratories (Bedford, OH). Matrigel (Cat. # 354234) and Cell culture inserts (Cat. # 353182) for invasion and motility assay were obtained from BD Biosciences (Franklin Lakes, NJ). ROCK1 primary antibody (Cat. # A300-457A) was from Bethyl Laboratories (Montgomery, TX). RPMI 1640 (Cat. # 11835-030) was from Gibco (Life Technologies, Grand Island, NY), fetal bovine serum (FBS, Cat. # SH30088.03) was from HyClone (Thermo Fisher Scientific, Pittsburg, PA), Penicillin-Streptomycin Solution (Cat. # 30-001-CI) was from Cellgro (Manassas, VA).

**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) fetal bovine serum, penicillin (10 U/ml)-
streptomycin (10 U/ml) at 37°C in humidified 5%CO2 atmosphere.

**Animal studies:** 5-6 weeks old female athymic nude-Foxn1nu mice (Harlan Laboratories,
Indianapolis, IN) 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. 1x10^6 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 9mg/kg, DOX 9mg/kg and
GGA, GGA and saline. Doxorubicin has been reported to induce cardiotoxicity in a wide range of
dosages (4mg/kg to 25mg/kg)(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 (1mg /g
body weight) was given 48 hour before doxorubicin per os method (pipette). GGA was previously
shown to elicit a protective response when given 24-48 hours prior to stressor(30-35) in dosages
from 200mg/kg to 1000mg/kg (orally or intraperitoneally)(34-36).
Tumor progression was evaluated by palpation and tumor size measurements with calipers. Tumor
volumes were calculated by the following formula: [1/2xLxWxH] (37), where L-length, W-width,
H-height. All mice were housed under a 12 hours 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 National Institutes of Health. The protocol was approved by the Animal
Care and Use Committee of the Johns Hopkins Medical Institutions (Animal Welfare Assurance #
A-3273-01).
**Echocardiography:** Trans-thoracic echocardiography was performed on conscious mice using Acuson Sequoia C256 ultrasound machine (Siemens Corps, Mountain View, CA) equipped with the 15MHz 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: inter-ventricular 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: \( \text{FS (\%)} = \left[ \frac{\text{LVEDD} - \text{LVESD}}{\text{LVEDD}} \right] \times 100 \).

**Necropsy:** Mice were euthanized (CO\(_2\)) 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 RIPA buffer (25mM Tris-HCl; 150mM NaCl; 1% NP-40; 1% sodium deoxycholate; 0.1% SDS; 1x proteinase inhibitor (Roche, cat.#11697498001); 1x phosphatase inhibitors (Sigma, cat.#P5726 and P0044)) and centrifuged at 12,000 \( \times \) g at 4°C for 15 min. Protein measurements were performed using a Bio-Rad protein assay (BioRad, Hercules,
CA); equal amounts of total protein (40 μg) were used per lane. Proteins were denatured in SDS gel-loading buffer (0.125 M Tris, pH 6.8, 20% glycerol, 5% mercaptoethanol, 4% SDS, and 0.002% bromophenol blue) at 100°C for 5 min and separated on a 4-12% SDS-PAGE gradient gel using an XCell SureLock™ Mini-Cell Electrophoresis System with Kaleidoscope prestained molecular weight standards (Bio-Rad, Hercules, CA). After electrophoresis, the proteins were transferred to a PVDF membrane. Blots were blocked with 5% nonfat milk in 0.1% TBST (50 mM Tris-HCl, pH 7.4, 150 mM 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 IgG; bound antibodies were detected using a Western blot chemiluminescence reagent kit (Pierce, Rockford, IL).

**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 μm, 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 μm, and stained with hematoxylin-eosin (H&E). Lung sections with 5 lobes were scanned by Aperio and evaluated for the presence of tumor metastases.

**TUNEL:** Staining was performed using DeadEnd Fluorometric TUNEL system (Promega, Madison, WI) according to the manufacturer's instructions, as described previously(40). Counterstaining with DAPI aided in the morphological 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-mm² field in LV 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 sec, 4000 RPM), the supernatant was added to a lucigenin (5 μM) solution containing NADPH (100μM). 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. NOS-dependent superoxide generation was measured by adding L-NAME (100μM) 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 x 10⁴/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) (25μM) was applied to stimulate the invasion of the cancer cells through Matrigel and LPA-induced invasiveness was compared to the basal level of cell invasiveness. 72 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 x 10⁴/well) and treated with specified concentrations of doxorubicin, GGA or both drugs for 24 hours. MTT stock solution was prepared (5mg of MTT (Sigma M-5655) to 1mL of the growth media), further diluted 1:10 with growth media and the cells were incubated in MTT solution for 2-3 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 570nM.

**Statistical methods:** GraphPad Prism software (GraphPad, La Jolla, CA) was used to perform statistical analysis. After determining means and standard deviations, the unpaired Student’s *t*-test or one-way analysis of variance (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, since 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 (9mg/kg) alone and doxorubicin combined with GGA. We chose to use the dosing schedule, where GGA treatment is initiated 48 hours before the oxidative stress, since 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 fractional shortening (FS, %) (Figure 1A, 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, since GGA treatment alone had no effect on systolic function compared to saline treatment. GGA did not have a significant effect on the weight loss seen with doxorubicin treatment (Figure 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 (9mg/kg) alone and doxorubicin combined with GGA. We found that doxorubicin treatment resulted in significant
increase in cell death in the hearts, while GGA pre-treatment significantly reduced the doxorubicin-induced cell death (Figure 2A,B), consistent with the protective effects seen with measurements of systolic function (Figure 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 (Figure 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 (RHO-associated coiled-coil protein kinase 1) 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, while 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 doxorubicin treatment group, accompanied by decreased full-length ROCK1 (top band) (160 kDa), compared to saline group. The combination of GGA with doxorubicin treatment significantly reduced the level of ROCK1 cleavage comparable to saline treatment. Hearts of mice treated with GGA alone showed further decrease of cleaved ROCK1 (Figure 2 D,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 to saline controls. GGA treatment reduced the basal level of superoxide production and when given prior to doxorubicin, significantly abolished the doxorubicin induced superoxide production (Figure 3).

Doxorubicin induces uncoupling of nitric oxide synthase (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, providing a NOS-mediated mechanism for GGA’s protection against doxorubicin toxicity. L-NAME treatment did not result in a complete reduction of superoxide production in the doxorubicin treated mice, suggesting that other sources of superoxide production exist in doxorubicin treated hearts not related to NOS (Figure 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 anti-neoplastic 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 anti-neoplastic 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 to saline controls, GGA control mice did not have a significant difference in tumor volumes. But remarkably, GGA given with doxorubicin significantly enhanced the anti-tumor effect of doxorubicin compared to doxorubicin treatment alone (Figure 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 (Figure 4B). Representative lung sections are provided (Figure 4C). GGA alone slightly inhibited the number of tumor nodules per lung section; although, the differences did not reach statistical significance (p=0.2446) due to high individual variability of the number of tumor nodules per lung section. Furthermore, these tumors were highly responsive to doxorubicin alone, and thus any added effect of GGA on decreasing tumor growth or metastasis could not be readily appreciated at the doses of doxorubicin used in our experiments.

GGA inhibited LPA-induced tumor cell motility and invasion in breast cancer cells.
To explore possible cellular mechanisms for the anti-cancer effects of GGA, we examined effects of GGA on tumor cell migration and invasiveness using lysophosphatidic acid RHO signaling stimulus. Lysophosphatidic acid (LPA) exerts its biological 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 MDA-MB-231 breast cancer cells that express a high level of RHO A (45). In motility experiments, GGA reduced cell motility in the presence of LPA (*Figure 5A*). GGA treatment alone reduced the basal level of invasion, while GGA in combination with LPA, further reduced invasion of MDA-MB-231 cells (*Figure 5B*).

Since GGA inhibits 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 (geranylgeraniol) 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 (*Figure 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 \textit{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, while GGA in combination with doxorubicin did not result in a significant change compared to doxorubicin alone (Figure 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, pre-treatment with geranylgeranylacetone (GGA) blocked doxorubicin cardiac toxicity by 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 anti-neoplastic 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. Since 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 breast cancer patients when doxorubicin dose exceeds 300mg/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 anti-cancer 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 pro-apoptotic RHO/ROCK1 pathway in the heart with 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. Additionally, 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, since 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, since L-NAME did not decrease superoxide production neither in GGA only treatment group, nor in 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 (non-motile, non-dividing 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). Since 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 lysophosphatidic acid (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 anti-tumor effect \textit{in vivo} can be attributed to GGA negative effect on cancer cells viability, as shown in our \textit{in vitro} study and in previously published studies\cite{61}. We did not see an additive effect in combined GGA-doxorubicin treatment groups \textit{in vitro}, however, \textit{in vivo} multiple factors contribute to the tumor progression, which may not be accounted for with \textit{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\cite{62}. RHO GTPases, and RHO-associated kinases (ROCKs) inhibition is also reported to affect angiogenesis \cite{63}. In addition, Rho/ROCK1 pathway is involved in cancer cells survival and propagation through various functions, including cell adhesion, motility, and migration \cite{24}. ROCK1 is found in MDA-MB-231 cells, although in low quantities, and GGA negative effects on RHO/ROCK1 pathway may also contribute to overall GGA effects on cancer progression, observed in our study\cite{64}.

Our studies demonstrate that GGA has both important cardioprotective properties and anti-neoplastic properties, which together can be therapeutically effective when GGA is used in combination with doxorubicin. Since GGA has been used for thirty 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.

References


LEGENDS

Figure 1. Effect of GGA on cardiac function in murine model of doxorubicin-induced cardiotoxicity

A. Representative M-mode echocardiograms of the mice from saline, GGA, doxorubicin and doxorubicin in combination with GGA treatment groups. Doxorubicin (9mg/kg) given once every two weeks for 4 cycles induces contractility deficit at 6 wks from initial injection. GGA administration (48 hours before doxorubicin) preserves cardiac function in doxorubicin treatment group, GGA alone does not have an effect. B. Scatter plot of fractional shortening percentage (FS, %). Doxorubicin causes reduction of FS, % (p<0.0001), and addition of GGA to the treatment improves FS, % (p<0.0001), GGA alone had no effect on FS, %. C. Histogram of mouse body weights in grams. The data are presented as means ± SD, n=9-12 mice per group. *** p<0.001.

Figure 2. Effects of GGA treatment on cardiac cell death, ROCK1 cleavage, and superoxide production in the hearts of doxorubicin-treated mice.

A. Histogram and B. Representative images of TUNEL-positive nuclei. Mice were euthanized at 6 weeks after the initial injection of doxorubicin. Doxorubicin (9mg/kg) was given once every two weeks for 4 cycles. GGA administration was given 48 hours before doxorubicin. Total number of cells per x20 field was quantified by DAPI staining and TUNEL-positive nuclei were expressed as a percentage of total cells (nuclei) in each heart with five fields per heart analyzed. Cell death in the hearts of the animals treated with doxorubicin was significantly higher than in controls (p=0.0491). In the group which received combinatory therapy, cell death was significantly lower than in doxorubicin group (p=0.0124). Cell death in GGA group was not significantly different from control group. The data are presented as means ± SD, n= 3-8 mice per group.* p<0.05. C. Representative H&E stained heart sections from mice treated with saline, GGA, DOX or
combination 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 mice. 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 mice hearts. Doxorubicin treatment significantly increased cleaved ROCK1 levels (p=0.0054), while addition of GGA to doxorubicin therapy reduced ROCK1 cleavage (p=0.0002). GGA treatment did not affect cleaved ROCK1 levels. The data are presented as means ± SD, n=3 mice per group. ** p<0.01, *** p<0.001.

Figure 3. Effects of doxorubicin and GGA treatment on total and eNOS-dependent superoxide generation. Doxorubicin treatment significantly increased superoxide levels in murine left ventricles (p=0.0159). L-NAME had no effect at superoxide generation in controls, but significantly reduced doxorubicin-mediated overproduction of superoxide in treated animals (p=0.0159). GGA had no effect at superoxide production by itself, but abolished doxorubicin-induced superoxide production (p=0.0077). L-NAME did not result in further reduction of superoxide production in doxorubicin-treated compared to controls. The data are presented as means ± SD, n=3-5 mice per group.* p<0.05, ** p<0.01.

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 (9mg/kg) was given once every two weeks for 4 cycles. GGA administration was given 48 hours before doxorubicin. Mice were euthanized at 6 wks after the initial injection of doxorubicin. GGA alone did not affect tumor volumes, but significantly enhanced anti-tumor effect of doxorubicin (p=0.0012). The data are presented as means ± SD, n=5-7 mice per group.** p<0.01. B, 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. The data are presented as means ± SD, n=9-13 per group.

**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. Lysophosphatidic acid (LPA) was applied to stimulate the motility or invasion of the cancer cells and LPA-induced motility and invasiveness was compared to 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). The data are presented as means ± 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. The data are presented as means ± SD, n=3 wells per group.* p<0.05, ** p<0.01. C. GGA decreases MDA-MB-231 cells viability in vitro, while not significantly altering doxorubicin effects in MTT assay. The data are presented as means ± SD, n=7 wells per group. **** p<0.0001.
Figure 3

Lucigenin-enhanced chemiluminescence (cpm/mg)

GGA  -  -  +  +  -  -  -  +  +
DOX  -  -  -  -  +  +  +  +  +
L-NAME  -  +  -  +  -  +  -  +  +
Geranylgeranylaceotide blocks doxorubicin-induced cardiac toxicity and reduces cancer cell growth and invasion through RHO pathway inhibition

Polina Sysa-Shah, Yi Xu, Xin Guo, et al.

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