

Award Number:
W81XWH-07-1-0330

TITLE:
Estrogen and the dietary phytoestrogen tesveratrol as regulators of the Rho
GTPase Rac in breast cancer research

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REPORT DATE:
June 2009

TYPE OF REPORT:
Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

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1. REPORT DATE 06.01.2009		2. REPORT TYPE Annual		3. DATES COVERED 07 MAY 2008 - 6 MAY 2009	
4. TITLE AND SUBTITLE Estrogen and the Dietary Phytoestrogen resveratrol as Regulators of the Rho GTPase Rac in Breast Cancer Research				5a. CONTRACT NUMBER W81XWH-07-1-0330	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Surangani Dharmawardhane Email: surangi@uccaribe.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Universidad Central del Caribe Avenida Laurel and Santa Juanita Bayamon, PR 00956				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Our hypothesis is that estrogen (E2) and low concentrations of resveratrol promote breast cancer invasion and metastasis while high concentrations of resveratrol prevent breast cancer metastasis via regulation of the signaling protein Rac. Specific Aim1 proposed to test the effect of E2, resveratrol, or a small molecule Rac-specific inhibitor NSC23766 on Rac activity, cell migration/ invasion, and cell cycle progression of metastatic breast cancer cells. We show that dependent on estrogen receptor (ER) status, E2 and resveratrol have differential effects on Rac activity, cell migration/invasion, and cell growth. The Rac inhibitor NSC23766 only had a modest inhibitory effect on Rac activity or cell migration of breast cancer cell lines. Therefore, we developed novel derivatives of NSC-223766 and show that EHop-017 and EHop-023 are more efficient inhibitors of Rac activity and cell migration than NSC-23766. EHop-023 also inhibits mammary tumor growth in vivo. Aim 2 proposed to test the effect of these compounds on breast cancer progression in immunocompromised nude mice from mammary tumors established from fluorescent protein-tagged breast cancer cells. Our studies show that at low concentrations, resveratrol alone did not affect breast cancer progression. Interestingly, resveratrol in combination with other grape polyphenols reduced breast cancer growth and metastasis to bone and liver (Castillo-Pichardo et al., 2009). Therefore, E2 and low concentrations of resveratrol promote while high concentrations of resveratrol inhibit breast cancer progression in a breast cancer cell line that express ERβ. However, in the ER (-) cell line, estrogen had no effect on Rac activity or cell invasion while resveratrol was inhibitory at all concentration tested.					
15. SUBJECT TERMS Estrogen, resveratrol, breast cancer, metastasis, Rac					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
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Table of Contents

	<u>Page</u>
Introduction.....	5
Body.....	5
Key Research Accomplishments.....	12
Reportable Outcomes.....	13
Conclusion.....	13
References.....	15
Appendices.....	18

Introduction

The focus of this research project is the role of the hormone estrogen (E_2) and the structurally similar natural compound resveratrol on breast cancer invasion and metastasis. E_2 is important for initiation and progression of breast cancers (1;2;2). Increased $ER\alpha$ levels are associated with 50-80% of breast tumors and inhibition of $ER\alpha$ has become a major strategy for prevention and treatment of breast cancer (3-6). During breast cancer progression, cancer cells become resistant to antiestrogen therapy because malignant breast cancers express only $ER\beta$ or no ER and overexpress EGFR isoforms EGFR1 or Her-2 (7). Therefore, it is important to test alternative therapies that can be used effectively to treat ER (-) breast cancer.

Resveratrol is a natural compound from grapes and peanuts that is structurally similar to E_2 and interacts with both $ER\alpha$ and $ER\beta$ (8-11). Resveratrol has proapoptotic, antigrowth, anti-inflammatory, antiangiogenic, and anti-invasive properties that makes it an attractive anticancer compound (12-14). Much of the data on potential anticancer properties of resveratrol has been shown *in vitro* with high concentrations of resveratrol ranging from 30-200 μ M (15-21). We and others have shown that resveratrol at 50 μ M can inhibit cell migration and invasion (22-25). Resveratrol can exert biphasic effects where low concentrations are estrogenic while high concentrations are antiestrogenic (16;18;26;27). However, the effects of resveratrol in $ER\alpha$ (-) or $ER\alpha\beta$ (-) breast cancers are not well understood. Therefore, the **purpose** of this study is to investigate the effects of resveratrol on breast cancer progression in metastatic breast cancers that have lost $ER\alpha$. We and others have demonstrated that activity of the Rho GTPase Rac is necessary for breast cancer invasion and metastasis (28;29). Our preliminary data demonstrated that the effects of E_2 and resveratrol on cell functions relevant for metastasis such as actin cytoskeletal rearrangement to form motile structures, cell migration, and invasion may be mediated by the action Rac. Therefore, we formulated the **hypothesis** that high concentrations of resveratrol prevent breast cancer invasion and metastasis while E_2 and low concentrations of resveratrol promote breast cancer invasion and metastasis via Rac-regulated mechanisms. Our **objective** is to analyze the effect of varying concentrations of E_2 , resveratrol, or a Rac inhibitor on breast cancer invasion and metastasis using human metastatic breast cancer cell lines with no $ER\alpha$ expression.

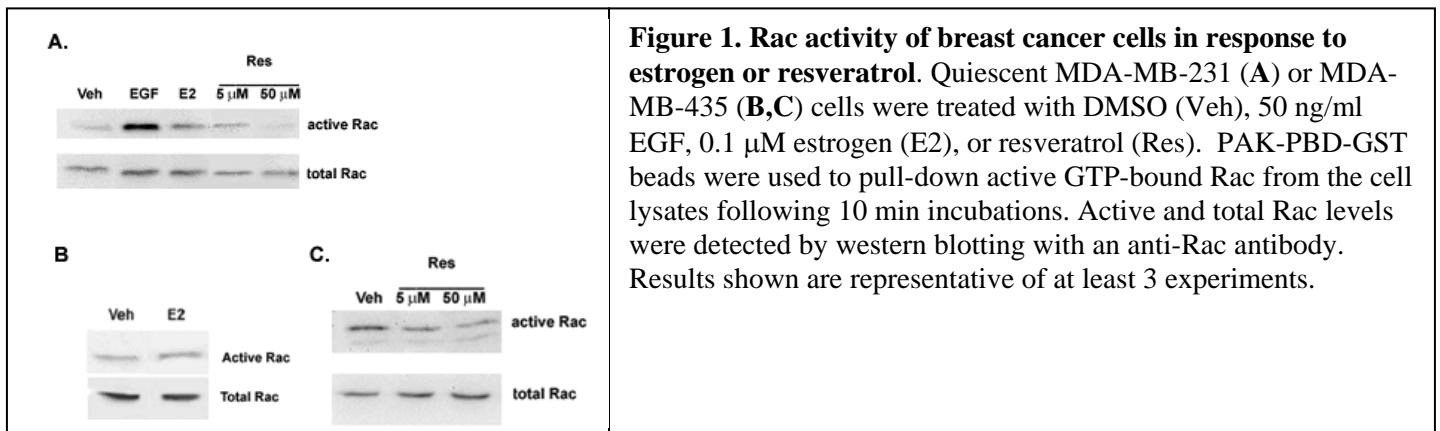
Body

Specific Aim 1 (Task 1). Determine the effect of estrogen and resveratrol on metastatic breast cancer cell lines *in vitro* (Months 1-24)

I. Analyze activities of Rho GTPases, Rac GEFs, and Rac GAPs.

The objective of this task was to treat $ER\alpha$ (-) β (+) low metastatic MDA-MB-231 and $ER\alpha$ (-) β (-) high metastatic MDA-MB-435 human breast cancer cell lines with vehicle control; resveratrol, E_2 , or Rac-specific inhibitor NSC23766 and determine changes in cell functions known to affect breast cancer metastasis.

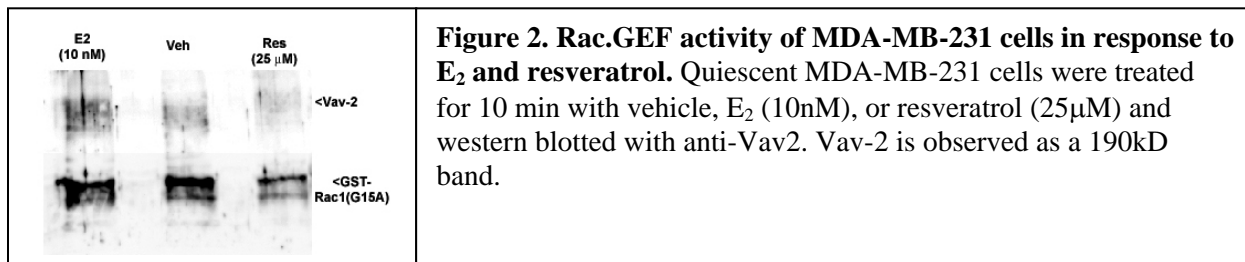
Analysis of Rac activity in response to E_2 and resveratrol:



In the last annual report, using MDA-MB-231 human metastatic breast cancer cells, the effect of vehicle, EGF (+ control), E₂ (100 nM), 5 or 50 μM resveratrol on Rac activity was determined using a pulldown assay that determines the amount of active GTP-bound Rac that is co-precipitated with a GST-fusion protein from the Rac.GTP binding domain of a downstream effector PAK (PBD), methods as in (30). The data shows that as per our hypothesis, E₂ and low concentrations of resveratrol (5 μM) activated Rac while high concentrations resveratrol (50 μM) inhibited Rac activity in this ERβ (+) MDA-MB-231 breast cancer cell line (Fig. 1A). Interestingly, when Rac activity was tested in the ER (-) MDA-MB-435 cell line, we found that E₂ had no effect on Rac activity compared to vehicle control in quiescent cells (Fig. 1B). However, unlike with the ERβ (+) cells, resveratrol decreased Rac activity at both 5 and 50 μM (Fig. 1C). Therefore, the E₂ effect appears to be ER-dependent while resveratrol may have alternative mechanisms of action in ER (-) breast cancer cells.

Analysis of Rac.GEF activity in response to resveratrol:

Since Rac activity and not expression is enhanced in metastatic breast cancers and a metastatic variant of MDA-MB-435 (28), we hypothesized that regulation of Rac activity was important for breast cancer progression. Rho GTPases are activated by guanine nucleotide exchange factors (GEF) and are inactivated by GTPase activating proteins (GAP) (31). To test the hypothesis that oncogenic exchange factors for Rac were involved with Rac activation in metastatic breast cancer, we used the strategy of using Rac(G15A), a dominant negative nucleotide-free form of Rac1 that forms a high-affinity complex with active GEFs to pulldown Rac.GEFs (32). This construct was obtained from the laboratory of Dr. Keith Burrige (University of North Carolina, Chapel Hill) and glutathione-S-transferase (GST)-fusion proteins were expressed and isolated from E.coli. GST-Rac1(G15A) were then coupled to glutathione sepharose beads and incubated with cell lysates following 10 min with vehicle, E₂, or resveratrol and western blotted with specific antibodies to the Rac.GEF Vav-2. Fig. 2 shows the exciting result where E₂ treatment increased the amount of Vav-2 bound to Rac1 (G15A) compared to vehicle while resveratrol at 25 μM reduced the interaction of Vav-2 with Rac (G15A).



During the past year, the PI trained graduate student Tanialis Ortiz-Rivera in Rac and Rac.GEF activity assays using pulldowns. Now that the techniques have been established, Ortiz-Rivera will spend the next year completing the proposed Rac activity and Rac.GEF and Rac.GAP assays in response to E₂ and resveratrol using MDA-MB-231 and MDA-MB-435 cell lines for her Ph.D. Dissertation.

II. Analyze cell migration and invasion in response to E₂ and resveratrol

Since Rac activity is known to regulate cell migration and invasion, the effect of E₂ and resveratrol on cell migration and invasion of MDA-MB-231 cells was tested and shown in the last annual report. Cell migration assays were conducted with quiescent MDA-MB-231 cells on the top well of a Transwell (CoStar) while the bottom well contained vehicle, E₂ (10 nM), or 5 or 50 μM resveratrol. For invasion assays, the top surface of the top well was coated with Matrigel, a basement membrane substrate. Herein, we show that at low concentrations, resveratrol (0.5μM) increased cell migration of MDA-MB-231 cells while 5μM did not affect cell migration. Both concentrations of resveratrol did not affect chemotactic migration of the ER (-) MDA-MB-435 cells (Fig. 3). In invasion assays (Fig. 4), 50μM resveratrol inhibited MDA-MB-231 cell invasion by ~30% compared to controls. E₂ exerted an opposite effect to 50 μM resveratrol (significant at p<0.04) by increasing

cell migration/invasion 2-fold compared to controls. Interestingly, 5 μM resveratrol acted in a similar manner to E_2 by increasing cell invasion. Similarly, we observed a $\sim 40\%$ decrease in MDA-MB-231 cell invasion across a Matrigel matrix in response to 50 μM resveratrol and a ~ 1.6 -fold increase in invasion in response to E_2 or 5 μM resveratrol (Fig. 4A). When the MDA-MB-435 ER (-) cells were subjected to a similar analysis, we show that E_2 had no effect on MB-435 cell invasion while 5, 25, and 50 μM resveratrol inhibited invasion by $\sim 70\%$ (Fig. 4B). These results indicate that similar to Rac activity, metastatic breast cancer cells respond to E_2 only if $\text{ER}\beta$ is expressed. ER (-) cells were unresponsive to E_2 and resveratrol at all concentrations tested inhibited MDA-MB-435 cell invasion.

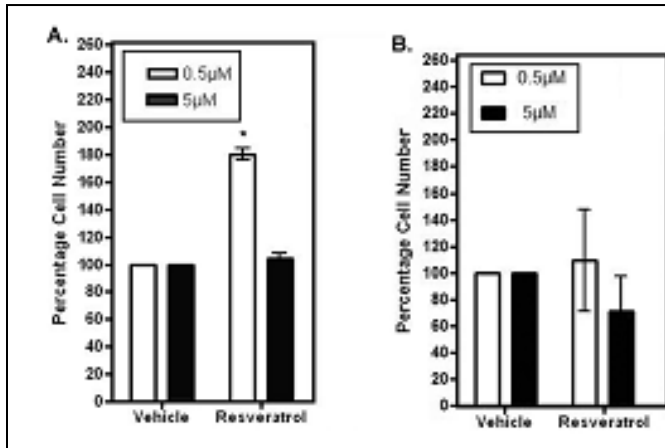


Fig. 3. Effect of resveratrol on MDA-MB-231 and MDA-MB-435 cell migration. Quiescent MDA-MB-231 (A) or MDA-MB-435 (B) cells were placed on the top well of Transwell chambers in serum-free, phenol red-free media and the number of cells that migrated through the membrane of the top well in response to various treatments was quantified relative to control. Data (\pm SEM) are quantified from analysis of 25 microscopic fields/treatment ($n=3$). The bottom well contained the following for 8 hours: vehicle, 0.5 μM or 5 μM resveratrol. An asterisk indicates statistical significance ($p \leq 0.05$) when compared to vehicle.

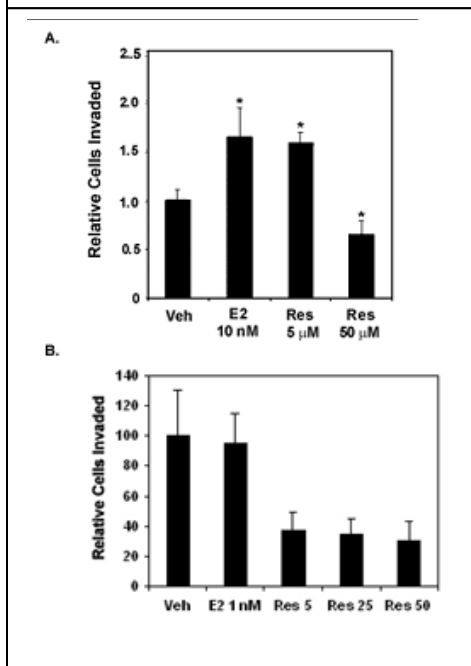


Figure 4. Effects of estrogen or resveratrol on MDA-MB-231 (A) and MDA-MB-435 (B) cell invasion.

Quiescent cells were placed on the top well of Transwell chambers where the top membrane was coated with Matrigel. The bottom well contained the following for 8 hours: DMSO control (Veh), 10 nM estrogen (E_2), or 5, 25, or 50 μM resveratrol (Res). Number of cells that migrated through the membrane of the top well was quantified relative to control. Data are quantified from analysis of 25 microscopic fields/treatment from 6 experiments and expressed as mean relative cells migrated \pm SEM. An asterisk indicates statistical significance ($p \leq 0.05$) when compared to vehicle.

II. Analysis of cell proliferation and cell cycle progression in response to resveratrol

Next we analyzed the effect of resveratrol on breast cancer cell proliferation and cell cycle progression. MDA-MB-231 or MDA-MB-435 cells were treated with vehicle or resveratrol at 0.5, 5, or 20 μM every 48h for 96h and cell number and cell cycle stage determined by quantification and flow cytometric analysis of propidium iodide (PI)-stained nuclei (Fig.5). At 0.5 μM , resveratrol did not cause significant changes in MDA-MB-231 or MDA-MB-435 cell number. At 5 and 20 μM , resveratrol significantly decreased MDA-MB-231 cell proliferation and the decrease in cell proliferation at 20 μM was reflected in a significant S phase cell cycle arrest (Fig. 3A, B). Resveratrol at low concentrations (0.5 and 5 μM) did not affect cell proliferation or cell

cycle progression of MDA-MB-435 cells. Cell cycle changes and effects of apoptosis by resveratrol at pharmacological concentrations (20-50 μM) are currently under analysis. These data demonstrate that resveratrol is a more effective inhibitor of cell growth in the ER β (+) MDA-MB-231 cell line compared to the ER (-) MDA-MB-435 cell line.

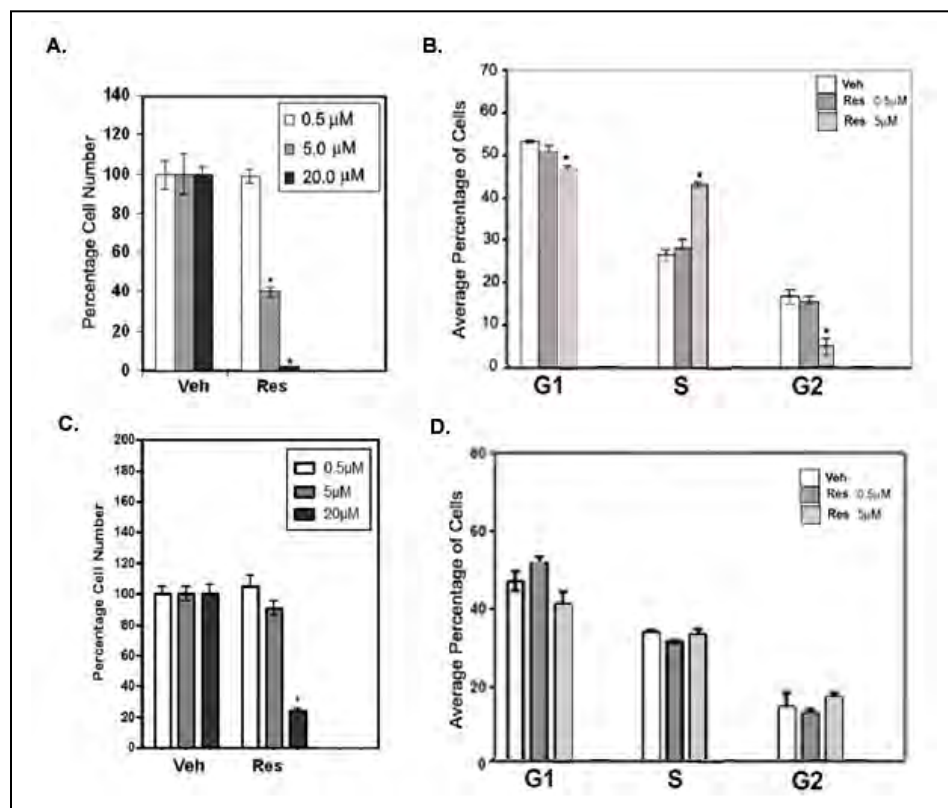
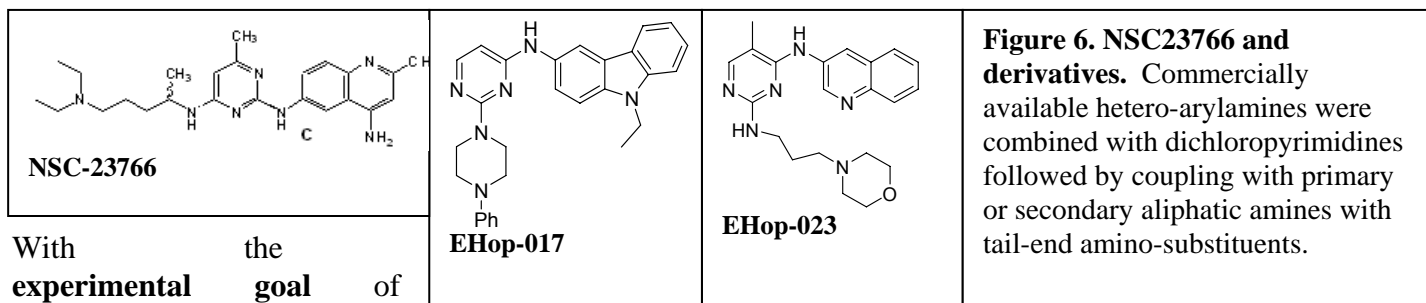


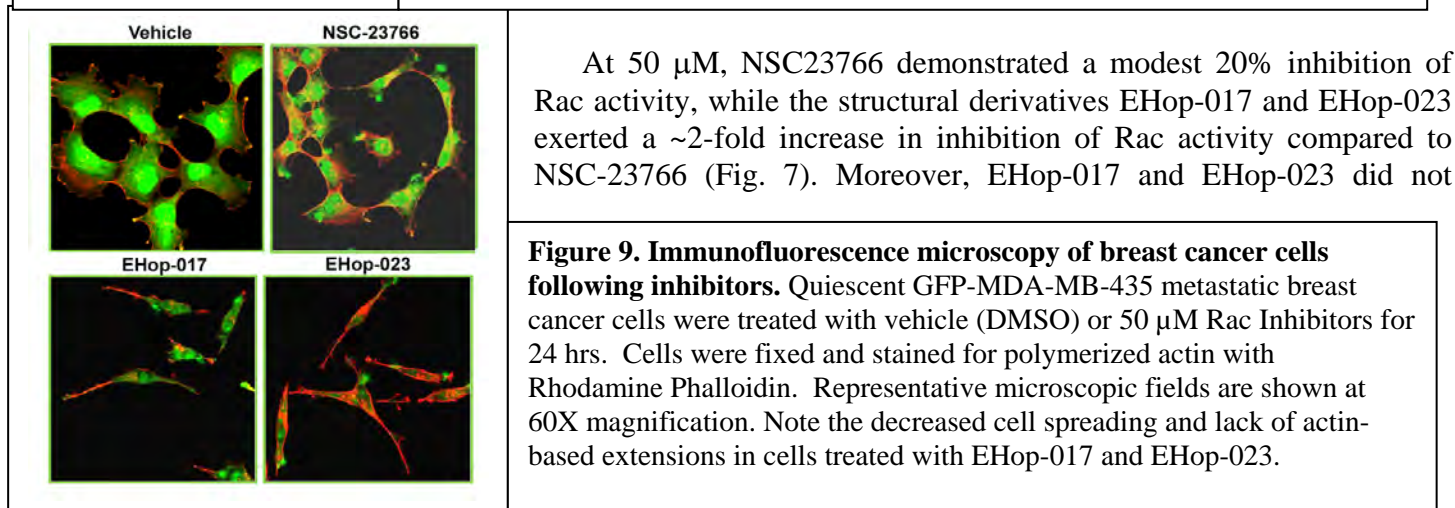
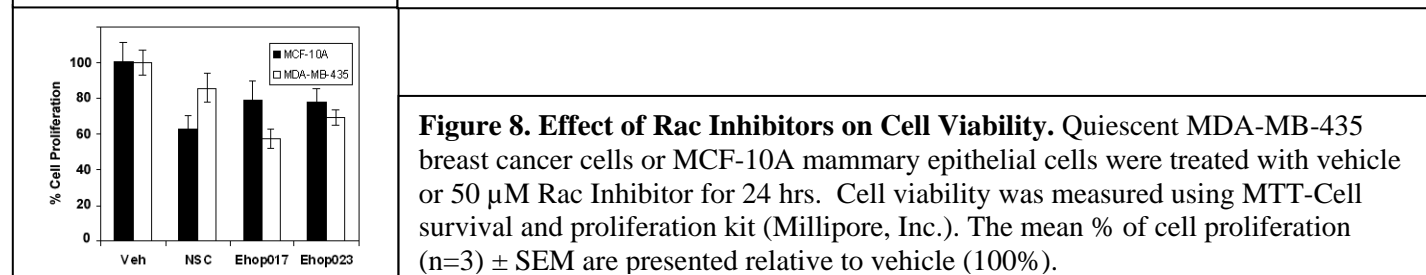
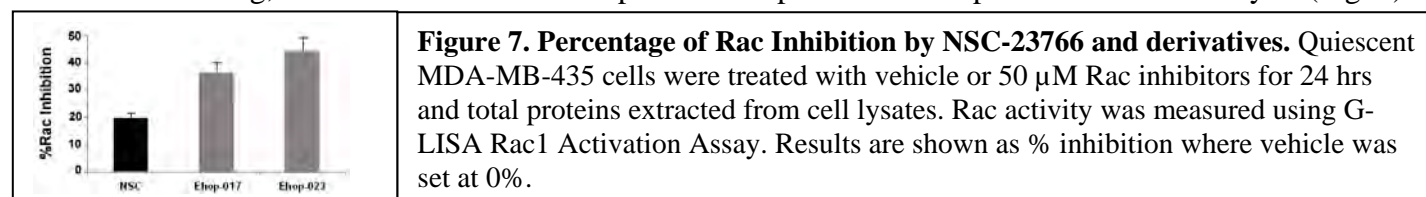
Figure 5. Effect of resveratrol on breast cancer cell proliferation and cell cycle progression. MDA-MB-231 (A) or MDA-MB-435 (C) cells in 5% serum and phenol red-free media were treated with vehicle, 0.5, 5, or 20 μM resveratrol every 48h for 96h. Cell number was quantified from PI-stained intact (non-apoptotic) nuclei. Percentage of viable cells \pm SEM for 20 microscopic fields/treatment is shown (n=3). MDA-MB-231 (B) or MDA-MB-435 (D) cells treated with vehicle, 0.5 or 5 μM resveratrol were subjected to Flow cytometry analysis of percentage of PI stained cells at each cell cycle phase \pm SEM for three individual experiments (n=3). An asterisk indicates statistical significance ($p \leq 0.05$) when compared to vehicle.

Development of Rac-specific inhibitors:

In the original proposal, we planned to use NSC23766, a commercially available Rac-specific inhibitor for a direct analysis of the inhibition of Rac interaction with Rac.GEFs. NSC23766 is a small molecule compound that was identified from the NCI chemical database as a putative Rac inhibitor (33). Subsequently, this compound was shown to specifically inhibit Rac1 binding and activation by the Rac-specific GEFs Trio or Tiam1 and fit into the surface groove of Rac1 known to be critical for GEF binding (34). Therefore, NSC-23766 cannot inhibit binding of other Dbl family members like Vav or DOCK family GEFs (35). Since we have shown that Vav-2 may be an important Rac.GEF in regulation of cancer cell function by E₂ and resveratrol (Fig. 2), NSC-23766 may be limited in use as an inhibitor of Rac-mediated breast cancer metastasis. In the last annual report, we reported our strategy for developing more efficient derivatives of NSC23766 that can inhibit a wider range of Rac.GEFs. Using the G-LISA Rac Activation Assay (Cytoskeleton, Inc., Denver, CO), we described previously that in highly metastatic breast cancer cells with hyperactive Rac activity, the IC₅₀ for action of NSC-23766 on Rac activity was as high as 95 μM (data not shown).

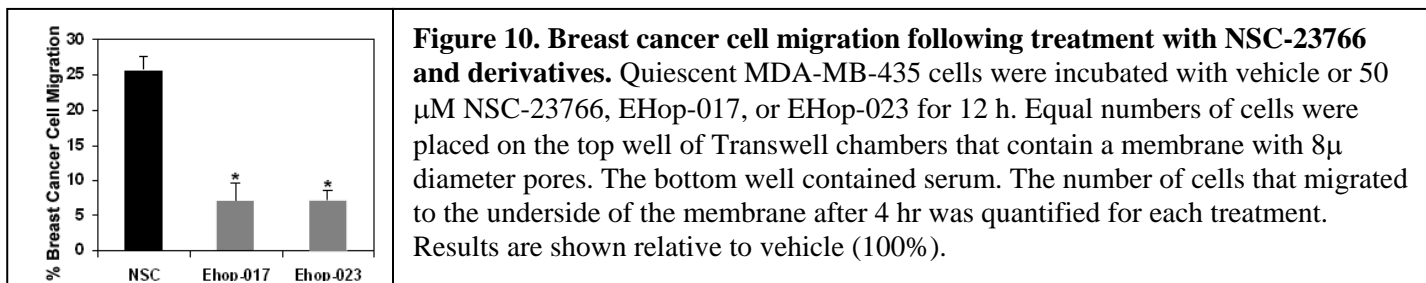


With the experimental goal of identifying more efficient structural derivatives of NSC-23766, research collaborators Drs. Cornelis Vlaar and Eliud Hernandez (Department of Pharmacology, University of Puerto Rico, San Juan, PR) initiated the synthesis of novel derivatives of NSC-23766. A facile two-step synthesis for the preparation NSC23766 derivatives was developed by combining commercially available (hetero)-arylamines with dichloropyrimidines. Subsequent coupling with primary or secondary aliphatic amines with or without a tail-end amino-substituent provided NSC-23766 derivatives. In the previous annual report we showed the analysis of a range of inhibitors (42 compounds) for more efficient inhibition of Rac activity without toxicity to normal mammary epithelial cells. From this screening, we have selected two compounds EHop-017 and EHop-023 for further analysis (Fig. 6).

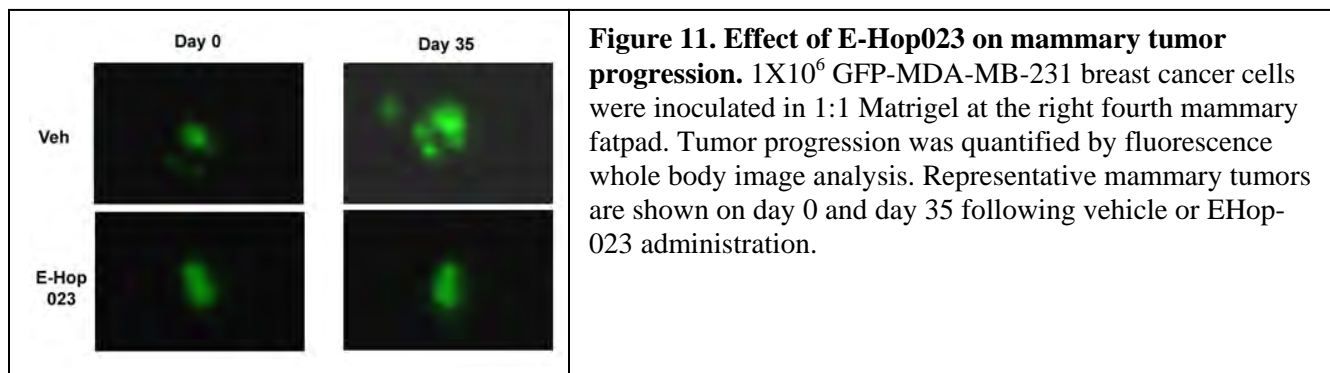


affect the viability of mammary epithelial cells. EHop-017 reduced MDA-MB-435 cell number by 40% while EHop-023 reduced MDA-MB-435 cells by 30% indicating that these compounds exert specific inhibitory effects on breast cancer cell proliferation (Fig. 8). To determine whether inhibition of Rac activity has an effect on cell functions relevant for metastasis that are under Rac regulation, the effects of NSC23766 and selected

derivatives on formation of motile actin structures and directed cell migration were determined. MDA-MB-435 cells were incubated with vehicle or 50 μ M NSC23766, EHop-017, or EHop-023 for 24h, fixed, and stained for polymerized actin (red). Fluorescence microscopy demonstrated a marked decrease in actin rich structures called lamellipodia and cell spreading (functions under Rac regulation) in cells treated with EHop-017 or EHop-023 compared to cells treated with vehicle or NSC23766 (Fig. 9). When MDA-MB-435 cells were subjected to Transwell migration assays following incubation of EHop-017 or E-Hop-023 at 50 μ M, both compounds inhibited cell migration by 80% and 95% respectively compared to vehicle while NSC-23766 inhibited cell migration by 75% (Fig. 10).



To test the effect of EHop-023 on mammary tumor progression *in vivo* and to determine toxicity of this compound, we conducted a preliminary study where nude mice were inoculated with green fluorescent protein (GFP) tagged MDA-MB-231 cells at the mammary fat pad. After 1 week of tumor establishment, mice (6/experimental group) received intraperitoneal injections of control or EHop-023 at 10 mg/kg body weight (BW) 3X week for 35 days. Data show that E-Hop023 did not have toxic effects on mice where the average weight of mice that received the vehicle was 25.2 \pm 1.5 and the mice that were administered EHop-023 had an average weight of 24.9 \pm 2.3. Fig. 11 shows that compared to vehicle, EHop-023 treatment inhibited primary tumor growth of MDA-MB-231 mammary tumors.



Therefore, EHop-023 appears to be a viable Rac inhibitor that inhibits Rac activity, cell migration, lamellipodia extension, and primary tumor growth. ***In the original proposal, all Specific Aims proposed using NSC-23766 as a Rac inhibitor in vitro and in vivo. We now feel that E-Hop-023 would be a better choice for this purpose.***

Tasks 2 and 3. Determine the effect of estrogen and resveratrol on breast cancer progression (Months 25-36)

These tasks proposed testing the effect of E2, resveratrol, and a Rac inhibitor on breast cancer progression of fluorescent protein (FP)-tagged MDA-MB-231 ER α (-) ER β (+) low metastatic human breast cancer cell line and MDA-MB-435 ER (-) high metastatic cell line in nude mice.

In the previous annual report, we showed that xenografts of GFP-MDA-MB-231 cells at 1-2X10⁵ cells/inoculation can be maintained up to 4 months as tumors in the mammary fatpads of female athymic nude mice. The fluorescent tumors were analyzed for fluorescent area and integrated density by *in situ* whole body fluorescence image analysis (Fig. 12). As measured by twice weekly fluorescence image analysis, tumor growth remained linear during the study (36). This low metastatic variant was not very efficient with forming distant metastases and therefore, is not a good model for investigation of the effect of resveratrol on metastasis.

Next we determined that ability of the more aggressive GFP-MDA-MB-435 cells to develop primary and secondary metastases in female athymic nude mice. 10 mice/group

were inoculated with 5X10⁵ GFP-MDA-MB-435 cells at the mammary fatpad. One day following tumor cell inoculation, the mice were orally gavaged with vehicle (90% oil, 10% ethanol), 10 mg/kg BW resveratrol, or 5 mg/kg each resveratrol, quercetin, and catechin (RQC), the major polyphenols of grapes and red wine. The concentration of resveratrol used did not have a significant effect on primary mammary tumor progression or metastasis (Fig. 12.A, B). This may be because we used a low-medium

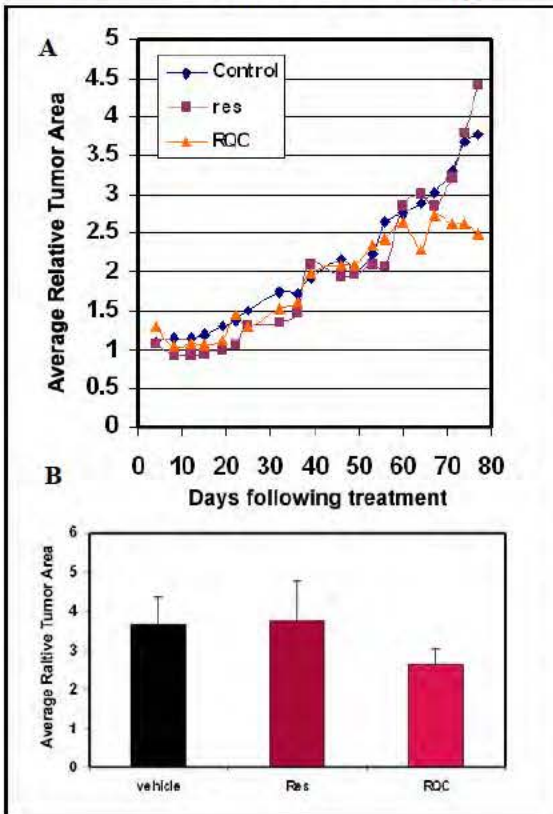


Figure 12. Response of GFP-MDA-MB-435 mammary tumors to dietary resveratrol. A. Relative GFP-MDA-MB-435 mammary tumor progression was determined by *in situ* whole body fluorescence image analysis from day 1 of tumor plantation and compared with images acquired 2X a week for 77 days. Average relative tumor area as calculated from integrated fluorescence intensity of mammary tumors from vehicle (Veh) or 10 mg/kg BW resveratrol (Res)-treated (8 mice/group) or 5mg/kg BW each resveratrol, quercetin, and catechin (RQC) B. Average relative tumor area (integrated density of mammary tumor on day 77/ integrated density of mammary tumor on day 01) on day 77 for Veh, Res, or RQC treated mice.

concentration of resveratrol (10 mg/kg BW). Interestingly, a combination of 5 mg/kg BW resveratrol, quercetin, and catechin reduced primary mammary tumor growth by 35% and drastically reduced bone and lung metastases (37).

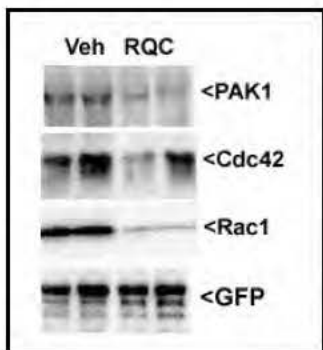


Figure 13. Expression of Rac, Cdc42, and their downstream effector PAK1 in mammary tumors following vehicle or grape polyphenol diets. The primary mammary tumors established from GFP-tagged MDA-MB-231 cells following 120 days of vehicle or a combination of 5 mg/kg BW each resveratrol, quercetin, and catechin (RQC) were excised and prepared for SDS-PAGE by homogenization, lysis, and dissolving in sample buffer. Gels were run using equal protein concentrations of vehicle (N=2) or RQC (N=2) and western blotted with specific antibodies for PAK1, Cdc42, Rac1, and GFP (as a loading control and to demonstrate that the proteins are from GFP-tagged human breast cancer cells).

Interestingly, western blotting of excised MDA-MB-231 mammary tumors for Rac1, close homolog Cdc42, and their downstream effector PAK1 showed that Rac1 and PAK1 protein levels were downregulated in the tumors from RQC treated mice compared to vehicle treated mice (Fig. 13). RQC treatment in this experiment resulted in a 70% reduction in tumor size compared to vehicle controls (36). This intriguing result demonstrates the relevance of Rac1 and PAK expression and activity to mammary tumor progression.

During the last year of the funding period, we will test the effect of E₂, 5 and 50 mg/kg BW resveratrol, and 10 mg/kg BW EHop-023 (Rac inhibitor) on breast cancer progression of mice with GFP-MDA-MB-231 or GFP-MDA-MB-435 mammary tumors.

Key Research Accomplishments

- Analysis of Rac activity of ER β (+) MDA-MB-231 breast cancer cells demonstrated that low concentrations of resveratrol act similar to estrogen and increased Rac activity while high concentrations inhibited Rac activity.
- Rac activity of ER (-) MDA-MB-435 cells did not change in response to estrogen but was decreased by both low and high resveratrol concentrations.
- Rac.GEF activity for the Oncogene Vav-2 showed that in MDA-MB-231 cells, Vav-2 association with Rac is increased by estrogen and decreased by high concentrations of resveratrol.
- Analysis of cell migration and invasion of MDA-MB-231 breast cancer cells demonstrated that low concentrations of resveratrol act similar to estrogen and increased cell migration and invasion while high concentrations inhibited cell migration and invasion.
- Analysis of cell migration and invasion of MDA-MB-435 breast cancer cells demonstrated that these ER (-) cells are unresponsive to estrogen and cell migration and invasion are inhibited by both low and high concentrations of resveratrol.
- Analysis cell proliferation and cell cycle progression showed that resveratrol is a more effective inhibitor of cell growth in the ER β (+) MDA-MB-231 cell line compared to the ER (-) MDA-MB-435 cell line. Resveratrol even at low concentrations reduced MDA-MB-231 cell growth and arrested the cell cycle at S/G2 transition. Higher concentrations of resveratrol were required to inhibit growth of MDA-MB-435 cells.
- Novel derivatives of NSC-23766 were developed because this parent compound was not an efficient Rac inhibitor of breast cancer cells with high endogenous Rac activity. EHop-023 was selected as a more efficient inhibitor of Rac activity that did not affect cell viability but inhibited Rac-mediated lamellipodia formation and cell migration of MDA-MB-435 breast cancer cells and inhibited primary mammary tumor growth of MDA-MB-231 cells.
- The effect of a medium concentration of dietary resveratrol was assessed in mice with GFP-MDA-MB-231 mammary tumors. Resveratrol at 10 mg/kg BW did not affect primary mammary tumor growth or metastases. Combined resveratrol, quercetin, and catechin (major polyphenols in grape) at 5 mg/kg BW reduced mammary tumors from both MDA-MB-231 and MDA-MB-435 cells and reduced metastasis to bone and liver from MDA-MB-435 mouse mammary tumors.

Reportable Outcomes

- Funding was requested from DoD/BCRP Idea Award Program to further develop novel Rac inhibitors EHop-023 and EHop-017 as anti breast cancer invasion compounds.

Date of Submission: 04/08/2009

Title: Novel small molecule inhibitors that target Rac and Cdc42 as metastatic breast cancer therapeutics

- Funding was also requested from DoD/BCRP Idea Award Program to investigate a breast cancer metastasis inhibitory role for combined grape polyphenols resveratrol, quercetin, and catechin.

Date of Submission: 04/08/2009

Title: Potential of grape polyphenols in prevention and chemosensitization of breast cancer metastasis to the bone

- See Appendix for attached manuscript: Castillo-Pichardo L, Martínez-Montemayor MM, Martínez JE, Wall KM, Cubano LA, Dharmawardhane S. Inhibition of metastases to bone and liver by dietary grape polyphenols. Clin Exp Metastasis. 2009, in press

Conclusions

The proposed *hypothesis* that high concentrations of resveratrol prevent breast cancer invasion and metastasis while E₂ and low concentrations of resveratrol promote breast cancer invasion and metastasis via Rac-regulated mechanisms was validated, but shown to be dependent on ER expression. Our results show that low concentrations of resveratrol act similar to estrogen and increases Rac activity and cell migration/invasion while high concentrations inhibit Rac activity, Rac/Rac.GEF association, and cell migration/invasion of ER β (+) metastatic breast cancer cells. However, in ER (-) MDA-MB-435 cells, estrogen had no effect and resveratrol at all concentration tested inhibited Rac activity and cell migration/invasion. Resveratrol also reduced cell proliferation and inhibited cell cycle progression at S-phase in MDA-MB-231 cells but this inhibitory effect was not as pronounced in MDA-MB-435 cells. This data suggests a novel mechanism of regulation for resveratrol in inhibition of aggressive ER (-) breast cancer metastasis.

Interestingly, in both MDA-MB-231 and MDA-MB-435 metastatic cancer cells, a combination of resveratrol, quercetin, and catechin at 0.5 and 5 μ M significantly decreased cell proliferation and induced cell cycle arrest and apoptosis. Therefore, we tested the effect of combined grape polyphenols on tumor growth at the mammary fatpad and metastasis using the highly metastatic ER (-) cell line MDA-MB-435. As shown by our previous studies using MDA-MB-231 cells (36), this study with MDA-MB-435 cells showed that combined grape polyphenols at 5mg/kg body weight each resveratrol, quercetin, and catechin reduced mammary tumor growth. We also recently reported that combined resveratrol, quercetin, and catechin can specifically inhibit metastasis to bone and liver (37). This manuscript (Castillo-Pichardo, et al., 2009, Clin. Exp. Met., in press) is included in the appendix because it was partially funded by this DoD award.

We also developed novel Rac inhibitors that were more efficient than the commercially available NSC-23766 Rac inhibitor that we intended to use in the original proposal. However, this inhibitor was not sufficient to inhibit all of the Rac activity of the breast cancer cell lines with high endogenous Rac activity. Therefore, we developed and identified a NSC-23766 derivative EHop-023 that can be used for the proposed experiments.

In the last year of funding, we expect to complete all tasks and demonstrate that 5 mg/kg BW resveratrol treatment would increase while 50 mg/kg BW of resveratrol would decrease breast cancer progression of MDA-MB-231 cells. We recently (June 01, 2009) initiated this study at Universidad Central del Caribe (UCC) where nude mice with MDA-MB-231 xenografts will receive vehicle, 0.5 mg/kg BW 17 β -estradiol, 5 or 50 mg/kg BW resveratrol, or 10 mg/kg BW EHop-023. This study is expected to be completed in August/September 2009. The PI will be moving her laboratory in July 2009 to the nearby University of Puerto Rico, Medical Sciences

Campus (UPR-MSU). However, she will remain as adjunct faculty and will have access to the UCC animal care facility to complete the recently initiated animal study at UCC. We will submit an animal protocol to conduct a similar study on mice bearing MDA-MB-435 tumors to the IUCAC of the new institution UPR-MSU and expect to initiate that study in January 2010.

As per our *in vitro* data, MDA-MB-435 breast cancer progression may be inhibited by all concentrations of resveratrol used. This may be due to an ER-independent alternative mechanism of regulation by resveratrol. To delineate the molecular mechanisms of resveratrol action in ER (-) breast cancer, we plan to excise mouse mammary tumors at the end of these studies and perform PCR arrays and western blotting for genes and proteins associated with Rac signaling and invasion/metastasis.

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Inhibition of mammary tumor growth and metastases to bone and liver by dietary grape polyphenols

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Received: 13 October 2008 / Accepted: 4 March 2009
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Abstract The cancer preventive properties of grape products such as red wine have been attributed to polyphenols enriched in red wine. However, much of the studies on cancer preventive mechanisms of grape polyphenols have been conducted with individual compounds at concentrations too high to be achieved via dietary consumption. We recently reported that combined grape polyphenols at physiologically relevant concentrations are more effective than individual compounds at inhibition of ER α (-), ER β (+) MDA-MB-231 breast cancer cell proliferation, cell cycle progression, and primary mammary tumor growth (Schlachterman et al., *Transl Oncol* 1:19–27, 2008). Herein, we show that combined grape polyphenols induce apoptosis and are more effective than individual resveratrol, quercetin, or catechin at inhibition of cell proliferation, cell cycle progression, and cell migration in the highly metastatic ER (-) MDA-MB-435 cell line. The combined effect of dietary grape polyphenols (5 mg/kg each resveratrol, quercetin, and catechin) was tested on progression of mammary tumors in nude mice created from green fluorescent protein-tagged MDA-MB-435 bone metastatic variant. Fluorescence image analysis of primary tumor growth demonstrated a statistically significant decrease in tumor area by dietary grape polyphenols. Molecular analysis of excised tumors demonstrated that

reduced mammary tumor growth may be due to upregulation of FOXO1 (forkhead box O1) and NFKBIA (I κ B α), thus activating apoptosis and potentially inhibiting Nf κ B (nuclear factor κ B) activity. Image analysis of distant organs for metastases demonstrated that grape polyphenols reduced metastasis especially to liver and bone. Overall, these results indicate that combined dietary grape polyphenols are effective at inhibition of mammary tumor growth and site-specific metastasis.

Keywords Breast cancer · Catechin · Metastasis · Quercetin · Resveratrol

Introduction

Breast cancer is the most commonly diagnosed form of cancer and the second major cause of death from cancer in women [1, 2]. Recent clinical advances have remarkably increased the survival rates from primary breast cancer; however, the prognosis of breast cancer patients is still limited by metastases that can occur years after initial diagnosis and potential cure. Malignant breast cancers often overexpress epidermal growth factor receptor (EGFR) isoforms such as Her-2 that further confound effective treatment of metastatic breast cancer [3]. Therefore, investigation of the effect of dietary alternatives and their mechanisms of action specifically on Her-2 overexpressing metastatic cancers can lead to alternative therapeutic strategies.

Grape skins and thus red wine, contain many polyphenols that have anticancer properties [4, 5]. Grape polyphenols have been implicated in cancer protection in numerous *in vitro* studies due to antioxidant and pro-apoptotic effects as well as inhibition of a number of tumorigenic pathways

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[6–8]. Combined grape polyphenols extracted from red wine have been shown to specifically inhibit the growth of breast cancer cells with low cytotoxicity towards normal mammary epithelial cells [9]. However, the effects of grape polyphenols on metastatic breast cancer remain to be investigated.

Resveratrol, quercetin, and catechin, grape polyphenols selected for this study, represent about 70% of the total polyphenols in red wine and have been shown to be the most effective anticancer compounds in red wine [8, 10]. Resveratrol is found in low, but significant amounts in red wine and comprises about 1% of total polyphenols [10, 11]. In breast cancer, resveratrol has been implicated in prevention of multistage carcinogenesis [12, 13]. Quercetin comprises about 6% of total polyphenols in red wine [10] and has been reported to decrease Her-2 expression [14]. Her-2 is often overexpressed in metastatic cancers including the MDA-MB-435 cell line that was used for this study. The monomeric form of catechin constitutes up to 65–70% of total red wine polyphenols and has been shown to delay tumor initiation [10, 15, 16]. Resveratrol, quercetin, and catechin are all viable chemopreventives because they are absorbed and metabolized rapidly *in vivo* and can be detected in plasma and urine samples in the intact form in humans and rodent models [17–20].

Individually, resveratrol, quercetin, or catechin induce cell cycle arrest and apoptosis in cancer cells [21–23], prevent breast carcinogenesis and cancer progression in rodent models [24–26], and inhibit angiogenesis [27]. Much of the data on the cancer preventive effects of grape polyphenols have been generated from estrogen receptor (ER) (+) tissue culture cell lines and rodent models using pharmacological concentrations of individual polyphenols [16, 24–26, 28, 29]. We previously reported that in ER α (–), ER β (+) MDA-MB-231 breast cancer cells, resveratrol is inhibitory at high pharmacological concentrations and acts similar to estrogen by increasing cell functions and signaling relevant for metastasis at low dietary levels [30, 31]. However, the effects of combined grape polyphenols at low, dietary concentrations are only now beginning to be assessed.

Recently, we reported that combined resveratrol, quercetin, and catechin (RQC) treatment at physiologically relevant concentrations was more efficient than individual grape polyphenols at inhibition of cell proliferation, cell cycle progression, and primary mammary tumor growth of MDA-MB-231 cells [1]. In this study, we were limited in investigation of the role of grape polyphenols as potential metastasis preventives because the low metastatic MDA-MB-231 cell line formed only a few lung metastases. Most breast cancers preferentially metastasize to bone and liver, where ~80% of patients with advanced breast cancer develop bone cancer, causing severe morbidity and mortality [32]. Therefore, for the current study, we selected a

bone metastatic variant of the highly metastatic cancer cell line, MDA-MB-435 [33], to test the effect of grape polyphenols on cell proliferation, cell cycle progression, apoptosis, cell migration, tumor growth, and metastatic progression. Mammary fat pad tumors were established in nude mice and we show that dietary grape polyphenols inhibit both primary tumor growth and metastatic cancer progression from the breast to bone and liver. Our results show that this inhibition may be due to upregulation of caspase 3 activity and expression of FOXO1 transcription factor and NFKBIA; molecules known to regulate cancer progression [34–36].

Materials and methods

Cell culture

Human metastatic cancer cell lines MDA-MB-231 (ER α –, ER β +) (American Type Culture Collection, Manassas, VA, USA) and a bone metastatic variant of MDA-MB-435 (ER–) stably expressing GFP were used for the study (kind gift of Dr. Danny Welch, The University of Alabama at Birmingham, AL, USA) [37]. Cells were cultured in DMEM with 10% heat-inactivated FBS as described in [1, 33].

Cell proliferation and cell cycle progression

MDA-MB-435 (2×10^5) cells in 5% charcoal-stripped FBS were treated every 48 h for 96 h with vehicle (0.2–0.5% DMSO), 0.5, 5, or 20 μ M resveratrol, quercetin, or catechin or a combination RQC at 0.5, 5, or 20 μ M each. Cells were fixed, nuclei stained with PI and cell proliferation quantified as the number of cells with intact nuclei. Cell cycle stage of MDA-MB-435 cells was determined by flow cytometry of PI-stained cells as previously described in [1], following treatment with 5 μ M resveratrol, quercetin, or catechin or 5 μ M RQC every 48 h for 96 h.

Caspase 3 activity assay

Apoptosis was analyzed by the caspase 3 activity of cell lysates following vehicle (0.2% DMSO) or 0.5 or 5 μ M RQC for 48 h using a Caspase-3 Colorimetric Assay Kit as per manufacturer's instructions (Sigma Aldrich, St Louis, MO, USA). Briefly, the *p*-nitroaniline (pNA) moiety resulting from hydrolysis of acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-pNA) by caspase 3 activity was detected at 405 nm ($\epsilon_{\text{mM}} = 10.5$) after incubating the reaction mixture at 37°C for 22 h. The concentration of the pNA released from the substrate was calculated from the absorbance values at 405 nm using a calibration curve prepared with pNA standards. Concentration of pNA was

further converted to caspase 3 activity in μmol of pNA $\text{min}^{-1} \text{ml}^{-1}$.

Annexin V staining

Apoptotic cells were detected by fluorescence microscopy of Annexin V-Cy3-18 stained cells as per manufacturer's instructions (Sigma Aldrich, St Louis, MO, USA). Briefly, MDA-MB-435 cells grown on coverslips were treated with vehicle (0.2% DMSO) or 5 μM RQC for 48 h and stained with Annexin V-Cy3-18 in binding buffer (10 mM HEPES/NaOH, pH 7.5, 0.14 M NaCl, 2.5 mM CaCl_2) for 15 min at room temperature. Coverslips were washed in binding buffer and fixed with 3.7% paraformaldehyde prior to fluorescence microscopy. Images were digitally acquired from an Olympus inverted fluorescence microscope using Metamorph software (Molecular Devices, Sunnyvale, CA, USA) and quantified from ten random microscopic fields ($20\times$ mag.)/coverslip.

Cell migration

Equal numbers of viable quiescent GFP-tagged MDA-MB-231 or MDA-MB-435 cells (1×10^5) were placed in the top well of Transwell chambers where the bottom well contained vehicle (0.2% DMSO), 0.5 or 5 μM resveratrol, quercetin, or catechin or 0.5 or 5 μM RQC in serum-free and phenol red-free media. Following 8 h incubation, the cells on top of the membrane of the inner well were removed and the number of cells that migrated to the underside of the membrane through 8 μm diameter pores quantified following PI staining as described in [31].

Animals

Female athymic nu/nu mice, 5–6 week old (Charles River Laboratories, Inc., Wilmington, MA, USA) were maintained under pathogen-free conditions in Hepa-filtered cages under controlled light (12 h light and dark cycle), temperature (22–24°C), and humidity (25%). Throughout the experiment, the animals were provided with autoclaved AIN 76-A phytoestrogen-free diet (Tek Global, Harlan Teklad, Madison, WI, USA) and water ad libitum. This project was approved by the Universidad Central del Caribe Institutional Animal Care and Use Committee.

Tumor model

GFP-MDA-MB-435 cells ($\sim 1 \times 10^6$) in Matrigel (BD Biosciences, San Jose, CA, USA) were injected into the fourth right mammary fat pad of female nude mice under isoflurane inhalation to produce orthotopic primary tumors as described in [38]. After tumor establishment (1 week

post-inoculation), the animals were randomly divided into experimental treatment groups. About 3–5 animals per group were eliminated due to failure of tumor take, small or too large tumor area in 1 week, or due to penetration of the peritoneum that resulted in immediate GFP fluorescence in the intestines. Mice with similar tumor area as quantified by integrated density of fluorescence images were selected for further study.

Diet administration

Nude mice ($n = 10$ /experimental group) were orally gavaged either with vehicle (90% corn oil, 10% ethanol) or a combination of 5 mg/kg body weight (BW) resveratrol, 5 mg/kg BW quercetin, and 5 mg/kg BW catechin (RQC) in a 100 μl volume three times per week. The number of mice/group is in the range of previously published similar studies that demonstrated statistically significant differences in dietary treatments [39–41].

Whole body fluorescence image analysis

Mammary tumor growth was quantified as changes in intensity and integrated density of GFP-fluorescence as per our previously described methods [1, 42]. Anesthetized mice were imaged immediately following breast cancer cell inoculation and two times per week thereafter. A 300 Watt power source with two optical delivery systems fitted with excitation filters (470/40 nm) was used for whole body imaging of GFP fluorescence (LT99D2, Lighttools Research, Encinitas, CA, USA). Images were captured with a Spot II charge-coupled device (CCD) camera (Diagnostic Instruments, Sterling Heights, MI, USA) mounted with a 530/25 nm emission filter (Chroma Technology, Rockingham, VT, USA).

Tumor fluorescence intensities were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The final images were acquired on day 77. Relative tumor area was calculated as the fluorescence intensity of each tumor on each day of imaging relative to the fluorescence intensity of the same tumor on day 1 of diet administration.

Analysis of metastases

Following sacrifice, lungs, kidneys, livers, femurs, and hearts were excised and immediately stored in liquid N_2 . Stored organs were thawed and analyzed using an Olympus MV10 fluorescence macro zoom system microscope and images acquired with an Olympus DP71 digital camera. Each organ was imaged on both sides. The fluorescent lesions (green component of RGB images) were quantified for integrated intensity and area using Image J software.

Pixel values ranging from 0 to 255 were detected and a signal cut off of 58 (approximately one standard deviation above the mean of the maximum noise) was used to separate background signal from GFP signal. To eliminate potential false positives, a minimum fluorescent area threshold of 0.003 mm^2 was used (roughly four pixels). Areas identified as metastases were also validated by visual inspection and false positives eliminated from further analysis.

Real time reverse transcriptase (RT²)-PCR analysis

At the end of the study, solid primary tumors at the mammary fat pad were immediately stored in “RNA later” (Ambion, Austin, TX, USA). Total RNA extraction and DNAase treatment was performed using the Qiagen RNeasy Kit (Qiagen, Valencia, CA, USA) following manufacturer’s protocol. RNA concentration was detected using a NanoDrop (Thermo Scientific, Wilmington, DE, USA), while RNA integrity and quality analysis were evaluated using the Experion automated electrophoresis system (BioRad, Hercules, CA, USA). C-13 kit (SA Biosciences, Frederick, MD, USA) was used to synthesize cDNA from the extracted RNA (0.5 μg) and used to investigate gene expression profiles by the commercially-available phosphoinositide 3-kinase (PI3-K) Pathway Finder RT² ProfilerTM PCR arrays (SA Biosciences, Frederick, MD, USA). This RT² ProfilerTM PCR Array is designed to simultaneously profile the expression of 84 PI3-K pathway-specific genes, plus five housekeeping genes and seven RNA quality controls. The spreadsheets, gene tables, and template formulas included with the PCR array package

were used to calculate relative changes in gene expression and perform statistical analyses. Reproducibility was maintained by using RNA from three tumors per treatment (three biological replicates).

Statistical analysis

Data are expressed as the mean \pm SEM. Statistical analyses were done using Microsoft Excel or GraphPad Prism 5 software. Differences between means were determined using Student’s *t*-Test and two-way ANOVA.

Results and discussion

Effect of grape polyphenols on metastatic breast cancer cells in vitro

Previously, we demonstrated that a combination of resveratrol, quercetin, and catechin at 0.5, 5, or 20 μM reduced cell number significantly from control and was more efficient than individual compounds in the MDA-MB-231 ER α (–) ER β (+) human low metastatic breast cancer cell line [1]. However, due to the low metastatic nature of this cell line, we did not observe adequate metastases in a nude mouse model to enable a statistical analysis of the role of grape polyphenols on metastasis. Therefore, we tested the effect of dietary RQC on a highly metastatic ER (–) cancer cell line, MDA-MB-435. The origin of the MDA-MB-435 cell line has been called into question by several recent microarray studies that show expression of melanoma-associated genes [43]. However, MDA-MB-435 cells

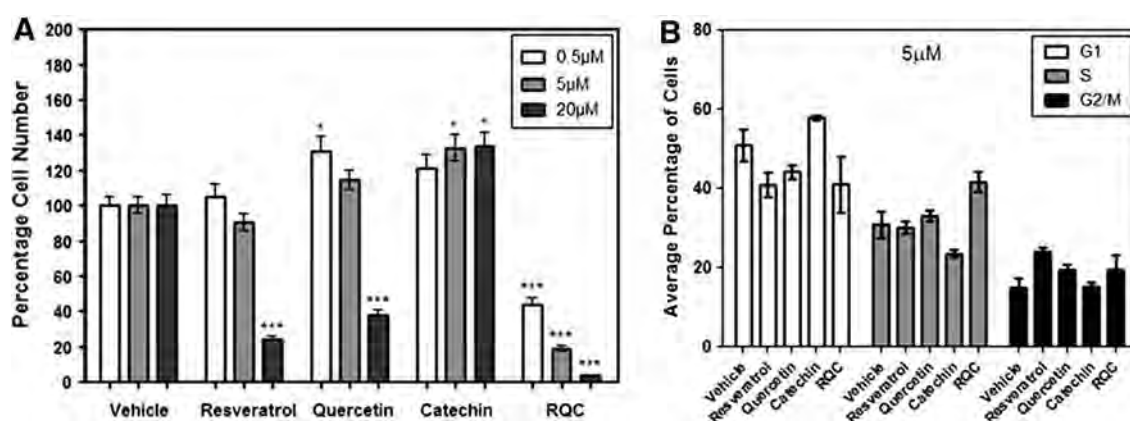


Fig. 1 Effect of grape polyphenols on MDA MB 435 cell proliferation and cell cycle progression. Cells in 5% serum and phenol red free media were treated with vehicle, 0.5, 5, or 20 μM resveratrol, quercetin, or catechin, or a combination of 0.5, 5, or 20 μM each (RQC) every 48 h for 96 h. Data was quantified from PI stained intact (non apoptotic) nuclei. **a** Cell proliferation. Percentage of viable

cells \pm SEM for 20 microscopic fields/triplicate treatments is presented. **b** Cell cycle progression. Cell cycle stage following 5 μM treatment with individual resveratrol, quercetin, or catechin or combined RQC. An asterisk indicates statistical significance of $P < 0.05$ and three asterisks indicate $P < 0.001$ when compared to vehicle

express breast differentiation-specific proteins and secrete milk lipids [44]. Since the patient had no evidence of melanoma but was diagnosed with only a breast carcinoma; and, since melanocytes do not produce milk, the simplest conclusion is that MDA-MB-435 is a very poorly differentiated breast carcinoma. This cell line has been extensively used to investigate metastasis from mammary fat pad inoculations, and remains as one of few models available for experimental metastasis of breast cancer in nude mice [33, 45].

As shown in Fig. 1a, 0.5 or 5 μM treatment with resveratrol, quercetin, or catechin alone did not decrease MDA-MB-435 cell proliferation. Resveratrol or quercetin at high concentrations (20 μM) significantly inhibited cell proliferation by 80 and 60% while catechin alone increases cell proliferation significantly. Therefore, the effects on cell proliferation at 20 μM RQC appear to be an additive effect of resveratrol and quercetin. The combined RQC treatment significantly inhibited MDA-MB-435 cell proliferation by ~ 50 , 80, and 90% at 0.5, 5, or 20 μM of each polyphenol (Fig. 1a). These compounds were more effective in the MDA-MB-231 cell line where both resveratrol and quercetin inhibited cell proliferation at 5 and 20 μM by ~ 60 and $\sim 95\%$; while combined resveratrol, quercetin, and catechin (RQC) treatment at 0.5, 5, and 20 μM each inhibited cell proliferation by ~ 60 , 85, and 98%, respectively, compared to vehicle controls [1]. Since combined RQC treatment induced a significant reduction on MDA-MB-435 cell proliferation, we then tested the cell cycle stage of MDA-MB-435 cells following 5 μM RQC treatment and found that these compounds arrested MDA-MB-435 cells at S phase as was shown before with MDA-MB-231 cells (Fig. 1b), [1]. However, unlike with the MDA-MB-231 cells, the S phase arrest of the MDA-MB-435 cells in response to RQC demonstrated a P value of 0.06.

The method we used to recover cells for analysis of cell cycle stage (i.e., trypsinization followed by centrifugation, fixing, staining, and washing) does not account for potentially apoptotic, non-adherent, or weakly adherent cells that may become removed during the repeated washings. Moreover, the observed increase in cells at S-phase does not correlate with the 80% decrease in cell number observed with 5 μM RQC (Fig. 1a, b). Therefore, we investigated the effect of RQC treatment on apoptosis of MDA-MB-435 cells by caspase 3 activity. This downstream effector caspase was selected to assess the effect of RQC on both receptor-regulated and mitochondrial apoptotic pathways. As shown in Fig. 2a, 5 μM RQC treatment increased caspase 3 activity by twofold at a $P < 0.06$ when compared to vehicle, while RQC at 0.5 μM did not affect caspase 3 activity in a significant manner. Similarly, Annexin V staining of MDA-MB-435 cells to detect phosphatidyl serines on the outer

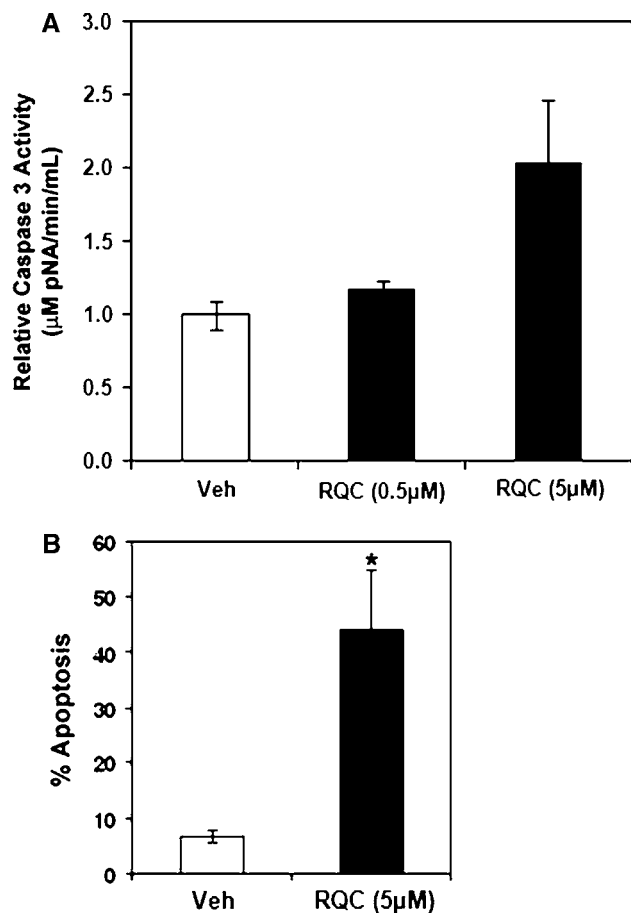


Fig. 2 Effect of grape polyphenols on apoptosis of MDA MB 435 cells. Apoptosis of MDA MB 435 cells was detected by caspase 3 activity assays (a) or fluorescence microscopy for Annexin V staining (b) following 48 h incubation with vehicle or RQC. **a** Average caspase 3 activity in $\mu\text{mol of pNA min}^{-1} \text{ml}^{-1}$ relative to vehicle ($n = 4 \pm \text{SEM}$) as quantified from absorbance at 405 nm of the pNA released by caspase 3 activity. **b** Percentage of cells undergoing apoptosis was calculated by Image J analysis of brightfield (total number of cells) and red fluorescence (apoptotic cells stained with Annexin V Cy3) from ten random microscopic fields/cover slip. Averages \pm SEM are shown for two separate experiments with duplicates for each experiment ($n = 4$). An asterisk indicates statistical significance ($P < 0.05$) when compared to vehicle

leaflet of the cell membrane indicated that at 48 h following 5 μM RQC, 44% of cells were significantly apoptotic ($P < 0.01$) compared to only 6.8% of vehicle-treated cells (Fig. 2b). Resveratrol and quercetin at high concentrations have been implicated in apoptosis of cancer cell lines by inducing caspase activity and inhibition of cell survival via PI3K/Akt pathways [23, 46, 47]. In MDA-MB-231 cells, by western blotting with total Akt and phospho-Akt^{ser-473} antibodies, 5 μM RQC (15 min) was found to decrease Akt activity by $\sim 50\%$ compared to vehicle (data not shown). Therefore, the observed decrease in breast cancer cell numbers in response to RQC treatment is thought to be due

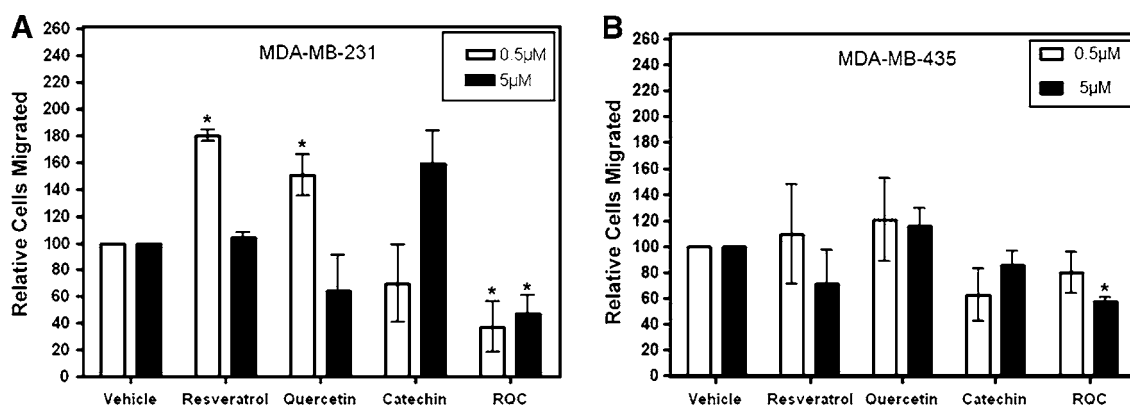


Fig. 3 Effect of grape polyphenols on breast cancer cell migration. Quiescent MDA MB 231 (a) or MDA MB 435 (b) cells were placed on the top well of Transwell chambers in serum free, phenol red free media and the number of cells that migrated through the membrane of the top well in response to various treatments was quantified relative

to control. Data are quantified from analysis of 25 microscopic fields/treatment ($n = 3 \pm \text{SEM}$). The bottom well contained the following for 8 h: vehicle, 0.5 or 5 μM resveratrol, quercetin, catechin or a combination of 0.5 or 5 μM each (RQC). An *asterisk* indicates statistical significance ($P < 0.05$) when compared to vehicle

to a block in cell cycle progression, increased apoptosis, and reduced cell survival signaling.

Since directed cell migration has been implicated with metastatic efficiency, we tested the effect of individual and combined grape polyphenols on cell migration. Migration assays were performed using Transwell chambers where individual resveratrol, quercetin, or catechin or combined RQC treatment was added to the bottom well while the inner well contained serum-starved MDA-MB-231 or MDA-MB-435 cells. In MDA-MB-231 cells, resveratrol and quercetin at 0.5 μM increased cell migration in a statistically significant manner (Fig. 3a). The effect of resveratrol is similar to our previous results that reported low concentrations of resveratrol to act comparable to estrogen and increase cell migration [31]. None of the other grape polyphenols significantly changed breast cancer cell migration. At 0.5 and 5 μM , combined RQC treatment significantly reduced MDA-MB-231 cell migration by $\sim 60\%$ when compared to vehicle controls; whereas, 0.5 μM combined RQC treatment reduced MDA-MB-435 cell migration by $\sim 20\%$, and 5 μM RQC significantly reduced cell migration by 40% (Fig. 3).

The lower response of the inhibitory effect of RQC treatment in the ER $(-)$ MDA-MB-435 cells compared to the ER $(+)$ MDA-MB-231 cells may be due to grape polyphenols acting as antiestrogenic compounds in the MDA-MB-231 cells. Also, since MDA-MB-435 cells are Her2 $^{++}$ it is possible that combined grape polyphenols are not as efficient at inhibiting the increased Her-2 signaling in this highly aggressive cancer cell line. However, mechanistic studies need to be conducted to further address the differences in response to grape polyphenols between these two cancer cell lines.

Effect of grape polyphenols on mammary tumor growth in vivo

To test the effect of resveratrol, quercetin, and catechin on metastatic breast cancer progression in vivo, we established mammary fat pad tumors from GFP-tagged highly metastatic MDA-MB-435 cancer cells as previously described [42]. As quantified from the integrated density of fluorescent images, mice ($n = 10$ per group) with similar initial tumor volumes (vehicle group = $9,036.6 \pm 654$ and RQC group = $9,825 \pm 501$) were selected for further study.

One week following tumor establishment, mice were gavaged with vehicle (90% oil, 10% ethanol) or 5 mg/kg BW RQC three times a week. This dietary concentration was selected based on our previous study where administration of 0.5, 5, or 20 mg/kg BW RQC demonstrated that the inhibitory effect on mammary tumor growth plateaued at 5 mg/kg BW [1]. Tumor progression was quantified by fluorescence image analysis twice a week. The relative tumor area was calculated as the fluorescence intensity of each tumor on day of imaging relative to the fluorescence intensity of the same tumor on day 1 of diet administration as described in [1]. As shown in Fig. 4a, tumor growth remained linear and similar for both vehicle and RQC treated mice for 60 days. After 60 days, the RQC-treated mice demonstrated reduced tumor growth compared to vehicle. At the end of the study on day 77, the mice following RQC diet demonstrated smaller tumors that were reduced by $\sim 37\%$ in a statistically significant manner (Fig. 4b). Previously, we reported a 69% decrease in MDA-MB-231 mammary tumor growth with 5 mg/kg BW RQC treatment [1]. The present data demonstrates that, as with the in vitro effects, dietary RQC treatment of nude

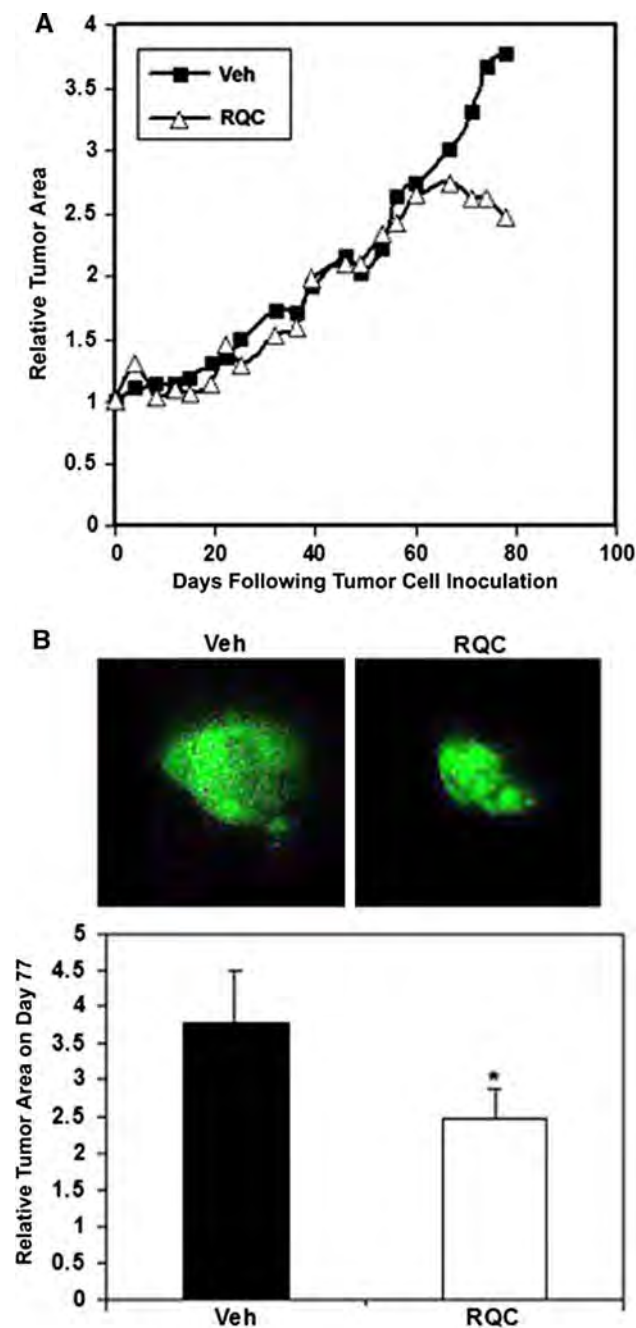


Fig. 4 Effect of grape polyphenols on the growth of MDA MB 435 mammary fat pad tumors. MDA MB 435 cells (10^6) in Matrigel were inoculated at the mammary fat pad of nude mice. One week following injection, mice were fed vehicle or a combination of 5 mg/kg BW Res, 5 mg/kg BW Quer, and 5 mg/kg BW Cat (RQC) three times a week by oral gavage. Whole body fluorescence images were acquired two times a week. **a** Average relative tumor area as a function of days following injection. Relative tumor area was calculated as the area of fluorescence, measured by fluorescence intensity on each day of imaging as a function of the fluorescence intensity of the same tumor on day 1. **b** GFP MDA MB 435 tumors following vehicle or RQC diets at day 77. Representative digital images and mammary tumor area as quantified from digital images acquired on day 77, made relative to same tumor image on day 1. Asterisk denotes statistical significance at $P < 0.05$

mice with MDA-MB-435 mammary tumors results in a significant inhibition of primary tumor growth but this effect is less compared to the effect of RQC treatment on MDA-MB-231 mouse mammary xenografts.

At the end of the study (77 days), there were no statistically significant differences in body weights from mice treated with vehicle ($24.35 \text{ g} \pm 1.6$) or RQC ($24.04 \text{ g} \pm 1.7$). This is similar to our previous report where 118 days of dietary RQC treatment at concentrations as high as 25 mg/kg BW did not significantly change mouse weights from vehicle controls [1]. Therefore, the decrease in tumor area at the end of the study was not due to toxic effects of dietary RQC. This data indicates that combined grape polyphenols can be safe and effective therapeutics and preventives of primary tumor growth of ER (-) breast cancer.

To initiate a molecular analysis of the effect of grape polyphenols on breast cancer, we analyzed changes in expression of PI3-K pathway genes because this pathway is a central regulator of cancer cell survival and invasion [48]. Interestingly, real-time PCR arrays for PI3-K pathway genes from tumor extracts revealed that expression of FOXO1 transcription factor was upregulated significantly by 1.87-fold in mouse mammary tumors following RQC treatment (Table 1). FOXO factors have been shown to function as tumor suppressors in a variety of cancers. They are actively involved in promoting apoptosis in a mitochondria-independent and -dependent manner by inducing the expression of death receptor ligands and pro-apoptotic Bcl-2 family members [35]. Forkhead proteins have been shown to be important for the anticancer activities of resveratrol [49]. This is the first time that elevation of death promoting genes have been implicated in vivo for a combination of dietary grape polyphenols in reducing mammary tumor growth. Since FOXO1 can be inactivated via Akt-mediated phosphorylation, elevation of FOXO1 transcripts may not necessarily result in increased protein activity. Interestingly, AKT1 expression was also decreased by threefold in the PCR array but this was not statistically significant. Moreover, RQC treatment of breast

Table 1 Effect of RQC treatment on expression of PI3 kinase pathway genes

Gene	Fold difference RQC/vehicle	<i>P</i> value
FOXO1, forkhead box 01A	1.87	0.007
NFKBIA, nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha	1.70	0.041
TLR4, toll like receptor 4	1.91	0.003

Only genes that demonstrated >1.5 fold difference and P value of <0.05 from RT² PCR arrays are shown

cancer cells significantly reduces active phospho Akt1 (p -Akt^{ser 473}) levels in 15 min without affecting total Akt1 levels (data not shown). The relative contribution of decreased phospho Akt1 levels and increased FOXO1 levels and caspase 3 activity to RQC-mediated effects on cell survival and apoptosis is currently under investigation.

Nf κ B transcription factor that regulates tumorigenic and immunomodulatory signaling is a potential target for the chemopreventive activity of grape polyphenols, resveratrol, and quercetin [50–53]. NFKBIA, which codes for I κ B, the subunit that sequesters Nf κ B in an inactive state, was also upregulated significantly by 1.7-fold in mammary tumors following RQC treatment (Table 1). This data indicates that inhibition of Nf κ B signaling may contribute to the observed reduced mammary tumor growth and metastasis by grape polyphenols. However, I κ B proteins can become inactivated via phosphorylation-induced, proteasome-mediated degradation by I κ B kinase (IKK) activity. Therefore, increased NFKBIA gene expression by RQC treatment may not reflect increased stable protein levels. Future experiments will determine the stability and phosphorylation status of I κ B in response to RQC. In addition, we also found a significant increase in Toll-like receptor 4 (TLR4) expression that have been implicated in cancer progression (Table 1). However, TLRs may also stimulate apoptosis under certain conditions [54] and can be negatively regulated by PI3-K signaling [55]. Therefore, the significance of this result warrants further investigation.

Previous *in vivo* studies have also supported a role for grape polyphenols in cancer prevention. Grape juice, grape seed extract, and red wine have been shown to significantly reduce cancer in rodent models [56–58]. Grape skin extract, which is concentrated in red wine, was recently shown to contain more growth inhibitory effects than grape pulp, juice, or seeds on mouse mammary tumor growth [59]. Since the effect of grape polyphenols on cancer metastasis remains to be evaluated, we next analyzed the

effect of dietary grape polyphenols on breast cancer metastasis.

Effect of grape polyphenols on metastasis

Our macro imaging system easily detected surface primary tumors, local invasion into the circulatory system, lymphatics, and metastatic tumors in the GI tract through the skin of nude mice. However, the resolution of this imaging system allows detection of only $\sim 10^4$ GFP-tagged cells and is thus limited in sensitivity for detection of micrometastases. Therefore, fluorescent metastatic lesions were quantified by microscopy following surgical removal of organs. Only eight mice/treatment were used for analysis of metastasis due to early death of two from the vehicle group and one from the RQC group. This number is similar to a previous study that reported the effect of dietary genistein on metastasis from MDA-MB-435 mammary tumors [39]. All of the mice following vehicle or RQC treatment presented with lung metastases. Therefore, lungs were further analyzed by Image J for quantification of the area of fluorescence. The number of metastatic lesions/lung was reduced in RQC-treated mice in a statistically significant manner when compared to vehicle treatment. However, the area of fluorescence calculated from these lesions was not statistically different for mice treated with vehicle or RQC (Fig. 5a, b, c; Table 2). Therefore, we conclude that RQC treatment does not block metastases to the lung from this cancer cell variant.

The MDA-MB-435 cell line used in this study was selected as a bone metastatic variant by intracardiac injection in nude mice [33]. Since breast cancers preferentially metastasize to the bone [60], this trend was simulated by inoculating the MDA-MB-435 bone metastatic variant into the mammary fat pad. The ability of the GFP-MDA-MB-435 mammary tumors to invade bone was investigated by fluorescent image analysis of excised, cleaned femurs from mice

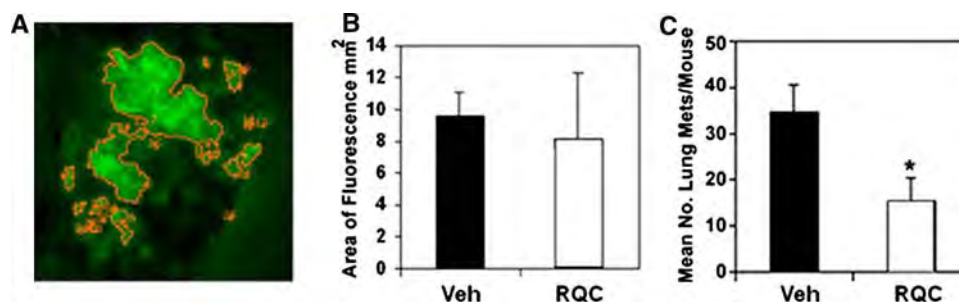


Fig. 5 Effect of grape polyphenols on lung metastasis. Following necropsy, lungs were excised from mice with GFP MDA MB 435 mammary tumors that received either vehicle or RQC diets and analyzed for metastases by fluorescence microscopy followed by quantitative image analysis. **a** Green fluorescence image of a representative lung demonstrating analysis of traced fluorescence

area. **b** Lung metastatic efficiency expressed as average area of fluorescence from lungs of vehicle or RQC treated mice \pm SEM ($n = 8$). **c** Average number of fluorescent metastatic foci/lung for vehicle or RQC treated mice. Asterisk denotes statistical significance at $P < 0.05$

Table 2 Distant metastases in mice following vehicle or combined resveratrol, quercetin, and catechin (RQC) treatment

	Number of mice with metastases (N = 8)		Number of metastatic lesions/organ with metastases	
	Veh	RQC	Veh	RQC
Bone	5	2	11.4	3
Heart	4	1	1.5	1
Kidney	3	3	15.7	1.33
Liver	8	2	22	11
Lung	8	8	34.7	15.7
Lymph node	3	3	2	1.7

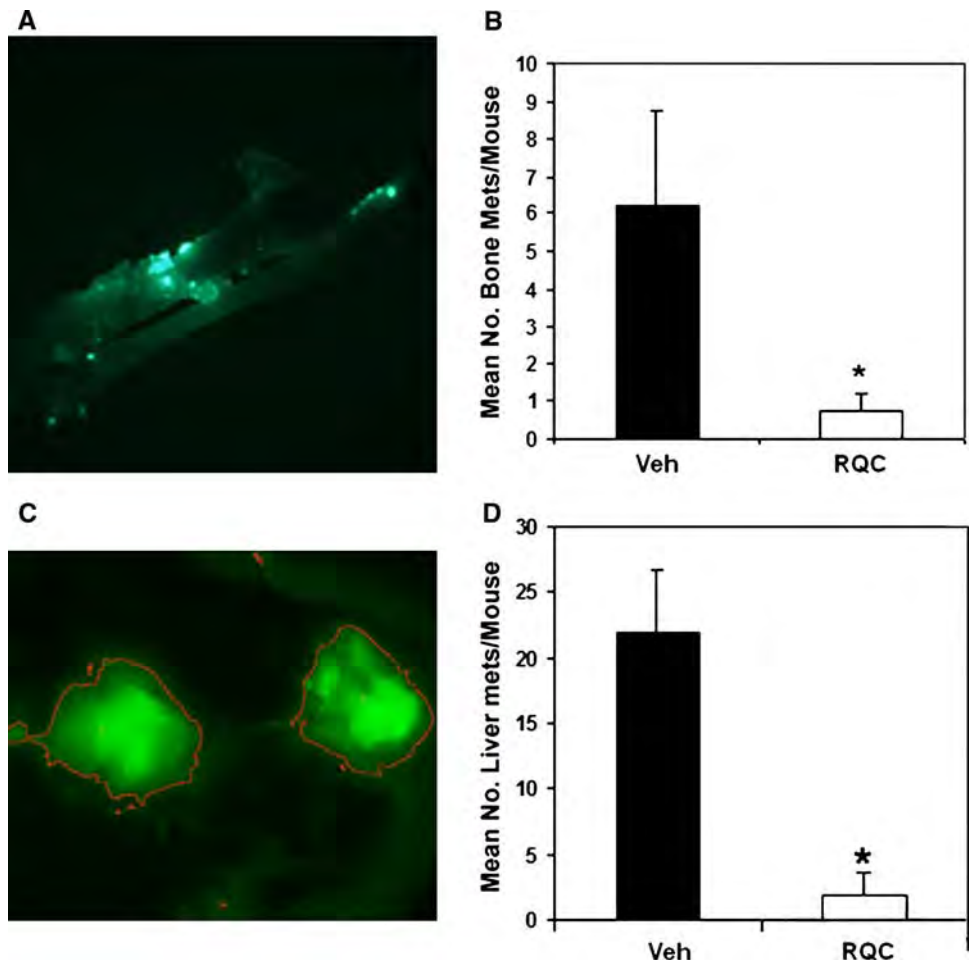
on vehicle control or RQC diets as described in [33]. In vehicle controls, 5/8 mice presented with bone metastases while only 2/8 mice following RQC treatment demonstrated fluorescent metastatic foci in femurs. Of the mice with bone metastases, the number of metastatic lesions were higher for vehicle treated mice compared to RQC treatments in a statistically significant manner (Fig. 6a, b; Table 2). Similarly, the number of mice with liver metastases and the number of metastatic lesions/liver were also significantly lower in RQC

treated mice compared to vehicle controls (Fig. 6c, d; Table 2). The mean fluorescent area or integrated density for a single metastatic lesion were similar for bone or liver metastases from vehicle or RQC treated mice (data not shown); however, very few the RQC-treated mice presented bone or liver metastases and exhibited lower numbers of metastatic foci/organ.

Table 2 also shows that less RQC treated mice presented with heart metastases when compared to vehicle treated mice. However, the number of metastatic foci/heart in RQC treated mice was only slightly less when compared to vehicle. When kidneys were examined for metastases, the number of mice with kidney metastases did not change but there were more metastatic lesions/kidney in the vehicle treated mice. Only three mice for vehicle or RQC treatments demonstrated lymph node metastases. Since the numbers of mice with heart, kidney, or lymph node metastases were low even for vehicle treatments, it is not possible to analyze these results in a statistically significant manner or derive definitive conclusions on the effect of RQC treatment compared to controls.

To our knowledge, this is the first report of an inhibitory effect of grape polyphenols on breast cancer metastasis. The differential effects of RQC on site-specific metastases

Fig. 6 Effect of grape polyphenols on bone and liver metastasis. Following necropsy, femurs and livers were excised from mice with GFP MDA MB 435 mammary tumors that received either vehicle or RQC diets and analyzed for metastases by fluorescence microscopy followed by quantitative image analysis. **a** Green fluorescence image of a representative femur from vehicle treated mouse. **b** Average number of fluorescent metastatic foci/femur for vehicle or RQC treated mice. **c** Green fluorescence image of a representative liver from vehicle treated mouse. **d** Average number of fluorescent metastatic foci/liver for vehicle or RQC treated mice. Asterisk denotes statistical significance at $P < 0.05$



indicate that RQC treatment did not inhibit metastatic cancer cells from being released to the lymphatics or the vascular system from the primary tumors at the mammary fat pad. RQC treatment also did not block the entry of cells into the lungs, where all of the mice in the study presented with lung metastases. Interestingly, subsequent metastases to the bone and liver were reduced by RQC treatment, indicating that these compounds may affect establishment of further metastases either by regulation of exit from the lung vasculature or at the entry points of localized signaling at liver and bone microenvironments. Our intriguing data that demonstrates upregulation of NF κ B levels in mammary tumors following RQC treatment implicates inhibition of NF κ B signaling by dietary grape polyphenols as a potential pathway that regulates breast cancer progression. Interestingly, NF κ B signaling has been associated with bone and liver metastasis [61–63]. The mechanistic basis for these interesting possibilities and the ability of grape polyphenols to specifically inhibit components of the bone and (or) liver molecular signature are currently under investigation.

Acknowledgments We acknowledge the excellent technical assistance of Alexander Schlachterman, Felix Valle, and Alina De La Mota Peynado with the animal protocols. This research was supported by grant numbers AICR IIG 03 31 06 and DoD/BCRP W81XWH 07 1 0330 to SD; DoD/BCRP W81XWH 08 01 0258 to LCP; NCCR/NIH 2G12RR003035, S06GM050695, and G11HD052352 to UCC; and NIH/RCMI G12 RR03051 and MBRS RISE 5R25GM061838 08 to UPR MSC. The content is solely the responsibility of the authors and does not necessarily represent the official views of NCCR, NICHD, NIGMS or the National Institutes of Health.

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