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14. ABSTRACT
This award proposed to test the hypothesis 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 was to test the effect of varying concentrations of E2, resveratrol, or a small molecule Rac-specific inhibitor NSC23766 on cell migration, invasion, and Rac activity of metastatic breast cancer cells. Aim 2 was 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. This first year report shows that at low concentrations, resveratrol acted similar to E2 and activated while at high concentrations resveratrol inhibited Rac and breast cancer cell migration. As proposed, we tested the efficiency of the commercially available Rac inhibitor NSC23766 in breast cancer cells. However, NSC23766 had only a modest inhibitory effect on Rac activity or cell migration of breast cancer cell lines. Therefore, we developed and tested novel more efficacious NSC23766 derivatives that will be used for the proposed study. For Aim 2, the effect of treatment with vehicle or resveratrol on mice with GFP-MDA-MB-435 mammary tumors was determined. Primary breast cancer progression and distant metastases as analyzed by whole body and microscopic fluorescence image analysis demonstrated that resveratrol reduced lung and liver metastases. Therefore, these experiments support our hypothesis that E2 and low concentrations of resveratrol promote while high concentrations of resveratrol inhibit breast cancer progression.

15. SUBJECT TERMS
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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. The ***rationale*** for this proposal comes from the vast body of work that has been done on the role of E_2 on initiation and progression of breast cancers (1;2). Increased ERα levels are associated with 50-80% of breast tumors. Consequently, inhibition of ERα has become a major strategy for prevention and treatment of breast cancer (3-6). Resveratrol is a natural compound from grapes and peanuts that is structurally similar to E_2 and interacts with both ERα and ERβ (7-10). Resveratrol has proapoptotic, antigrowth, anti-inflammmatory, antiangiogenic, and anti-invasive properties that makes it an attractive anticancer compound (11-13). Much of the data on potential anticancer properties of resveratrol has been shown *in vitro* with high concentrations of resveratrol ranging from 30-200 μM (14-20). We and others have shown that resveratrol at 50 μM can inhibit cell migration and invasion (21-24). Resveratrol can exert biphasic effects where low concentrations are estrogenic while high concentrations are antiestrogenic (15;17;25;26). Therefore, the ***purpose*** of this study is to investigate the effects of resveratrol on breast cancer progression to metastasis. We and others have demonstrated that activity of the Rho GTPase Rac is necessary for breast cancer invasion and metastasis (27;28). 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 is 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 breast cancer cell lines and a nude mouse model.

The following are our **Specific Aims**:

1. Determine the role of E_2 and resveratrol on Rho GTPase activity and cell functions relevant to breast cancer metastasis *in vitro*. Metastatic MDA-MB-231 and MDA-MB-435 human breast cancer cell lines will be treated with vehicle control, resveratrol, E_2, or Rac-specific inhibitor NSC23766 and the following investigated:

- **I.** Analyze activities of Rho GTPases, Rac GEFs, and Rac GAPs.
- **II.** Analyze cell migration and invasion
- **III.** Analyze cell cycle progression and apoptosis

2. Determine the role of E_2 and resveratrol on breast cancer metastasis *in vivo*. Breast tumors will be created using RFP-MDA-MB-231 or RFP-MDA-MB-435 cells in ovariectomized nude mice. Once primary tumors are established, the mice will be treated with vehicle control or varying concentrations of resveratrol, E_2, or Rac-specific inhibitor NSC23766 and the following investigated:

- **I.** Monitor primary tumor progression using fluorescence image analysis
- **II.** Monitor distant metastases using fluorescence image analysis
- **III.** Analyze primary and metastatic tumors by histopathology and immunocytochemistry

Body

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

The objective of this task is to treat ER alpha (-) beta (+) low metastatic MDA-MB-231 and ER alpha (-) beta (-) 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:

Using MDA-MB-231 human metastatic breast cancer cells, the effect of vehicle, EGF (+ control), E_2 (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) (Fig. 1). The data show that as per our hypothesis, E_2 and low concentrations of resveratrol activate Rac while high concentrations of resveratrol inhibit Rac activity.

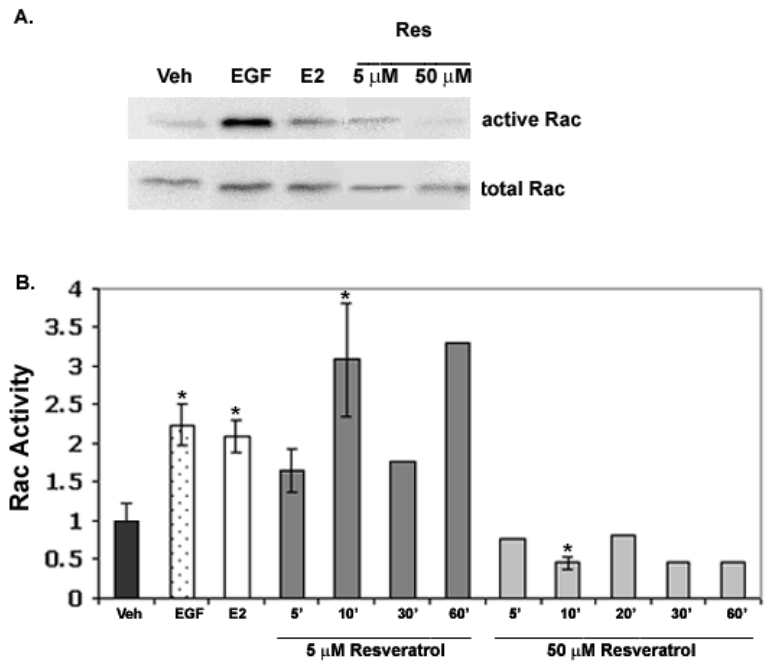

Figure 1. Rac activity of breast cancer cells in response to estrogen or resveratrol. Quiescent MDA-MB-231 cells were treated with DMSO (Veh), 50 ng/ml EGF, 0.1 μM estrogen (E2), or resveratrol (Res). PAK-PBD-GST beads were used to pull-down active GTP-bound Rac from the cell lysates. Active and total Rac levels were detected by western blotting with an anti-Rac antibody. A , Representative western blot of Rac activity of MDA-MB-231 cells in response to vehicle, EGF, E2, or resveratrol at 5 or 50 μM following a 10 min incubation. Results shown are representative of at least 3 experiments. *B,* Rac activity (active Rac/total Rac) relative to vehicle alone controls as quantified from densitometric scans of western blots. A star (*) denotes statistical significance from control. Error bars are SEMs from at least 3 experiments.

Analysis of cell migration and invasion in response to E_2 and resveratrol:

Since Rac activity is known to regulate cell migration and invasion, we tested the effect of E_2 and resveratrol on cell migration and invasion of MDA-MB-231 cells. 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, E_2 (100 nM), 5 or 50 μM resveratrol. For invasion assays, the top surface of the top well was coated with Matrigel, a basement membrane substrate. 50 μM resveratrol inhibited cell migration by ~30% compared to controls. E_2 exerted an opposite effect to 50 μM resveratrol (significant at $p<0.04$) by increasing cell migration 2-fold compared to controls. Interestingly, 5 μM resveratrol acted in a similar manner to E_2 by increasing cell migration. Similarly, we observed a ~ 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.

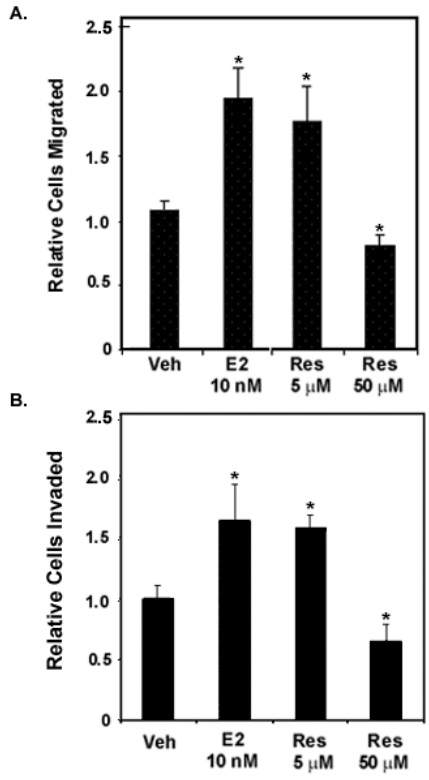

Figure 2. Effects of estrogen or resveratrol on cell migration and invasion of MDA-MB-231 cells.
A, Cell Migration. Quiescent cells were placed on the top well of Transwell chambers in serum- free media using the following as chemoattractants in the bottom well for 8 hours: DMSO control (Veh), 10 nM estrogen (E2), or 5 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 ± SEM. Treatments denoted by different letters indicate a significant difference between those treatments at $p<0.05$. *B,* Invasion. Quiescent cells were placed on the top well of Transwell chambers where the membrane was coated with Matrigel. The bottom well contained DMSO (Veh), 10 nM estrogen (E2), or 5 or 50 μM resveratrol (Res). Number of cells that migrated through the Matrigel matrix after a 24 hour incubation was quantified and made relative to control. Data are quantified from analysis of 25 microscopic fields/treatment from 4 experiments and expressed as mean relative cells migrated ± SEM. Treatments denoted by different letters indicate a significant difference between those treatments at $p<0.05$.

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 guanine nucleotide exchange factors (GEF) that activate Rac. NSC23766 is a small molecule compound that was identified from the NCI chemical database as a putative Rac inhibitor (29). 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 (30). The binding pocket of NSC23766 in Rac1 is located at the three-way junction site of switch I, switch II, and β loops of the effector region of Rac, where it provides the binding surface for Tiam1 or the related GEF Trio (29). Therefore, NSC-23766 cannot inhibit binding of other Dbl family members like Vav or DOCK family GEFs that interact with the switch I region of Rac as well as β2-β3 loops in the same effector loop (31). By western blotting, we determined that the cell lines that would be used for this study contained both Tiam-1 and Vav (Fig. 3). Thus, NSC-23766 may be limited in use as an inhibitor of Rac-mediated breast cancer metastasis.

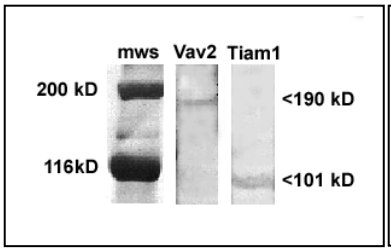

Figure 3. Expression of Rac.GEFs in breast cancer cells. MDA-MB-231 breast cancer cell lysates were western blotted with anti Vav-2 or anti Tiam-1 monospecific antibodies

Since, NSC23766 has been shown to be effective with an $IC_{50}\approx50\mu M$ in fibroblasts (29), we tested the effect of this compound on Rac activity of breast cancer cell lines that will be used for the proposed study using 100 μM NSC-23766 for 24 h. The G-LISA Rac Activation Assay (Cytoskeleton, Inc., Denver, CO), which measures the amount of active Rac following incubation of equal amounts of protein from cell lysates to the

Rac.GTP binding domain of a downstream effector was used to measure Rac activity in the presence or absence of NSC-23766. NSC23766 demonstrated ~30% inhibition at 100 μM when compared to the vehicle alone (control) at equal amounts of protein (Fig. 4). Therefore, this data indicated that NSC-23766 is not a good Rac inhibitor in aggressive breast cancer cells.

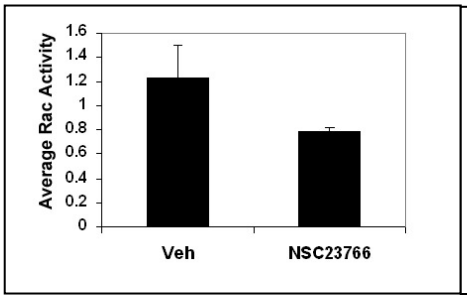

Figure 4. Rac activity in the presence of NSC23766. MDA-MB-435 cells were incubated for 24 h in vehicle or 100 μM NSC-23766 for 24 h. Cells were lysed and subjected to a G-LISA Rac activation assay to measure Rac activity. Data shows ± S.D., N=3.

Therefore, to develop more efficient Rac inhibitors that can inhibit Rac interaction with a wider range of GEFs, we initiated the synthesis of novel derivatives of NSC-23766 in collaboration with medicinal chemists Drs. Cornelis Vlaar and Eliud Hernandez (Department of Pharmacology, University of Puerto Rico-Medical Sciences Campus, San Juan, PR). A facile two-step synthesis for the preparation NSC23766 derivatives was developed and is represented in Fig.5. Thus far, about seven commercially available (hetero)-arylamines have successfully been combined with dichloropyrimidines. Subsequent coupling with primary or secondary aliphatic amines with or without a tail-end amino-substituent provided NSC-23766 derivatives. Several examples for which biological analysis have already been performed are represented in Fig. 5.

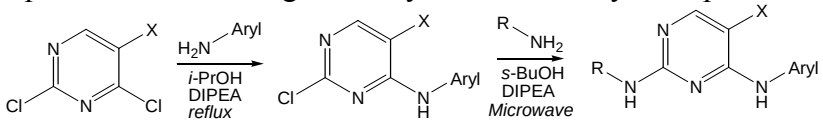

Figure 5: Synthesis of NSC23766 derivatives with the pyrimidine-core

Rac activity of MDA-MB-435 cells that demonstrate high intrinsic Rac activity was determined following incubation with NSC-23766 or 34 derivatives at 50 μM after 24 h incubation. Several compounds were more efficient inhibitors of Rac activity than NSC-23788, which only gave a 20% inhibition at 50 μM. As shown in Fig. 6, we have successfully identified NSC-23766 derivatives that do not affect cell viability but inhibit Rac activity. Compound EHop-016 that gave 100% inhibition of Rac activity also reduced cell viability of MDA-MB-435.Rac (T17N) cells that express dominant negative Rac (data not shown). Therefore, compounds EHop-028, EHop-023, and EHop-015 were selected for further testing because these compounds inhibited Rac activity >50% without affecting the viability of MCF-10 normal mammary epithelial cells or MDA-MB-435 cells (Fig. 7).

To determine whether inhibition of Rac activity has an effect on cell functions relevant for metastasis that are under Rac regulation, the effects of NSC-23766 and selected derivatives on formation of motile actin structures and directed cell migration were tested. MDA-MB-435 cells were incubated with vehicle, 50 μM NSC-23766, EHop-028, or EHop-023 for 24h, fixed, and stained for polymerized actin using Rhodamine Phalloidin. Fluorescence microscopy demonstrated a marked decrease in actin rich structures called lamellipodia that are under Rac regulation in cells treated with EHop-028 or EHop-023 compared to cells treated with vehicle or NSC-23766. Cells treated with EHop-028 and EHop-023 treatment were also less spread compared to control cells (Fig. 8 A). When MDA-MB-435 cells were subjected to Transwell migration assays following incubation of EHop-028 or E-Hop-023 at 50 μM for 24 h, both compounds inhibited cell migration by 80% and 95% respectively compared to vehicle control (Fig. 8B).

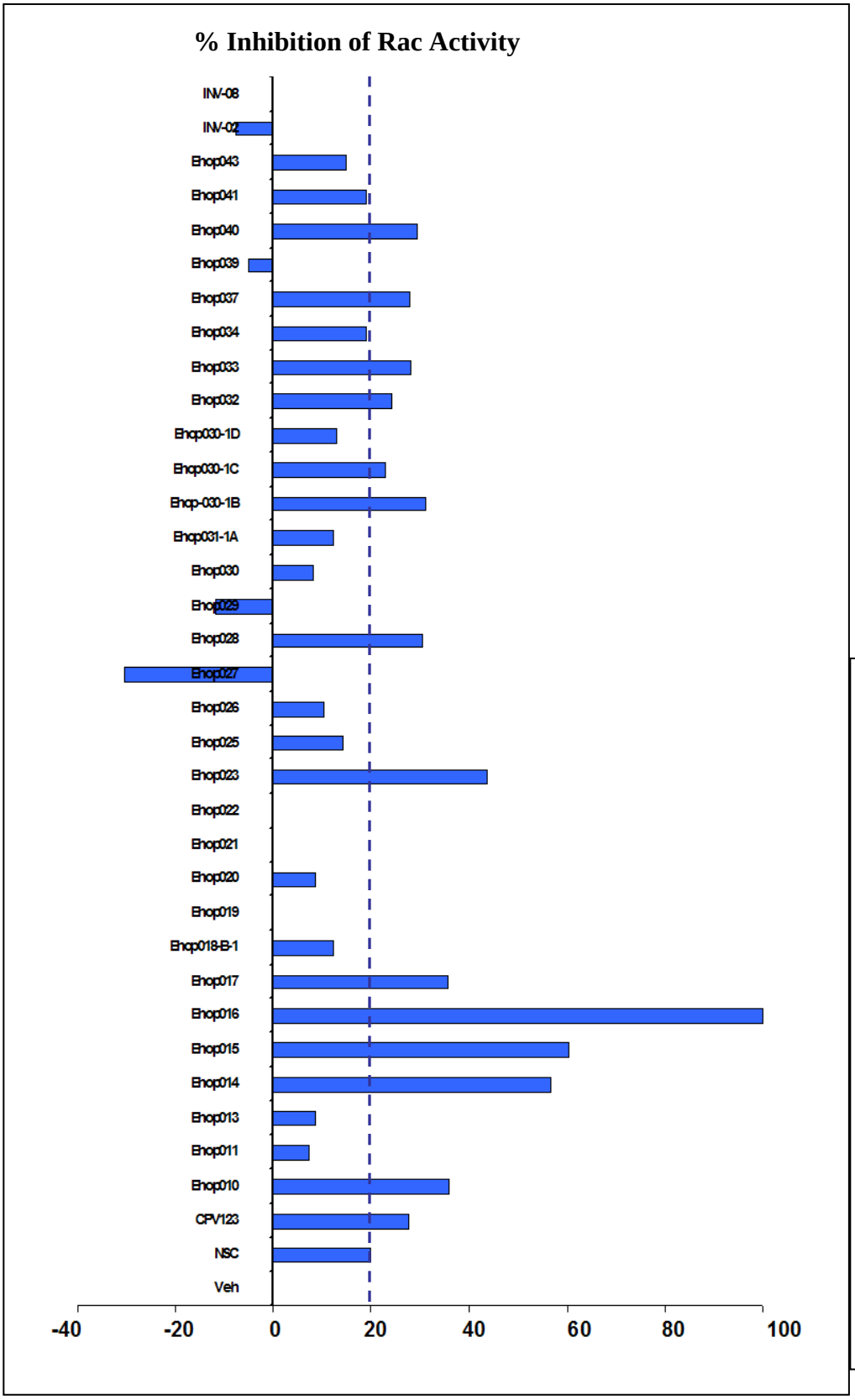

Figure 6. Percentage of Rac Inhibition by NSC-23766 and derivatives. Quiescent MDA-MB-435 cells were treated with vehicle, or 50 µM Rac Inhibitor for 24 hrs, and total protein extracts were obtained. Rac activity was measured using G-LISA Rac1 Activation Assay (Cytoskeleton, Inc.). Average chemiluminescence of each experimental sample, was measured from a GTP-Rac affinity plate. Results are shown as % inhibition where vehicle is set at 0%.
Dashed line represents % Rac inhibition of 20% by the parent compound NSC-23766.

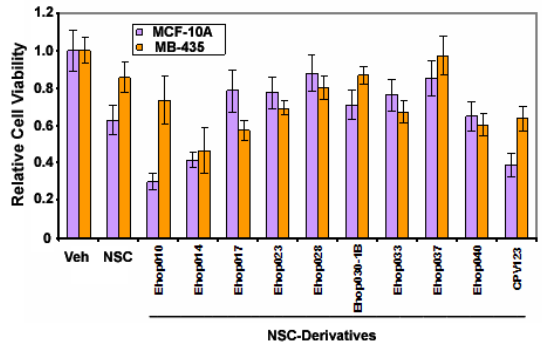

Figure 7. Effects of NSC-23766 or derivatives on cell viability. MCF10 mammary epithelial or MDA-MB-435 metastatic breast cancer cells were incubated with NSC-23766 or derivatives at 50 μM for 24hrs. MTT Proliferation assay was performed according to manufacturer (Millipore), and absorbance read using the Benchmark Plus Microplate Reader (Bio-Rad) at 570nm with a reference filter of 630nm. Relative cell viability compared to vehicle control (1.0) is shown.

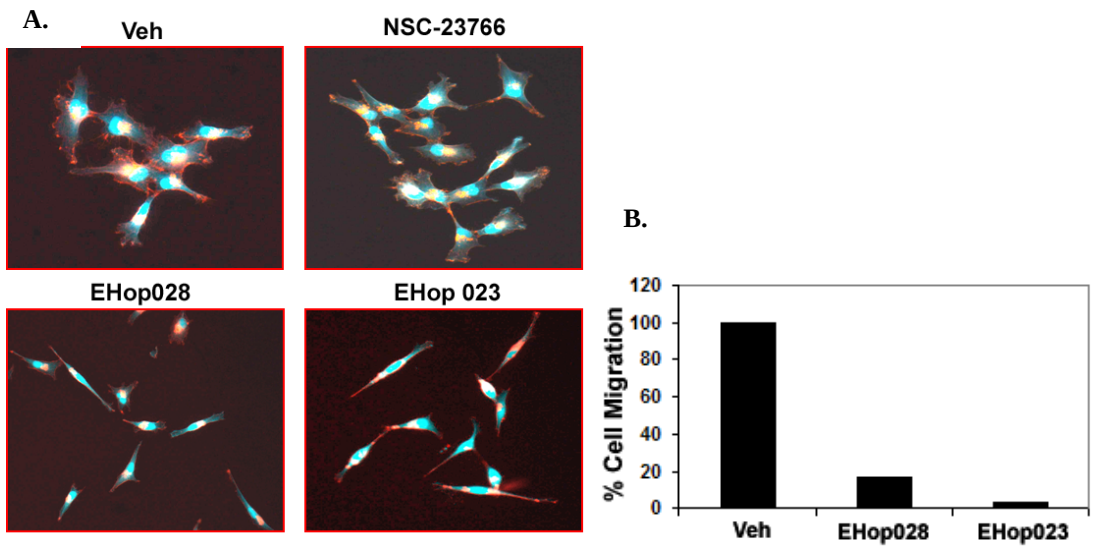

Figure 8. Breast cancer cell shape and migration following treatment with NSC-23766 and derivatives.

A. Fluorescent micrograph of MDA-MB-435 cells on coverslips treated with vehicle or 50μM each NSC-23766, EHop-028, or EHop-023 for 24h. Cells were fixed and stained with Rhodamine phalloidin to visualize F-actin. **B.** MDA-MB-435 cells were treated with vehicle or 50μM each NSC-23766, EHop-028, or EHop-023 for 24h. Equal numbers of cells were placed on the top well of Transwell chambers that contain a membrane with 8μ diameter pores. The bottom well contained serum. The number of cells that migrated to the underside of the membrane was quantified for each treatment. Results are shown relative to vehicle (100%).

Tasks 2 and 3. Determine the effect of estrogen and resveratrol on breast cancer progression

These tasks proposed the use of fluorescent protein (FP)-tagged MDA-MB-231 ERα (-) ERβ (+) low metastatic human breast cancer cell line and MDA-MB-435 in ovariectomized nude mice.

The tasks were:

I. Establish primary tumors from these cell lines by inoculation into mammary fat pads of female nude mice.

II. Administer treatments to mice. Once primary tumors are established (~3 $mm^{2,}$ about 1 week), the following treatments will be administered every other day by gavage for a maximum of 60 days. Treatments include vehicle control, 0.1 or 0.5 mg/kg body weight 17β-estradiol, resveratrol at 0.5, 5, or 50 mg/kg body weight (BW). NSC23766 (10 or 50 mg/kg BW) will be administered weekly by i.p. injection.

III. Monitor breast cancer progression and distant metastases by daily fluorescence image analysis.

To lay the groundwork for these experiments, we created GFP-tagged MDA-MB-231 and GFP-MDA-MB-435 cells by transfection of cells using a mammalian expression vector that contained the cDNA for eGFP and neomycin resistance. Positive clones were selected according to GFP fluorescence and neomycin resistance. GFP-expressing clones were passaged for a month in the absence of neomycin to ensure stable integration and used for inoculation into athymic nude mice. Mammary fat pad injections of GFP-MDA-MB-231 cells at 1-2X10^5 cells/inoculation resulted in a large variation in tumor take. Therefore, to ensure uniform tumor take and equal size of initial tumors, xenografts were established using ~5X10^6 cells. These donor xenografts were excised and divided into ~2mm^3 pieces that were implanted into 10 mice at the right mammary fat pad. These GFP-MDA-MB-231 xenografts 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. As measured by twice weekly fluorescence image analysis, tumor growth remained linear during the study (Fig. 9). This low metastatic variant was not very efficient with forming distant metastases. We found that lung metastases were not visible by macroscopic fluorescence image analysis. Microscopic image analysis of lung metastases demonstrated smaller metastatic foci (Fig. 10) that were present in 6/10 mice.

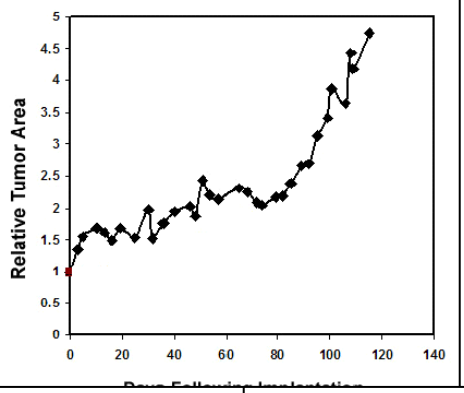

Figure 9. Growth of GFP-MDA-MB-231 mammary tumors. 2 mm^3 GFP-MDA-MB-231 xenografts were implanted at the right mammary fat pad of 10 female nude mice. Average tumor area as measured by fluorescence whole body image analysis followed by quantification of integrated intensity of digital images using ImageJ software is shown. Relative tumor area is the integrated pixel intensity on each day as a function of the pixel intensity on day 01 after xenograft implantation.

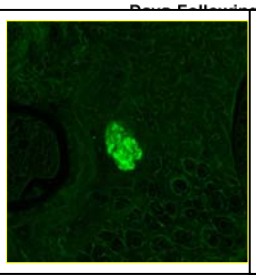

Figure 10. Lung metastasis from GFP-MDA-MB-231 mammary tumor of athymic nude mouse. A representative confocal micrograph of ~25 cell micrometastasis from an excised lung is shown (mag. 400X)

This experiment demonstrated that we can conduct the in vivo experiments with the MDA-MB-231 cell line for as long as four months. However, we will have to conduct a microscopic analysis of the micrometastases from excised lungs at the conclusion of the study.

Next we determined that ability of the GFP-MDA-MB-435 cells to develop primary and secondary metastases in female athymic nude mice. 7 mice/group were inoculated with $5X10^5$ 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) or 10 mg/kg BW resveratrol to determine the response to a medium concentration of resveratrol and to ensure that resveratrol treatment does not interfere with GFP expression in the fluorescent mammary tumors. This concentration of resveratrol did not change primary mammary tumor progression (Fig. 11.A, B). The mice treated with resveratrol showed a decreased trend in the number of lung (Fig. 11.C) and liver metastases (Fig. 11.D). However, these differences were not statistically significantly compared to vehicle controls. This may be because we used a medium concentration of resveratrol (10 mg/kg BW) or the smaller numbers of mice used. During the second year of the funding period we will test the effect of 0.5, 5, and 50 mg/kg BW resveratrol on mice with GFP-MDA-MB-231 or GFP-MDA-MB-435 mammary tumors, which are predicted to respectively increase and decrease breast cancer progression to metastasis dependent on the concentration of resveratrol.

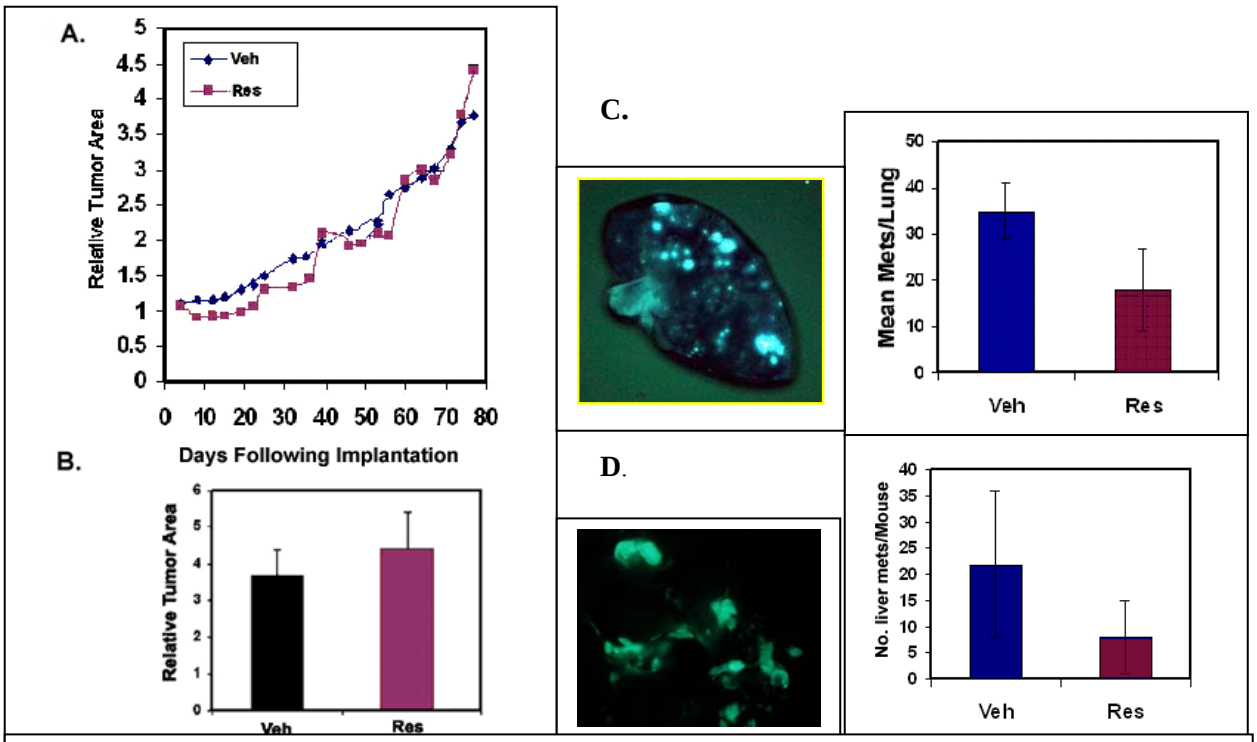

Figure 11. Response of GFP-MDA-MB-435 mammary tumors to dietary resveratrol. A. Relative GFP-MDA-MB-435 mammary tumor progression was determined by in situ whole body fluorescence image analysis from day 1 of tumor implantation and compared with images acquired 2X a week for 77 days. Average relative tumor area as calculated from integrated fluorescence intensity of mammary tumors from vehicle (Veh) or 10 mg/kg BW resveratrol (Res) treatment. **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 or Res treated mice. **C.** Example of an excised lung with fluorescent metastatic foci from vehicle-treated mouse. Histogram, Mean metastatic foci/lung for mice following Veh or Res (10 mg/kg BW) treatment. **D.** Example of an excised liver with fluorescent metastatic foci from vehicle-treated mouse. Histogram, Mean metastatic foci/liver for mice following Veh or Res (10 mg/kg BW) treatment

Key Research Accomplishments:

- Analysis of Rac activity of MDA-MB-231 breast cancer cells demonstrated that low concentrations of resveratrol act similar to estrogen and increase Rac activity while high concentrations inhibit Rac activity.
- Analysis of cell migration and invasion of MDA-MB-231 breast cancer cells demonstrated that low concentrations of resveratrol act similar to estrogen and increase cell migration and invasion while high concentrations inhibit cell migration and invasion.
- 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.
- Stable breast cancer cells expressing green fluorescent protein (GFP) were created and methodology was developed for the assessment of fluorescent breast cancer progression in female athymic nude mice using GFP-MDA-MB-231 and GFP-MDA-MB-435 cells.
- The effect of a medium concentration of dietary resveratrol was assessed in mice implanted with GFP-MDA-MB-435 mammary tumors. Resveratrol at 10 mg/kg BW did not affect primary mammary tumor growth but reduced lung and liver metastases.

Reportable Outcomes:

- These studies will be reported at the upcoming Era of Hope meeting.

Dharmawardhane, S, Azios, NG, Castillo-Pichardo, L, and De La Mota-Peynado, A.

Estrogen and resveratrol as regulators of the Rho GTPase Rac in breast cancer metastasis. Era of Hope, Deaprtment of Defense Breast Cancer Research Program Meeting, Baltimore, MD, June 25-27, 2008.

- Novel Rac inhibitors (~34 compounds) were developed from the commercially available parent compound NSC-23766 and 3 compounds were identified for further analysis.
- Funding was requested from DoD/BCRP to further develop these novel Rac inhibitors as anti breast cancer invasion compounds.

1. DoD/BCRP Concept Award Program
Date of Submission: 01/23/2008
Title: Novel Rac inhibitors as therapeutic agents for breast cancer metastasis
2. DoD/BCRP Synergy Idea Program
Date of Submission: 05/07/2008
Title: Small molecule inhibitors of Rac as anti-invasive breast cancer compounds

Conclusions:

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 was validated. 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 and cell migration/invasion of breast cancer cells.

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.

Development of whole body fluorescence image analysis methodology for an in situ analysis of breast cancer progression demonstrated that GFP-MDA-MB-231 and GFP-MDA-MB 435 mammary tumor progression can be successfully monitored and quantified in athymic nude mice. 10 mg/kg BW resveratrol treatment by oral gavage did not affect fluorescence of mammary tumors or distant metastases for as long as four months and reduced lung and liver metastases. Oral gavage with resveratrol in 90% oil, 10% ethanol 3X a week did not affect mouse weight or cause undue stress. We found that studies with MDA-MB-435 tumors have to be terminated in 2-2.5 months due to mice becoming moribund as a result of wide spread lung metastases and primary tumor burden. In the next two years of funding we expect 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.

References

1. Yager JD, Davidson NE. Estrogen carcinogenesis in breast cancer. *N.Engl.J.Med.* 2006;354:270-82.

2. Fuqua SA. The role of estrogen receptors in breast cancer metastasis. *J Mammary Gland Biol Neoplasia* 2001;6:407-17.

3. Platet N, Cathiard AM, Gleizes M, Garcia M. Estrogens and their receptors in breast cancer progression: a dual role in cancer proliferation and invasion. *Crit Rev.Oncol.Hematol.* 2004;51:55-67.

4. Lewis JS, Jordan VC. Selective estrogen receptor modulators (SERMs): Mechanisms of anticarcinogenesis and drug resistance. *Mutat.Res.* 2005;591:247-63.

5. Katzenellenbogen BS, Choi I, Delage-Mourroux R, Ediger TR, Martini PG, Montano M et al. Molecular mechanisms of estrogen action: selective ligands and receptor pharmacology. *J Steroid Biochem Mol Biol* 2000;74:279-85.

6. Park WC, Jordan VC. Selective estrogen receptor modulators (SERMS) and their roles in breast cancer prevention. *Trends Mol Med*2002;8:82-8.

7. Levenson AS, Gehm BD, Pearce ST, Horiguchi J, Simons LA, Ward JE, III et al. Resveratrol acts as an estrogen receptor (ER) agonist in breast cancer cells stably transfected with ER alpha. *Int.J.Cancer* 2003;104:587-96.

8. Bhat KP, Lantvit D, Christov K, Mehta RG, Moon RC, Pezzuto JM. Estrogenic and antiestrogenic properties of resveratrol in mammary tumor models. *Cancer Res* 2001;61:7456-63.

9. Harris DM, Besselink E, Henning SM, Go VL, Heber D. Phytoestrogens induce differential estrogen receptor alpha- or Beta-mediated responses in transfected breast cancer cells. *Exp.Biol.Med.(Maywood.)* 2005;230:558-68.

10. Bowers JL, Tyulmenkov VV, Jernigan SC, Klinge CM. Resveratrol acts as a mixed agonist/antagonist for estrogen receptors alpha and beta. *Endocrinology* 2000;141:3657-67.

11. Aggarwal BB, Shishodia S. Molecular targets of dietary agents for prevention and therapy of cancer. *Biochem.Pharmacol.* 2006;71:1397-421.

12. Signorelli P, Ghidoni R. Resveratrol as an anticancer nutrient: molecular basis, open questions and promises. *J.Nutr.Biochem.* 2005;16:449-66.

13. Le Corre L, Chalabi N, Delort L, Bignon YJ, Bernard-Gallon DJ. Resveratrol and breast cancer chemoprevention: molecular mechanisms. *Mol.Nutr.Food Res.* 2005;49:462-71.

14. Waffo-Teguo P, Hawthorne ME, Cuendet M, Merillon JM, Kinghorn AD, Pezzuto JM et al. Potential cancer-chemopreventive activities of wine stilbenoids and flavans extracted from grape (Vitis vinifera) cell cultures. *Nutr Cancer 20*03;40:173-9.

15. Shih A, Davis FB, Lin HY, Davis PJ. Resveratrol induces apoptosis in thyroid cancer cell lines via a MAPK- and p53-dependent mechanism. *J Clin Endocrinol Metab* 2002;87:1223-32.

16. She QB, Huang C, Zhang Y, Dong Z. Involvement of c-jun NH(2)-terminal kinases in resveratrol-induced activation of p53 and apoptosis. *Mol Carcinog* 2002;33:244-50.

17. Miloso M, Bertelli AA, Nicolini G, Tredici G. Resveratrol-induced activation of the mitogen-activated protein kinases, ERK1 and ERK2, in human neuroblastoma SH-SY5Y cells. ***Neurosci.Lett.*** 1999;264:141-4.

18. Pozo-Guisado E, Alvarez-Barrientos A, Mulero-Navarro S, Santiago-Josefat B, Fernandez-Salguero PM. The antiproliferative activity of resveratrol results in apoptosis in MCF-7 but not in MDA-MB-231 human breast cancer cells: cell-specific alteration of the cell cycle. ***Biochem.Pharmacol.*** 2002;64:1375-86.

19. Soleas GJ, Goldberg DM, Grass L, Levesque M, Diamandis EP. Do wine polyphenols modulate p53 gene expression in human cancer cell lines? ***Clin.Biochem.*** 2001;34:415-20.

20. Niles RM, McFarland M, Weimer MB, Redkar A, Fu YM, Meadows GG. Resveratrol is a potent inducer of apoptosis in human melanoma cells. ***Cancer Lett.*** 2003;190:157-63.

21. Azios NG, Dharmawardhane SF. Resveratrol and estradiol exert disparate effects on cell migration, cell surface actin structures, and focal adhesion assembly in MDA-MB-231 human breast cancer cells. *Neoplasia* 2005;7:128-40.

22. Pozo-Guisado E, Merino JM, Mulero-Navarro S, Lorenzo-Benayas MJ, Centeno F, Alvarez-Barrientos A et al. Resveratrol-induced apoptosis in MCF-7 human breast cancer cells involves a caspase-independent mechanism with downregulation of Bcl-2 and NF-kappaB. *Int.J Cancer* 2005;115:74-84.

23. Rodrigue CM, Porteu F, Navarro N, Bruyneel E, Bracke M, Romeo PH et al. The cancer chemopreventive agent resveratrol induces tensin, a cell-matrix adhesion protein with signaling and antitumor activities. *Oncogene* 2005.

24. Woo JH, Lim JH, Kim YH, Suh SI, Min dS, Chang JS et al. Resveratrol inhibits phorbol myristate acetate-induced matrix metalloproteinase-9 expression by inhibiting JNK and PKC delta signal transduction. *Oncogene* 2004;23:1845-53.

25. Klinge CM, Blankenship KA, Risinger KE, Bhatnagar S, Noisin EL, Sumanasekera WK et al. Resveratrol and estradiol rapidly activate MAPK signaling through estrogen receptors alpha and beta in endothelial cells. ***J.Biol.Chem.*** 2005;280:7460-8.

26. Pozo-Guisado E, Lorenzo-Benayas MJ, Fernandez-Salguero PM. Resveratrol modulates the phosphoinositide 3-kinase pathway through an estrogen receptor alpha-dependent mechanism: relevance in cell proliferation. ***Int.J.Cancer*** 2004;109:167-73.

27. Baugher PJ, Krishnamoorthy L, Price JE, Dharmawardhane SF. Rac1 and Rac3 isoform activation is involved in the invasive and metastatic phenotype of human breast cancer cells. ***Breast Cancer Res.*** 2005;7:R965-R974.

28. Chan AY, Coniglio SJ, Chuang YY, Michaelson D, Knaus UG, Philips MR et al. Roles of the Rac1 and Rac3 GTPases in human tumor cell invasion. *Oncogene* 2005;24:7821-9.

29. Gao Y, Dickerson JB, Guo F, Zheng J, Zheng Y. Rational design and characterization of a Rac GTPase-specific small molecule inhibitor. *Proc.Natl.Acad.Sci.U.S.A* 2004;101:7618-23.

30. Gao Y, Xing J, Streuli M, Leto TL, Zheng Y. Trp(56) of rac1 specifies interaction with a subset of guanine nucleotide exchange factors. *J.Biol.Chem.* 2001;276:47530-41.

31. Kwofie MA, Skowronski J. Specific recognition of Rac2 and Cdc42 by DOCK2 and DOCK9 guanine nucleotide exchange factors. *J Biol.Chem.* 2007.