

**Award Number:** W81XWH-07-1-0426

**TITLE:** Elucidating Mechanisms of Farnesyltransferase Inhibitor Action and Resistance in Breast Cancer by Bioluminescence Imaging

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**REPORT DATE:** July 2009

**TYPE OF REPORT:** Annual

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

**DISTRIBUTION STATEMENT:**

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# REPORT DOCUMENTATION PAGE

*Form Approved*  
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<b>1. REPORT DATE (DD-MM-YYYY)</b> 01-06-2009			<b>2. REPORT TYPE</b> Annual		<b>3. DATES COVERED (From - To)</b> 1 June 2008 - 31 May 2009	
<b>4. TITLE AND SUBTITLE</b>  Elucidating Mechanisms of Farnesyltransferase Inhibitor Action and Resistance in Breast Cancer by Bioluminescence Imaging					<b>5a. CONTRACT NUMBER</b>	
					<b>5b. GRANT NUMBER</b> W81XWH-07-1-0426	
					<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b>  David Piwnica-Worms, Ken Blumer  Email: PIWNICA-WORMSD@MIR.WUSTL.EDU					<b>5d. PROJECT NUMBER</b>	
					<b>5e. TASK NUMBER</b>	
					<b>5f. WORK UNIT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  Washington University  St. Louis, MO 63110					<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012					<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>  <b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>	
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b>  Approved for public release; distribution unlimited						
<b>13. SUPPLEMENTARY NOTES</b>						
<b>14. ABSTRACT</b> Prenyltransferase inhibitors (FTIs & GGTIs) block post-translational processing of Ras-like GTPases that have key roles in breast cancer. However, FTIs have yet to be used widely in breast cancer therapy because it is not yet possible to identify patients likely to be FTI-sensitive or to use combinatorial therapy to broaden the spectrum of patients that respond to FTIs. A new lentiviral-based prenylation responsive bioluminescence reporter system has been constructed and validated in human breast cancer cell lines. Our strategy should offer the opportunity to visualize over time the action of FTIs and GGTIs toward specific, biologically relevant prenylation-dependent proteins in tumors of living animals in vivo.						
<b>15. SUBJECT TERMS</b> Breast cancer, farnesyltransferase inhibitors, drug resistance, molecular imaging						
<b>16. SECURITY CLASSIFICATION OF:</b>			<b>17. LIMITATION OF ABSTRACT</b>	<b>18. NUMBER OF PAGES</b>	<b>19a. NAME OF RESPONSIBLE PERSON</b>	
<b>a. REPORT</b>	<b>b. ABSTRACT</b>	<b>c. THIS PAGE</b>			USAMRMC	
U	U	U	UU	8	<b>19b. TELEPHONE NUMBER (include area code)</b>	

## Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusion.....	8

## Introduction

Prenyltransferase inhibitors (FTIs & GGTIs) block post-translational processing of Ras-like GTPases that have key roles in breast cancer. In phase II trials, FTIs have exhibited clinical benefit toward a subset of breast cancer patients. However, FTIs have yet to be used widely in breast cancer therapy because it is not yet possible to identify patients likely to be FTI-sensitive or to use combinatorial therapy to broaden the spectrum of patients that respond to FTIs. GGTIs are also being developed for cancer therapy. To overcome these hurdles, mechanisms determining whether breast cancer tumors are sensitive or resistant to FTIs or GGTIs in vivo must be understood. These mechanisms remain elusive because signaling networks regulating proliferation, survival and migration of breast cancer cells in vitro versus in vivo can be strikingly different, and because conventional biochemical means of detecting FTI or GGTI action in tumors are insensitive, invasive, or correlate poorly with FTI or GGTI action. Therefore, novel means of elucidating mechanisms of FTI or GGTI activity in tumors are required. Accordingly, this project will develop molecular imaging strategies that for the first time specifically detect the ability of FTIs or GGTIs to inhibit prenylation in tumors of living animals.

## Body

The main objective is to identify mechanisms determining whether breast cancer tumors in living animals are sensitive or insensitive to FTIs or GGTIs. We will develop novel imaging technology to detect the action of clinically employed FTIs or GGTIs in human breast cancer cell lines in vitro and in mouse xenograft models of human breast cancer. With this system, the specific aims of the project were:

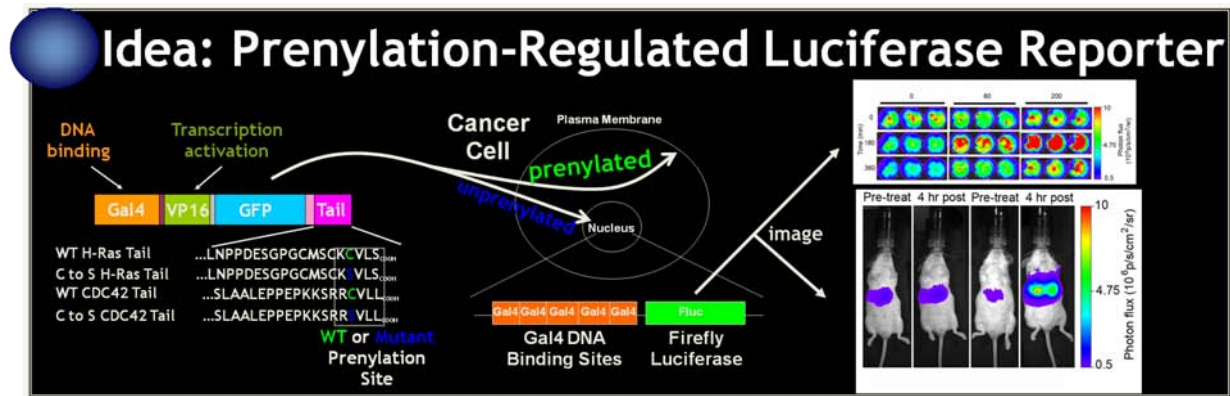
Aim 1--Determine whether FTIs or GGTIs inhibit prenylation in tumors.

Aim 2--Determine whether prenylation blockade occurs in FTI- or GGTI-sensitive vs. -insensitive tumors.

Aim 3--Determine whether FTase or GGTase knockdown or Erk, Akt or mTOR inhibition augments FTI or GGTI sensitivity in vitro or in vivo.

### **Aim 1: Determine whether FTIs inhibit farnesylation in tumors**

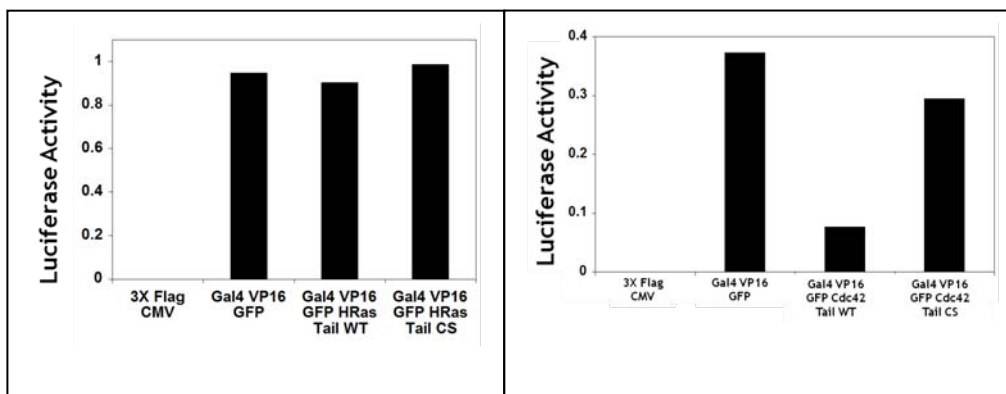
Year 1--The majority of our effort in the first 12 months of funding was directed toward developing, validating and calibrating the desired bioluminescence reporter. Our strategy to image FTI or GGTI action in vivo is shown in **Figure 1**, which summarizes the final strategy we have employed. Our initial proposed imaging strategy used a plasmid that constitutively expressed a chimeric transcription factor comprised of the DNA binding domain (DBD) of the yeast Gal4 transcription factor, the VP16 transcriptional activation domain (AD), a nuclear localization sequence (NLS), and H-Ras. However, repeated analysis with this construct in breast cancer cell lines yielded a high degree of variability and lack of durable signals with this construct. Thus, we sought an alternative strategy. Following an extensive series of experiments with various permutations of this basic construct, we found a chimeric transcription factor fused to the prenylation domains of the CaaX proteins H-Ras or Cdc42 which showed promise to localize to the nucleus upon inhibition of prenylation. We have now constructed plasmids



**Figure 1.** Concept of prenylation-regulated luciferase reporter.

that constitutively express chimeric transcription factors comprised of the DNA binding domain (DBD) of the yeast Gal4 transcription factor, the VP16 transcriptional activation domain (AD), a green fluorescent protein (GFP) scaffold and the CaaX domains of H-Ras or Cdc42. When farnesylated, the resultant Gal4-VP16-GFP-H-Ras-CaaX or Gal4-VP16-GFP-Cdc42-CaaX fusion proteins target the plasma membrane and thus, fail to activate a Gal4 promoter-driven luciferase reporter construct. However, when unfarnesylated, such as in response to FTI treatment, the fusion protein should now be imported into the nucleus and drive expression of the Gal4 promoter luciferase reporter. For further analysis via mutational inactivation of the CaaX box, we also engineered two mutant constructs comprising CaaX to SaaX tail substitutions as control positive probes of reporter function.

First, to document the cytotoxic effects of FTI's in the breast cancer cell lines of interest for our analysis, we first determined the cytotoxicity profiles of FTI-277 and GGTI-298, two prenylation inhibitors, on human MDA-MB-231 and MCF-7 breast cancer cells. Both compounds showed concentration-dependent cytotoxicity in both cell lines. The LD<sub>50</sub> values were 20  $\mu\text{M}$  and 0.01  $\mu\text{M}$  for FTI-277, and 5  $\mu\text{M}$  and 0.8  $\mu\text{M}$  for GGTI-298, in MDA-MB-231 and MCF-7 cells, respectively. Thus, MCF-7 cells were more sensitive to both compounds under cell culture conditions.

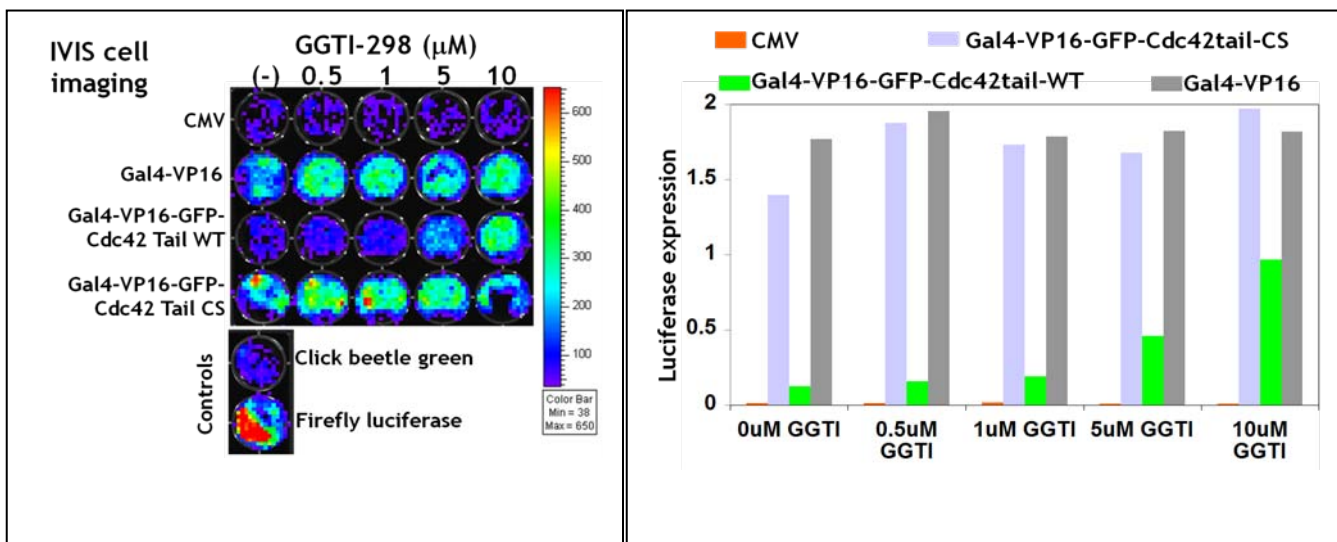


**Figure 2 (left) and Figure 3 (right).** Functional responsiveness of the Gal4-VP16-GFP-H-Ras-CaaX (Figure 2) and Gal4-VP16-GFP-Cdc42-CaaX (Figure 3) fusion transactivators in live cells.

Next, expression and function of the Gal4-VP16-GFP-H-Ras-CaaX and Gal4-VP16-GFP-Cdc42-CaaX was tested in MDA-MB-231 cells. As shown (Figure 2), the Gal4-VP16-GFP-H-Ras-CaaX was not sensitive to the C-S mutation

in the prenylation tail. In contrast, the wild-type Gal4-VP16-GFP-Cdc42-CaaX fusion showed low reporter signal under basal conditions and high signal with the C-S mutation, conforming to expectations for a prenylation responsive reporter (**Figure 3**).

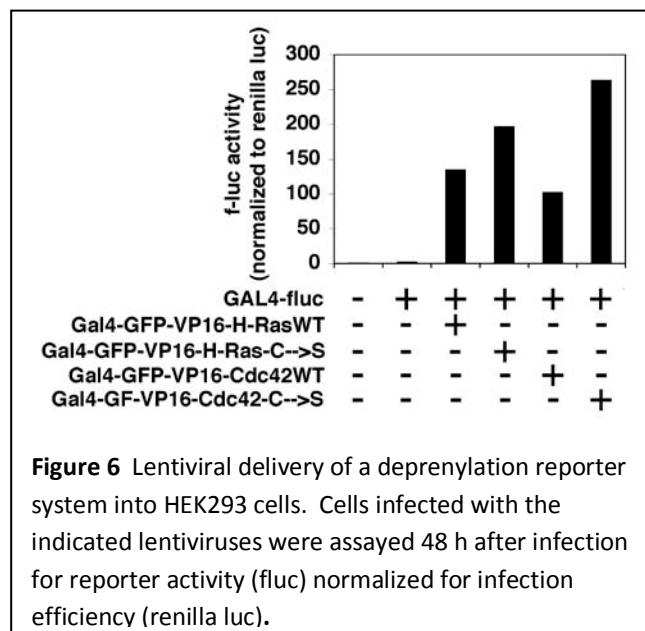
To test the effect of a prenylation inhibitor on the responsiveness of the reporter, we transiently expressed the wild-type Gal4-VP16-GFP-Cdc42-CaaX fusion in MDA-MB-231 reporter cells. Increasing concentrations of GGTI-298 produced graded increases in bioluminescence output of the luciferase reporter as expected (**Figures 4 and 5**). The  $EC_{50}$  was estimated as  $\sim 7 \mu\text{M}$ , correlating well with the  $LD_{50}$  value for compound-induced cytotoxicity. Importantly, there was no effect of the compound on the Gal4-VP16-GFP fusion control or the C-S tail mutant.



**Figure 4 (left) and Figure 5 (right).** Concentration-response of GGTI-mediated inhibition of prenylation in live MDA-MB-231 cells assessed with a Gal4-VP16-GFP-Cdc42-CaaX fusion transactivator of a Gal4-responsive luciferase reporter. Figure 4 shows live cell bioluminescence images and Figure 5 shows quantitative analysis of the photon flux.

**Year 2-**Encouraged by the preliminary results described above, we used the 2<sup>nd</sup> year of funding to develop an expression system that would yield highly reproducible levels of reporter expression, an absolute requirement for experiments planned in Aims 2 and 3. The reporter system described above would not meet this criterion because it involves cells that are transiently transfected with the reporter system on plasmids, which vary significantly in copy number from cell to cell and which can be lost stochastically over time as cells proliferate. In contrast, experiments in Aims 2 and 3 require cells that stably express the reporter system integrated into their chromosomes to yield reporter expression that is quantitatively reproducible from cell to cell and as cells proliferate, a precondition for conducting siRNA screens in cell culture and for imaging FTI or GGTI action in living animals. Accordingly, as described below we determined whether lentiviruses could be used to deliver a functional deprenylation-regulated reporter system.

To provide proof of concept, we infected HEK293 cells with a two-lentivirus system. One virus carried the GAL4-firefly luciferase (fluc) reporter. A second virus used the ubiquitin promoter to drive expression of Gal4-GFP-VP16 proteins fused to the wild type (WT) or mutant (C→S) prenylation sequences of H-Ras or Cdc42, and a downstream IRES (internal ribosome entry site) to express *Renilla* luciferase for normalization of infection efficiency. As shown in **Figure 6**, induction of Fluc reporter activity required expression of a Gal4-GFP-VP16 fusion protein (compare lane 2 with lanes 3-6). Moreover, induction of the Fluc reporter was more robust using non-prenylated mutant forms of the Gal4-GFP-VP16-H-Ras or -Cdc42 fusion proteins (lanes 4 and 6) relative to their wild type controls (lanes 3 and 5, respectively).



To streamline the generation of breast cancer cell lines in which the prenylation reporter system is stably integrated into chromosomal DNA, we next constructed a system in which a single lentivirus contained all the components of the system, as follows:

- i) Gal4 DNA binding sites that drive deprenylation-inducible expression of the firefly luciferase reporter to detect FTI or GGTI action;
- ii) the ubiquitin promoter driving constitutive expression of Gal4-GFP-VP16 fused to the H-Ras or Cdc42 prenylation sequence (either wild type, or a non-prenylated C→S mutant) to trigger deprenylation-regulated expression of the firefly luciferase reporter;
- iii) an internal ribosome entry site (IRES) downstream of Gal4-GFP-VP16-H-Ras/Cdc42 to drive constitutive expression of *Renilla* luciferase as a means of normalizing expression of the deprenylation-regulated firefly luciferase reporter to cell number;
- iv) the PGK promoter driving constitutive expression of a neomycin-resistance gene to select for cells bearing the integrated lentivirus.

In constructing these lentiviruses we had two hurdles to overcome. First, DNA fragments bearing each component noted above had to be small enough so that when combined into a single lentiviral plasmid they could be packaged into virus particles. Second, cloning strategies were limited by the paucity of cloning sites in lentiviral vectors. Despite these challenges, we have just completed construction of the one-virus system. We therefore are now ready to address Aims 2 and 3 in the coming year as a no-cost extension of this project.

**Aims 2 and 3 will be pursued as planned in no-cost extension year 3.** The lentivirally-delivered reporter system and novel imaging strategy will be coupled with molecular screening methods to identify

mechanisms determining FTI or GGTI sensitivity and resistance in tumors. The system now will be used to compare FTI and GGTI action toward tumor xenografts derived from breast cancer cell lines that are sensitive versus resistant to FTIs or GGTIs in vitro. This strategy should offer the opportunity to visualize over time the action of FTIs and GGTIs toward specific, biologically relevant prenylation-dependent proteins in tumors of living animals. We hope to address whether FTIs or GGTIs exert their adjuvant chemotherapeutic action by inhibiting prenylation of specific, biologically relevant targets such as Cdc42, and over the long-term, to define the mechanisms responsible for FTI or GGTI sensitivity and insensitivity in vivo.

### **Key Research Accomplishments**

- A new lentiviral-based prenylation responsive bioluminescence reporter system has been constructed and validated in human breast cancer cell lines.
- The imaging reporter responds in cell culture to a GGTI in a concentration-dependent manner.
- This system should provide a gain-of-function bioluminescence assay for imaging FTI action in vitro and in vivo.

### **Reportable Outcomes**

Abstract presentation, Era of Hope Annual Meeting, 2008, Baltimore, MD:

Penly, A., Pichler-Wallace, A., Blumer, K., Piwnica-Worms, D. Elucidating mechanisms of prenyltransferase inhibitor action and resistance in breast cancer by bioluminescence imaging.

### **Conclusions**

Prenyltransferase inhibitors (FTIs & GGTIs) block post-translational processing of Ras-like GTPases that have key roles in breast cancer. However, FTIs have yet to be used widely in breast cancer therapy because it is not yet possible to identify patients likely to be FTI-sensitive or to use combinatorial therapy to broaden the spectrum of patients that respond to FTIs. Our strategy should offer the opportunity to visualize over time the action of FTIs and GGTIs toward specific, biologically relevant prenylation-dependent proteins in tumors of living animals. We hope to address whether FTIs or GGTIs exert their adjuvant chemotherapeutic action by inhibiting prenylation of specific, biologically relevant targets such as H-Ras and Cdc42, and over the long-term, to define the mechanisms responsible for FTI or GGTI sensitivity and insensitivity in vivo.