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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: June 2010

TYPE OF REPORT: FINAL

PREPARED FOR: U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

x Approved for public release; distribution unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
06-30-2010	Final	1 JUN 2007 - 31 MAY 2010

4. TITLE AND SUBTITLE

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

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0426

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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One Brookings Drive. St. Louis, MO 63130-4862

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Material 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 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.

15. SUBJECT TERMS
Breast cancer, farnesyltransferase inhibitors, drug resistance, molecular imaging

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE	UU	10	19b. TELEPHONE NUMBER *(include area code)*
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Table of Contents

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 was to identify mechanisms determining whether breast cancer tumors in living animals are sensitive or insensitive to FTIs or GGTIs. We planned to develop a 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 strategy we 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 then constructed plasmids

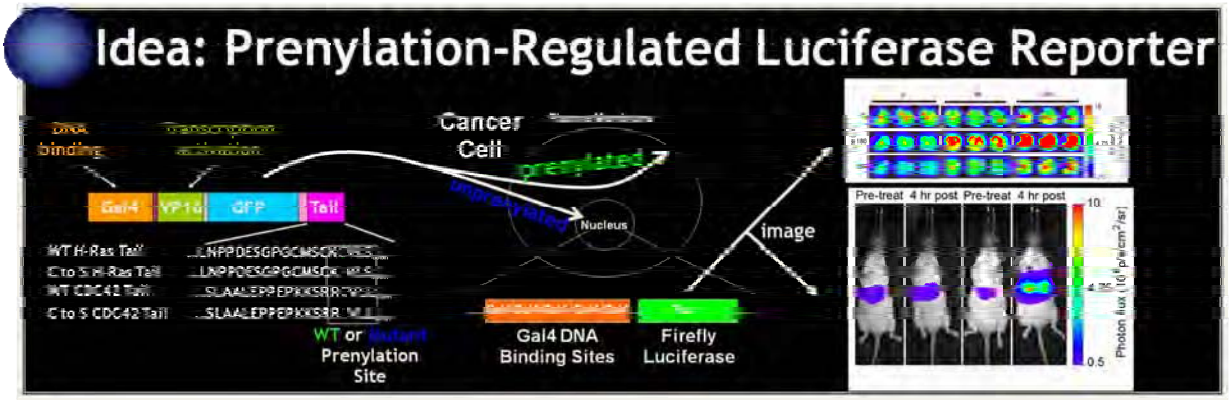
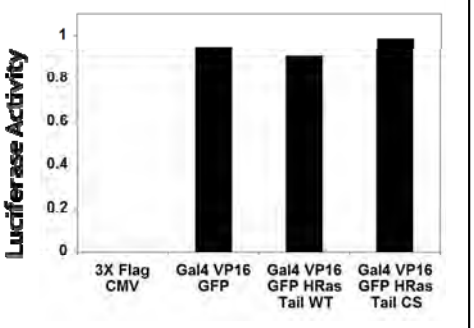


Figure 1. Concept of prenylation-regulated luciferase reporter.

that constitutively expressed 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 targeted the plasma membrane and thus, failed 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_{50} values were 20 μM and 0.01 μM for FTI-277, and 5 μM and 0.8 μM 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.



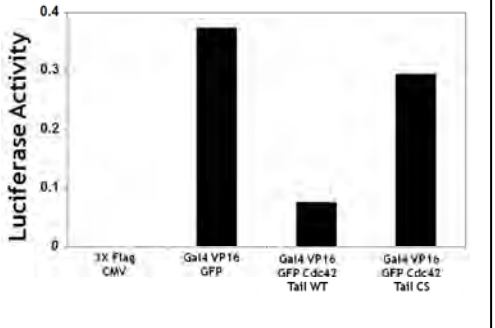


Figure 2 (left) and Figure 3 (right). Functional responsiveness of the Gal4-VP16-GFP-H-Ras-CaaX (Figure 2) and Gal4-VP16-GFP-Cdc42-CaaX (Figure 3) fusion transactivators in live cells.

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 ~7 μM, 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.

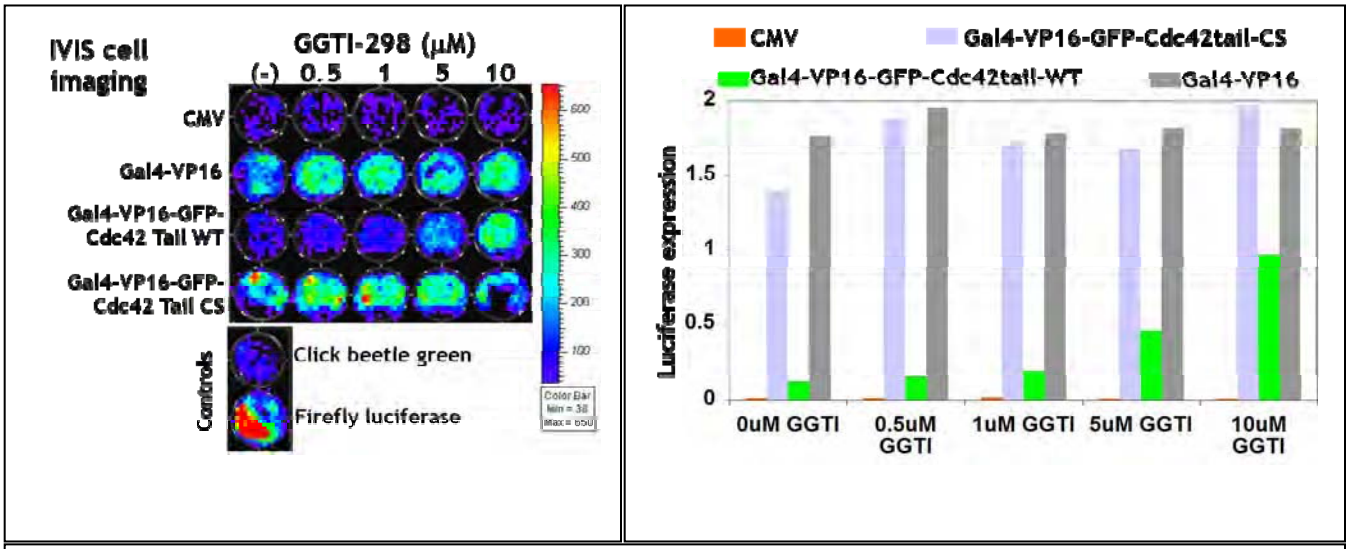


Figure 4 (left) and Figure 5 (right). Concentration-response of GGTI-mediated inhibition of prenylation in live MDA-MB-231 cells assessed with a Gal4-VP16-GFP-Cdc42-CaaX fusion transactivator of a Gal4-responsive luciferase reporter. Figure 4 shows live cell bioluminescence images and Figure 5 shows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

Year 2-Encouraged by the preliminary results described above, we used the 2nd 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).

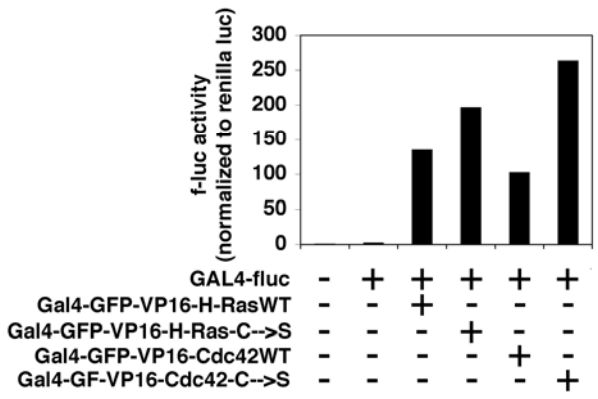


Figure 6 Lentiviral delivery of a deprenylation reporter system into HEK293 cells. Cells infected with the indicated lentiviruses were assayed 48 h after infection for reporter activity (fluc) normalized for infection efficiency (renilla luc).

To streamline the generation of breast cancer cell lines in which the prenylation reporter system was 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;

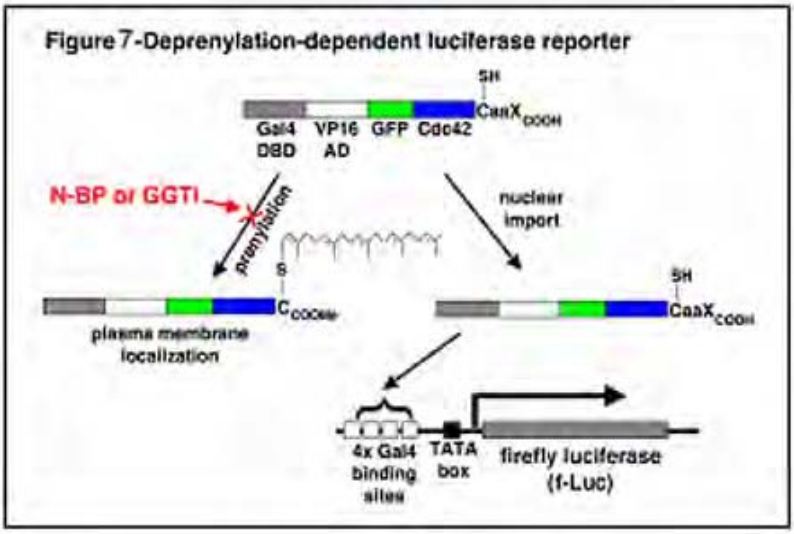
iii) an internal ribosome entry site (IRES) downstream of Gal4-GFP-VP16-H-Ras/Cdc42 to drive constitutive expression of *Renilla* luciferase as a means of normalizing expression of the deprenylation-regulated firefly luciferase reporter to cell number;

iv) the PGK promoter driving constitutive expression of a neomycin-resistance gene to select for cells bearing the integrated lentivirus.

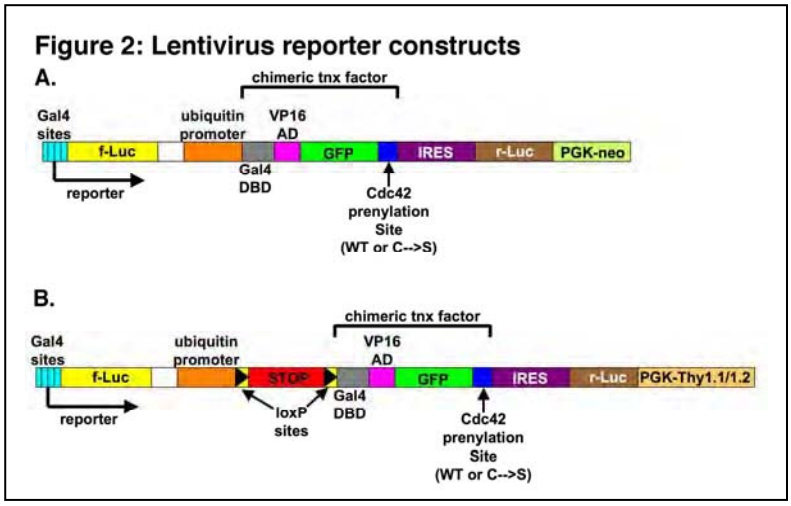
In constructing these lentiviruses we had two hurdles to overcome. First, DNA fragments bearing each component noted above had to be small enough so that when combined into a single lentiviral plasmid they could be packaged into virus particles. Second, cloning strategies were limited by the paucity of cloning sites in lentiviral vectors.

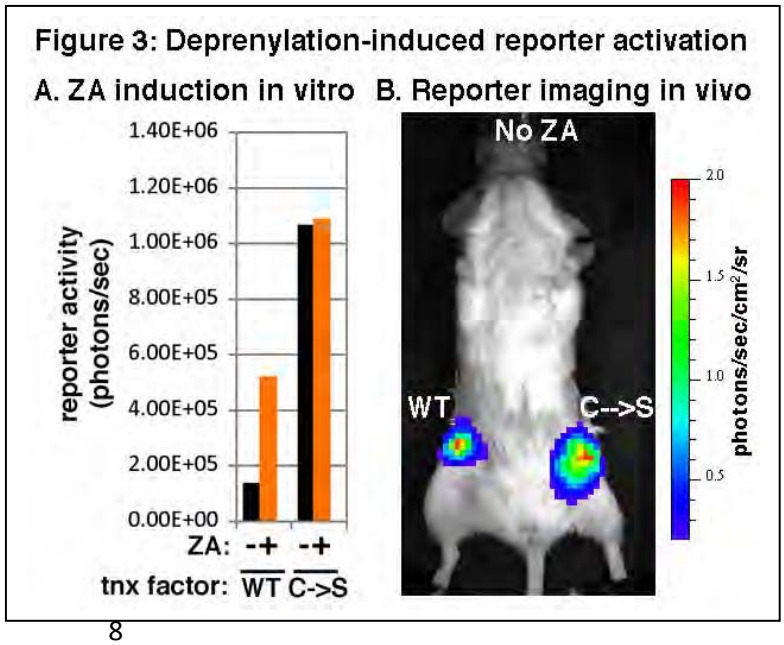
No-Cost-Extension Year 3

Accordingly, we modified our strategy to image inhibition of the second type of protein prenylation (geranylgeranylation), which would be blocked by nitrogen-containing bisphosphonates (N-BPs) or geranylgeranyltransferase inhibitors (GGTIs). **Figure 7 summarizes the strategy that eventually proved successful after investigating several alternatives.**



In preliminary experiments, we constructed a plasmid that constitutively expresses a chimeric transcription factor comprised of the DNA binding domain (DBD) of the yeast Gal4 transcription factor, the activation domain (AD) of the VP16 transcription factor, GFP, and either the wild type (WT) or inactive mutant (C→S) geranylgeranylation site of Cdc42, a Rho-type GTPase critical for ErbB1 signaling and breast cancer biology. After obtaining positive results in transient transfection assays (not shown), we designed a system for stably introducing the reporter system into tumor or host cells by lentviral transduction. We built a single lentivirus (**Extension Figure 2A**) with a 6 kb insert that contains: 1) the f-Luc reporter driven by Gal4 DNA binding site sequences and a TATA element; 2) a constitutive ubiquitin promoter that drives expresssion of the chimeric DBD-VP16-GFP-Cdc42 WT or C→S mutant transcription factor; 3) an IRES and *Renilla* luciferase (r-Luc) coding region to normalize f-Luc reporter activity for





differences in infection efficiency or cell number; and 4) a PGK-neo cassette to select for stably transduced cells. After preparing viral particles pseudotyped with VSV-G, we obtained clones of stably transduced human MDA-MB-231 breast cancer cells bearing reporter lentiviruses driven by either the chimeric WT or C→S mutant transcription factor. The WT fusion protein localized mainly to the plasma membrane via prenylation (not shown), and drove relatively low reporter activity as indicated by quantitative IVIS bioluminescence imaging of intact cells (**Extension Figure 3A**). In contrast, the C→S mutant protein, which cannot be prenylated, concentrated in the nucleus (not shown) and induced f-Luc expression ~8-fold higher than the WT control (**Extension Figure 3A**). In cells expressing the WT fusion protein, zoledronic acid (ZA) treatment (24 h at 20 μM, near its IC_{50}) induced reporter activity ~3-fold relative to the no drug control (**Extension Figure 3A**), as expected if ZA inhibited prenylation of the WT chimeric transcription factor, thereby shunting it to the nucleus. In contrast, cells expressing the C→S mutant protein exhibited similar reporter activity in the presence and absence of ZA (**Extension Figure 3A**), indicating that reporter induction by ZA required a functional prenylation site in the chimeric transcription factor. To determine whether photon fluxes driven by the reporter system could be imaged in vivo, we subdermally implanted 10^4 MDA-MB-231 cells transduced with the WT and C→S mutant reporters into opposite flanks of immunodeficient (Nod/Scid) mice. In the absence of ZA, signals produced by the WT and C→S mutant reporters were readily detected by IVIS imaging of anesthetized mice (**Extension Figure 3B**). Therefore, these pilot data demonstrated that this novel reporter system should be well suited for directly imaging the action of ZA in tumor and host cells 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.
- A second generation imaging reporter responds in cell culture to a bisphosphonate in a concentration-dependent manner.
- This system should provide a gain-of-function bioluminescence assay for imaging GGTIs and bisphosphonate 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.

Successful submission and activation of a DOD Breast Idea Expansion Award (BC096369; co-PIs: Piwnica-Worms and Blumer)

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. This grant established the foundation for the Breast Idea Expansion Award (BC096369) to identify, in breast cancer therapy, biologically relevant cellular targets of bisphosphonates (such as zoledronic acid or other N-BPs), compounds that target farnesylpyrophosphate (FPP) synthase in the isoprenoid lipid precursor pathway.