Reversion of RhoC GTPase-induced Inflammatory Breast Cancer Phenotype by Treatment with a Farnesyl Transferase Inhibitor

Kenneth L. van Golen, LiWei Bao, Melinda M. DiVito, Zhifeng Wu, George C. Prendergast, and Sofia D. Merajver


Abstract

Inflammatory breast carcinoma (IBC) is a highly aggressive form of locally advanced breast cancer that has the ability to invade and block the dermal lymphatics of the skin overlying the breast. In previous series of studies, our laboratory identified overexpression of RhoC GTPase in >90% of IBCs (K. L. van Golen et al., Clin. Cancer Res., 5: 2511–2519, 1999) and defined RhoC as a mammary oncogene involved in conferring the metastatic phenotype (K. L. van Golen et al., Cancer Res., 60: 5832–5838, 2000). RhoC GTPase is involved in cytoskeletal reorganization during cellular motility. Farnesyl transferase inhibitors (FTIs) were previously shown to be effective in modulating tumor growth in Ras-transformed tumor cells. Recently, studies have focused on RhoB as a putative non-Ras target of FTI action. In the present study, we assessed the effect of the FTI L-744,832 on RhoC overexpressing IBC and RhoC-transfected human mammary epithelial (HME-RhoC) cells. Treatment of the SUM149 IBC cell line and HME-RhoC transfectants with the FTI L-744,832 led to reversion of the RhoC-induced phenotype, manifested by a significant decrease in anchorage-independent growth, motility, and invasion. Although RhoC expression and activation were not affected, RhoB levels were increased by FTI treatment. Transient transfection of geranylgeranylated RhoB (RhoB-GG) into the same cells reproduced the effects of the FTI, thus suggesting that FTI-induced reversion of the RhoC phenotype may be mediated by an increase in RhoB-GG levels. These data provide direct evidence that FTIs may find use in the clinic when directed against RhoC-overexpressing tumors and suggest appropriate biological markers to evaluate during FTI treatment.

Introduction

The term IBC was first coined in 1924 by Drs. Lee and Tannenbaum to describe a phenotypically distinct form of locally advanced breast cancer (LABC) (1, 2). IBC is a fast-growing, highly invasive, and metastatic form of LABC, which is clinically characterized by primary skin changes (1–4). These primary skin changes are the result of blockage of the dermal lymphatics of the skin overlying the breast resulting in edema, peau d’orange, and nipple retraction (1–4). At the time of diagnosis, nearly all tumors have spread to the regional lymph nodes and on close inspection, more than one-third of patients have gross distant metastases (1–4). Despite aggressive multimodality treatments, the 5-year disease-free survival rate for women with IBC is <45%, making IBC the deadliest form of breast cancer (1–4).

During investigation of the genetic mechanisms responsible for the unique IBC phenotype, our laboratory identified overexpression of RhoC GTPase in >90% of IBCs (5). RhoC GTPase is a member of the Ras-homology family of small GTP-binding proteins and is responsible for cytoskeletal reorganization during cellular motility (6–10). RhoC belongs to a highly homologous subfamily comprised of RhoA, RhoB, and RhoC (11). Although these family members have >90% sequence homology to one another, their roles in the cell are distinct (11). To determine the contribution of RhoC GTPase overexpression to the IBC phenotype, we generated stable RhoC-overexpressing HME cell lines (HME-RhoC) (12). The HME-RhoC clones nearly recapitulated the invasive features of the IBC phenotype. Specifically, the cells grew under anchorage-independent growth conditions and produced tumors when orthotopically injected into athymic nude mice (12–14). The cells were highly motile and invasive and produced conditioned medium rich in pro-angiogenic cytokines in vitro (12–14). Taken together, these data demonstrate that overexpressed, active RhoC GTPase is a mammary oncogene leading to advanced disease.

Regulation of the GTPase activity of both the Ras and the Rho proteins is achieved through interactions of GAPs, GDIs, GDFs, and GEFs (15, 16). RhoA, RhoC, and a fraction of RhoB

Received 1/17/02; revised 4/4/02; accepted 4/15/02.

1 Supported by National Cancer Institute Grant R01 CA 77612 (to S. D. M.); Grant DAMO 17-00-1-0345, from The Department of Defense, United States Army Breast Cancer Program (to S. D. M.); and Grant ST32 CA 09537 (to S. D. M.) and a postdoctoral fellowship (to K. L. v. G.) from the Susan G. Komen Breast Cancer Foundation.

2 To whom requests for reprints should be addressed, at 7217 CCGC, 1500 East Medical Center Drive, Ann Arbor, MI 48109-0948. E-mail: smerajve@umich.edu.

3 The abbreviations used are: IBC, inflammatory breast cancer; FTI, farnesyl transferase inhibitor; HME, human mammary epithelial cells; FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR; PI, propidium iodide; RhoB-GG, geranylgeranylated RhoB; GADH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; PISK, phosphatidylinositol 3-kinase; β-gal, β-galactosidase; GAP, GTPase-activating proteins; GDI, GDP-dissociation inhibitor; GDF, GDI dissociation factor; GEF, guanine nucleotide exchange factor; SEM, scanning electron microscopy.
are geranylgeranylated, and the remaining portion of RhoB is farnesylated (17–19). For the Rho proteins to enter the GDP/GTP cycle they must be transported and localized to the membrane (19, 20), GTP binding produces a conformational change in the GTPase, thereby allowing interaction with downstream effector proteins (15, 21). Hydrolysis of GTP to GDP by the intrinsic Rho GTPase activity modulates the interaction with the effector protein (22, 23). The GTPase activity is greatly increased by activated GAPs, thus leading to increased hydrolysis of GTP (24). The entire process is balanced by the GDIs, which prevent GDP dissociation by binding to the prenylation group of the GTPase and sequestering the complex in the cytoplasm (25). The GTPase is subsequently liberated from the GDI by GDFs, closing the cycle (25).

In light of evidence demonstrating that FTIs can target non-Ras molecules, such as the RhoB protein, and recent work suggesting that RhoB alterations, specifically, the accumulation of RhoB-GG, may interfere with transforming Rho signals, we sought to test the effect of FTIs on RhoC-transformed breast cells.

Materials and Methods

Cell Culture. Cell lines were maintained under defined culture conditions for optimal growth in each case as described previously (26–28). E6/E7 immortalized HME cells (29) were grown in 5% FBS (Sigma Chemical Co., St. Louis, MO)-supplemented Ham’s F-12 medium (JRH Biosciences, Lenexa, KS) containing insulin, hydrocortisone, epidermal growth factor, and cholera toxin (Sigma Chemical Co.). Stable HME transfectants containing either the human RhoC GTPase or control β-gal genes were maintained in the described medium supplemented with 100 μg/ml hygromycin (LifeScience Technologies; Gaithersburg, MD) as described previously (12, 13). The SUM149 IBC cell line was grown in 5% FBS-supplemented Ham’s F-12 medium containing insulin and hydrocortisone. The HME cells were characterized as being keratin 19 positive, thus ensuring that they are from the same differentiation lineage as the SUM149 IBC tumor cell line. For FTI treatment, actively growing cells were treated with 25 μM FTI L-744,832 and harvested 48 h later. Cell viability was assessed prior to transfections using a trypsin blue exclusion assay. Harvested cells were washed in 10 ml of HBSS (LifeScience Technologies). A 100-μl aliquot was taken, diluted 1:1 with prediluted trypsin blue (Sigma Chemical Co.), and counted on a hemacytometer.

Transient transfections were performed by growing cells in 100-mm plates until reaching 50% confluence. Expression constructs for wild-type RhoB, RhoB-GG, and a geranylgeranyl-deficient RhoB mutant were generated as described previously (30–32). The RhoB containing vectors or a vector control were introduced into the cells using FuGene6 transfection reagent (Roche, Indianapolis, IN) as described previously (12). Transient transfectants were used in biological assays 24 h after transfection.

Anchorage-independent Growth and Focus Formation. For anchorage-independent growth assays, a 2% stock of sterile low-melt agarose was diluted 1:1 with 2× MEM. Further dilution to 0.6% agarose was made using 10% FBS-supplemented Ham’s F-12 medium complete with growth factors, and 1 ml was added to each well of a six-well plate as a base-layer. The cell layer was then prepared by diluting agarose to 0.3% and 0.6% with 103 cells (either untreated or 25 μM FTI L-744,832 for 24 h) in 2.5% FBS-supplemented Ham’s F-12/1.5 ml/well. A 1-ml layer of medium was maintained on top of the agar to provide nutrients and, in the case of the treated cells, additional inhibitor. Colonies ≥100 μm in diameter were counted after a 2-week incubation at 37°C in a 10% CO2 incubator.

A modified focus formation assay was performed by harvesting treated and untreated cells and plating at dilutions of 1000, 500, and 100 cells/35-mm dish. The cells were then cultured for 2 weeks at 37°C in a 10% CO2 incubator. The plates were washed with 10 ml of PBS, fixed for 10 min with ice-cold methanol, and stained for 10 min with 2% methylene blue in 50% ethanol, and visible foci were counted.

Western Blot and RhoC Activation Analysis. Proteins were harvested from cell cultures using radiolabeled assay buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 0.3 mg/ml aprotinin; Sigma Chemical Co.) Ten-μg aliquots were mixed with Laemmli buffer, heat-denatured for 3 min, separated by SDS-PAGE, and transferred to nitrocellulose. Nonspecific binding was blocked by overnight incubation with 2% powdered milk in Tris-buffered saline with 0.05% Tween 20 (Sigma Chemical Co.). Immobilized proteins were probed using antibodies specific for RhoC GTPase (33), or RhoB GTPase (Cytoskeleton Inc. Denver, CO). Protein bands were visualized by ECL (Amersham-Pharmacia Biotech, Piscataway, NJ).

A RhoC activation assay was performed as described previously (34, 35). Cells grown to 40% confluence were incubated in the presence or absence of 25 μM FTI L-744,832 for 24 h. Proteins were harvested using GST-FISH buffer (34) and were centrifuged. The supernatant was mixed with a slurry of GST-rhotekin fusion protein bound to glutathione-sepharose beads. Only GTP-bound Rho binds to the GST-rhotekin fusion protein. The mixture was centrifuged, separated by SDS-PAGE, transferred to nitrocellulose and probed using a RhoC-specific antibody (33). Protein bands were visualized by ECL and exposed to Hyperfilm (Amersham).

Semiquantitative RT-PCR. Total RNA was harvested from actively growing cells at 50% confluence using Trizol Reagent (Life Technologies), and cDNA was made using the AMV-reverse transcriptase kit (Promega, Madison, WI). RhoC and RhoB transcripts were PCR amplified from aliquots of cDNA using a 1:100 dilution of Rho-specific primers mixed with GAPDH primers. PCR products were then separated on a 1.2% TAE-agarose gel and were visualized by ethidium bromide. The relative intensity of the Rho and GAPDH bands was measured using an Alpha Imager 2200 (Alpha Innotech Co., San Leandro, CA).

Motility and Invasion Assays. Random motility was determined using a gold-colloid assay (36). Gold colloid was layered onto glass coverslips and placed into 6-well plates. Cells were seeded onto the coverslips and allowed to adhere for 1 h at 37°C in a CO2 incubator (12,500 cells/3 ml in serum-free medium). To stimulate the cells, the serum-free
medium was replaced with 5% FBS containing Ham’s F-12 supplemented with growth factors and allowed to incubate for 3 h at 37°C. The medium was aspirated and the cells fixed using 2% glutaraldehyde (Sigma Chemical Co.). The coverslips were then mounted onto glass microscope slides and areas of clearing in the gold colloid corresponding to phagokinetic cell tracks were counted.

The invasion assay was performed as described previously with minor modification (37). A 10-μl aliquot of 10 mg/ml Matrigel (BD Biosciences, Bedford, MA) was spread onto a 6.5-mm Transwell filter with 8 μm pores (Costar, Corning, NY) air-dried in a laminar flow hood, and reconstituted with a few drops of serum-free medium. The lower chamber of the Transwell was filled with either serum-free or serum-containing media. Cells were harvested and resuspended in serum-free medium with 0.1% BSA at a concentration of 3.75 × 10⁵ cells/ml, and 0.5 ml was added to the top chamber. The chambers were incubated for 24 h at 37°C in a 10% CO₂ incubator. The cell suspension was aspirated, and excess Matrigel was removed from the filter using a cotton swab. The filters were then cut away from the Transwell assembly and fixed, gel side down, with methanol to a glass microscope slide, stained with H&E, and 20 random ×40 magnification fields were counted. The number of cells that had invaded into the serum-free medium-containing lower chambers were considered background and were subtracted from the number of invaded cells in the serum-containing samples.

Statistical analysis was performed using a two-tailed Student’s t-test.

**Apoptosis Assay.** Cytofluorometric analysis of cell cycle distribution and apoptosis was performed by PI staining of nuclei as reported previously (38, 39). Briefly, cells were treated with 25 μM FTI L-744,832 (Merck) alone, 10 μM LY294002 (Calbiochem, San Diego, CA) alone, or a combination of both FTI L-744,832 and LY294002. Untreated and treated cells, 1 × 10⁶, were harvested from 35-mm wells, washed once with ice-cold PBS (Fisher Scientific, Pittsburgh, PA) and pelleted; supernatants were removed and 500 μl of PI-hypotonic lysis buffer [0.1% sodium citrate, 0.1% Triton X, 100 μg/ml RNAse type I-A, 50 μg/ml PI (SIGMA)] were added. Samples were analyzed by flow cytometry after a 20-min incubation at 25°C.

**Rhodamine-Phalloidin Staining of Actin Filaments.** Visualization of actin filaments was accomplished by staining the cells with a rhodamine-conjugated phalloxin. Briefly, cells were grown on glass coverslips for 48 h and washed with PBS followed by fixation with 1:1 ice-cold acetone and methanol. After a 30-min incubation in PBS containing 1% BSA, 5 μl of methanol rhodamine-phalloidin stock (Molecular Probes, Eugene, OR) were added to each coverslip and allowed to stain for 20 min at room temperature. After repeated washing with PBS, the coverslips were mounted onto glass microscope slides using Gel/Mount (Biomedica Co., Foster City, CA). Cells were visualized under a Zeiss scanning laser confocal microscope equipped with a 573-nm fluorescence filter.

**SEM.** Cells (12,000) were fixed with buffered 2.5% glutaraldehyde for 1 h, rinsed, and post-fixed for an additional hour with buffered osmium tetroxide. After dehydration in ascending strengths of ethanol, the cells were critical-point dried, mounted onto standard SEM stubs, and gold-sputter coated. They were viewed using an AMRAY 1000-B Scanning Electron Microscope.

**Results**

**Effect of FTI Treatment on RhoC-overexpressing Breast Cells.** The ability of cells to grow in soft agar is a hallmark of malignant transformation (40). Previously, we found that RhoC overexpression led to the growth of mammary epithelial cells under anchorage-independent conditions (12, 14). As demonstrated in Fig. 1A, treatment of RhoC-overexpressing HME cells and the SUM149 IBC cell line with 25 μM FTI L-744,832 resulted in a significant decrease in anchorage-independent growth. Although the HME-β-gal control-transfected cells did not readily grow under anchorage-independent conditions, they were slightly affected by FTI treatment. The 80% decrease in anchorage-independent growth of the Rho-expressing cells did not correlate with a decrease in monolayer growth rate as determined by a 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (data not shown). It has been suggested that FTI treatment sensitizes cells to apoptotic death on treatment with the PI3K inhibitor LY294002 (31). However, in our system, we did not observe an increase in apoptosis in cells treated with FTI L-744,832 alone, LY294002 alone, or a combination of the two, as has been observed for Ras-transformed cells (data not shown).

To evaluate the effect of FTI treatment on RhoC-mediated cellular motility, we assessed the same treated cell lines in colloidal-gold random motility assay. Cells were seeded onto glass coverslips overlayed with a gold colloid and stimulated with serum to induce motility. Discernable and quantifiable tracks were left as the cells moved and phagocytized the gold colloid. At 24 h after stimulation, the SUM149 and HME-RhoC cells treated with FTI L-744,832 were 1.8- to 2-fold less motile than their untreated counterparts (Fig. 1B). Both the HME-β-gal control and the MCF10AT c1 (an MCF10A clone transfected with a constitutively active Ras; Ref. 41) were unaffected by FTI treatment. As determined by a trypan blue dye exclusion assay, the reduction in cell motility was not caused by a decrease in the number of viable cells (data not shown).

As shown in Fig. 1C, when the FTI-treated cells were tested for their ability to invade through a Matrigel-coated filter in response to a chemoattractant, it was found that the SUM149 and HME-RhoC cells were 2-fold less invasive than the untreated cells. Again, the control cell lines, HME-β-gal and MCF10AT c1, were unaffected by FTI treatment.

Taken together, these data suggest that treatment of RhoC-overexpressing cells with a FTI leads to the inhibition of RhoC-mediated anchorage-independent growth, motility, and invasion without significantly affecting cell growth or viability.

As demonstrated in Fig. 2, C and E, rhodamine-phalloidin staining for actin filaments shows a highly organized and polarized cytoskeleton in the RhoC-overexpressing cells. These actin bundles are lost or diminished on treatment with
FTI L-744,832 (Fig. 2, D and F). Numerous focal adhesions were visible on the periphery of the treated cells. However, loss of cytoskeletal polarity led to morphological changes towards a rounded shape, as demonstrated by laser scanning confocal microscopy and scanning electron microscopy (Fig. 2, G–J). The morphology of the control HME-β-gal cells was also similarly affected, albeit to a lesser degree, by FTI treatment, as these cells became dissociated and flattened (Fig. 2, A and B).

**Rho Protein Levels Increase as a Result of FTI Treatment.** To determine the effect of FTI treatment on RhoC expression, we performed semiquantitative RT-PCR and Western blot analysis. As shown in Fig. 3A, RhoC mRNA expression increased in all of the cell lines on FTI treatment. A concordant increase in RhoC protein levels was also observed, as determined by Western blot analysis using a RhoC-specific antibody developed in our laboratory (33). The activity of RhoC was assessed using a GST-pulldown assay (34, 35). This assay utilizes a GST-fusion protein of a Rho-binding domain motif found in a variety of Rho-effector proteins. GTP-bound Rho is in its active state and can bind the Rho-binding domain (35). Using this assay, we found that in the SUM149 and HME-RhoC cell, the levels of GTP-bound RhoC were not affected by FTI treatment, which indicated that RhoC activation itself was unaffected by RhoB and RhoC accumulation. As expected from the mRNA and protein levels, activated RhoC was elevated in all of the FTI-treated cells, including the HME-β-gal control cells.

Because previous studies suggested a role for RhoB in reverting the malignant phenotype of Ras-transformed cells treated with FTI (42), we performed semiquantitative RT-PCR and Western blot analysis for RhoB. As shown in Fig. 3B, RhoB mRNA levels markedly increased 24 h after treatment with FTI L-744,832 in all of the cell lines tested. Furthermore, RhoB protein levels were also significantly increased.

These results support earlier observations that the accumulation of RhoB, likely RhoB-GG but not farnesylated RhoB, leads to a reversion of the malignant phenotype by FTI (30, 42, 43). The mechanism of FTI inhibition of the RhoC-induced phenotype appears to be independent of direct action of the FTI on geranylgeranylated RhoC. This is demonstrated by an accumulation of RhoC protein and no change in its activity.

**Expression of RhoB-GG Recapitulates the FTI-mediated Effects on RhoC-overexpressing Breast Cells.** In light of the previous experiments, we hypothesized that the accumulation of RhoB, likely RhoB-GG but not farnesylated RhoB, leads to a reversion of the malignant phenotype by FTI (30, 42, 43). The mechanism of FTI inhibition of the RhoC-induced phenotype appears to be independent of direct action of the FTI on geranylgeranylated RhoC. This is demonstrated by an accumulation of RhoC protein and no change in its activity.
vector-specific primers demonstrated mRNA expression in all of the cell lines tested.

Table 1 shows the results of a focus formation assay for the transfectants. The SUM149 and HME-RhoC cells, transiently transfected with RhoB-GG, formed significantly fewer foci than did the RhoB-GG-deficient mutant transfectants or the nontransfected cells. The morphology of the RhoB-GG-transfected cells were similar to their FTI-treated counter-
parts (Fig. 2, K–L). Similarly, the RhoB-GG transfectants were less motile when tested in the colloidal-gold assay (Fig. 4). Although statistical significance was not reached for the transfectants, the trends indicate that expression of RhoB-GG leads to decreased motility in RhoC-overexpressing breast cells. These data provide evidence for a role for RhoB-GG as a mechanism for inhibiting or reverting the RhoC-induced phenotype in these cells.

Discussion

The observation that H-Ras was inactive and not localized to its specific membrane compartment after FTI treatment led to the idea that these inhibitors could be used therapeutically (44–48). Like Ras, the Rho GTPases are posttranslationally modified to locate them to their distinct cellular compartment, so that they can carry out their specific function (17–19). Each Rho protein contains a COOH-terminal CAAX domain that determines prenylation and polybasic residues in the hypervariable domain, upstream of the CAAX domain, which dictate proper membrane localization (49).

Table 1  Mean focus formation and SD after transient transfection with RhoB-GG, a geranylgeranyl-deficient RhoB (RhoB-A3), and vector alone

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Vector alone</th>
<th>RhoB-GG</th>
<th>RhoB-A3</th>
</tr>
</thead>
<tbody>
<tr>
<td>HME-β-gal</td>
<td>48.5 ± 5.5</td>
<td>24.5 ± 1</td>
<td>36.5 ± 2.5</td>
</tr>
<tr>
<td>HME-RhoC</td>
<td>85 ± 3</td>
<td>23.5 ± 1</td>
<td>69 ± 6.5</td>
</tr>
<tr>
<td>SUM149 cell line</td>
<td>19.5 ± 1.5</td>
<td>5 ± 0.5</td>
<td>20.5 ± 1</td>
</tr>
<tr>
<td>MCF10AT c1</td>
<td>53.5 ± 0.5</td>
<td>25.5 ± 6</td>
<td>46 ± 1.5</td>
</tr>
</tbody>
</table>

The number of foci were assessed in triplicate 35-mm plates. The data presented are from a seeding density of 100 cells/plate. However, similar trends were seen when cells were seeded at 1000 or 500 cells/plate. Focus formation was dramatically reduced in the RhoC-overexpressing HME-RhoC and SUM149 cells by expression of geranylgeranylated RhoB, but not an unprenylated mutant (RhoB-A3). Although a trend was noted, statistical analysis by a Kruskal-Wallis test did not demonstrate a significant difference between the groups.
Membrane localization and trafficking of the Rho GTPases are complex phenomena. Rho proteins are, in their inactive state, localized in the cytosol, sequestered there by specific RhoGD (25). On activation, the GTP-bound protein is prenylated and transported to its specific membrane compartment. The type of prenylation is dependent on the Rho protein (50–53). RhoC GTPase is geranylgeranylated, whereas RhoB is both geranylgeranylated and farnesylated (17–19, 50, 51).

It has been demonstrated that in vitro, Rho GTPases can self-aggregate (54). Cdc42 and Rac2 homodimer formation has been implicated in the negative regulation of the activity of those proteins (55). RhoB and RhoC GTPase have been found to exist, not as homodimers, but as either monomeric or oligomeric complexes. In addition, RhoC has been shown to have an arginine finger motif COOH-terminal to the CAAAX domain, which imparts self-activated GTPase regulatory function (54). Specifically, GTP-bound RhoC, when in complex with itself, can self-convert to RhoC-GDP. In contrast, RhoB does not contain this arginine domain and, therefore, does not have intrinsic GAP activity. It is yet unknown whether RhoB and RhoC can form heterodimers either in vitro or in vivo. Because FTI cannot directly block geranylgeranyl RhoC function, one possible explanation for FTI suppression of RhoC function is that accumulation of RhoB-GG leads to the oligomerization of RhoC, which leads to increased intrinsic GAP activity and GTPase deactivation.

The promise of FTIs as a potent therapeutic reagent has been supported by in vivo studies. Mammary tumors that develop in K-Ras transgenic mice can be growth inhibited by FTI treatment (56). Lebowitz et al. (30) and Prendergast et al. (57–59) have provided evidence that FTI suppression of Ras transformation was accomplished by interfering with Rho activity, because Rho was shown to be critical in Ras-induced transformation. Subsequent experiments demonstrated that a shift from farnesylated RhoB GTPase to RhoB-GG occurred on FTI treatment (30, 31, 60, 61). The shift in the specific forms of prenylated RhoB is accompanied by the accumulation and mislocalization of RhoB-GG, which is normally a short-lived protein (with a half-life of 2–4 h in cells; Refs. 30, 62). Our present data support these observations. In this study, we observed increased expression and accumulation of RhoB, presumably RhoB-GG, on treatment with FTI L-744,832. We also demonstrated that transient transfection of RhoC-overexpressing breast cells with RhoB-GG, recapitulated the effects of FTI treatment, inhibiting focus formation and random motility, whereas transfection with the RhoB GG-deficient mutant failed to mimic FTI effects.

In Ras-transformed cells, the effects of RhoB-GG may be attributable to a “gain-of-function” and relocalization of the protein (42, 43, 59, 63). Normally, RhoB GTPase is involved in vesicular and receptor trafficking (64). However, after FTI treatment, the inhibition of farnesylated RhoB and the accumulation of RhoB-GG, may lead to altered functions. The biosynthesis of geranylgeranyl PP, is the next step after the synthesis of farnesyl PP, in the acetyl-CoA pathway of cholesterol synthesis (reviewed by Cohen et al.; Ref. 48). Therefore, FTI treatment may provide more substrate for the geranylgeranyl PP, synthase to produce geranylgeranyl PP, and, ultimately, functionally geranylgeranylated Rho. Furthermore, several investigators suggest that a membrane receptor may exist that binds to the Rho prenyl-group, thereby helping to specifically localize it to a membrane compartment (51, 65–67). In this scenario, the accumulation of RhoB-GG may compete with RhoC, displacing it and possibly preventing it from interacting with downstream effector molecules.

In our experiments, we demonstrate that on FTI-treatment, RhoC levels also increase; however, the ratio of RhoB:RhoC remains increased over pretreatment levels. Furthermore, we speculate that RhoC may be accumulating in the cytoplasm, or, if it is reaching the inner membrane, its effect on the cell is attenuated by RhoB-GG. These ideas have yet to be tested. As demonstrated by labeling the actin cytoskeleton with a rhodamine-labeled phalloidin, the polarized actin bundles associated with the motile RhoC cells are nearly lost on FTI treatment. Although focal adhesions are visible in both treated and untreated cells, they are located exclusively around the outside edges of the FTI-treated cells. Both laser confocal and scanning electron microscopy demonstrate that, as observed previously (57), the cells have lost their polarity and are flattened.

In contrast with Ras-transformed cells, the growth rate of the RhoC-overexpressing cells was only slightly affected by FTI treatment. Cell viability was also unaffected. Again, this is in contrast to previous experiments, which demonstrated that a combination of FTI and LY294002 (an inhibitor of P13K) led to increased apoptosis in Ras-transformed cells (31) but not in RhoC-overexpressing cells.

Taken together, these data suggest that FTIs may prove a potent novel therapeutic agent against tumors that overexpress RhoC GTPase. This is the first report of FTI inhibition of the cancer phenotype induced specifically by overexpression of RhoC GTPase. The mechanism of FTI action in RhoC-overexpressing IBC and HME transfectants may be similar to that previously described for Ras-transformed cells, namely, that the effect is mediated by the accumulation of RhoB-GG. However, as described above, there are notable differences in how FTI treatment affects cell growth in Ras-transformed versus RhoC-overexpressing cells. In addition to IBC, it has been demonstrated that aggressive noninflammatory, metastatic breast cancers, advanced pancreatic cancer, and metastatic melanoma overexpress RhoC GTPase, and this event significantly contributes to their clinical behavior; therefore, FTI treatment may be effective against these aggressive cancers (33, 68, 69). Our data support testing whether FTI treatment is efficacious in these aggressive RhoC-driven malignancies.

Acknowledgments
We thank Lisa Robbins for help in preparation of the manuscript and Satoru Hayasaka for performing statistical analysis. The reagents for the Rho-activation assay were kindly provided by Dr. John Collard of the Netherlands Cancer Institute (Amsterdam, the Netherlands).
References


Molecular Cancer Therapeutics

Reversion of RhoC GTPase-induced Inflammatory Breast Cancer Phenotype by Treatment with a Farnesyl Transferase Inhibitor 1 Supported by National Cancer Institute Grant R01 CA 77612 (to S. D. M.); Grant DAMD 17-00-1-0345, from The Department of Defense, United States Army Breast Cancer Program (to S. D. M.); and Grant 5T32 CA 09537 (to S. D. M.) and a postdoctoral fellowship (to K. L. v. G.) from the Susan G. Komen Breast Cancer Foundation.

Kenneth L. van Golen, LiWei Bao, Melinda M. DiVito, et al.