Genetic and Pharmacologic Inhibition of eIF4E Reduces Breast Cancer Cell Migration, Invasion, and Metastasis

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Abstract

The translation initiation factor eIF4E is an oncogene that is commonly overexpressed in primary breast cancers and metastases. In this article, we report that a pharmacologic inhibitor of eIF4E function, ribavirin, safely and potently suppresses breast tumor formation. Ribavirin administration blocked the growth of primary breast tumors in several murine models and reduced the development of lung metastases in an invasive model. Mechanistically, eIF4E silencing or blockade reduced the invasiveness and metastatic capability of breast cancer cells in a manner associated with decreased activity of matrix metalloproteinase (MMP)-3 and MMP-9. Furthermore, eIF4E silencing or ribavirin treatment suppressed features of epithelial-to-mesenchymal transition, a process crucial for metastasis. Our findings offer a preclinical rationale to explore broadening the clinical evaluation of ribavirin, currently being tested in patients with eIF4E-overexpressing leukemia, as a strategy to treat solid tumors such as metastatic breast cancer. Cancer Res; 75(6); 1102–12. ©2015 AACR.

Introduction

Despite major advances in breast cancer diagnosis and treatment as well as the understanding of breast cancer biology, this disease remains a significant clinical challenge. Metastasis is the main cause of breast cancer fatality, so understanding and targeting metastatic disease is critical for better therapeutic success. The eukaryotic translation initiation factor 4E (eIF4E) is an oncogene, which is frequently overexpressed in cancer, including at least 50% of breast cancers (1–3). Overexpression and activity of eIF4E is associated with tumor formation, metastatic disease, and increased tumor invasion in mice (4–6). eIF4E function is essential for cap-dependent translation of specific mRNAs, including many critical to cell division, cell survival, and angiogenesis (2, 7). Importantly, cancer cells that overexpress eIF4E may develop an oncogene addiction to eIF4E, rendering these cells vulnerable to eIF4E inhibition, whereas normal cells are relatively insensitive (8–10). We have reported that eIF4E is overexpressed in primary breast tumors and in metastases (3), and studies have indicated that high levels of eIF4E in the primary tumor correlate with clinical progression (11, 12). This suggests that eIF4E could promote a metastatic phenotype, and knockdown of eIF4E has in fact been shown to reduce breast cancer cell migration and invasion (13). Moreover, metastasis is reduced when eIF4E cannot be phosphorylated (14).

The epithelial-to-mesenchymal transition (EMT) is a fundamental cellular process that is vital for metastasis. During EMT, activation of transcription factors leads to altered expression of cell surface proteins, production of extracellular matrix (ECM) degrading enzymes, including matrix metalloproteinase (MMP)-2, -3, and -9 (15–17), culminating in the formation of a migratory and invasive cell (18). The transcriptional events involved in EMT are well characterized, but the role of posttranscriptional regulation is not well defined. One important regulator of EMT is the cytokine TGFβ (19–21). We recently described a novel model wherein EMT induced by TGFβ requires translational activation via the noncanonical TGFβ signaling branch acting through eIF4E phosphorylation (14).

The only drug to date that has been shown to exhibit antitumor activity linked to inhibition of eIF4E in patients is the antiviral drug ribavirin (22). Ribavirin has been shown to mechanistically inhibit eIF4E by competing with the 7-methylguanosine mRNA cap (23, 24) and has antitumor activity in tumor cells with elevated levels of eIF4E (3, 23, 25). In a proof-of-principle clinical trial, ribavirin triggered dramatic clinical improvements in patients with poor prognosis, eIF4E-overexpressing acute myelogenous leukemia (AML; ref. 22). Strikingly, clinical response correlated with eIF4E inhibition, as shown by relocalization of eIF4E and reduced protein levels of eIF4E targets (22). We have previously demonstrated that ribavirin suppresses proliferation and clonogenic potential of breast cancer cells in vitro in an eIF4E-dependent manner (3). To extend these results, we assessed the effects of ribavirin in vivo and found a remarkable inhibition of primary tumor growth. In addition, we show that inhibiting eIF4E, via siRNA-mediated downregulation or using ribavirin, can suppress the expression of mesenchymal proteins and MMPs, specifically MMP-3 and -9, and reduce invasion of both...
mesenchymal cancer cells and normal mammary epithelial cells induced to undergo EMT. Ultimately, we show that ribavirin can reduce metastasis in vivo.

Materials and Methods

**Cells and reagents**

Mammary epithelial NMuMG cells were cultured in DMEM with 10% FBS, 10 μg/mL insulin (Sigma), and antibiotics. MT2186 cells, derived from a tumor that developed in a MMTV-PyMT transgenic mouse (26), were obtained from Dr. Josie Ursini-Siegel (Lady Davis Institute, Montreal, QC, Canada) and maintained in DMEM (MDA-MB-231) or RPMI-1640 and antibiotics. Human breast cancer cells were obtained from Dr. Ursini-Siegel and cultured in RPMI-1640 with 10 % FBS and antibiotics. Human breast cancer cells were obtained from the ATCC and maintained in DMEM (MDA-MB-231) or RPMI-1640 (BT474) with 10 % FBS and antibiotics. Culture media, FBS, and antibiotics were purchased from Wisent. Lyophilized ribavirin (Kemprotex Ltd.) was dissolved in H2O and sterile-filtered before use. Aliquots were kept at −80°C and thawed only once. TGFβ1 was purchased from PeproTech.

**Animal models**

Female, 4 to 6 weeks old, FVB/NCrl, Balb/cAnNCrl and Ctrl-Nu-Fox1tm1 mice were purchased from Charles River laboratories.

All animal care and experiments were carried out according to rules and regulations established by the Canadian Council of Animal Care and protocols were approved by the University Animal Care Committee. A total of 5 × 105 MT2186 or 66c4 cells were injected into the mammary fat pad of FVB or Balb/c mice, respectively. A total of 5 × 103 BT474 cells were resuspended in a mix of Matrigel (BD) and PBS (1:3 ratio) before injection into the mammary fat pad of nude mice, who had estradiol pellets (0.72 mg, 60-day release) implanted subcutaneously 1 week before tumor cell injection. Tumors were allowed to form and mice with palpable tumors were randomized into groups receiving vehicle (H2O) or ribavirin (3-mg ribavirin/mouse/d, administered orally by gavage 5 d/wk). At endpoint, tumors were divided into three parts that were snap frozen for protein and RNA isolation, fixed in 10% formalin for immunohistochemistry, and mechanically dissected for assessment of clonogenic capacity. Lungs were formalin-fixed for hematoxylin and eosin (H&E) staining.

**Ribavirin concentration in mice**

Ribavirin plasma concentrations were assessed by mass spectrometry (Aprecia) in samples collected during treatment (pool of five samples from individual mice) or at the time of sacrifice (1–2 hours after the last ribavirin dose).

**Clonogenic assay**

Cells isolated from each tumor were homogenized and passed through a 25-G needle until resuspended as single cells and then seeded at 500 cells per well in 6-well plates. After 14 days, cells were fixed in 10 % TCA and stained with sulfonated B. Visible colonies were counted using a Gel Count colony counter (Oxford Optronix).

**Immunohistochemistry**

Tumor sections were stained for Ki67 (abcam, 1:1,000) and counterstained with 20 % Harris-modified hematoxylin (Fisher).

Slides were scanned and Ki67-positive nuclei assessed using Spectrum (Aperio Technologies). Batch analysis of 10 selected areas of 5.8 × 102 mm² (2.4 × 10³ pixels) were run. Percent positive nuclei per section were determined by addition of areas until the average percent positive nuclei for one section did not change. One tumor section per animal was analyzed, and at least three areas of healthy tumor tissue were taken into consideration for the percent positive nuclei result.

**H&E staining and quantification of metastasis**

A total of 5 × 50 μm serial step sections were prepared and stained for each mouse. Slides were scanned and metastases were counted by hand. Percent burden and average metastasis size were determined using Spectrum (Aperio Technologies). Percent burden per animal was calculated over the five sections as metastasis area divided by total lung area. Average metastasis size was calculated per animal as the average size of all metastasis areas found within the five sections.

**RNAi**

Mature and human scrambled control siRNA and siRNAs specific for elf4E and MMP-3 were obtained from IDT. The siRNAs were introduced into NMuMG, MT2186, and MCF10A using RNAiMAX (Invitrogen) according to the manufacturer’s protocol.

**Western blotting**

Cells were lysed in protein lysis buffer (50 mmol/L Tris-HCl, pH 8, 150 mmol/L NaCl, and 1 % Triton X-100) or RIPA buffer (150 mmol/L Tris-HCl, pH 7, 150 mmol/L NaCl, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % SDS) supplemented with protease and phosphatase inhibitors. Twenty to 50 μg of protein was used to detect total elf4E, E-cadherin, fibronectin, N-cadherin, and vimentin (antibodies from BD Biosciences); phospho-elf4E, phospho-SMAD2, SMAD2/3, and Snail1 (Cell Signaling); as well as cyclin D1 (Santa Cruz), MMP-3 (abcam), and ZO-1 (Invitrogen). β-Actin (Sigma) or Lamin A (Santa Cruz) were used to confirm equal protein loading. Protein expression was quantified using ImageJ (NIH).

**Immunofluorescence**

NMuMG cells were cultured on glass coverslips in 12-well plates. After 24 hours, the cells were fixed with ethanol:acetic acid at −20°C, incubated with anti-ZO-1 (Invitrogen), then incubated with Alexa Fluor 488–conjugated goat anti-rabbit IgG (Molecular Probes). Nuclei were stained with Hoechst. The mounted samples were scanned with a Leica DM LB2 microscope. Differences between samples were quantified by selecting a defined area corresponding to edges between two cells and by calculating the ImageJ parameter “RawIntDen,” which is the sum of the pixel values in the selected area.

**Quantitative PCR**

RNA was prepared using TRIzol (Invitrogen). cDNA was prepared from 1 μg of total RNA, using iScript CDNA Synthesis Kit (Bio-Rad). Snail1 mRNA expression was quantified using the Applied Biosystems 7500 Fast Real-Time PCR System with SYBR Green–based detection using the following primers: 5’-GCGGGAGCCAAAACCTATAGC-3’ and 5’-AGGGCTGCTGGAAGGTG-3’. 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following primers: 5’-GTGAA-3’ and 36B4 mRNA was quantified using the following.
primers: 5′-GGCCACGGCAGCCACGT3′ and 5′-TCATCCAG-CAGGTCGTTCACA-3′. 18S was quantified using a predesigned TaqMan assay (Applied Biosystems).

**Wound-healing assay**

Cells subjected to eIF4E knockdown or pretreated with 20 μmol/L ribavirin for 24 to 48 hours were seeded in 6-well plates. The next day, a wound was made in the confluent cell layer using a 200 μl tip and the cells were gently washed with PBS to remove all floating cells. Fresh complete media and drugs were added to the cells (5 ng/mL TGFβ, 20 μmol/L ribavirin). Pictures of the wounds were taken at the time of the wounding and after 24 hours. The area not filled by cells was quantified using ImageJ.

**Matrigel invasion assay**

Cells were pretreated with 20 μmol/L ribavirin for 24 to 48 hours and prestained in 1% PBS for 24 hours. Phenol red-free Matrigel and 12-well plates with inserts with 8-μm porous membranes were purchased from BD Biosciences. For 66cl4 cells, Matrigel was replaced with collagen I (Life technologies). Complete media in the bottom chamber acted as chemoattractant, whereas the cells were resuspended in serum-free media ± ribavirin (20 μmol/L) and seeded on top of the matrix. Eighteen hours later, cells that had migrated through the matrix and the membrane were fixed in 5% glutaraldehyde and stained with crystal violet for visualization and counting.

**Migration and invasion Transwell assay**

The Transwell migration and invasion assays were performed as previously described (28, 29). In brief, two independent NMuMG-ErbB2 explants (NT118L and NT118R) were transfected with control siRNA or eIF4E-specific siRNAs and incubated in the presence or absence of TGFβ for 24 hours before plating into Boyden chambers. Cells that migrated or invaded to the underside of the Transwell were fixed and stained. Rates of cell migration and invasion were assessed over the course of 24 hours in a RTCA DP Analyzer (Roche Applied Science) and calculated according to manufacturer’s instructions using the xCELLigence RTCA software (Roche Applied Science).

**Gelatin zymography for MMP activity**

Proteins from concentrated culture medium, plasma, or protein extracts were mixed 1:1 with loading dye (0.1 mol/L Tris-HCl, pH 6.8, 4.5% SDS, 22% glycerol, bromophenol blue) and incubated at 37°C for 15 minutes before separation in 7.5% acrylamide gels containing 0.1% gelatin A (Fisher). Subsequently, the gel was washed with 2.5% Triton X-100 and incubated for 24 hours at 37°C in reaction solution (50 mmol/L Tris-HCl, pH 7.4, 5 mmol/L CaCl2, 200 mmol/L NaCl) with gentle shaking. The reaction was stopped by fixing the gel for 5 minutes in 30% methanol + 10% acetic acid, followed by staining with 0.25% Coomassie blue. Bands were visualized using the chemigenius 2 bioimaging system (Syngene) and quantified using ImageJ.

**Statistical analysis**

All in vitro experiments were performed at least 3 times and quantitative data are shown as the average of all replicates. Each in vivo experiment was performed once with the number of animals indicated in the figures. Statistical analyses were performed using GraphPad Prism. The type and stringency of the tests were determined according to the data characteristics and determined before experimentation. Details are given in each figure legend.

**Results**

**Ribavirin blocks growth of mammary tumors in vivo**

To assess the anti-tumor activity of ribavirin in vivo, we first used mammary tumor–derived MT2186 cells to generate tumors in syngeneic FVB mice. MT2186 cells are growth inhibited by ribavirin in vitro, and as previously shown in human breast cancer lines, this is due to cell-cycle arrest rather than cell death (3). When tumors reached 500 mm3, mice were randomized to receive vehicle (H2O) or ribavirin. Ribavirin 3 mg orally/mouse/d resulted in plasma concentrations of 35 (±10) μmol/L as measured in non–tumor-bearing FVB mice 1 to 2 hours posttreatment (Supplementary Table S1). Importantly, similar concentrations were measured in plasma from patients with AML treated with a well-tolerated dose of ribavirin either alone (22) or in combination with low-dose Ara-C (unpublished data), confirming that the dose is clinically relevant. Animal weight was unaffected, indicating that the dose is well tolerated (Supplementary Fig. S1A). Importantly, we observed greatly reduced tumor growth in the ribavirin-treated group (Fig. 1A), which translated to improved survival in this group (Fig. 1B). Strikingly, 5 of 10 ribavirin-treated MT2186 tumors regressed and 3 did not increase in size during the first 25 days of treatment (Supplementary Fig. S1B). However, all tumors except one appeared to gain some degree of resistance between days 25 and 40. At the time of sacrifice, a part of each tumor was mechanically dissociated to obtain a single-cell suspension for analysis of clonogenic capacity. Cells from ribavirin-treated tumors showed a greatly reduced ability to form colonies ex vivo (Fig. 1C), despite the fact that the tumors were growing at similar rates at the time of excision, as confirmed by Ki67 staining (Supplementary Fig. S1C). We examined the lungs of all mice at endpoint, following H&E staining, but no metastases were observed, despite the fact that these cells are invasive in vivo (see below). To further examine the early response to ribavirin in vivo, thus avoiding the emergence of drug resistance mechanisms, we treated mice carrying MT2186 tumors with vehicle or ribavirin for only 5 days. Remarkably, all tumors in the ribavirin-treated group stopped growing or regressed during these 5 days (Fig. 1D), and this was associated with a decrease in Ki67-positive nuclei compared with the control group (Fig. 1E). Tumor-derived protein extracts from each of the 2 experiments were analyzed by western blotting to assess the effect of ribavirin on eIF4E and its known target cyclin D1. In tumors that had regressed following 5-day treatment with ribavirin, reduced levels of cyclin D1 were observed; although due to lower tumor take in this experiment, the numbers were too small to perform statistical analysis (Supplementary Fig. S1D and S1E). The total level of eIF4E was not affected by ribavirin (Fig. 2A and C). However, phosphorylation of eIF4E was significantly reduced in tumors treated with ribavirin for either 5 or 40 days (Fig. 2B and D; Supplementary Fig. S1F and S1E). Moreover, the level of eIF4E phosphorylation showed a positive correlation with tumor growth rate, as determined by the Pearson r test (Fig. 2E and F), suggesting that the in vivo efficacy of ribavirin could be related to its ability to reduce phospho-eIF4E in these tumors.

As a complement to these studies, we tested the in vivo activity of ribavirin in a human xenograft model. Nude mice carrying BT474 tumors were treated as described for the FVB mice and, although
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 Ribavirin blocks primary tumor growth in vivo. MT2186 cells were injected into the mammary fat pad of syngeneic FVB mice and treatment began once the tumors reached a size of 500 mm\(^3\). A, tumor growth was monitored twice a week for 40 days. B, survival of the two groups plotted as a Kaplan-Meier plot. Mice were sacrificed when tumors reached a maximal size of 2,000 mm\(^3\). C, clonogenic capacity of tumor cells isolated at the time of sacrifice. Cells were allowed to form colonies on plastic for 14 days. D, percent tumor change was calculated over a 5-day treatment period. E, Ki67 staining of tumors collected after 5 days treatment was quantified as described in Materials and Methods. Two-way ANOVA was performed to assess whether the curves are significantly different in A. An unpaired Student \(t\) test was performed to compare groups in C–E.

Figure 1.

the experiment had to be terminated prematurely due to estradiol toxicity (seen in both the control and ribavirin groups), a significant reduction in tumor growth was observed in response to ribavirin (Supplementary Fig. S2).

Ribavirin reduces migration and invasion of mammary tumor cells in vitro

It has been reported that knockdown of eIF4E can reduce breast cancer cell migration and invasion (13). Ribavirin is the only clinically used drug with documented activity against eIF4E (22), and having established that ribavirin has potent in vivo activity against mammary tumors, we wanted to test whether ribavirin reduces the migratory and invasive properties of breast cancer cells. Notably, ribavirin treatment of MT2186 cells significantly reduced cell motility (Fig. 3A) and invasion through Matrigel (Fig. 3B). Moreover, it reduced the invasive capacity of the human triple-negative breast cancer cell line MDA-MB-231 (Fig. 3C) and murine metastatic 66cl4 cells (27) (Fig. 3D). Importantly, suppression of cell migration and invasion was not due to reduced overall cell number, as equal numbers of cells were re-seeded into the wells after the pretreatment period, and migration or invasion was assessed within 24 hours. We further assessed whether reducing or inhibiting eIF4E affects other characteristics of invasive cells, such as the expression and activity of MMPs. First, MMP-2/9 activity was assessed in conditioned media from MT2186 cells. MMP-9, a reported eIF4E target (30), was confirmed to be down-regulated by either eIF4E knockdown or ribavirin treatment, whereas MMP-2 was not reduced (Supplementary Fig. S3). We recently reported that translation of MMP-3 is dependent on phosphorylated eIF4E (14). Consistent with this, we observed a considerable reduction in MMP-3 protein in MT2186 and 66cl4 cells transfected with siRNA targeting eIF4E or pretreated with ribavirin (Fig. 3E and F). Knockdown of MMP-3 in MT2186 cells resulted in a modest suppression of Matrigel invasion (Fig. 3G), suggesting that the reduced level of MMP-3 following ribavirin treatment may account for part of the anti-invasive effect.

Knockdown or functional inhibition of eIF4E suppresses TGFβ-induced EMT

TGFβ is known to stimulate breast cancer cell invasiveness via upregulation of MMP activity, in concert with EMT. Thus, we asked whether inhibiting eIF4E, chemically or genetically, could block characteristics of TGFβ-induced cell invasiveness. We used NMuMG mammary epithelial cells, a well-defined model that undergoes EMT and MMP production in response to TGFβ (31, 32). Following eIF4E knockdown, the cells were exposed to TGFβ, and epithelial and mesenchymal markers were assessed by Western blotting. As expected, TGFβ reduced expression of the epithelial markers E-cadherin and ZO-1 and increased the mesenchymal markers N-cadherin, fibronectin, and vimentin. Knockdown of
eIF4E attenuated these changes (Fig. 4A; Supplementary Fig. S4A). Similar results were obtained in human, normal mammary epithelial MCF10A cells (Supplementary Fig. S4B). Importantly, knockdown of eIF4E did not reduce the induction of SMAD2 phosphorylation by TGFβ, indicating that the negative effect on EMT is independent of suppression of the canonical TGFβ signaling pathway (Fig. 4A). Immunofluorescent staining for ZO-1, which is a component of the intercellular epithelial cell junctions, showed that TGFβ stimulation leads to a loosening of the cell junctions, and this is prevented by siRNA targeting eIF4E (Supplementary Fig. S5). We detected robust induction of MMP-9 activity in TGFβ-treated NMuMG cells, and this was reduced by knockdown of eIF4E (Fig. 4B). To further assess the effects of eIF4E knockdown on the biologic outcome of EMT in these cells, we also performed a wound-healing assay to evaluate motility. TGFβ treatment significantly increased the ability of the cells to migrate and fill in the wound, as expected (33), and this was prevented by eIF4E knockdown (Fig. 4C). Furthermore, we tested whether eIF4E knockdown would affect TGFβ-stimulated migration and invasion in activated ErbB2-transformed NMuMG cell lines, NT11L, and NT11R (29, 28). Knockdown of eIF4E prevented TGFβ-induced cell migration and invasion in NT11L and NT11R, as determined in Transwell assays (Fig. 4D and E). We then tested whether ribavirin treatment could recapitulate some of the effects of eIF4E knockdown on TGFβ-induced EMT. Specifically, NMuMG cells were pretreated with 20 μmol/L ribavirin for 24 hours before the addition of TGFβ, and changes to the expression of mesenchymal markers was determined. Indeed, pretreatment with ribavirin suppressed the induction of both N-cadherin and fibronectin, and similar to eIF4E knockdown, did not reduce the
induction of SMAD2 phosphorylation by TGFβ (Fig. 4F). MMP activity was assessed, and ribavirin pretreatment reduced induction of MMP-9 by TGFβ (Fig. 4G).

A master regulator of EMT and metastasis, Snail, is controlled by eIF4E phosphorylation (14). This prompted us to look at the expression of Snail in both eIF4E siRNA-transfected and ribavirin-treated cells. Induction of Snail has been reported to be sufficient to induce EMT in mammary epithelial cells (34). We first showed that induction of Snail1 protein by TGFβ was suppressed in NMuMG and MCF10A cells transfected with siRNA targeting eIF4E or pretreated with ribavirin (Fig. 5A and B; Supplementary Fig. S4). The reduction was confirmed to occur at the posttranscriptional stage, as Snai1 mRNA was unaffected (Fig. 5C). Moreover, we observed less Snail1 protein in MDA-MB-231, MT2186, and 66cl4 breast cancer cells treated with ribavirin (Fig. 5D), whereas Snail1 mRNA was not reduced (Fig. 5E). We found that the decrease in Snail1 protein was accompanied by an increase in ZO-1 and decreased fibronectin and vimentin in MDA-MB-231 cells, suggesting a mesenchymal-to-epithelial transition (Supplementary Fig. S6). However, we did not observe a change in cell morphology (not shown). In summary, reduced eIF4E activity represses the invasive characteristics of both TGFβ-treated mammary epithelial cells and mesenchymal-like breast cancer cell lines, and this may at least in part be explained by reduced induction of the critical regulator of EMT and invasiveness, Snail.

Ribavirin reduces pulmonary metastases

To extend our analysis of the anti-invasive activity of ribavirin to an in vivo model, we used 66cl4 cells, which form tumors in syngeneic Balb/c mice that are known to metastasize to the lungs (27). Starting on day 11 post-tumor cell injection, the mice were treated orally with H2O or ribavirin as described above, which resulted in plasma concentrations similar to those measured in the FVB mice [23 (±5) μmol/L; see Supplementary Table S1]. A modest delay in tumor growth was evident in the ribavirin-treated mice (Fig. 6A). On day 27, all mice were sacrificed and tumors and lungs were preserved for analysis. Importantly, the ribavirin-treated mice had significantly reduced tumor burden in the lungs (Fig. 6B). The heavy tumor burden in the lungs of several mice made it hard to distinguish individual metastases, but there was a clear trend toward reduced number of metastases as well as smaller average metastasis size in the ribavirin group (Fig. 6C and D), accounting for the decreased metastatic burden. There was no correlation between metastatic burden and either primary tumor size or tumor growth rate, as determined by Pearson r correlation (not shown). Consistent with the MT2186 model
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Ribavirin did not alter the level of eIF4E protein in the tumors, as determined by Western blotting but reduced the level of eIF4E phosphorylation (Supplementary Fig. S7A and S7C). eIF4E phosphorylation showed a significant correlation with tumor growth (percent change in tumor size) over the course of the experiment (Supplementary Fig. S7B). Our in vitro data showed that ribavirin reduced the levels of MMP-3 and -9, so we wanted to assess if this also occurred in vivo. MMP activity has been associated with invasiveness in breast cancer models, including 66cl4 cells (35). We collected plasma from 66cl4 tumor-bearing mice over time (Fig. 7A). This increase was significantly less MMP-9 per total protein than tumors from the control group (Fig. 7C; Supplementary Fig. S8A). MMP-3 levels were also assessed in the plasma and in tumor lysates (both collected on day 27) and found that ribavirin-treated tumors contained significantly less MMP-9 per total protein than tumors from the control group (Fig. 7C; Supplementary Fig. S8A). MMP-3 levels were also assessed in the plasma and in tumor lysates (both collected on day 27) and the pro-form as well as the processed, active forms were significantly reduced in the ribavirin-treated tumors (Fig. 7D and E; Supplementary Fig. S8B and S8C). Of note, MMP-9 mRNA levels did not differ significantly between the 2 groups, whereas MMP-3 mRNA was reduced in the ribavirin-treated tumors, indicating some regulation at the transcript level (Supplementary Fig. S8D and S8E).

Finally, to determine whether MMP levels, quantified by densitometry, correlate with lung metastasis in this experiment, we used the Pearson r one-tailed correlation test. Notably, positive correlations were observed between percent metastatic burden and both plasma MMP-9 (r = 0.39, P = 0.04) and MMP-3 (r = 0.39, P = 0.04) levels and both plasma MMP-9 (r = 0.39, P = 0.04) and MMP-3 (r = 0.39, P = 0.04) levels.
tumor MMP-9 \((r = 0.44, P = 0.03)\). In addition, there was a weaker correlation between active MMP-3 in the tumor and metastatic burden \((r = 0.37, P = 0.05)\). We noted that one mouse with high levels of MMP-3 and -9 had very few metastases, which strongly reduced the statistical significance. This was possibly due to a somewhat delayed tumor onset in this mouse. Overall, our results suggest that the activity of MMP-3 and -9 in the tumor is important for its metastatic potential, and thus it is plausible that the ability of ribavirin to reduce the expression and secretion of these MMPs is involved in its anti-metastatic activity. Moreover, the levels of both MMP-3 and -9 in the tumor correlated with the level of phospho-eIF4E (Fig. 7F and C), again suggesting that suppression of eIF4E phosphorylation by ribavirin is functionally important.

**Discussion**

Metastatic breast cancer is an important clinical challenge. Our understanding of the underlying molecular mechanisms that regulate metastatic spread remains limited, and improved knowledge will certainly bring new treatment options. We hypothesized that modulation of eIF4E, a translation factor that is frequently overexpressed in breast cancer and regulates the expression of many proteins with important functions in metastatic cancer (2), might affect invasiveness in part by regulating EMT. In fact, we recently found that eIF4E phosphorylation modulates EMT and metastasis via translational control of Snail and MMP-3 (14). We thus postulated that ribavirin, a drug that has been shown to suppress eIF4E activity in both cells (3, 23, 25, 36) and in patients (22), would show antimetastatic activity in breast cancer models in vitro and in vivo. We showed that reducing eIF4E activity, using siRNA or ribavirin, could suppress migration and invasion in mammary tumor–derived cells in vitro, and this was associated with reduced levels of MMP-3 and -9 (Fig. 3; Supplementary Fig. S3). MMP-3 is known to promote mammary tumor development and metastasis, through its ability to alter the stromal compartment and to directly induce EMT (16, 17, 37, 38), and several studies have shown that MMP-9 can stimulate migration and invasion (35, 39). From these data, we concluded that suppression of MMP-3 and -9 may be partly responsible for reducing the invasive behavior of cells following ribavirin treatment, but postulated that modulation of additional factors may also play a role. Indeed, we established that either down-regulation of eIF4E or ribavirin pretreatment suppressed TGFβ-induced EMT, correlating with reduced induction of MMP-9 as well as reduced motility, migration, and invasion (Fig. 4).

Moreover, we found that induction of Snail was reduced, providing a possible mechanism for the blunting of pro-invasive activity of TGFβ (Fig. 5). Of note, the ability of TGFβ to activate SMAO transcriptional pathways was not altered, suggesting that eIF4E-mediated translation facilitates TGFβ-mediated EMT. We therefore conclude that the mechanism by which ribavirin exerts anti-invasive effects in these tumor cells involves reducing the eIF4E-dependent translation of several mRNAs encoding important regulators as well as effectors of EMT, including MMP-9 (30) Snail, and MMP-3 (14). These proteins could thus be direct ribavirin targets contributing to its...
Ribavirin reduces lung metastasis. 66cl4 cells were injected into the mammary fat pad of syngeneic Balb/c mice and treatment began on day 11 postsurgery, once tumors were palpable. A, tumor growth was monitored twice a week. B, metastatic lung burden was calculated as percentage of tumor area/total lung area for each mouse. C, number of metastasis counted for each mouse. Two-way ANOVA was performed to assess whether the curves are significantly different in A. Student t tests were performed to determine whether groups were significantly different in B–D.

In vivo, we used the highly aggressive 66cl4 model to assess the affect of ribavirin on metastasis. Despite only a modest inhibition of tumor growth by ribavirin in this model, a reduction in lung metastases was evident (Fig. 6). The difference in average metastatic burden was quite striking; 18% versus 4% of total lung area. We evaluated both the number of metastases per lung and the average metastasis size, and although there was a high degree of variability, especially in number of metastasis per lung, both values were reduced in the ribavirin group. Because ribavirin significantly reduces the production by the tumor of two important matrix remodeling enzymes, MMP-3 and -9, we propose that both the tumor cells and, perhaps indirectly, the surrounding stroma are altered by ribavirin to suppress and delay metastatic spread. Future experiments to determine the time course of lung colonization and to profile changes to the tumor microenvironment, both in the mammary gland and in the lung, will help elucidate the contribution of ribavirin induced changes in the different compartments. It is important to note that the level of MMP-9 produced by the tumor, and to a lesser extent MMP-3, showed a correlation with metastatic burden, suggesting in fact that suppression of these enzymes by ribavirin are important for its antimetastatic activity. This is consistent with published data obtained from the genetic knockout of MMP-9 in PyMT transgenic mice, which resulted in a dramatic reduction in lung metastasis (40). Of note, it has also been reported that MMP-9–null mice display improved response to doxorubicin due to increased vascular leakage (41), stressing the possibility that suppression of MMPs by ribavirin may not only reduce metastasis but also enhance response of the primary tumor to chemotherapy.

Intriguingly, we found that ribavirin consistently reduces eIF4E phosphorylation in all in vivo tumor models of breast cancer, and the level of phospho-eIF4E at end point correlates with tumor growth rate (Fig. 2). We find this particularly interesting, as phosphorylation of eIF4E has been shown to be essential for its tumorigenic function (42, 43). In addition, the level of eIF4E phosphorylation in the 66cl4 tumors correlated significantly with levels of both MMP-3 and -9, further supporting the notion that suppression of this posttranslational modification is important for the in vivo activity of ribavirin. It has previously been shown that inhibition of the eIF4E kinase Mnk can reduce lung metastasis in a colon cancer model (44). A recent article also reported that concomitant treatment with an Mnk inhibitor and the mTORC1 inhibitor RAD001 is required for efficient inhibition of protein synthesis and proliferation in a glioma model, further supporting a crucial role for eIF4E phosphorylation and its inhibition in cancer therapy (45).

Because ribavirin is a widely available, generic drug with limited toxicity, its potential use as a breast cancer therapeutic is of great interest. In this study, we have confirmed its activity against solid tumors in vivo, using 3 different mammary tumor models, including the metastatic 66cl4, where antimetastatic activity was observed despite only a minor effect on the primary tumor growth. An ongoing phase I/II trial of ribavirin monotherapy in patients with metastatic cancers expressing high levels of eIF4E (ClinicalTrials.gov NCT01309490) will define the maximum tolerated dose and ultimately assess molecular response in biopsies collected from accessible metastatic sites after 15 days of treatment. We anticipate that this will further support the future inclusion of ribavirin, or other inhibitors of eIF4E function, in breast cancer therapy.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


Genetic and Pharmacologic Inhibition of eIF4E Reduces Breast Cancer Cell Migration, Invasion, and Metastasis

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