Title: Genetic and pharmacologic inhibition of eIF4E reduces breast cancer cell migration, invasion and metastasis

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Keywords: eIF4E, Ribavirin, Breast cancer, EMT and metastasis

Conflict of interest: The authors have no conflicts of interest to disclose.
ABSTRACT

Translation initiation factor eIF4E is an oncogene that is commonly overexpressed in primary breast cancers and metastases. In this report, 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 MMP-3 and MMP-9. Further, eIF4E silencing or ribavirin treatment suppressed features of epithelial-to-mesenchymal transition (EMT), 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.
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, while 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 correlates 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 post-transcriptional regulation is not well defined. One important regulator of EMT is the cytokine transforming growth factor beta (TGF-β) [19-21]. We recently described a novel model wherein EMT induced by TGF-β requires translational activation via the non-canonical TGF-β 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 AML [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. Additionally, 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.

METHODS & MATERIALS

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) and cultured in DMEM with 5% FBS, mammary epithelial growth supplement (MEGS, Wisent, Quebec, Canada) and antibiotics. 66cl4 cells [27] were obtained from Dr. Ursini-Siegel and cultured in RPMI1640 with 10% FBS and antibiotics. Human breast cancer cells were obtained from the ATCC and maintained in DMEM (MDA-MB-231) or RPMI1640 (BT474) with 10% FBS and antibiotics. Culture media, FBS and antibiotics were purchased from Wisent. Lyophilized ribavirin (Kemprotec
Ltd, UK) was dissolved in H₂O and sterile-filtered before use. Aliquots were kept at -80°C and thawed only once.

TGF-β1 was purchased from Peprotech.

**Animal models**

Female, 4-6 weeks old, FVB/NCrI, Balb/cAnNCrI and Crl:NU-Foxn1nu mice were purchased from Charles River laboratories (Quebec, Canada). 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. 5x10⁵ MT2186 or 66cl4 cells were injected into the mammary fat pad of FVB or Balb/c mice, respectively. 5x10⁶ BT474 cells were resuspended in a mix of Matrigel (BD) and PBS (1:3 ratio) prior to injection into the mammary fat pad of nude mice, who had estradiol pellets (0.72 mg, 60-day release) implanted s.c. one week prior to tumor cell injection. Tumors were allowed to form and mice with palpable tumors were randomized into groups receiving vehicle (H₂O) or 3 mg ribavirin/mouse/day, administered p.o. by gavage 5 days/week. At end point, tumors were divided into 3 parts that were snap frozen for protein and RNA isolation, fixed in 10 % formalin for immunohistochemistry, and mechanically dissociated for assessment of clonogenic capacity. Lungs were formalin fixed for hematoxylin & eosin (H&E) staining.

**Ribavirin concentration in mice**

Ribavirin plasma concentrations were assessed by mass spectrometry (Aprenda, Watertown, MA) in samples collected during treatment (pool of 5 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, then seeded at 500 cells/well in 6-well plates. After 14 days, cells were fixed in 10 % TCA and stained with sulphorhodamine B. Visible colonies were counted using a Gel Count colony counter (Oxford Optronix).
**Immunohistochemistry (IHC)**

Tumor sections were stained for Ki67 (abcam, 1:1000) 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 $5.8 \times 10^{-2}$ mm$^2$ (2.4 x 10$^5$ 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 3 areas of healthy tumor tissue were taken into consideration for the percent positive nuclei result.

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

5 x 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 5 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 5 sections.

**RNAi**

Murine and human scrambled control siRNA and siRNAs specific for eIF4E 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 mM Tris-HCl, pH 8, 150 mM NaCl and 1 % Triton X-100) or RIPA buffer (150 mM Tris-HCl pH 7, 150 mM NaCl, 1 % NP-40, 1 % Sodium deoxycholate, 0.1 % SDS) supplemented with protease and phosphatase inhibitors. 20-50 micrograms of protein were used to detect total eIF4E, E-cadherin, fibronectin, N-cadherin and vimentin (antibodies from BD Biosciences), phospho-eIF4E, phospho-SPHAD2, 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 Image J (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 Image J parameter “RawIntDen”, which is the sum of the pixel values in the selected area.

**Quantitative PCR (qPCR)**

RNA was prepared using Trizol (Invitrogen). cDNA was prepared from 1 μg of total RNA, using iScript™ cDNA synthesis kit (Bio-Rad). Snai1 mRNA expression was quantified using the Applied Biosystems 7500 fast real-time PCR system with SYBR® Green-based detection using the following primers: 5’-GCCGGAAGCCCAACTATAGC-3’ and 5’-AGGGCTGCTGGAAGGTGAA-3’. 36B4 mRNA was quantified using the following primers: 5’-GGCACCGAGGCAACAGTT-3’ and 5’-TCATCCAGCAGGTGTTTGACA-3’. 18S was quantified using a predesigned Taqman® assay (Applied Biosystems).

**Wound healing assay**

Cells subjected to eIF4E knockdown or pretreated with 20 μM ribavirin for 24-48 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 TGF-β, 20 μM ribavirin). Pictures of the wounds were taken at the time of the wounding and after 24 h. The area not filled by cells was quantified using Image J.
Matrigel invasion assay

Cells were pre-treated with 20 µM Ribavirin for 24-48 hours and pre-starved in 1 % FBS for 24 h. Phenol red-free Matrigel™ and 12-well plates with inserts with 8um 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 while the cells were resuspended in serum-free media -/+ Ribavirin (20 µM) and seeded on top of the matrix. 18 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 TGF-β for 24 hours prior to 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 media, plasma or protein extracts were mixed 1:1 with loading dye (0.1 M Tris-HCl pH 6.8, 4.5 % SDS, 22 % Glycerol, Bromophenol blue) and incubated at 37°C for 15 minutes prior to 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 mM Tris-HCl pH 7.4, 5 mM CaCl₂, 200 mM NaCl) with gentle shaking. The reaction was stopped by fixing the gel for 5 minutes in 50 % 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 Image J.
Statistics

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 prior to 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 utilized 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. 3 mg ribavirin p.o./mouse/day resulted in plasma concentrations of 35 (+/-10) µM as measured in non-tumor bearing FVB mice 1-2 hours post treatment (Supplemental Table S1). Importantly, similar concentrations were measured in plasma from AML patients 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 (Supplemental 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, five out of ten ribavirin treated MT2186 tumors regressed and three did not increase in size during the first 25 days of treatment (Supplemental Fig. S1B). However, all tumors except one appeared to gain some degree of resistance between day 25 and day 40. At the time of sacrifice, a part of each tumor was mechanically dissociated in order to obtain a single cell suspension for analysis of clonogenic capacity. Cells from ribavirin
treated tumors showed a greatly reduced ability to form colonies \textit{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 (Supplemental Fig. 
S1C). We examined the lungs of all mice at end-point, following H&E staining, but no metastases were observed, 
despite the fact that these cells are invasive \textit{in vitro} (see below). To further examine the early response to 
ribavirin \textit{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 to the control group (Fig. 1E). Tumor-derived protein extracts from each of the two 
experiments were analyzed by western blot, 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 (Supplemental Fig. S1D-E). The total level of eIF4E was not affected by ribavirin (Fig. 2A,C). 
However, phosphorylation of eIF4E was significantly reduced in tumors treated with ribavirin for either 5 or 40 
days (Fig. 2B, D and supplemental Fig. S1E-F). Moreover, the level of eIF4E phosphorylation showed a positive 
correlation with tumor growth rate, as determined by Pearson’s $r$ test (Fig. 2E-F), suggesting that the \textit{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 \textit{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 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 (Supplemental Fig. S2).

\textit{Ribavirin reduces migration and invasion of mammary tumor cells \textit{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 \textit{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 pre-treatment 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 downregulated by either eIF4E knockdown or ribavirin treatment, while MMP2 was not reduced (Supplemental 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-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 TGF-β induced EMT**

TGF-β 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 TGF-β induced cell invasiveness. We used NMuMG mammary epithelial cells, a well-defined model that undergoes EMT and MMP production in response to TGF-β [31, 32]. Following eIF4E knockdown, the cells were exposed to TGF-β and epithelial and mesenchymal markers were assessed by western blot. As expected, TGF-β 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 and Supplemental Fig. S4A). Similar results were obtained in human, normal mammary epithelial MCF10A cells (Supplemental Fig. S4B).
Importantly, knockdown of eIF4E did not reduce the induction of SMAD2 phosphorylation by TGF-β, indicating that the negative effect on EMT is independent of suppression of the canonical TGF-β signaling pathway (Fig. 4A). Immunofluorescence staining for ZO-1, which is a component of the intercellular epithelial cell junctions, showed that TGF-β stimulation leads to a loosening of the cell junctions, and this is prevented by siRNA targeting eIF4E (Supplemental Fig. S5). We detected robust induction of MMP-9 activity in TGF-β treated NMuMG cells, and this was reduced by knockdown of eIF4E (Fig. 4B). To further assess the effects of eIF4E knockdown on the biological outcome of EMT in these cells, we also performed a wound-healing assay to evaluate motility. TGF-β 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 TGF-β stimulated migration and invasion in activated ErbB2-transformed NMuMG cell lines, NT11L and NT11R [29, 34]. Knockdown of eIF4E prevented TGF-β induced cell migration and invasion in NT11L and NT11R, as determined in transwell assays (Fig. 4D-E). We then tested if ribavirin treatment could recapitulate some of the effects of eIF4E knockdown on TGF-β induced EMT. Specifically, NMuMG cells were pretreated with 20 µM ribavirin for 24 hours before the addition of TGF-β, 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 TGF-β (Fig. 4F). MMP activity was assessed, and ribavirin pretreatment reduced induction of MMP-9 by TGF-β (Fig 4G).

A master regulator of EMT and metastasis, Snail, is controled 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 [35]. We first showed that induction of Snail1 protein by TGF-β was suppressed in NMuMG and MCF10A cells transfected with siRNA targeting eIF4E or pretreated with ribavirin (Fig. 5A-B and Supplemental 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), while Snai1 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 (Supplemental 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 TGF-β 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 ribavirin’s anti-invasive activity 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 p.o. with H2O or ribavirin as described above, which resulted in plasma concentrations similar to those measured in the FVB mice (23 (+/-5) µM, see Supplemental 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 towards reduced number of metastases as well as smaller average metastasis size in the ribavirin group (Fig. 6C-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’s *r* correlation (not shown). Consistent with the MT2186 model, ribavirin did not alter the level of eIF4E protein in the tumors, as determined by Western blot, but reduced the level of eIF4E phosphorylation (Supplemental Fig. S7A, C). eIF4E phosphorylation showed a significant correlation with tumor growth (percent change in tumor size) over the course of the experiment (Supplemental 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 [36]. We collected plasma from 66cl4-tumor bearing mice at different time points after tumor cell injection,
to analyze circulating MMP activity over the course of the experiment. Gelatin zymography was used to assess the levels of MMP-2 and -9, and we found a striking, selective increase in MMP-9 activity in the plasma of tumor bearing mice over time (Fig. 7A). This increase was suppressed in the ribavirin treated mice (Fig. 7A-B).

Moreover, we assessed MMP-9 levels in tumor lysates (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 and Supplemental Figure 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-E and Supplemental Fig. S8B-C). Of note, MMP-9 mRNA levels did not differ significantly between the two groups, while MMP-3 mRNA was reduced in the ribavirin treated tumors, indicating some regulation at the transcript level (Supplemental Fig. S8D-E). Finally, to determine whether MMP levels, quantified by densitometry, correlate with lung metastasis in this experiment, we used Pearson’s 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 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 MMP-3 and -9 activity in the tumor are 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-G), 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, 37] and in patients [22], would show anti-metastatic 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 and supplemental 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, 38, 39], and several studies have shown that MMP-9 can stimulate migration and invasion [36, 40]. 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 downregulation of eIF4E or ribavirin pretreatment suppressed TGF-β 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 TGF-β’s pro-invasive activity (Fig. 5). Of note, the ability of TGF-β to activate SMAD transcriptional pathways was not altered, suggesting that eIF4E mediated translation facilitates TGF-β 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 anti-invasive effect. Additionally, a wealth of experimental data link EMT to the generation of cancer stem cells (CSCs), and
this study raises the possibility that eIF4E is implicated in the generation or maintenance of such cells. We note that ribavirin treated tumors showed significantly reduced clonogenic capacity \textit{ex vivo} (Fig. 1C), which could be indicative of less CSCs within the tumor. Our preliminary data also suggest that eIF4E inhibition reduces the formation of mammospheres, another feature of CSCs. Future studies are clearly needed to define whether eIF4E indeed promotes the development of CSCs, which may spearhead the development of therapeutic strategies designed to interfere with eIF4E signaling and switch off EMT programs.

\textit{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\% vs. 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 anti-metastatic 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 [41]. Of note, it has also been reported that MMP-9 null mice display improved response to doxorubicin due to increased vascular leakage [42], 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 [43, 44]. Additionally, 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 ribavirin’s in vivo activity. It has previously been shown that inhibition of the eIF4E kinase Mnk can reduce lung metastasis in a colon cancer model [45]. A recent article also reported that concomitant treatment with a 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 [46]. We have also recently reported that MMTV-PyMT transgenic mice deficient for phosphorylated eIF4E display reduced metastasis, and this is associated with reduced mRNA translation of MMP-3 and Snail [14]. The mechanism by which ribavirin reduces eIF4E phosphorylation in vivo is not clear; however we did not observe a decrease in either phosphorylation or total level of Mnk1 (not shown).

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 three different mammary tumor models, including the metastatic 66cl4, where anti-metastatic 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.

ACKNOWLEDGEMENTS
We are thankful to Dr. Josie Ursini-Siegel for cell lines and tumor models, and for valuable discussions. We also thank Dr. Maryse Lemaire, Dr. Alicia Bolt, Cynthia Guilbert, Luis Fernando and Dr. Koren Mann for their help with sample processing during the 66cl4 in vivo experiment. This project was funded by CIHR operating grant MOP-115002 and a grant from the Canadian Cancer Research Society.

REFERENCES


FIGURE LEGENDS

Figure 1. 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-Meyer plot. Mice were sacrificed when tumors reached a maximal size of 2000 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 Methods & Materials. Two-way ANOVA was performed to assess whether the curves are significantly different in A. Unpaired student’s t-test was performed to compare groups in C-E.

Figure 2. Ribavirin reduces eIF4E phosphorylation in tumors in vivo. Densitometry analysis of western blots of protein extracts from snap frozen tumors harvested after 5 or 40 days of ribavirin treatment. A, C) Total eIF4E normalized to Lamin A. B, D) Phosphorylated eIF4E normalized to total eIF4E. E, F) Pearson’s r correlation analyses show positive correlations between phosphorylated eIF4E and tumor growth rate over the course of the treatment. The lines represent linear regression. Unpaired student’s t test was performed to compare groups in A-D. *Outlier removed in C using Grubb’s test.

Figure 3. Ribavirin reduces invasion and MMP-3 production in breast cancer cell lines. A) Cell motility was assessed in a wound-healing assay using MT2186 cells (n=12 per treatment). B-D) Cell invasion through Matrigel (B, C) or collagen I (D) were assessed in MT2186, MDA-MB-231 and 66cl4 cells, as indicated (n=12 per treatment). E) MMP-3 levels were assessed in whole cell extracts or conditioned media (secreted MMP-3) from MT2186 cells transiently transfected with siRNA or treated with 20µM ribavirin as indicated in the figure. F) MMP-3 levels were assessed in whole cell extracts from 66cl4 cells treated as indicated. *indicates a glycosylated form of pro-MMP-3. G) MT2186 cells were transiently transfected with siRNA targeting MMP-3 and...
invasion through Matrigel was assessed \((n=6 \text{ per group})\). Right: western blot showing MMP-3 knockdown 72 hours post-transfection. To compare groups, unpaired student’s \(t\) test was performed in A-D and one-way ANOVA in G; n.s. is not significant.

**Figure 4. Genetic or functional inhibition of eIF4E suppresses induction of EMT by TGF-\(\beta\).** A) Western blot analysis of epithelial and mesenchymal proteins, as well as SMADs, in TGF-\(\beta\) treated NMuMG cells transfected with siRNA targeting eIF4E or a scrambled control. The cells were treated with 5 ng/ml TGF-\(\beta\) for the indicated times. B) MMP-9 activity was assessed by gelatin zymography in NMuMG cells treated with 5 ng/ml of TGF-\(\beta\) for 24h. C) Cell motility of NMuMG cells assessed with the wound-healing assay in NMuMG cells treated as in (B). D, E) NMuMG cells transformed with the neu oncogene, NT11L and R, were transfected with siRNA targeting eIF4E or a scrambled control and cell migration and invasion were assessed in transwell assays over 24 hours. F) NMuMG cells were pretreated with 20\(\mu\)M ribavirin, followed by 4 ng/ml TGF-\(\beta\), and mesenchymal proteins and SMADs were assessed. G) MMP-9 activity was assessed as in B, in cells treated as in F. Numbers above the blots in (A), (B), (F) and (G) represent densitometry measurements of the bands shown, normalized to Lamin A in (A) and \(\beta\)-actin in (F). Unpaired student’s \(t\) test was performed to compare groups in C-E.

**Figure 5. Genetic or functional inhibition of eIF4E blunts Snail protein expression.** A) Snail1 protein expression in NMuMG cells treated with 5 ng/ml TGF-\(\beta\) following transfection with siRNA targeting eIF4E or a scrambled control. B) Snail1 protein expression in NMuMG cells pretreated with 20\(\mu\)M ribavirin for 48 h, followed by TGF-\(\beta\) for 24 h. C) Snai1 mRNA was assessed in NMuMG cells treated as in A. D) Snail1 protein expression in MDA-MB-231, MT2186, and 66cl4 cells treated with 20\(\mu\)M ribavirin for 48 hours. E) Snai1 mRNA in cells treated as in D. Numbers above the blots in (A) and (B) represent densitometry measurements of the bands shown, normalized to Lamin A and \(\beta\)-actin, respectively.
Figure 6. Ribavirin reduces lung metastasis. 66cl4 cells were injected into the mammary fat pad of syngeneic Balb/c mice and treatment began on day 11 post-surgery, once tumors were palpable. A) Tumor growth was monitored twice a week. B) Metastatic lung burden was calculated as % tumor area/total lung area for each mouse. C) Number of metastases counted over 5x50 μm step sections for each mouse. D) Average size of all metastases counted for each mouse. Two-way ANOVA was performed to assess whether the curves are significantly different in A. Student’s t-tests were performed to determine if groups were significantly different in B-D.

Figure 7. Suppression of metastasis is associated with reduced MMP-3 and -9 in vivo. A) MMP-2/-9 activity present in the plasma of 66cl4 tumor bearing mice, treated as indicated, was measured by zymography. B, C) MMP-9 activity in the plasma (B) and tumors (C) of all mice at end-point was detected by zymography and quantified by densitometry. D, E) MMP-3 protein expression was assessed by Western blot in the plasma (D) and tumors (E) of all mice at end-point. F, G) Pearson’s r correlation analyses show positive correlations between phosphorylated eIF4E and MMP-9 activity (F) and MMP-3 expression (G) in the tumor. The lines represent linear regression. Unpaired student’s t test was performed to compare groups in B-E.
FIGURE 2

A

B

eIF4E/lamin A protein

Control Ribavirin (5 days)

p=0.2

p=0.01

C

D

eIF4E/lamin A protein

Control Ribavirin (5 days)

p=0.4

p=0.02

E

F

P-eIF4E/eIF4E

Percent tumor change

r=0.71

p=0.01

r=0.54

p=0.02

5 days

40 days
FIGURE 3
FIGURE 4

A

B

C

D

E

F

G

Author Manuscript Published OnlineFirst on January 21, 2015; DOI: 10.1158/0008-5472.CAN-14-1996

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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FIGURE 6

A

Tumor volume (mm$^3$)

Control (n=10)
Ribavirin (n=10)

Days after surgery

p=0.008

B

Metastatic burden (% tumor/lung area)

Control
Ribavirin

p=0.03

C

Number of metastases per lung

Control
Ribavirin

p=0.08

D

Average size of metastases (μm$^2$)

Control
Ribavirin

p=0.04
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*Cancer Res* Published OnlineFirst January 21, 2015.

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