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MEK inhibition increases lapatinib sensitivity via modulation of FOXM1

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Abstract

The standard targeted therapy for HER2-overexpressing breast cancer is the HER2 monoclonal antibody, trastuzumab. Although effective, many patients eventually develop trastuzumab resistance. The dual EGFR/HER2 small molecule tyrosine kinase inhibitor lapatinib is approved for use in trastuzumab-refractory metastatic HER2-positive breast cancer. However, lapatinib resistance is a problem as most patients with trastuzumab-refractory disease do not benefit from lapatinib. Understanding the mechanisms underlying lapatinib resistance may ultimately facilitate development of new therapeutic strategies for HER2-overexpressing breast cancer. Our current results indicate that MEK inhibition increases lapatinib-mediated cytotoxicity in resistant HER2-overexpressing breast cancer cells. We genetically and pharmacologically blocked MEK/ERK signaling and evaluated lapatinib response by trypan blue exclusion, anchorage-independent growth assays, flow cytometric cell cycle and apoptosis analysis, and in tumor xenografts. Combined MEK inhibition and lapatinib treatment reduced phosphorylated ERK more than single agent treatment. In addition, Western blots, immunofluorescence, and immunohistochemistry demonstrated that the combination of MEK inhibitor plus lapatinib reduced nuclear expression of the MEK/ERK downstream proto-oncogene FOXM1. Genetic knockdown of MEK was tested for the ability to increase lapatinib-mediated cell cycle arrest or apoptosis in JIMT-1 and MDA361 cells. Finally, xenograft studies demonstrated that combined pharmacological inhibition of MEK plus lapatinib suppressed tumor growth and reduced expression of FOXM1 in HER2-overexpressing breast cancers that are resistant to trastuzumab and lapatinib. Our results suggest that FoxM1 contributes to lapatinib resistance downstream of MEK signaling, and supports further study of pharmacological MEK inhibition to improve response to lapatinib in HER2-overexpressing trastuzumab-resistant breast cancer.

Keywords

lapatinib; HER2; erbB2; breast cancer; resistance; MEK

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CONFLICTS OF INTEREST

The authors declare that there are not any conflicts of interest pertaining to the work discussed in this article.

INTRODUCTION

The human epidermal growth factor receptor (HER) tyrosine kinase family is comprised of four members: HER1 (human epidermal growth factor receptor, EGFR), HER2, HER3, and HER4. Upon ligand binding, receptor dimerization activates multiple signaling pathways including the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) pathways [1–3]. HER2 is overexpressed in about 20% of metastatic breast cancers and is associated with a poor prognosis [4,5]. Trastuzumab (Herceptin), a monoclonal antibody targeted against the extracellular domain of HER2, is a highly effective treatment for patients with HER2-overexpressing breast cancers. However, as is the case with many targeted therapies, a majority of patients will eventually develop resistance to trastuzumab, resulting in disease progression.

The dual EGFR/HER2 tyrosine kinase inhibitor lapatinib (Figure 1) (Tykerb) is approved for use in trastuzumab-refractory tumors. Lapatinib binds to the ATP-binding pocket of HER2, thereby preventing receptor phosphorylation and subsequent activation of downstream pathways. However, response to single agent lapatinib in trastuzumab-resistant cancers is less than 25% [6,7]. Understanding the molecular mechanisms that lead to lapatinib resistance may ultimately improve the clinical benefit that patients received from lapatinib-based treatment.

Previous studies have focused primarily on the role of PI3K/mTOR signaling in lapatinib resistance [8–10]. However, inhibition of MEK appears to also play a critical role in mediating lapatinib cytotoxicity. Tanizaki et al. [11] showed that lapatinib-mediated inhibition of MEK is critical for induction of apoptosis. Lapatinib treatment of HER2-overexpressing cells inhibited MEK/ERK signaling, leading to up-regulation of the pro-apoptotic protein Bcl-2-interacting mediator of cell death (BIM). Further, transfection of oncogenic Ras into HER2-overexpressing, lapatinib-sensitive SKBR3 and BT474 cells has been shown to activate MEK/ERK signaling and abrogate response to lapatinib [12]. The Forkhead box transcription factor M1 (FOXM1) is downstream of the MEK signaling pathway and its up-regulation is associated with HER2 overexpression [13–15]. Upon MEK/ERK phosphorylation, FOXM1 is activated and translocates to the nucleus to stimulate proliferation and cell survival by modulating expression of cell cycle and apoptosis regulators including the cyclin-dependent kinase inhibitor p27^{kip1} and the anti-apoptotic protein survivin [16].

In the current study, we examine the role of MEK/ERK signaling in HER2-positive trastuzumab-resistant cells that exhibit reduced response to lapatinib. We demonstrate that an inability to block ERK phosphorylation is associated with reduced response to lapatinib. Pharmacologic inhibition or knockdown of MEK in combination with lapatinib induced cell cycle arrest and/or apoptosis in association with reduced expression of nuclear FOXM1 in resistant cells. Further, knockdown or pharmacologic inhibition of FOXM1 increased response of resistant cells to lapatinib, whereas transfection of FOXM1 into sensitive cells resulted in resistance. Finally, co-treatment of xenografts of HER2-positive, trastuzumab-resistant breast cancer cells with lapatinib and the MEK inhibitor selumetinib suppressed tumor growth, reduced Ki-67 staining, suppressed ERK phosphorylation, and reduced expression of FOXM1 in comparison to xenografts treated with single agents or vehicle control. Thus, MEK inhibition should be studied further as a strategy for increasing response to lapatinib in HER2-positive breast cancers that have progressed on trastuzumab.

MATERIALS AND METHODS

Materials

Lapatinib was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Selumetinib (Figure 1) was purchased from LC Laboratories (Woburn, MA). PD0325901 was purchased from Cayman Chemical (Ann Arbor, MI). Thiostrepton was purchased from Sigma-Aldrich (St. Louis, MO). All drugs were dissolved in DMSO at a stock concentration of 10 mM except thiostrepton, which was dissolved at a stock concentration of 50 mM. Lapatinib for *in vivo* studies was purchased from the Winship Cancer Institute pharmacy and dissolved in a buffer containing 1% Tween-80 and 5% hydroxypropyl methylcellulose just before use.

Cell culture

JIMT-1 cells were purchased from DSMZ (Braunschweig, Germany); all other cell lines were purchased from American Type Culture Collection (Manassas, VA). HCC1419 and HCC1954 cells were maintained in RPMI with 10% fetal bovine serum (FBS); MDA-MB-361 cells were maintained in DMEM with 20% FBS; JIMT-1 and BT474 cells were maintained in DMEM with 10% FBS. All cells were maintained in 1% penicillin/streptomycin and cultured in humidified incubators at 37°C with 5% CO₂.

Matrigel cultures and growth assays

Cells were plated at 1×10^4 in a 12-well plate format in 250 μ L matrigel (BD Biosciences; Franklin Lakes, NJ) diluted 1:1 (media:matrigel). The matrigel-cell suspension was allowed to solidify for 2 hours at 37°C followed by the addition of 1 mL of media containing either lapatinib, PD0325901, selumetinib, or DMSO control. Media plus drug was changed twice a week for approximately two weeks. Photographs were taken with an Olympus IX50 inverted microscope at 4 $\times$ magnification. Matrigel was then digested using dispase (BD Biosciences), and viable cells were counted by trypan blue exclusion. Experiments were repeated on at least two independent occasions to ensure reproducibility. For anchorage-dependent growth, cells were plated at 3×10^4 in a 12-well plate format. After 24 hours, media plus drug was added for 48 hours for the lapatinib plus PD0325901 treatments, and 72 hours for the lapatinib plus selumetinib treatments. Viable cells were then counted by trypan blue exclusion. Experiments were repeated three times for reproducibility.

Transfection

Cells were plated in antibiotic-free media. The next day, cells were transfected using Lipofectamine 2000 (Invitrogen; Carlsbad, CA) with either 100 nM MEK1 siRNA plus 100 nM MEK2 siRNA or control siRNA (Santa Cruz Biotechnology) according to the manufacturer's protocol. After 24 hours, cells were treated with vehicle control or lapatinib for an additional 24 hours, at which point cells were counted by trypan blue exclusion. Knockdown was confirmed after 48 hours of transfection by Western blotting. In an independent set of experiments, 100 nM FOXM1 siRNA (Cell Signaling; Danvers, MA) or control siRNA (Santa Cruz Biotechnology) was transfected using Lipofectamine. After 24 hours, cells were treated with vehicle control or lapatinib for an additional 48 hours, at which point cells were counted by trypan blue exclusion. Knockdown was confirmed after 72 hours of transfection by Western blotting. Experiments were repeated twice for reproducibility.

Cell Cycle Analysis and Apoptosis Detection

For cell cycle analysis, cells were harvested, washed twice with DPBS+10% FBS, fixed in ice-cold 80% ethanol, and stored at -20°C for at least 24 hours. Fixed cells were incubated in 50 μ L of PI buffer (20 μ g/mL PI (Sigma), 0.1% Triton-X 100, 200 μ g/mL RNaseA

(Promega) in DPBS) for 30 minutes in the dark. The cells were then resuspended in 400 μ L of DPBS for flow analysis. For apoptosis detection, cells were harvested and stained for annexin V-FITC and propidium iodide using the TACS Annexin V kit (Trevigen; Gaithersburg, MD) according to the manufacturer's protocol. All samples were analyzed using a BD FACS Canto II cytometer (BD Biosciences; San Jose, CA) with BD FACS Diva software. All experiments were performed in triplicate.

Western blotting

Cells were lysed in RIPA buffer (consists of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin; Cell Signaling) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich). Total protein extracts (30 μ g) were run on SDS-PAGE and blotted onto nitrocellulose. Blots were probed overnight using the following antibodies from Cell Signaling: p-S473 Akt-XP used at 1:1000, and polyclonal antibodies against total Akt (1:1000), p-Thr202/Tyr204 p42/p44 ERK1/2 (1:1000), total p42/p44 ERK1/2 (1:1000), and FOXM1 (D12D55) XP (1:1000). β -actin monoclonal AC-15 (Sigma-Aldrich) at 1:10,000 was used as a loading control. Protein bands were detected using the Odyssey Imaging System (Li-Cor Biosciences; Lincoln, NE). Experiments were repeated at least three times to ensure reproducibility.

Isolation of Nuclear and Cytosolic Fractions

Nuclear-cytoplasmic fractionation of JIMT-1 cells was conducted using the NE-PER nuclear and cytoplasmic extraction reagents kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

Real-Time PCR

Total RNA was extracted using the RNeasy purification kit (Qiagen; Valencia, CA) and treated with DNase (Invitrogen). Total RNA was used to prepare cDNA using random primers and the Superscript III first strand synthesis Kit (Invitrogen). Real-time quantitative PCR master mix (4304437, Applied Biosystems; Carlsbad, CA) was used to synthesize cDNA. Primers for RPLPO (Hs99999902_M1), FOXM1 (Hs01073586_M1), and p27 (Hs00153277_B1) were obtained from Applied Biosystems (Taq-Man Gene Expression Assays). The RPLPO internal control was used to normalize samples and compared as arbitrary units, represented as mean $\pm$ SD. Samples were run in triplicate, and experiments were repeated at least twice to ensure reproducibility.

Immunofluorescence

Cells were plated on glass coverslips in 24-well cell culture plates. After 24 hours, cells were treated with 1000 nM lapatinib, 1000 nM selumetinib, combination 1000 nM lapatinib plus selumetinib, or vehicle control for 24 hours. Cells were fixed with 4% formaldehyde and permeabilized with 0.3% Triton X-100. Coverslips were blocked for one hour in blocking buffer (10% goat serum in TBS-T) and incubated with primary antibody in blocking buffer overnight at 4°C. Antibodies against FOXM1 (Santa Cruz) and p27^{kip1} (Dako; Carpinteria, CA) were each used at a 1:25 dilution. The next day coverslips were incubated with secondary antibodies for one hour at room temperature, and signal amplification was performed using tyramide (Invitrogen). Cells were mounted using Prolong Gold (Invitrogen) and examined using a Zeiss LSM 510 Meta confocal system.

Xenograft studies

Female athymic *nu/nu* mice (Harlan; Indianapolis, IN) were used to establish flank xenografts by s.c. injection with 1×10^6 JIMT-1 cells (isolated from female patient) and 50%

Matrigel (BD Biosciences). Tumor volumes were calculated using the formula $(\text{length} \times \text{width}^2)/2$. Animals were administered vehicle diluent, lapatinib (75 mg/kg), selumetinib (50 mg/kg), or combination lapatinib and selumetinib by oral gavage. All treatments were done daily for 5 days, then off for 2 days, for a total of 42 days. All animal procedures were done in accordance with institutional IACUC regulations under an approved IACUC protocol and per the guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health.

Immunohistochemistry

IHC was performed on tumor tissues from two animals per treatment group using a standard immunoperoxidase procedure as previously described [17]. The treatment groups were vehicle diluent, lapatinib (75 mg/kg), selumetinib (50 mg/kg), or combination lapatinib and selumetinib. The tissue sections on glass slides were deparaffinized by heating at 60°C for 10 minutes, followed by cooling for 30 minutes while in the same buffer. Quenching of the endogenous peroxidase was achieved by incubating slides with 0.3% H₂O₂ in methanol for 15 minutes. The tissues were then washed with water and PBS/TBS and incubated in 10% swine serum (Dako) for 1 hour to eliminate non-specific background staining. Tissue sections were stained for FOXM1 (Santa Cruz, SC500, dilution 1:100), pERK (Cell Signaling, CS9101, 1:500) Ki67 (Thermo Scientific, SP6, 1:400). Biotinylated anti-rabbit antibody (Dako) was used as secondary antibody, and positive staining was detected by incubation with 3,3 diaminobenzidine solution (DAB + chromogen; Dako) with hematoxylin as a counterstain. The slides were then washed in water and dehydrated by passing through alcohol grades and xylene. The slides were mounted with permanent mounting medium Permount (Fisher Scientific; Pittsburgh, PA). After drying completely, the slides were viewed under a light microscope, and pictures were taken at 10X.

Statistics

P-values were determined for experimental versus control treatments by two-tailed student's t-test, * $p < 0.05$, ** $p < 0.005$. For statistical analysis of drug combination and combination index (C.I.), the method of Chou and Talalay [18] was used with the commercial software package Calcsyn (Biosoft, Cambridge, United Kingdom). C.I. > 1 indicates antagonism between drugs; C.I. = 1 indicates additive effects; C.I. < 1 indicates drug synergy.

RESULTS

Sustained MEK/ERK signaling is associated with reduced sensitivity to lapatinib

The ability of lapatinib to suppress ERK phosphorylation was examined in the HER2-overexpressing breast cancer lines BT474, HCC1419, JIMT-1, and MDA361 (Figure 2A). Lapatinib achieved dose-dependent inhibition of ERK phosphorylation in BT474 and HCC1419 cells, which we previously showed are sensitive to lapatinib [9]. In contrast, concentrations of lapatinib as high as 1000 nM failed to reduce ERK phosphorylation in JIMT-1 and MDA361 cells, which we previously showed have a lower sensitivity to lapatinib [9].

Next, Western blots for phosphorylated and total ERK protein were performed in BT474, HCC1419, JIMT-1, and HCC1954 cell lysates to determine if lapatinib-resistant cells show increased baseline signaling of this pathway. However, levels of phosphorylated ERK were similar in lapatinib-sensitive and lapatinib-resistant lines (Figure 2B). Thus, sustained phosphorylation of ERK is associated with reduced response to lapatinib but does not appear to be due to endogenous hyper-activation of MEK signaling.

Pharmacologic MEK inhibition increases sensitivity to lapatinib

To investigate whether MEK inhibition improves response to lapatinib, we treated lapatinib-resistant JIMT-1 cells with 1000 nM lapatinib plus either 10 nM or 1000 nM of the MEK inhibitor PD0325901 (PD) [19]. The combination of PD and lapatinib inhibited ERK phosphorylation to a greater degree than single agents (Figure 3A). Furthermore, although PD at 1000 nM induced Akt phosphorylation, lapatinib co-treatment overcame this compensatory signaling.

Next, the combination of lapatinib plus MEK inhibition was tested in clonogenic growth assays. Growth of JIMT-1 cells in matrigel was partially reduced by lapatinib alone (Figure 3B). This is in contrast to adherent growth assays, in which lapatinib did not suppress growth of JIMT-1 cells, and may be due to prolonged exposure to the drug (3 weeks versus 3 days in adherent assays). However, PD alone did not significantly reduce growth of JIMT-1 cells. The most striking result was that co-treatment with PD plus lapatinib almost completely blocked survival of JIMT-1 cells in matrigel. Colonies that were still visible showed reduced colony size and number versus single agent treatment groups. Interestingly, statistical analysis of drug combination effects by the method of Chou and Talalay with CalcuSyn software showed synergy between lapatinib and PD in JIMT-1 cells but not in BT474 cells (Table 1). These results are an indication that co-treatment with lapatinib and a MEK inhibitor achieved synergistic cytotoxicity in JIMT1 HER2-positive breast cancer cells, which showed a lower level of inhibition by single-agent lapatinib relative to BT474 cells.

Next, we combined lapatinib with the MEK inhibitor, selumetinib [20,21], which is being developed for clinical use. Co-treatment of HCC1419, JIMT-1, and HCC1954 cells with selumetinib plus lapatinib inhibited ERK phosphorylation in comparison to single agent treatments (Figure 4A). Lapatinib alone significantly reduced survival of BT474 cells (Figure 4B), whereas the viability of adherent JIMT-1 cells was not affected by lapatinib or selumetinib as single agents. In contrast, combined treatment with lapatinib plus selumetinib significantly reduced survival of JIMT-1 cells in a dose-dependent manner. Similar to the combination of PD and lapatinib, co-treatment with selumetinib plus lapatinib significantly decreased JIMT-1 colony size and number growth in matrigel (Figure 4C). Finally, cell cycle profiling of JIMT-1 cells showed that the combination of selumetinib and lapatinib induced G1 arrest (Figure 4D) and reduced the percentage of cells in S phase by half (Table 2). Thus, co-treatment with a MEK inhibitor improved lapatinib sensitivity in HER2-positive breast cancer cells.

Knockdown of MEK in combination with lapatinib induces apoptosis of JIMT-1 cells

Since the combination of pharmacological MEK inhibition and lapatinib suppressed ERK phosphorylation and cellular proliferation, we examined the effects of MEK knockdown on lapatinib resistance. Knockdown of MEK was achieved by simultaneously transfecting siRNA oligonucleotides against MEK1 and MEK2 and was confirmed by Western blotting (Figure 5A). The combination of lapatinib plus MEK knockdown reduced phosphorylation of ERK in JIMT-1 cells (Figure 5A) and significantly reduced cell survival (Figure 5B). Flow cytometric analysis of annexin-stained cells showed increased apoptosis of cells that were transfected with MEK1/2 siRNA and treated with lapatinib (Figure 5C). Apoptosis was induced in almost 63% of cells treated with lapatinib plus MEK knockdown, which was approximately 2-fold higher than when cells were treated with lapatinib and control siRNA (Table 3).

Co-treatment with selumetinib and lapatinib alters FOXM1 expression

FOXM1 functions as a transcription factor in the nucleus where it represses expression of cell cycle inhibitors including p27^{kip1}. Immunofluorescence was performed to determine the cellular localization of FOXM1 in different treatment groups. FOXM1 was localized to the nucleus and cytoplasm in the majority of control HCC1419 cells (Figure 6A). Treatment with lapatinib, selumetinib, or the combination of drugs did not alter FOXM1 localization, i.e., all cells still showed FOXM1 staining in the nucleus and cytoplasm. In contrast, FOXM1 was exclusively nuclear in JIMT-1 cells treated with vehicle control alone. Single agent lapatinib or selumetinib did not alter this localization. However, upon treatment with the combination of lapatinib plus selumetinib, FOXM1 was detected in both the nucleus and cytoplasm of the majority of JIMT-1 cells, indicating that co-treatment induced translocation of FOXM1 into the cytoplasm (Figure 6B). Nuclear and cytoplasmic fractioning confirmed that JIMT-1 cells expressed FOXM1 primarily in the nucleus at baseline, and that lapatinib alone or selumetinib alone did not alter this localization (Figure 6C). Combination lapatinib plus selumetinib reduced expression of FOXM1. Consistent with these findings, the transcript level of the FOXM1 target p27^{kip1}, which is normally repressed by FOXM1, was induced by lapatinib by was dramatically more increased upon co-treatment with lapatinib plus selumetinib (Figure 6D). These results suggest that combining lapatinib with MEK inhibition results in reduced FOXM1 nuclear staining, reduced FOXM1 expression, and reduced transcript level of target gene p27^{kip1}, consistent with the induction of growth arrest.

FOXM1 knockdown increases lapatinib sensitivity

To gain additional evidence that modulation of FOXM1 improves response to lapatinib, we knocked down FOXM1 and assessed cell survival. FOXM1 knockdown resulted in significantly decreased cell viability on its own. A significantly greater reduction in viability was observed when FOXM1 knockdown was combined with lapatinib compared to lapatinib treatment in FOXM1 expressing cells (Figure 7A).

The thiazole antibiotic thiostrepton selectively reduces FOXM1 mRNA and protein expression in a dose- and time-dependent manner [22,23]. FOXM1 down-regulation by thiostrepton was found to induce apoptosis and repress migration and metastasis in breast cancer cells. Treatment of JIMT-1 cells with thiostrepton resulted in a significant decrease in viability (Figure 7B). The combination of thiostrepton and lapatinib treatment resulted in significantly reduced colony survival of HCC1954 and JIMT-1 cells grown in matrigel (Figure 7C). Conversely, transfection of a FOXM1 expression plasmid into lapatinib-sensitive HCC1419 cells abrogated lapatinib-induced cytotoxicity (Figure 7D). These data suggest that FOXM1 expression levels regulate sensitivity to lapatinib, and that down-regulation of FOXM1 improves lapatinib sensitivity.

The combination of selumetinib plus lapatinib suppresses tumor growth of JIMT-1 xenografts

Next, JIMT-1 cells were injected to form flank xenografts in athymic mice. Mice were treated with vehicle control, 75mg/kg oral lapatinib, 50mg/kg oral selumetinib, or a combination of lapatinib and selumetinib. Co-treatment with lapatinib plus selumetinib achieved a statistically significant greater inhibition of tumor growth in comparison to either drug alone (Figure 8A). Tumors from the combination treatment group showed lower levels of the proliferation marker Ki67, decreased phosphorylation of ERK, and reduced FOXM1 expression (Figure 8B). These results support the concept that MEK inhibition plus lapatinib suppresses phosphorylation of ERK and FOXM1 expression, consistent with *in vitro* results. Further, the results suggest that the combination of MEK inhibitor selumetinib plus lapatinib suppresses growth of trastuzumab-resistant HER2-overexpressing breast cancer.

DISCUSSION

HER2 overexpression is frequently observed in metastatic breast cancer and is associated with aggressive disease and poor prognosis [5,24]. The dual EGFR/HER2 small molecule tyrosine kinase inhibitor, lapatinib, has been approved for use in HER2-overexpressing breast cancers that have progressed on trastuzumab. Response rates to single agent lapatinib range between 12 % and 25%, making it necessary to elucidate the precise mechanisms that confer resistance [6,25,26]. Our data demonstrate that sustained MEK/ERK signaling results in poor lapatinib response despite similar baseline levels of phosphorylated ERK. Combination MEK inhibition and lapatinib treatment suppressed MEK/ERK signaling, increasing overall cytotoxicity, apoptosis, and inhibition of tumor growth relative to the single agent treatments (Figure 9). Combination MEK inhibition and lapatinib treatment also led to a greater decrease in ERK phosphorylation and reduced expression of the proto-oncogene FOXM1 when compared to either single agent treatment. These findings provide potential rationale for combining pharmacologic inhibition of MEK with lapatinib in HER2-overexpressing breast cancers that have progressed on trastuzumab.

In an effort to better understand the mechanisms behind growth inhibition of adherent and matrigel-suspended cells observed with combination MEK inhibition and lapatinib treatment, we performed cell cycle analysis experiments. Our results showed that pharmacological MEK inhibition combined with lapatinib treatment promoted G0/G1 cell cycle arrest and decreased the percentage of cells in S phase. Induction of p27^{kip1} in the combination treatment group versus either single agent groups was consistent with induction of G1 arrest and reduced proliferation. Similarly, addition of lapatinib to cells that had been transfected with small interfering RNA (siRNA) against MEK1 and MEK2 (MEK1/2) also reduced ERK phosphorylation and viability. In contrast to pharmacological inhibition, however, MEK1/2 knockdown plus lapatinib induced a significant level of apoptosis. Thus, it is possible that off-target effects of MEK inhibition elicit different cellular effects than specific knockdown of MEK1 and MEK2. *In vivo* pharmacological inhibition of MEK effectively increased lapatinib response, suppressing proliferation (as measured by Ki-67 staining) and leading to inhibition of tumor growth, supporting *in vitro* findings.

The forkhead transcription factors FOXO1, FOXO3a, and FOXM1 are downstream of MEK/ERK and play pivotal roles in tumorigenesis. FOXO1 and FOXO3a are upstream negative regulators of FOXM1 and behave as tumor suppressors. In contrast, FOXM1 functions as an oncogene. FOXM1 expression decreased upon treatment with the combination of MEK inhibition and lapatinib when compared to either single agent treatment. These data suggest that FOXM1 is a critical downstream effector of MEK involved in conferring lapatinib resistance.

FOXM1 has been implicated in multiple human cancers including breast, lung, esophageal, and pancreatic cancer [16,27–30]. In breast cancer, FOXM1 expression is associated with poor prognosis and has been tightly correlated with HER2 status [13,31,32]. The role of MEK-FOXM1 has not previously been investigated in HER2-overexpressing breast cancer, but this pathway has been targeted in ovarian cancer with promising results. Chan et al. showed that ERK/FOXM1 inhibition by U0126 or thiostrepton resulted in decreased cancer cell growth *in vitro* as well as *in vivo* in ovarian cancer models [33]. Our current data support inhibition of MEK-FOXM1 in combination with lapatinib as a potentially effective strategy for HER2-overexpressing breast cancers that are resistant to trastuzumab, suggesting a role for this pathway in lapatinib resistance. Our data indicate that pharmacologic and genetic inhibition of FOXM1 increases lapatinib sensitivity in resistant HER2-overexpressing breast cancer cells, providing evidence for the role of FOXM1 in lapatinib resistance. Indeed, overexpression of FOXM1 in lapatinib-sensitive cells abrogated

sensitivity to lapatinib. The present study is the first to show that decreased expression of FOXM1 due to MEK inhibition is required for optimal lapatinib response in trastuzumab-resistant HER2-overexpressing breast cancers.

We also showed that combinatorial MEK/ERK inhibition and lapatinib treatment results in decreased FOXM1 nuclear expression. This finding is consistent with published data demonstrating that FOXM1 is ubiquitously expressed in proliferating cells and is dependent on MEK/ERK signaling for activation and nuclear translocation [16,34]. Our data showed a positive correlation between decreased FOXM1 expression and induction of the FOXM1 target p27^{kip1} after treatment with combination lapatinib and MEK inhibition. Thus, induction of p27 may serve as a biomarker of lapatinib response. Additional FOXM1 downstream signaling molecules could be involved in the growth arrest and apoptosis induced by this combination. Future studies will investigate the role of FOXM1 downstream effectors in lapatinib resistance.

Our *in vivo* data with xenografts of lapatinib-resistant HER2-overexpressing breast cancer cells showed that co-treatment with a MEK inhibitor increases response to lapatinib, as demonstrated by suppressed tumor growth when compared to either single agent treatment. Tumor suppression was associated with decreased phosphorylation of ERK, reduced FOXM1, and decreased staining for the proliferation marker Ki-67. These data indicate the importance of simultaneously inhibiting MEK-FOXM1 signaling with lapatinib treatment in HER2-overexpressing breast cancers that have progressed on trastuzumab.

CONCLUSION

Our *in vitro* and *in vivo* results demonstrate that sustained MAPK signaling can be therapeutically targeted to increase sensitivity to lapatinib in trastuzumab-resistant HER2-overexpressing breast cancer cells (Figure 9).

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LIST OF ABBREVIATIONS

HER	human epidermal growth factor receptor
MAPK	mitogen-activated protein kinase
FOXM1	Forkhead box transcription factor M1
IHC	immunohistochemistry
siRNA	small interfering RNA

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Figure 1.

The structures for lapatinib and selumetinib were drawn in ChemDraw Ultra 12.0 (CambridgeSoft Corporation) and are shown here.

Figure 2. Sustained MEK Signaling in lapatinib-resistant cells

(A) BT474, HCC1419, JIMT-1, and MDA361 cells were treated with the control vehicle (C), 50, 100, 500, or 1000 nM lapatinib for 24 hours. Whole cell protein lysates were immunoblotted for p-ERK, total ERK or actin loading control. Blots were repeated at least three times with reproducible results. Lapatinib did not reduce ERK phosphorylation in JIMT-1 and MDA361 cells. (B) Untreated BT474, HCC1419, JIMT-1, and HCC1954 cells were lysed and immunoblotted for basal p-ERK, total ERK and actin loading control. Blots were repeated at least three times with reproducible results. No differences in baseline phosphorylated ERK were observed between lines.

Figure 3. MEK inhibition by PD0325901 (P) increases lapatinib (L) response

(A) In the blots to the left, JIMT-1 cells were treated with 1000 nM L, 10 nM P, combination 1000 nM L and 10 nM P, or vehicle control (C) for 24 hours. In the blots to the right, JIMT-1 cells were treated with 1000 nM L, 1000 nM P, combination 1000 nM L and 1000 nM P, or vehicle control (C) for 24 hours. Whole cell protein lysates were immunoblotted for p-ERK, total ERK, p-Akt, total Akt, or actin loading control. Blots were repeated at least twice with reproducible results. Combined lapatinib plus P suppressed phosphorylation of ERK to a greater degree than either drug alone and suppressed compensatory up-regulation of Akt phosphorylation. (B) JIMT-1 cells were plated in matrigel and treated with vehicle control, 1000 nM L, 10 nM P, or a combination of 1000 nM L and 10 nM P. Media plus drugs were changed every 3 days for approximately 2 weeks. Representative photos of colony growth in each treatment group are shown at 4× magnification. (C) Matrigel from the growth assays in (B) was dissolved with dispase, and the number of cells was counted by trypan blue. Viability is presented as a percentage of DMSO control, and reflects an average of three replicates per treatment group. Error bars represent the standard deviation between replicates. Experiments were repeated at least three times with reproducible results. Lapatinib plus P reduced growth in matrigel versus lapatinib alone; *p<0.05.

Figure 4. MEK inhibition by selumetinib (S) increases lapatinib (L) response

(A) HCC1419, JIMT-1, and HCC1954 cells were treated with the control vehicle (DMSO), 1000 nM L, 1000 nM S, or a combination of 1000 nM L and 1000 nM S (LS) for 24 hours. Whole cell protein lysates were immunoblotted for p-ERK, total ERK, or actin loading control. Blots were repeated on at least three separate occasions with reproducible results. Combined lapatinib plus selumetinib suppressed phosphorylation of ERK to a greater degree than either drug alone. (B) BT474 and JIMT-1 cells were plated at 3×10^4 in a 12-well plate format. After 24 hours, cells were treated with either L (10, 100, or 1000 nM), S (10, 100, or 1000 nM), or combination L plus S. Viable cells were counted by trypan blue exclusion after 72 hours and are reported as a percentage of control cells. Experiments were repeated twice

with reproducible results. Lapatinib plus selumetinib reduced survival of JIMT-1 cells versus lapatinib or selumetinib alone; * $p < 0.05$. **(C)** JIMT-1 cells were plated in matrigel and treated with vehicle control DMSO, 1000 nM L, 10 nM S, or a combination of 1000 nM L and 10 nM S (LS). Media plus drugs were changed every 3 days for approximately two weeks. Representative photos of colony growth in each treatment group are shown at 4 $\times$ magnification. Matrigel was dissolved with dispase, and viable cells were counted by trypan blue. Viability is presented as a percentage of DMSO control group, and reflects an average of three replicates per treatment group. Error bars represent the standard deviation between replicates. Experiments were repeated twice with reproducible results. Lapatinib plus selumetinib reduced growth of JIMT-1 cells in matrigel versus lapatinib alone; ** $p < 0.005$. **(D)** JIMT-1 cells were treated with DMSO control (C), 1000 nM L, 1000 nM S, or LS for 48 hours, fixed, stained with propidium iodide, and analyzed by flow cytometry. Cell cycle profiles are displayed. Triplicate cultures were run per treatment group, and the experiment was repeated three times.

Figure 5. MEK knockdown increases lapatinib (L) response

(A) JIMT-1 cells were transfected with 100 nM control siRNA (si-C) or MEK1/2 siRNA (si-MEK) for 24 hours, and then treated with DMSO control (C), or 1000 nM L for 48 hours. Total protein lysates were immunoblotted for MEK1/2, p-ERK, total ERK, and actin loading control to confirm knockdown. Knockdown of MEK was confirmed. The combination of MEK knockdown plus lapatinib suppressed ERK phosphorylation to a greater degree than MEK knockdown or lapatinib alone. (B) JIMT-1 cells were transfected with 100 nM control siRNA (si-C) or MEK1/2 siRNA for 24 hours and then treated with DMSO control (C) or 1000 nM L. After 48 hours, surviving cells were counted by trypan blue exclusion. Viability is presented as a percentage of DMSO-treated, si-C-transfected cells and reflects the average of 3 replicates per treatment group. Error bars represent standard deviation between replicates. P-values were determined by t-test for MEK1/2 knockdown plus lapatinib versus control siRNA plus lapatinib. MEK knockdown plus lapatinib showed significantly reduced cell survival versus lapatinib and control siRNA; $**p < 0.005$. Experiments were repeated at least three times with reproducible results. (C) JIMT-1 cells were transfected with 100 nM control siRNA (si-C) or MEK1/2 siRNA (si-MEK) for 24 hours and then treated with vehicle control (C) or 1000 nM L for 24 hours. Cells were stained with annexin V-FITC and propidium iodide and analyzed by flow cytometry. The top panels show dot plots of the cells. Quadrant 2 (Q2) shows late apoptotic cells (positive for both Annexin V-FITC and propidium iodide staining). Quadrant 3 (Q3) shows normal viable cells. Quadrant 4 (Q4) shows early apoptotic cells (Annexin V-FITC positive). The bottom graph shows quantification of the mean percentages of cells in each quadrant with Q4 showing increased apoptosis in cells treated with lapatinib plus selumetinib.

Figure 6. FOXM1 localization and expression in response to lapatinib plus selumetinib treatment (A) HCC1419 and (B) JIMT-1 cells were plated on glass coverslips for 24 hours before being treated with vehicle control, 1000 nM L, 1000 nM S, or a combination of 1000 nM L plus 1000 nM S (LS) for 24 hours. Immunofluorescence was performed to determine the cellular location of total FOXM1. Cells were observed at a magnification of 40 $\times$. Graphs are a quantification of the IF data from 10 samples per treatment demonstrating the percentage of cells expressing FOXM1 in the nucleus or nucleus plus cytoplasm versus the percentage of cells expressing FOXM1 in the cytoplasm or cytoplasm plus nucleus. The data indicated that lapatinib and selumetinib did not alter localization of FOXM1 in lapatinib-sensitive HCC1419 cells. In contrast, JIMT-1 cells, which expressed FOXM1 primarily in the nucleus

at baseline, showed an increased percentage of cells staining positive for FOXM1 in the cytoplasm when treated with lapatinib plus selumetinib. **(C)** Western blot analysis detecting FOXM1 from the nuclear and cytoplasmic fractions of JIMT-1 cells treated with DMSO control, 1000 nM lapatinib (L), 1000 nM selumetinib (S), or combination LS for 24 hours. Lamin B (nuclear) and eEF2 (cytoplasmic) were used as fractionation controls. The combination reduced expression of nuclear FOXM1. **(D)** Real-time PCR was performed for FOXM1 downstream target p27 in JIMT-1 cells that had been treated with DMSO control, 1000 nM L, 1000 nM selumetinib S, or combination LS for 24 hours. Values reflect the fold change in transcript normalized to RPLPO housekeeping gene. Lapatinib induced p27 transcript levels. The combination caused an even greater induction in p27 levels.

Figure 7. Modulation of FOXM1 expression affects lapatinib sensitivity

(A) JIMT-1 cells were transfected with 100 nM control siRNA (si-C) or FOXM1 siRNA (si-FM1) for 24 hours, and then treated with DMSO control vehicle (C) or 1000 nM Lapatinib (L) for 24h. Total protein lysates were immunoblotted for FOXM1 to confirm knockdown and actin. Alternatively, after 48 hours of drug treatment, surviving cells were counted by trypan blue exclusion. Viability is presented as a percentage of DMSO-treated control siRNA-transfected cells and reflects the average of 3 replicates per treatment group. Error bars represent standard deviation between replicates. P-values were determined by t-test for FOXM1 knockdown plus L versus si-C plus L; $**p < 0.005$. Experiments were repeated three times with reproducible results. FOXM1 knockdown reduced viability of JIMT-1 cells, with addition of lapatinib further reducing cell survival. (B) JIMT-1 cells were plated at 3×10^4 in a 12-well plate format. After 24 hours, cells were treated with DMSO control, 1000 nM L, 2000 μ M thiothrepton (T), or combination L plus T. Viable cells were counted after 48 hours by trypan blue exclusion and are reported relative to control cells. Error bars represent standard deviation between replicates. Experiments were repeated three times with reproducible results. P-values were determined by t-test for L treatment versus FOXM1 inhibition plus L. Viability was significantly reduced by lapatinib plus thiothrepton versus lapatinib alone; $**p < 0.005$. (C) HCC1954 and JIMT-1 cells were plated in matrigel and

treated with control, 1000 nM L, 2000 nM T, or combination 1000 nM L and 2000 nM T. Media plus drugs were changed every 3 days for approximately 2 weeks. Matrigel was dissolved with dispase, and viable cells were counted by trypan blue. Viability is presented as a percentage of DMSO control, and reflects an average of 3 replicates per treatment group. Error bars represent the standard deviation between replicates. Experiments were repeated three with reproducible results. P-value was determined by t-test for combination treatment versus lapatinib alone. Clonogenic growth was significantly reduced by lapatinib plus thioestrepton versus either agent alone in HCC1954 (**p<0.005) and JIMT-1 (*p<0.05) cells. **(D)** In the graph on the left, real-time PCR was performed for FOXM1 in HCC1419 cells that had been transfected with either 5µg pCMV-FOXM1 plasmid (pFM1) or control CMV plasmid (pCMV). Values reflect the fold change in transcript normalized to RPLPO housekeeping gene. In blot to the right, HCC1419 cells were transfected with either pCMV (C) or 2µg pFM1 for 24 hours. Total protein lysates were immunoblotted for FOXM1 to confirm overexpression and actin loading control. In the graph on the right, HCC1419 cells were transfected with 2 µg pCMV or pFM1 for 24 hours and then treated with DMSO control (C) or 1000 nM lapatinib (L) for another 48 hours. Viability measured by trypan blue exclusion is presented as a percentage of DMSO-treated pCMV and reflects the average of 3 replicates per treatment group. Error bars represent standard deviation between replicates. Experiments were repeated three times with reproducible results. P-values were determined by t-test for FOXM1 plus L versus vector control plus L; **p<0.005

Figure 8. The combination of selumetinib plus lapatinib suppresses tumor growth of JIMT-1 trastuzumab-resistant HER2-overexpressing breast cancer xenografts

JIMT-1 cells were injected s.c. in the flank of athymic mice. After palpable tumors formed, tumors were treated daily (5 days on, 2 days off) with vehicle control (n=3), 75 mg/kg oral lapatinib (n=3), 50 mg/kg oral selumetinib (n=3), or combination lapatinib plus selumetinib (n=3). **(A)** The top panel is a representative picture of an animal from each treatment group and the bottom panel is a representative picture of the excised tumor from one animal in each treatment group. In the graph to the right, mean tumor volume is shown per treatment group, with error bars representing the standard deviation between replicates. P-value was determined by t-test for combination treatment versus lapatinib alone for each week that measurements were taken; *p<0.05. Bottom IHC shows a representative picture of an animal from each treatment group stained for Ki67. **(B)** Staining for FOXM1 and pERK in a representative picture of an animal from each treatment group. All IHC photos were taken at 10× magnification.

Figure 9. Proposed model and summary

Lapatinib, a dual EGFR/HER2 kinase inhibitor, inhibits cell proliferation and survival by blocking the PI3K/Akt/mTOR (previously demonstrated by Gayle et al [9]) and MEK/ERK pathways. Based on our data, sustained activation of the MEK/ERK pathway in the presence of lapatinib is associated with reduced response to lapatinib. In this study, we inhibit MEK1/2 genetically using siRNA and pharmacologically using PD0325901 and selumetinib. FOXM1 was inhibited genetically using siRNA, pharmacologically using thiostrepton, and overexpressed using a FOXM1-CMV plasmid.

Table 1

Combination Index (C.I.) values for lapatinib + PD (1:1)

Cell line	ED50	ED75	ED90	Dm	m	r
BT474	353.13702	70.36036	14.63920	0.22133	-1.9126	0.93267
JIMT	0.00044	0.73971	1276.61516	0.61114	-1.5085	0.94372

Cells were treated with lapatinib (10, 100, or 1000 nM), MEK inhibitor PD (10, 100, or 1000 nM), or combination lapatinib plus PD. After 48 hours, cell viability was measured by trypan blue exclusion assay. The fraction of viable cells relative to DMSO control-treated cells was determined, and C.I. values were determined for the combination using CalcuSyn software. C.I. values are listed for effective doses at which 50%, 75%, or 90% (ED50, ED75, and ED90, respectively) of cells were killed. Statistically, drug synergy is defined by C.I. values less than 1.0, and very strong synergy is defined by C.I. values less than 0.1. Dm, the median-effect (ED50) drug concentration; $m < 1$ indicates a negative sigmoidal shape to the dose-effect curve; r states the linear correlation coefficient.

Table 2
Combination MEK inhibition and lapatinib treatment results in G0/G1 cell cycle arrest and apoptosis

Quantification of mean percentages + standard error among triplicates of JIMT1 cells treated with vehicle control DMSO, 1000nM lapatinib (L), 10nM selumetinib (S), or a combination of 1000nM L and 10nM S (LS) and analyzed by flow cytometry after fixing and staining with propidium iodide.

	%G0/G1	%S	%G2/M
C	55±0.48	18±0.08	28±0.54
L	54±0.78	16±0.18	30±0.63
S	57±1.66	15±0.78	29±0.89
LS	63±0.19	9±0.62	28±0.78

Table 3
Combination MEK knockdown and lapatinib treatment results in increased apoptosis in lapatinib-resistant cells

Quantification of mean percentages + standard error in triplicates of JIMT-1 cells transfected with 100 nM control siRNA (si-C) or MEK1/2 siRNA (si-MEK) for 24 hours and then treated with DMSO control (C) or 1000nM Lap for 48 hours. Cells were analyzed by flow cytometry for Annexin V-FITC and propidium iodide staining.

	% Late Apoptotic	% Normal	% Early Apoptotic
si-C +C	2.87±0.33	69±2.58	27.84±1.5
si-C+L	1.14±0.97	55.3±10.53	27.8±11.5
si-MEK+C	4.69±6	68.5±0.63	36±0.32
si-MEK +L	4.9±0	32.2±0.32	62.9±1.88