Combinatorial effects of lapatinib and rapamycin in triple-negative breast cancer cells

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Abstract

Triple negative (TN) breast cancers, which lack estrogen receptor, progesterone receptor and HER2/neu overexpression, account for approximately 15% of breast cancers, but occur more commonly in African-Americans. The poor survival outcomes seen with TN breast cancers patients are, in part, due to a lack of therapeutic targets. Epidermal growth factor receptor (EGFR) is over-expressed in 50% of TN breast cancers, but EGFR inhibitors have not been effective in patients with metastatic breast cancers. However, mTOR inhibition has been demonstrated to reverse resistance to EGFR inhibitors. We examined the combination effects of mTOR inhibition with EGFR inhibition in TN breast cancer in vitro and in vivo. The combination of EGFR inhibition using lapatinib and mTOR inhibition with rapamycin resulted in significantly greater cytotoxicity than the single agents alone and these effects were synergistic in vitro. The combination of rapamycin and lapatinib significantly decreased growth of TN breast cancers in vivo compared to either agent alone. EGFR inhibition abrogated the expression of rapamycin-induced activated Akt in TN breast cancer cells in vitro. The combination of EGFR and mTOR inhibition resulted in increased apoptosis in some, but not all, TN cell lines, and these apoptotic effects correlated with a decrease in activated eukaryotic translation initiation factor (eIF4E). These results suggest that mTOR inhibitors could sensitize a subset of TN breast cancers to EGFR inhibitors. Given the paucity of effective targeted agents in TN breast cancers, these results warrant further evaluation.
Introduction

Triple-negative (TN) breast cancers which lack expression of estrogen receptor (ER), progesterone receptor (PR) and HER2/neu (HER2) account for about 15% of all diagnosed breast cancers (1). We and others have noted a two-fold higher incidence of TN breast cancers in African-American patients compared to their Caucasian counterparts, regardless of age at diagnosis (2, 3). In African-American patients under the age of 40 TN breast cancers account for 50% of all diagnosed breast cancer cases (3). TN breast cancers are commonly high-grade and run an aggressive course, with a significant risk of developing metastases in the 5 years following diagnosis (4). Survival for patients with TN breast cancers is consequently poor, especially in African-American patients (3). The poor survival associated with TN breast cancers is, at least in part, due to a lack of effective targeted agents, which have positively impacted outcomes for patients with other subtypes of breast cancers (5, 6).

Genetic and immunohistochemical analyses demonstrate that 50% of basal-like breast cancers, which account for about three quarters of TN breast cancers, express epidermal growth factor receptor (EGFR) (7) and that EGFR expression has been associated with poor prognosis (8). However, the use of tyrosine kinase inhibitors directed towards EGFR in patients with unselected metastatic breast cancers produced little efficacy (9, 10). More recently, the use of single agent cetuximab (an EGFR monoclonal antibody) in metastatic TN breast cancers patients resulted in a response rate of only 6% and a clinical benefit rate of 20% (11). The addition of chemotherapy to cetuximab marginally increased the response rate to 17% (11). Given these disappointing results, it appears that EGFR
inhibition alone will not prove to be an effective therapeutic approach for patients with TN breast cancers.

The mTOR inhibitors, temsirolimus and everolimus, are currently approved for the treatment of metastatic renal carcinoma. The use of single agent mTOR inhibitors in patients with unselected metastatic breast cancers has not demonstrated encouraging results (12). The suboptimal outcomes obtained from the use of single agent mTOR inhibitors, like rapamycin and its analogues (or rapalogues), in the treatment of metastatic solid tumors is thought to be due partly to an increase in phosphorylated Akt levels following exposure to these rapalogues (13). mTOR inhibitor-induced Akt activation can be abrogated by the inhibition of upstream growth factors such as insulin-like growth-factor I receptors (13, 14). Given the fact that many TN breast tumors express EGFR (1), another upstream regulator in the PI3K/Akt pathway, we postulated that mTOR inhibitors would sensitize TN breast cancer cells to upstream inhibitors of the EGFR family. In support of this hypothesis, everolimus has been demonstrated to reverse resistance to trastuzumab in patients with trastuzumab-resistant HER2-positive metastatic breast cancers (15). The approach of targeting mTOR and EGFR concurrently has been evaluated previously pre-clinically in breast and other cancers (13, 16) but has not been specifically evaluated in TN breast cancers \textit{in vitro} and \textit{in vivo}.

Based on these data, we assessed the effects of co-inhibition of mTOR (using rapamycin) and EGFR (using lapatinib) (Fig. 1A) in TN breast cancer cell lines and nude mice models. Our results show that co-targeting mTOR and EGFR was synergistic in decreasing cell survival and resulted in increased apoptosis in some but not all TN breast cancer cell. Interestingly, the apoptotic effects noted were associated with changes in the
expression of activated eIF4E following combined treatment with EGFR and mTOR inhibitors. Furthermore, combined EGFR and mTOR inhibition down-regulated rapamycin-induced activation of Akt in vitro. These findings suggest that mTOR inhibition could improve the efficacy of EGFR inhibition in some TN breast cancers, and that the combination of an mTOR inhibitor with an EGFR inhibitor could warrant further evaluation in patients with TN breast cancers.

Materials and Methods:

Cell lines, Antibodies and Reagents:

The MDA-MB-231 breast cancer cell line was purchased from American Type Culture Collection (Manassas, VA). The breast cancer cell lines HCC1806 and MDA-MB-468 and lung cancer cell line A549 were generously provided by Drs. Sean Kimbro, Paula Vertino and Wei Zhou, respectively, at the Winship Cancer Institute, Emory University. These cell lines were not authenticated. MDA-MB-231, HCC1806 and A549 cells were routinely maintained in RPMI 1640 medium supplemented with 5% FBS. MDA-MB-468 cells were maintained in Dulbecco’s Modification of Eagle’s Medium (DMEM) supplemented with 5% FBS. The antibodies against p-Akt (S473), pS6 (S235/236), p-p44/42 MAPK (p-ERK, Thr202/Tyr204), and caspase-3 were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Antibody against p-EGFR (Tyr1173) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibody against p-eIF4E (pS209) was from Epitomics, Inc. (Burlingame, CA). Rapamycin (Rapa) was purchased from LC Laboratories (Woburn, MA). Erlotinib (Tarceva, Genetech, San Francisco, CA) and Lapatinib (Tykerb, GlaxoSmithKline, Research Triangle, NC) are
commercially available. For in vivo studies, lapatinib was dissolved in 1% Tween-80 (Sigma, MO) and rapamycin was dissolved in 2% ethyl alcohol. Protease inhibitor cocktail was purchased from Sigma (Saint Louis, MO)

**Cell Survival Assay:**

MDA-MB-231 and MDA-MB-468 cells were seeded at a density of 5000 cells/well in 96-well plates. They were grown overnight before treatment with various concentrations of rapamycin (0-100nM) and lapatinib (0-20μM) alone and in combination for 72 hours. Cell viability was assessed by the sulforhodamine B (SRB) assay following procedures described previously (17).

**Combination Index Assay:**

Combination index (CI) equations allow for the quantitative measurement of dose-effect relationships of single drugs and their combinations to determine synergistic drug interactions (18). The synergistic interactions between rapamycin and lapatinib were analyzed by CalcuSyn software (Biosoft, Ferguson, MO), which is based upon the Chou and Talalay method (18, 19). We further tested synergy of these drugs at several combinations using the method of Laska et al (20). MDA-MB-231 and MDA-MB-468 were seeded in 96-well plates at a density of 5000 cells per well overnight prior to drug treatment. After incubation with 1:2 serial dilutions of the drug based on their IC_{50} for rapamycin alone, lapatinib alone, or rapamycin in combination with lapatinib, the cells were subject to SRB assays (17). Data from SRB assays were expressed as fraction of cells with growth affected (FA) (comparison of drug-treated cells versus untreated ones). Combination index (CI) was calculated using CalcuSyn software. A CI>1 indicates antagonism, CI=1 indicates additivity, and CI<1 indicates synergism.
Western Blotting:
Breast cancer cell lines MDA-MB-231, HCC1806 and MDA-MB-468 and lung cancer cell line A549 were harvested and lysed in lysis buffer containing protease inhibitors (Sigma, Saint Louis, MO). Twenty micrograms of whole cell protein lysate were separated by SDS-PAGE followed by Western blot analysis with antibodies following procedures described in manufacturer’s instruction. The signals were detected with enhanced chemiluminescence (ECL) reagents (GE-Amersham, Piscataway, NJ), exposed on Hyblot CL autoradiography films (Denville Scientific, Metuchen, NJ) and developed using Konica SRX-101A medical film processor (Konica Medical & Graphic Corporation, Wayne, NJ).

Apoptosis Assay:
Apoptotic MDA-MB468 and MDA-MB-231 cells were determined by using Annexin V-phycoerythrin (PE) and 7-amino-actinomycin D (BD Biosciences). Cells were treated with rapamycin (25nM) alone, lapatinib (5uM) alone and in combination for 72 hours. Both floating and adherent cells were collected and labeled followed by fluorescence-activated cell sorting (FACS) analysis. The student t-test was used to evaluate p-values.

In Vivo Xenograft Tumor Model:
The animal protocol was approved by Emory University Institutional Animal Care and Use Committee. Female nude mice (athymic, nude-foxn1 nu, Harlan) aged 4-5 weeks were injected with 5x10^6 MDA-MB-231 or MDA-MB-468 cells into the mammary fat pad and were randomized into 4 groups and treated as follows: For the mice inoculated with MDA-MB-231, there are vehicle (1% Tween-80, p.o. five days a week and 2% Ethyl Alcohol, i.p. twice a week, n=6), rapamycin (3mg/kg, i.p. twice a week, n=10), lapatinib
(75mg/kg, p.o. five days a week, n=10) and the combination of rapamycin (3mg/kg, i.p. twice a week) and lapatinib (75mg/kg, p.o. five days a week) treatment group (n=12). For the study with MDA-MB-468, both treatment and dosage are the same as MDA-MB-231. But the sample size is 10 mice in each of the four groups. For both studies, treatment was started one week after the cells were injected. Mouse weight and tumor sizes were measured twice weekly. Tumor volume was calculated using the equation V (mm³) = largest diameter x smallest diameter²/2. The mice were sacrificed following treatment. Tumors were harvested, weighed, and snap frozen or placed in formalin for immunohistochemistry studies.

**Immunohistochemistry:**

Serial sections of 4μm thick tumor tissues were cut from the formalin-fixed, paraffin embedded tissue blocks. Antigen retrieval was performed in 1x EDTA buffer (pH 8.0) using the LabVision PTmodule. The immunohistochemistry assay was performed using DAKO LSAB 2 kit in a DAKO Autostainer (DakoCytomation, Glostrup, Denmark). The endogenous peroxidase was blocked with 3% hydrogen peroxide followed by incubation with primary antibodies for cleaved caspase-3 (1:500 dilution) and (Epitomics, Burlingame, CA) for 30 min at room temperature. The tissues were then incubated with biotinylated secondary antibody (DakoCytomation, CA) for 30 minutes followed by enzyme labeling with freshly prepared horseradish peroxidase-labeled streptavidin (DakoCytomation, CA). The developing chromogen DAB+ solution (DakoCytomation, CA) was added for 2 min and then the sections were lightly counterstained (1:6 dilution) with Hematoxylin in dH₂O (Richard-Allan Scientific, Kalamazoo, MI). The negative control consisted of nonimmune mouse or rabbit IgG. Digital images were captured
using the Aperio ScanScope XT slide scanner (Aperio Technologies, Vista, CA). Three images of different tissue sections were scored based on intensity level (0: negative; 1: weak; 2: moderate and 3: strong) multiplied by percentage of area staining (0-100). Student t-test was used to calculate the p-values.

**Statistical Analysis:**
Synergy of rapamycin and lapatinib was tested at the dose combinations listed in Table 1 using the method of Laska et al. (1994). For each dose combination \((r^*, l^*)\), we tested the null hypothesis that \(e(r^*, l^*) \leq e(r^*+l^*/m,0)\) or \(e(r^*, l^*) \leq e(0, mr^*+l^*)\) versus the alternative hypothesis that \(e(r^*, l^*) > e(r^*+l^*/m,0)\) and \(e(r^*, l^*) > e(0, mr^*+l^*)\), where \(e(r^*, l^*)\) denotes the fraction affected at \((r^*, l^*)\). Here, we took \(m = 0.2\) because each dose combination \((r^*, l^*)\) in Table 1 is the midpoint of the line joining the points \((r^*+l^*/m,0)\) and \((0, mr^*+l^*)\) and the fractions affected at these points are available from the experiments. Laska et al. (1994) showed that if the null hypothesis \(H_0\) is rejected in favor of the alternative hypothesis \(H_1\), then the combination is synergistic at \((r^*, l^*)\). The hypotheses were tested using 2 two-sample t-tests using four replicates at each point and the higher of the two p-values were reported in Table 1. Repeated measure ANOVA was used to compare the mean tumor volumes between the four different groups. Bonferroni correction to the p-values was adopted when comparing pairs of treatment groups.

**Results**
Combination of mTOR and EGFR inhibition is synergistic in TN breast cancer cells
EGFR and erbB2 expression levels were determined by Western blotting in MDA-MB-231, MDA-MB-468 and HCC1806 TN cell lines (Fig. 1B). ErbB2 expression levels were undetectable in MDA-MB-468 and HCC1806 whereas MDA-MB-231 had extremely low expression of erbB2 compared to erbB2-positive SKBR3 cell lines. MDA-MB-468 showed strong expression of EGFR, compared to MDA-MB-231 and HCC1806, which expressed moderate levels of EGFR. Given the differences in EGFR expression levels and response to lapatinib, we chose MDA-MB-231 and MDA-MB-468 cell lines for further evaluation.

We next compared the combined effects of rapamycin with erlotinib or lapatinib on activated EGFR protein levels in both MDA-MB-231 and MBA-MD-468 cells. Lapatinib alone and in combination with rapamycin decreased expression of activated EGFR more effectively in both cell lines, compared to erlotinib alone or in combination with rapamycin (Fig. 1C). Based on these findings, we selected lapatinib for subsequent experiments. The fact that the TN cells express no or extremely low levels of erbB2 suggests that the growth inhibitory effects of lapatinib are mediated through EGFR and not erbB2.

To determine the sensitivity of TN cells to mTOR inhibition in combination with EGFR inhibition, MDA-MB-231 and MDA-MB-468 TN breast cancer cell lines were treated with rapamycin (0.78-100 nM), lapatinib (0.156-20 uM) or both agents in combination for 72 hours. Two-fold serial dilutions were performed for both drugs. As shown in Figure 2A and 2B, rapamycin alone had very limited cytotoxic effect and cell survival rate remained between 85% to 100% for MDA-MB-231 and 75% to 90% for MDA-MB-468 after a 72-hour treatment. Lapatinib alone produced a gradual dose-
dependent growth inhibition in both cell lines. The inhibitory effect of combined treatment with rapamycin and lapatinib in two TN breast cancer cell lines was determined using a combination index (CI) and formal statistical tests. Table 1 gives the p-values for testing the synergy between rapamycin and lapatinib at each of seven dose combinations and for each cell line. Using Bonferroni correction for multiple testing, we conclude that for the MDA-MB-231 cell line, the combination is synergistic at (25,5), (12.5,2.5), (6.25,1.25), (3.125,0.625), (1.56,0.3125), and (0.78,0.156). There is no evidence of synergy at (50,10). For the MDA-MB-468 line, the combination is synergistic at (25,5), (12.5,2.5), (6.25,1.25), (3.125,0.625). There is no evidence of synergy at (50,10), (1.56,0.3125), and (0.78,0.156). Synergy at these dose combinations is also confirmed by the CI value in Figure 2C. We also note that the software CalcuSyn truncated cell survival fractions over 100% to values just below 100%. Also, the combined erlotinib and rapamycin treatments have been verified with CI to synergistically reduce cell growth in MDA-MB-468 TN cells (data not shown). These experiments indicate a synergistic interaction between rapamycin and lapatinib in suppressing growth of TN breast cancer cells.

**Effects of combined mTOR inhibition and EGFR inhibition on downstream signaling pathways**

mTOR inhibition may promote pharmacological mechanisms of resistance in cancer cells via feedback activation of the PI3K/Akt/mTOR and RAF/MEK/ERK signaling pathways (21). Rapamycin and a number of clinically available mTOR inhibitors activate Akt while inhibiting mTOR and downstream signaling (13, 14), and the precise mechanism of rapamycin-induced Akt activation remains unknown. This
aberrant Akt upregulation may partly explain the modest clinical responses elicited by these single agents in the treatment of many solid tumors, including breast cancer (12, 22). We explored the possibility that rapamycin-induced Akt activation could be repressed with combined EGFR and mTOR inhibition in TN breast cancer cells. Cells were treated with vehicle, rapamycin alone, lapatinib alone, or with rapamycin in combination with lapatinib. Lapatinib decreased the expression of p-Akt and p-ERK in both TN breast cancer cell lines (Fig. 3A). As expected, rapamycin increased the expression of p-Akt while abolishing mTOR signaling, as evidenced by decreased expression of the downstream target pS6 (Fig. 3A). Interestingly, rapamycin also increased the expression of upstream p-EGFR in MDA-MB-231 cells (Fig. 1B), possibly due to a similar feedback loop that resulted in increased Akt activation. In all cell lines, the combination of inhibitors decreased rapamycin-induced activation of p-Akt, and reduced p-ERK and pS6 expression levels (Fig. 3A).

Besides activating p-Akt, mTOR inhibition has been demonstrated to increase phosphorylated elf4E levels, a protein that plays a key role in cell proliferation and apoptotic resistance (23-25). Activated elf4E attenuates apoptosis (23, 24), and EGFR inhibition suppresses rapamycin-induced p-elf4E in lung cancer cell lines (25). We observed that elf4E activity differed in the two TN breast cancer cell lines following combined EGFR and mTOR inhibition. We found that p-elf4E expression was decreased following rapamycin plus lapatinib treatment in MDA-MB-468 cells (Fig. 3A). In contrast, dual inhibition of mTOR and EGFR in MDA-MB-231 breast cancer cells increased the expression of activated p-elf4E, similar to what has been described for
A549 non-small cell lung cancer cells (19). Expression of p-eIF4E activity is closely associated with apoptotic resistance (17, 18).

Given the fact that the combination of lapatinib and rapamycin decreased p-eIF4E levels in MDA-MB-468 cells, but not in MDA-MB-231 cells, we evaluated whether apoptosis was stimulated in response to the combination therapy. As expected from our data with p-eIF4E, rapamycin combined with lapatinib induced cleavage of caspase-3 in MDA-MB-468 cells but not in MDA-MB-231 cells (Fig. 3B). This data suggests that the failure to induce apoptosis in MDA-MB-231 treated with combined EGFR and mTOR inhibitors may be due to acquired apoptotic resistance via activated eIF4E.

To quantitatively determine whether the combination of mTOR and EGFR inhibition increased apoptosis, MDA-MB-231 and MDA-MB468 cells were treated with rapamycin alone, lapatinib alone or the combination of rapamycin and lapatinib for 72 hours. The percentage of apoptotic cell death was measured with Annexin V-PE and 7-AAD staining followed by FACS analysis (Fig. 3C). We noted a significant increase in apoptotic MDA-MB-468 cells treated with the combination of rapamycin and lapatinib (25.96%), compared to rapamycin (5.29%) and lapatinib (17.0%) alone (lapatinib versus combination p<0.01). There was no increase in apoptosis in MDA-MB-231 cells treated with either single agent alone or rapamycin and lapatinib in combination (p=0.54). Interestingly, the fact that both cell lines had less apoptotic dead cells than the growth-inhibited cells detected by SRB assay indicated that other inhibition mechanisms, like cytostasis, could also play roles in the observed inhibitory effects induced by the combined treatment.

**EGFR and mTOR inhibition suppresses growth of TN breast tumor xenografts**
To confirm our *in vitro* results, TN breast cancer cells MDA-MB-231 and MDA-MB-468 were injected into the mammary fat pads of athymic mice in two independent experiments. Mice were randomized into four treatment groups and administered rapamycin, lapatinib, combination of rapamycin and lapatinib, or vehicle. Tumor diameters were measured bi-dimensionally twice weekly. At the end of the overall treatments, neither lapatinib (mean tumor size 183mm$^3$, p=0.87) nor rapamycin (mean tumor size 133mm$^3$, p=0.098) alone significantly decreased the volume of MDA-MB-231 tumors compared to vehicle treated animals (mean tumor size 188 mm$^3$). In contrast, the combination of lapatinib and rapamycin (mean tumor size 76mm$^3$) significantly inhibited MDA-MB-231 tumor progression (60%), compared to lapatinib alone (4%, p<0.0001), rapamycin alone (29%, p=0.0096), and vehicle-treated animals (p=0.0005) (Fig. 4A). All p-values were adjusted using Bonferroni correction since we are comparing 5 pairs of groups.

Initially, MDA-MB-468 xenograft tumor growth was inhibited to a similar degree by each single agent and combined treatments. However, at the end of continued treatment, the combination of rapamycin and lapatinib significantly limited tumor growth (45%, mean tumor size 168mm$^3$), compared to the vehicle-treated group (mean tumor size 305 mm$^3$, p<0.0001), whereas the rapamycin was only able to inhibit the tumor growth by16% (mean tumor size 254mm$^3$, p<0.0001) and the lapatinib alone had no inhibition effect at the end (mean tumor size 306mm$^3$, p=0.03) (Fig. 4B). All p-values were adjusted using Bonferroni correction.

There was no significant difference in mouse weight between the treatment groups in either experiment, suggesting that the combination of an EGFR and mTOR
inhibitor does not significantly increase toxicity (data not shown). In summary, in TN xenografts breast cancer models, lapatinib significantly enhances the effect of rapamycin on tumor progression.

**Effects of EGFR and mTOR inhibition on apoptosis and proliferation *in vivo***

In order to evaluate the apoptotic effects of combined and single agent treatments on both MDA-MB-231 and MDA-MB-468 tumors, we examined caspase-3 activity by IHC (Fig. 5A and 5B). Basal levels of caspase-3 were low in both MDA-MB-231 and MDA-MB-468 tumor tissues and remained low in rapamycin-treated tumor tissues. The combination of lapatinib and rapamycin resulted in a greater increase in caspase-3 expression in MDA-MB-468 tumors (p<0.01). Even though we saw a trend in increased caspase-3 induced by combination treatment, there was no statistical difference in expression level in MDA-MB-231 treated groups (p=0.1). Interestingly, caspase-3 expression was increased in MDA-MB-468 (p<0.01) xenografts exposed to lapatinib alone, despite the fact that single agent lapatinib was less effective in inhibiting tumor growth than the combination of lapatinib and rapamycin *in vivo* (Fig. 5B). These results suggest that apoptosis may play only a partial role in the tumor inhibitory effects noted with combined EGFR and mTOR inhibition.

As tumor development is regulated by both decreased apoptosis and increased proliferation, we assessed the expression of Ki67 (Fig. 5A and 5B). Ki67 expression was markedly decreased in MDA-MB-468 tumors (p<0.001) and MDA-MB-231 tumors (p<0.01) treated with the combination of rapamycin and lapatinib, indicating that the
combination therapy decreased tumor proliferation. In contrast, neither rapamycin nor lapatinib alone was able to affect Ki67 expression in both TN tumors (Fig. 5B).

Discussion

The outcome for metastatic hormone receptor-positive and HER2-positive cancers has improved over the past 15 years with the availability of targeted therapies, such as tamoxifen, aromatase inhibitors and trastuzumab (26, 27). The median survival time for patients with metastatic hormone receptor-positive breast cancers is approximately 4 years, while the median survival for patients with HER2-positive metastatic breast cancers treated with trastuzumab-based chemotherapy approaches 3 years (26, 27). TN breast cancers pose a significant therapeutic problem because of a lack of targeted therapies, and the median survival for patients with metastatic estrogen receptor-negative breast cancers is less than 12 months (26). Chemotherapy remains the mainstay of therapy for metastatic TN breast cancers, but resistance is common and can develop rapidly (11). Response rates have been demonstrated to be lower and time to progression shorter in patients with TN breast cancers treated with single or combination chemotherapy compared to any of the other subtypes (28), suggesting that TN breast cancers are intrinsically more chemo-resistant than other breast cancer subtypes. Anti-angiogenic approaches, when added to chemotherapy, appear promising for patients with metastatic TN breast cancers (29). The use of the PARP inhibitor, iniparib, has been demonstrated to prolong progression free and overall survival in patients with TN metastatic breast cancers, compared to chemotherapy alone (30) in the Phase 2 setting, though these results were not confirmed in a larger Phase 3 clinical
trial. There is, therefore, a critical need for new therapeutic approaches in TN breast cancers.

EGFR is upregulated and over-expressed in a significant percentage of TN or basal-like cancers (1). However, previous trials in which patients with unselected metastatic breast cancers were treated with tyrosine kinase inhibitors targeting EGFR produced disappointing results, with response rates of 2% and time to progression of less than 2 months (9, 10). Response rates to single agent cetuximab are only 6%, and increased to 17% with the addition of carboplatin, in patients with TN metastatic breast cancer (11). Notably, in many patients, cancers progressed so rapidly that they were taken off the study before their first staging assessment. The addition of cetuximab increased response rates from 30% to 49% in patients with TN breast cancers but prolonged time to progression by only one week (31). Based on these trials, EGFR inhibitors alone or in combination with chemotherapy do not appear to be particularly effective in TN breast cancers.

mTOR inhibitors activate the Akt pathway, possibly through a feedback mechanism (13). Treatment of breast and lung cancer cells lines with mTOR inhibitors increases expression of activated Akt (16, 32). This paradoxical activation of Akt is believed to be one possible resistance mechanism to mTOR inhibitors by cancer cells and may explain the disappointing results to date reported with these agents when used as single therapies (12). However, recent clinical data suggest that mTOR inhibition can sensitize resistant HER2-positive breast cancers to the EGFR inhibitor, trastuzumab(15).
We, therefore, postulated that mTOR inhibition could sensitize TN breast cancer cells to EGFR inhibitors. We noted that the combination of rapamycin with lapatinib was synergistic in inhibiting TN breast cancer cell survival. We demonstrated that the combination of mTOR and EGFR inhibition increased apoptosis in some TN cell lines compared to either treatment given alone. Combined rapamycin and lapatinib also resulted in significant suppression of TN breast cancers in vivo compared with either agent alone. Lapatinib did not inhibit growth of TN breast cancers in vivo, which is in keeping with clinical trials, where minimal efficacy is noted in HER2-negative metastatic breast cancers (33). Consistent with our in vitro results, the combination of lapatinib and rapamycin induced moderate apoptosis in MDA-MB-468 tumors, while induction of apoptosis was not significant in MDA-MB-231 tumors. Combination therapy markedly decreased the expression of Ki67 in MDA-MB-468 tumors and MDA-MB-231 tumors. These results suggest that the inhibitory effects of combined mTOR and EGFR inhibition on the growth of MDA-MB-468 tumors are due to effects on both apoptosis and cell proliferation. In contrast, the growth inhibition noted in MDA-MB-231 tumors treated with combined therapy is probably due to reduced cellular proliferation. Taking our in vitro and in vivo data together, a subset of TN breast cancer appear to be more susceptible to concurrent inhibition of EGFR and mTOR. Lapatinib decreased the increase in activated Akt observed with the use of mTOR inhibitors in vitro. This finding potentially explains the synergistic effects we noted with combination therapy in vitro, though we were unable to show this conclusively in vivo (data not shown).

In summary, mTOR inhibitors in combination with EGFR inhibitors result in synergistic effects in TN breast cancer models in vitro and suppressed TN tumor growth.
in vivo, which warrant further evaluation. Given the lack of targeted agents and the rapid onset of chemo-resistance in metastatic TN breast cancers, there is a critical need for novel approaches to target these tumors. The mTOR inhibitor, everolimus has been combined with erlotinib in a Phase 1 clinical trial, demonstrating varying degrees of toxicity (34), but overall confirming the validity of this approach. Given the very poor outcomes for patients with metastatic TN breast cancers, we believe the combination of an mTOR inhibitor and an EGFR inhibitor warrants clinical investigation, and we are currently accruing to a trial evaluating the combination of everolimus and lapatinib patients with minimally pre-treated metastatic TN breast cancers.
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Table 1. *p*-values for testing synergy at selected dose combinations. Two-sample t-test was used to evaluate the synergistic effects at seven different dose-combinations. The *p*-values are summarized in the table.

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Figure Legends

Figure 1. Rapamycin plus lapatinib combined treatments reduce phosphorylation of EGFR in human TN breast cancer cell lines. (A) Chemical structures of rapamycin and lapatinib (used with permission from LC Laboratories). (B) Basal levels of erbB2 and EGFR expression among HCC1806, MDA-MB-231, MDA-MB-468, and SKBR3 breast cancer cell lines were compared by Western blotting. (C) MDA-MB-231 and MDA-MB-468 were treated with rapamycin (100nM), lapatinib (10µM), erlotinib (10µM), rapamycin plus lapatinib, or rapamycin plus erlotinib for 3 hours. Cells were then harvested and whole cell protein lysates were collected and analyzed by SDS-PAGE and Western blotting. Phosphorylation of EGFR (p-EGFR) was detected and compared among all treatment conditions in both cell lines. Representative blots of three independent experiments are shown. β-actin was used as a loading control.

Figure 2. Rapamycin combined with lapatinib synergistically inhibits survival of TN breast cancer cells. (A) MDA-MB-231 and (B) MDA-MB-468 were treated with 2-fold serial dilution of rapamycin alone (from 0.78nM to 100nM), lapatinib alone (from 0.156µM to 20µM), and the agents in combination for 72 hours and cell survival was analyzed using SRB assays. (C) Combination index (CI) was calculated via CalcuSyn software. Lapatinib plus rapamycin combined data of cell growth inhibition rate (1-survival rate) from SRB assay was expressed as fraction of affected cells (FA). CI value > 1 indicates antagonism, CI value = 1 indicates additivity, and CI value < 1 indicates synergism. The assay was prepared in quadruplicates. The error bars represent the standard error of replicates.
Figure 3. Effects of lapatinib and rapamycin on signaling and apoptosis in TN breast cancer cells. (A) TN breast cancer cell lines MDA-MB-231, MDA-MB-468 and lung cancer cell line A549 were treated with vehicle, rapamycin (100nM), lapatinib (10µM) and rapamycin combined with lapatinib (same doses) for 3 hours. Protein lysates from each treatment group were analyzed for the expression of p-Akt, p-ERK, pS6, p-eIF4E, and β-actin. (B) MDA-MB-231 and MDA-MB-468 cells were treated with vehicle, rapamycin, lapatinib or rapamycin combined with lapatinib at the above concentrations for 48 hours followed by SDS-PAGE and Western blotting analysis of caspase-3 activity. (C) MDA-MB-231 and MDA-MB468 cells were treated with rapamycin alone, lapatinib alone, and the two drugs in combination for 72 hours. Cells were then stained with Annexin V-PE and 7-AAD and analyzed by FACSorting analysis. The group of four graphs showed for each cell line is a one-time experiment representative of the four repeated ones. The bold number indicated in the middle of the graph is the mean of four replicates plus or minus standard error. The student t-test was used to calculate p-value.

Figure 4. Inhibitory effects of EGFR plus mTOR inhibitors on TN breast cancers in vivo. Nude mice were injected with 5x10^6 MDA-MB-231 cells or 5x10^6 MDA-MB-468 cells. Animals were treated with vehicle (1% Tween-80, p.o. and 2% ethyl alcohol, i.p., n=6/10), rapamycin alone (3 mg/kg, i.p., n=10/10), lapatinib alone (75 mg/kg, p.o., n=10/10) and their combination (same doses and route, n=12/10). Rapamycin was given twice a week and lapatinib was given 5 days a week. Mice injected with (A) MDA-MB-231 cells were treated for a total of 3 weeks, and mice injected with (B) MDA-MB-468 cells were treated for a total of 7 weeks. Tumor volumes of MDA-MB-231 and MDA-
MB-468 xenografts were measured twice weekly. Tumor sizes were compared among treatment groups. Vertical bars on the tumor growth curve chart indicate the standard error.

**Figure 5. Effects of combined mTOR/EGFR inhibition on proliferation and apoptosis in vivo.** (A) Representative images of cleaved caspase-3 and Ki-67 immunostaining for formalin-fixed, paraffin-embedded tumor tissues in MDA-MB-231 and MDA-MB-468 formed tumors. (B) Comparison of caspase-3 and ki67 expression level among control and treatment groups for MDA-MB-231 and MDA-MB-468 cells. Overall intensity level (ranging from 0 to 300, intensity score times percentage of area staining) is used to evaluate the protein expression level. Error bars represent standard error of scores. The student t-test was used to calculate p-values.
**Figure 1**

A. 

<table>
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<tr>
<th>Protein</th>
<th>HCC-1806</th>
<th>MDA-MB-231</th>
<th>MDA-MB-468</th>
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<tr>
<td>erbB2</td>
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<td>EGFR</td>
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<tr>
<td>β-actin</td>
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B. 

<table>
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<th>Compound</th>
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<tbody>
<tr>
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<td>+</td>
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<tr>
<td>erlotinib (10uM)</td>
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<td>+</td>
</tr>
<tr>
<td>lapatinib (10uM)</td>
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<td>+</td>
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<tr>
<td>p-EGFR</td>
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<td>β-actin</td>
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Figure 2
**A**

<table>
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<th>MDA-MB-231</th>
<th>MDA-MB-468</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Rapamycin (100μM)</td>
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<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Lapatinib (10μM)</td>
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<td>+</td>
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<tr>
<td>p-Akt</td>
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<td>p-S6</td>
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<tr>
<td>p-eIF4E</td>
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<tr>
<td>β-actin</td>
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**B**

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Rapamycin (100μM)</td>
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<td>-</td>
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<tr>
<td>Lapatinib (10μM)</td>
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<tr>
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<td>β-actin</td>
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**Figure 3**
**MDA-MB-231**

- Control: 1.30% ± 0.11%
- Rapamycin (R): 1.49% ± 0.45%
- Lapatinib (L): 1.28% ± 0.27%
- R+L: 1.12% ± 0.26%

**MDA-MB-468**

- Control: 5.45% ± 0.37%
- Rapamycin (R): 5.29% ± 0.73%
- Lapatinib (L): 17% ± 1.28%
- R+L: 25.96% ± 1.75%

Figure 3
Figure 4
Figure 5

(A) Immunohistochemical analysis of MDA-MB-231 and MDA-MB-468 cells treated with control, rapamycin (R), lapatinib (L), and R+L.

- **caspase 3**
- **Ki67**

**MDA-MB-231**

- Control
- Rapamycin (R)
- Lapatinib (L)
- R+L

**MDA-MB-468**

- Control
- Rapamycin (R)
- Lapatinib (L)
- R+L
Figure 5: Graphs showing the expression levels of caspase-3 and Ki67 for different groups (control, rapa, lapa, R+L) and cell lines (MDA-MB-231, MDA-MB-468).
Combinatorial effects of Lapatinib and rapamycin in triple-negative breast cancer cells

Tongrui Liu, Rami Yacoub, LaTonia D Taliaferro-Smith, et al.

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