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Inhibition of Mammalian Target of Rapamycin Is Required for Optimal Antitumor Effect of HER2 Inhibitors against HER2-Overexpressing Cancer Cells

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Abstract

Purpose: A significant fraction of HER2-overexpressing breast cancers exhibit resistance to the HER2 antibody trastuzumab. Hyperactivity of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway confers trastuzumab resistance, and mammalian target of rapamycin (mTOR) is a major downstream effector of PI3K/AKT. Therefore, we examined whether mTOR inhibitors synergize with trastuzumab.

Experimental Design: Immunocompetent mice bearing HER2$^+$ mammary tumors were treated with trastuzumab, the mTOR inhibitor rapamycin, or the combination. Mice were imaged for tumor cell death using an optical Annexin-V probe and with [^{18}F] FDG positron emission tomography. The signaling and growth effects of the mTOR inhibitor RAD001 on HER2$^+$ cells treated with trastuzumab or lapatinib were evaluated.

Results: Treatment of mice with trastuzumab plus rapamycin was more effective than single-agent treatments, inducing complete regression of 26 of 26 tumors. The combination induced tumor cell death (Annexin-V binding) and inhibited FDG uptake. Rapamycin inhibited mTOR and tumor cell proliferation as determined by phosphorylated S6 and Ki-67 immunohistochemistry, respectively. In culture, the combination of RAD001 plus trastuzumab inhibited cell growth more effectively than either drug alone. Trastuzumab partially decreased PI3K but not mTOR activity. Knockdown of *TSC2* resulted in HER2-independent activation of mTOR and dampened the response to trastuzumab and lapatinib. Treatment with the HER2 inhibitor lapatinib decreased phosphorylated S6 and growth in *TSC2*-expressing cells but not in *TSC2*-knockdown cells.

Conclusions: Inhibition of PI3K and mTOR are required for the growth-inhibitory effect of HER2 antagonists. These findings collectively support the combined use of trastuzumab and mTOR inhibitors for the treatment of HER2$^+$ breast cancer. (Clin Cancer Res 2009;15(23):7266–76)

Amplification and/or overexpression of the *HER2/erbB2* oncogene occurs in 20% to 30% of human breast cancers and is associated with poor prognosis (1, 2). The humanized monoclonal antibody trastuzumab (Herceptin), which binds the extracellular region of human HER2 (3), is approved for the treatment of HER2$^+$ breast cancer. Adjuvant trastuzumab therapy significantly improves patient outcome overall. However, in the metastatic setting, only a fraction of patients with HER2$^+$ disease responded to trastuzumab therapy (4–8), implying that many advanced cancers are trastuzumab-resistant. HER2$^+$ breast tumors showed increased caspase-3 activation following 1 week of neoadjuvant trastuzumab treatment (9), indicative of

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Translational Relevance

Although trastuzumab therapy has significantly improved outcome for patients with advanced HER2$^+$ breast cancer, a fraction of these cancers exhibit *de novo* or acquired resistance. Activation of the phosphatidylinositol 3-kinase signaling pathway has been linked with trastuzumab resistance, making this pathway a therapeutic target of interest. The mTORC1 serine/threonine kinase complex is a major effector in the phosphatidylinositol 3-kinase pathway, and mammalian target of rapamycin (mTOR) inhibitors suppress cancer cell growth in various model systems. We show that mTOR inhibitors synergize with trastuzumab to induce complete regression of HER2$^+$ mouse mammary tumors *in vivo* and to suppress the growth of HER2$^+$ human breast cancer cells *in vitro*. These findings support the use of combinations of trastuzumab and mTOR inhibitors for the treatment of patients with HER2$^+$ breast cancer.

apoptosis. Although the antitumor mechanism(s) of trastuzumab action remains unclear, the immunologic effects of this antibody are thought to be critical, likely invoking antibody-dependent cellular cytotoxicity (10).

HER2 dimerizes with members of the epidermal growth factor receptor (EGFR) tyrosine kinase family (EGFR, HER3, HER4) to induce downstream signaling cascades. The most oncogenic heterodimer is HER2/HER3, which engages downstream signaling pathways including phosphatidylinositol 3-kinase (PI3K)/AKT. Trastuzumab induces a cytostatic effect on HER2$^+$ breast cancer cell lines in culture (11) correlating with partial inhibition of AKT activation (12). However, HER2$^+$ primary breast tumors did not exhibit significant trastuzumab-induced downregulation of phosphorylated AKT (pAKT) or phosphorylated ERK (9), suggesting that trastuzumab is not a potent inhibitor of HER2 signaling *in vivo*.

Mutational activation of the PI3K pathway by activating mutations in *PIK3CA* (encoding the PI3K p110α catalytic subunit) or loss-of-function mutations in *PTEN* (encoding a lipid phosphatase that antagonizes PI3K) has been linked to trastuzumab resistance in cancer cells and primary tumors (13, 14). We reported that HER2$^+$ BT474 human breast cancer cells selected for trastuzumab resistance *in vivo* maintained PI3K activity and were sensitive to pharmacologic inhibitors of receptors upstream of PI3K (15). Trastuzumab inhibits PI3K signaling in parental but not trastuzumab-resistant BT474 cells, suggesting that these cells escape trastuzumab action through PI3K activation (15).

The mTORC1 complex [hereafter called mammalian target of rapamycin (mTOR)] is a major downstream effector in the PI3K/AKT pathway (16). Additionally, mTOR may be modulated by an AKT-independent mechanism via protein kinase C (17). mTOR inhibitors [e.g., rapamycin and RAD001 (everolimus)] are being tested clinically for the treatment of breast and other cancers (18, 19). Rapamycin blocked PTEN-deficient tumor formation in mice (20). mTOR inhibitors block cancer cell proliferation in culture (21, 22) and decrease tumor angiogenesis and vessel permeability *in vivo* (23). Results of phase I trials in patients with trastuzumab-resistant (24) and heavily pretreated (25) breast cancers have shown that the combination of RAD001 and trastuzumab may be a promising treatment option. We speculated that the inability of trastuzumab to completely block PI3K/AKT/mTOR signaling would permit synergy of trastuzumab with mTOR inhibitors to suppress the growth of HER2$^+$ cancer cells. Additionally, mTOR inhibitors may have effects on host (non-tumor) cells that contribute to their synergy with trastuzumab.

Materials and Methods

Tumor studies. Female mice expressing a MMTV promoter-driven human *HER2* transgene develop mammary gland tumors (26). Tumors were harvested from MMTV/HER2 females (gift from Sharon Erickson, Genentech). Two-millimeter pieces were transplanted subcutaneously near mammary gland 1 of syngeneic wild-type FVB females (5-7 weeks old) as described (27). Palpable tumors arose after 4 to 8 weeks. Tumors were measured two times a week, and volume was calculated as width^2 × length / 2. To account for the variability in response to drug treatment seen in this model (26, 27), transplanted tumors were derived from a different transgenic donor for each experiment. Tumor-bearing mice were randomized to treatment with vehicle(s), trastuzumab (30 mg/kg in PBS administered intraperitoneally two times a week; Vanderbilt University Hospital Pharmacy), rapamycin (1 mg/kg in 5% polyethylene glycol-400/5% Tween 80/0.9% NaCl administered intraperitoneally three times a week; Eton Biosciences), or the combination. Mouse studies were approved by the Vanderbilt Institutional Animal Care and Use Committee.

Immunohistochemistry. Formalin-fixed tumors were paraffin-embedded. Sections (5 μm) were used for H&E staining and immunohistochemistry using antibodies against Ki-67 (Biocompare), pHER2$_{Y1248}$ (scoring described in Supplementary Methods), pS6$_{S235/236}$, and pAKT$_{S473}$ (Cell Signaling). Ki-67 was scored as % positively stained cells in 10 fields (×400 magnification) of viable tumor. pS6 and pAKT staining was evaluated by an expert pathologist (M.G.O.) as described (28).

Cell proliferation. BT474 and SKBR3 cells (American Type Culture Collection) were maintained in IMEM/10% fetal bovine serum and McCoy's 5A medium/15% fetal bovine serum (Life Technologies), respectively. HR5 and HR6 cells were maintained in IMEM/10% fetal bovine serum plus 10 μg/mL trastuzumab as described (15). Cells were seeded in triplicate (4 × 10^4 per well, 12-well plates) and then treated with or without trastuzumab (21 μg/mL), RAD001 [20 nmol/L; gift from Carlos Garcia-Echeverria, Novartis (29)], 0360263-1 [AKTi, 1 μmol/L, AKT1/2 inhibitor (30)], lapatinib ditosylate (1 μmol/L; GW-572016; LC Laboratories), or combinations. For small interfering RNA experiments, cells were transfected with small interfering RNA targeting *tuberous sclerosis 2* (*TSC2*; Qiagen) or nonsilencing control (Qiagen) and then reseeded for cell proliferation assays and immunoblotting. Media plus drugs were replenished every 2 to 3 days, and cells were trypsinized and counted using a Coulter counter after 6 to 7 days.

Immunoblotting. Cells and tumor samples were lysed in 1% NP-40 buffer containing protease and phosphatase inhibitors. Samples were sonicated for 10 s and centrifuged at 14,000 rpm for 5 min, and protein concentrations were quantitated using BCA assay (Pierce). Lysates were used for immunoblotting with S6, pS6$_{S235/236}$, AKT, pAKT$_{S473}$, pAKT$_{T308}$, phosphorylated mitogen-activated protein kinase (pMAPK), MAPK, pHER3$_{Y1289}$, pEGFR$_{Y1173}$, pHER2$_{Y1248}$ (Cell Signaling), HER3 (Santa Cruz Biotechnology), HER2 (Neomarkers), and actin (Sigma) antibodies.

***In vivo* imaging.** Mice were imaged using NIR700-Annexin-V (details provided in Supplementary Methods) at baseline (pretreatment) when tumor size reached ≥400 mm^3, randomized to treatment with trastuzumab, rapamycin, or the combination, and reimaged 40 h later. Differences between pretreatment and post-treatment absolute tumor fluorescence were calculated. Mice were imaged using [^{18}F] FDG positron emission tomography at baseline, randomized to drug

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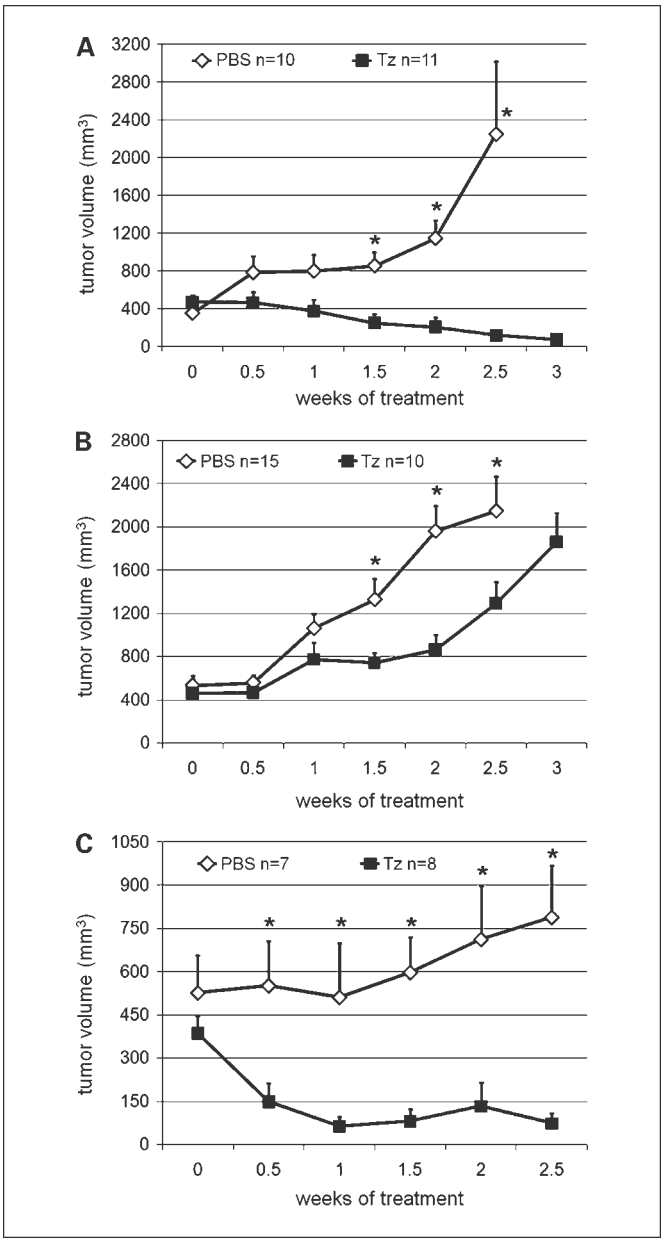

Fig. 1. Trastuzumab inhibits MMTV/HER2 tumor growth. Three groups of mice (*A-C*) bearing MMTV/HER2 tumors derived from three transgenic donors were randomized to trastuzumab (*Tz*) or PBS when tumor volume reached ≥200 mm^3. Average ± SE volume. *, $P < 0.05$, Mann-Whitney *U* test comparing trastuzumab and PBS groups at each time point.

treatment on days 0, 2, 4, and 6, and reimaged on days 3 and 7. Percent changes in [^{18}F]FDG tumor uptake comparing pretreatment versus post-treatment were calculated.

Statistics. Mann-Whitney *U* test was used to determine the significance of differences between groups in tumor volumes, % Ki-67$^+$ cells, NIR700-Annexin-V, and [^{18}F]FDG uptake. *t* test was used to determine the significance of changes in cell proliferation. $P < 0.05$ was considered significant.

Results

Rapamycin in combination with trastuzumab induces complete regression of MMTV/HER2 tumors. We transplanted tumors from three MMTV/HER2 transgenic female mice into three groups of 30 wild-type female mice each (called groups A-C). Tumor-bearing mice were randomized to treatment with trastuzumab or PBS once tumor volumes reached ≥200 mm^3. Whereas tumor volume continued to increase in PBS-treated mice, trastuzumab treatment inhibited tumor growth in all three groups (Fig. 1). In groups A and C, trastuzumab treatment for 3 weeks induced complete tumor regression in 7 of 10 and 6 of 8 mice, respectively (Fig. 1A and C; Supplementary Fig. S1). Whereas trastuzumab treatment significantly slowed the growth of group B tumors (Fig. 1B), 9 of 10 group B tumors continued to grow during trastuzumab therapy, and 25% to 30% of tumors in groups A and C grew or did not regress completely even after 4 weeks, suggesting that a fraction of MMTV/HER2 tumors are trastuzumab-resistant. In agreement with prior findings in MMTV/HER2 tumors (26), tumors from groups A to C showed strong HER2 immunoreactivity by immunohistochemistry (data not shown), and trastuzumab treatment did not alter pHER2$_{Y1248}$ immunoreactivity (Supplementary Fig. S1D).

We next determined whether cotreatment with the mTOR inhibitor rapamycin synergizes with trastuzumab to inhibit MMTV/HER2 tumor growth. We found previously that larger MMTV/HER2 tumors were less sensitive to trastuzumab therapy (27), so we allowed group D tumors to grow to ≥400 mm^3 before initiating treatment. Treatment with trastuzumab or rapamycin alone significantly inhibited tumor growth to similar extents compared with vehicle control (Fig. 2A and B; all $P < 0.05$); 4 weeks of single-agent treatments induced complete regression in only 3 of 10 and 1 of 10 cases, respectively. In contrast, the combination treatment significantly decreased tumor volume within 0.5 weeks compared with baseline ($P < 0.005$), induced complete regression in all cases within 3.5 weeks, and was significantly more inhibitory than either drug alone (trastuzumab versus combination $P < 0.05$ at 1 week and thereafter; rapamycin versus combination $P < 0.001$ at 1.5 weeks and thereafter). The treatment combination also more effectively inhibited tumor growth and induced complete regression than either drug alone when initiated with tumors ≥200 mm^3 (Fig. 2C and D).

Treatment with rapamycin decreases tumor cell proliferation and mTOR activity. To determine the effects of these treatments on tumor cell proliferation and survival, we randomized mice bearing tumors ≥400 mm^3 to trastuzumab, rapamycin, or the combination. Mice were treated on days 0 and 2, and tumors were harvested 24 h later on day 3. Immunohistochemical analysis showed that the percentage of Ki-67$^+$ tumor cells decreased on treatment with rapamycin alone (1.8 ± 1.7%, average ± SE) or in combination with trastuzumab (2.6 ± 1.7%), whereas trastuzumab alone (20 ± 11.5%) had no significant effect on the proportion of Ki-67$^+$ cells compared with controls (24.3 ± 9.8%; Fig. 3A and B; $P < 0.0005$). This suggests that rapamycin suppressed cell proliferation and confirms previous observations that trastuzumab does not alter tumor cell proliferation *in vivo* (9, 31). Immunohistochemical analysis of cleaved caspase-3 in tumors harvested after 3 and 6 days of treatment showed apoptosis surrounding prominent necrotic regions, but we did not detect differences in caspase-3 immunoreactivity or the extent of areas of necrosis between treatment groups (data not shown). However, we recently showed that 2 weeks of trastuzumab treatment induces apoptosis in MMTV/HER2 tumors when assessed by optical imaging (31). We speculate that the difference in timing and the large areas of necrosis observed herein may have obscured effects of trastuzumab on apoptosis as measured previously by cleaved

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caspase-3 immunohistochemistry in clinical specimens (9). Although mTOR inhibitors may have antiangiogenic effects (23), CD31 staining did not reveal changes in blood vessel number, size, or architecture between treatment groups (data not shown).

We next assessed the effects of trastuzumab and rapamycin on HER2 and mTOR signaling. Rapamycin alone or with trastuzumab effectively blocked S6 ribosomal protein phosphorylation (pS6) as measured by immunohistochemistry of tumor sections or by immunoblot of tumor homogenates (Fig. 3A and C), consistent with inhibition of mTOR and its downstream target S6 kinase (32). Trastuzumab did not decrease pS6, modestly decreased pHER3, but did not alter pAKT, pMAPK, pHER2, or HER2. pAKT immunoreactivity was not altered by drug treatment (Supplementary Fig. S2). These results suggest that rapamycin, but not trastuzumab, blocks mTOR activity *in vivo*.

Trastuzumab plus rapamycin treatment decreases tumor viability and glucose metabolism. We next determined the effects of treatment on the induction of cell death by *in vivo* imaging using NIR-labeled Annexin-V, a 35-kDa phosphatidylserine-binding protein. Phosphatidylserine is externalized to the outer leaflet of the plasma membrane during programmed cell death. We showed previously that NIR700-Annexin-V detects apoptosis in MMTV/HER2 tumors after 2 weeks of trastuzumab treatment (31).

Mice bearing MMTV/HER2 tumors were imaged using NIR700-Annexin-V to establish baseline tumor uptake (Fig. 4A). Mice were then randomized to a single treatment with trastuzumab, rapamycin, or the combination and reimaged 40 h later. The changes in absolute tumor fluorescence induced by drug treatment were plotted (Fig. 4A). Single-agent treatments did not alter tumor NIR700-Annexin-V uptake compared with controls, but the combination significantly increased absolute tumor fluorescence ($P < 0.05$), suggesting that tumor cell death occurs within 1.5 days of combination treatment. These results contrast with the data obtained with cleaved caspase-3 immunohistochemistry and suggest that this last method, which was evaluated at 3 and 6 days after treatment initiation, could have missed the time of maximally detectable treatment-induced cell death.

AKT activation stimulates glucose import and metabolism (33), and most glycolytic enzyme genes are transcriptionally regulated by AKT and mTOR signaling (34). To evaluate the effects of trastuzumab and rapamycin on glucose metabolism, we measured [^{18}F]FDG uptake by positron emission tomography. Baseline tumor [^{18}F]FDG uptake was determined, and mice were randomized to drug treatment on days 0, 2, 4, and 6 and reimaged on days 3 and 7. Whereas the single-agent treatments induced an apparent trend toward decreased [^{18}F]FDG tumor

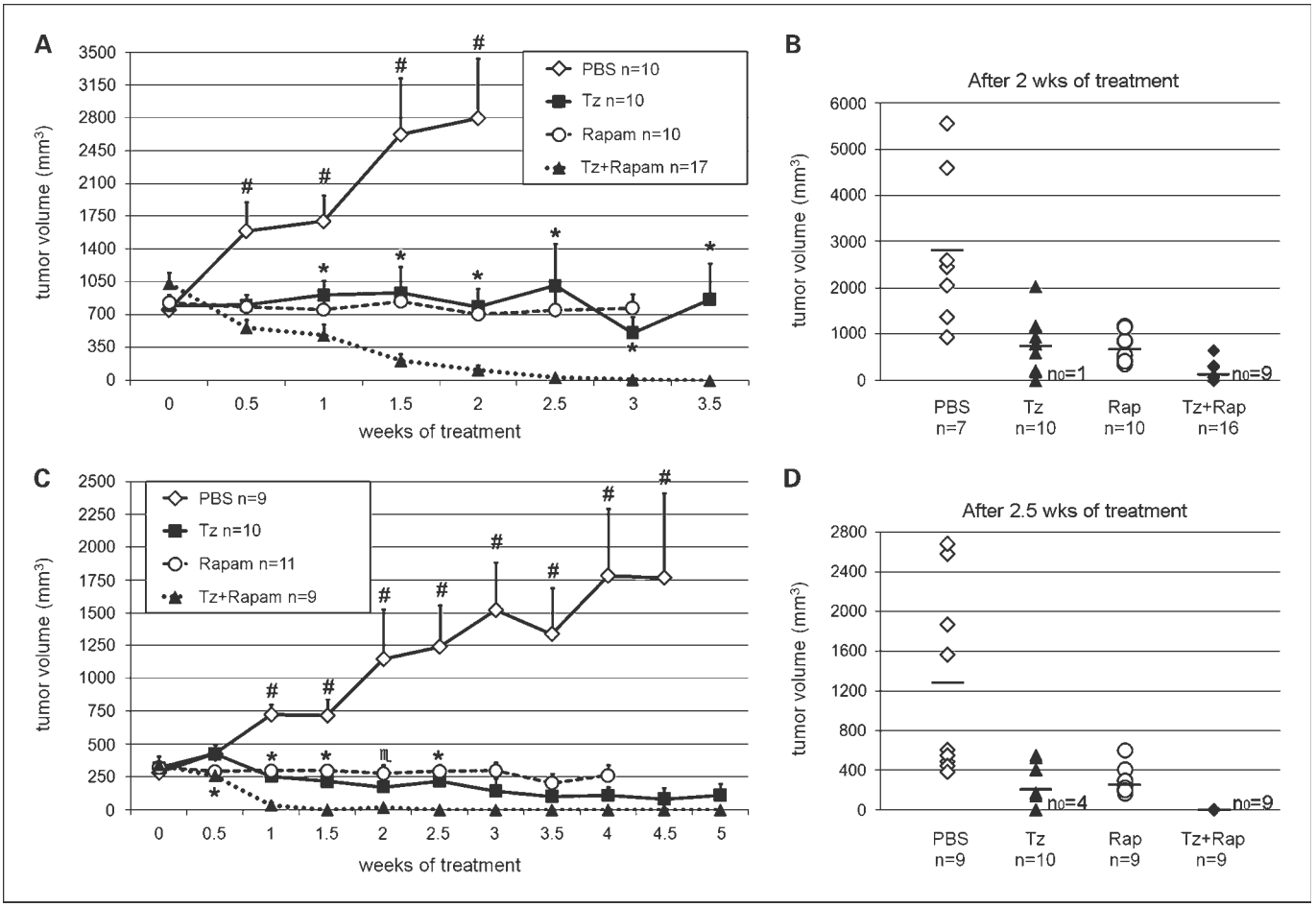

Fig. 2. Rapamycin synergizes with trastuzumab to induce complete tumor regression. Two groups of MMTV/HER2 tumor-bearing mice (groups D and E) were randomized to trastuzumab, rapamycin, or the combination once tumor volume reached ≥400 mm^3 (*A* and *B*) or ≥200 mm^3 (*C* and *D*). *A* and *C*, average ± SE volume. *, $P < 0.05$; ℳ, $P = 0.052$, Mann-Whitney *U* test comparing trastuzumab and combination groups at each time point; #, $P < 0.05$, all three Mann-Whitney *U* tests comparing vehicle with each other treatment group. *B* and *D*, tumor volume for each mouse after 2.5 wk (*B* shows group D) and 2 weeks (*D* shows group E) of treatment. Bars indicate average volumes. n_0 = number of mice with tumors that completely regressed. Numbers of animals decreased slightly at later time points due to excessive tumor burden.

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uptake, these groups were not significantly different from controls (Fig. 4B). However, the combination treatment induced a strong trend toward decreased [^{18}F]FDG tumor uptake compared with controls after 7 days (P = 0.0586; Fig. 4B); significant changes were not observed on day 3 (data not shown). A separate set of tumor-bearing mice was treated on days 0, 2, and 5, and tumors were harvested on day 6 for immunoblot analysis. Tumors from combination-treated mice showed decreased levels of several signaling proteins [HER3, HER2, and insulin receptor substrate-1 (IRS-1); Supplementary Fig. S3]. Therefore, the decreased [^{18}F]FDG tumor uptake at day 7 may have been linked to cell death in agreement with the decrease in tumor size seen after 0.5 weeks of combination treatment (Fig. 2).

mTOR inhibitor RAD001 synergizes with trastuzumab to inhibit HER2$^+$ breast cancer cell growth. To determine whether mTOR inhibition directly potentiates the effects of trastuzumab in a tumor cell-autonomous manner, we evaluated the effects of the combination on the proliferation of SKBR3 and BT474 cells. Both lines harbor *HER2* gene amplification and wild-type *PTEN*. SKBR3 cells have wild-type *PIK3CA*, whereas BT474 carry a weakly transforming *PIK3CA* mutation (35). Whereas treatment with trastuzumab or the mTOR inhibitor RAD001 alone significantly inhibited BT474 and SKBR3 cell proliferation (Fig. 5A), the combination was significantly more effective than either agent alone (all $P < 0.005$). However, the combination only marginally increased apoptosis in BT474 cells after 3 days compared with control or trastuzumab-treated cells (3.41%, 2.15%, and 2.1%, respectively; Supplementary Fig. S4). Therefore, trastuzumab and RAD001 appear to have a primarily cytostatic effect in culture.

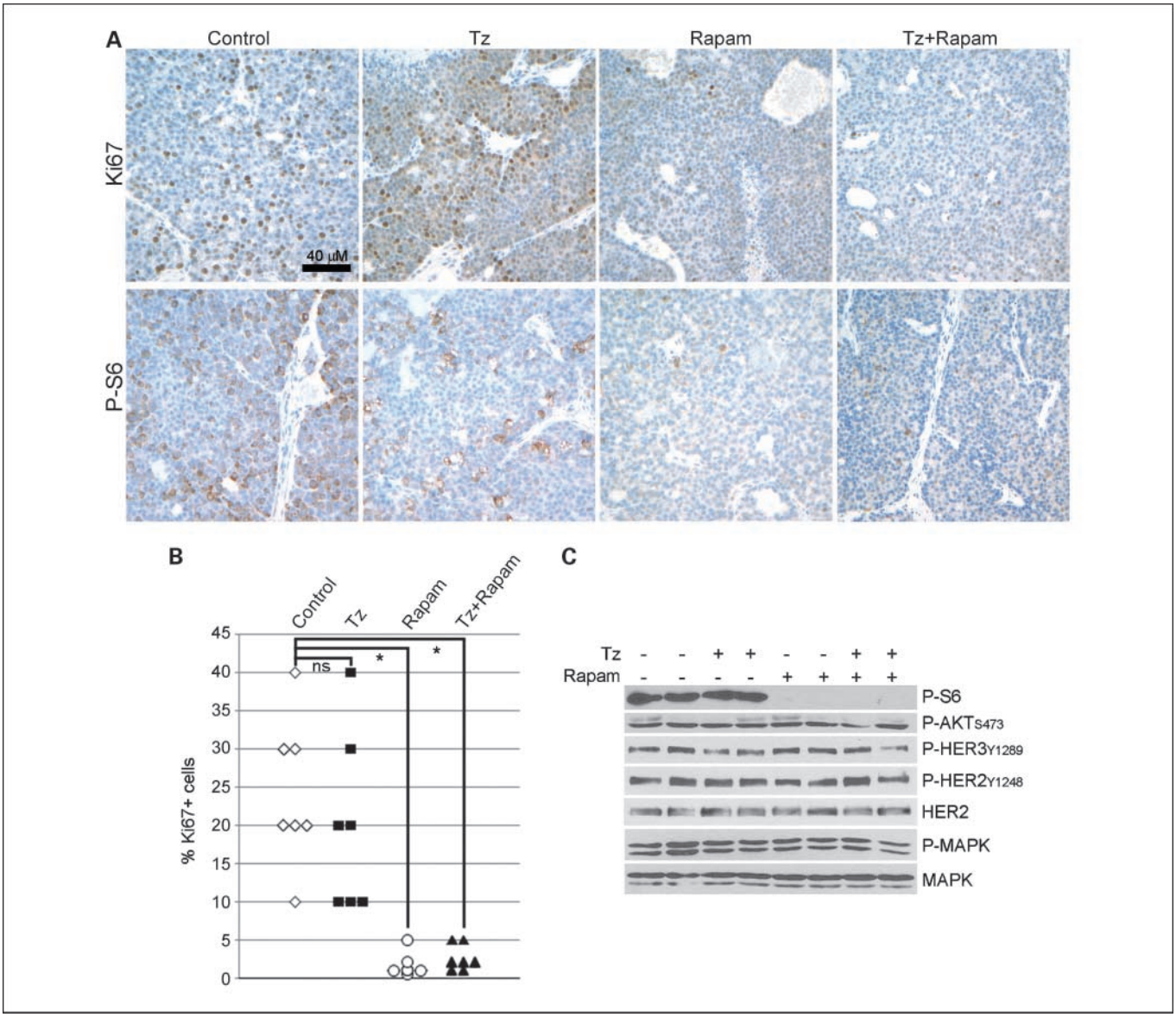

Fig. 3. Rapamycin inhibits mTOR and tumor cell proliferation. Tumor-bearing mice were treated with trastuzumab, rapamycin, or the combination (n = 6-7 per group) on days 0 and 2, and tumors were harvested 24 h later. *A,* immunohistochemical analysis of Ki-67 (*top row*) and pS6 (*bottom row*) in tumor sections. *B,* quantitative comparison of % Ki-67$^+$ tumor cells from *A*. *, $P < 0.005$, Mann-Whitney U test comparing control with each treatment group. ns, nonsignificant. *C,* immunoblot analysis of lysates from tumors used in *A*. Each lane contains equal amounts of protein pooled from 3 to 4 tumors. Blots were probed with indicated antibodies.

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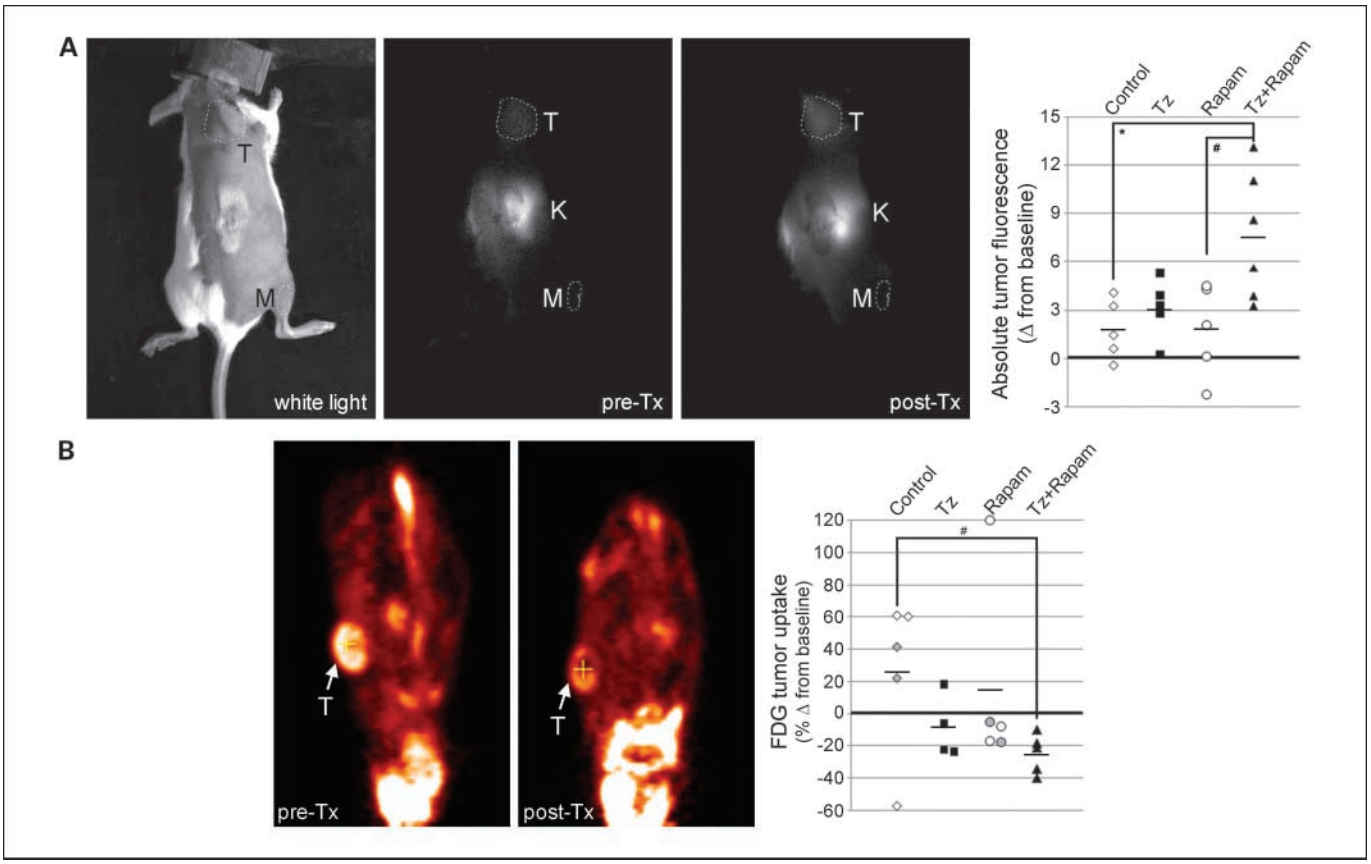

Fig. 4. Rapamycin synergizes with trastuzumab to decrease tumor viability and glucose metabolism. *A*, tumor-bearing mice were imaged at baseline using NIR700-Annexin-V, randomized to trastuzumab, rapamycin, or the combination (n = 5-6 per group), and reimaged 40 h post-treatment. Representative image shows tumor location (*white light*) and NIR700-Annexin-V signals at pretreatment and post-treatment. T, tumor; M, hind limb reference muscle (internal control); K, kidney (Annexin-V is cleared via kidney). Plot of the change in absolute tumor fluorescence induced by drug treatment for each mouse is shown (*right*). Bars indicate group averages. *, $P < 0.05$; #, $P = 0.052$, Mann-Whitney U test between treatment groups. *B*, tumor-bearing mice were baseline imaged by positron emission tomography for [^{18}F]FDG uptake, treated as above on days 0, 2, 4, and 6, and reimaged on day 7 (n = 4-5 per group). Images from a representative mouse show [^{18}F]FDG uptake pretreatment and post-treatment. Plot of the % change in [^{18}F]FDG uptake at day 7 compared with baseline is shown (*right*). Gray diamonds/circles are indicative of two tumors within the same mouse. #, $P = 0.0586$, Mann-Whitney U test between treatment groups.

We then analyzed the effects of trastuzumab and RAD001 on HER2 and mTOR signaling *in vitro*. Whereas trastuzumab treatment had only a modest inhibitory effect on mTOR activity (indicated by pS6 levels), RAD001 completely blocked it (Fig. 5B). In agreement with prior findings (12, 36, 37), trastuzumab partially decreased pAKT, indicating partial inhibition of PI3K signaling. pHER3 activates PI3K, and HER3 function is required for the transforming effects of overexpressed HER2 (38). In agreement with our prior observations (12), the trastuzumab-induced inhibition of PI3K is associated with decreased pHER3, supporting the notion that HER2 primarily transactivates HER3 in HER2$^+$ cells. Inhibition of mTOR has been shown to activate the insulin-like growth factor-I receptor/IRS-1 (39) and MAPK (40) pathways through derepression of negative feedback loops. Here, RAD001 treatment increased $pAKT_{S473}$, $pAKT_{T308}$, pMAPK, and pHER3 but did not alter IRS-1 levels (Fig. 5B and D). These RAD001-induced effects were partially blocked by cotreatment with trastuzumab (Fig. 5B), suggesting that feedback to activate HER3, PI3K, and MAPK was partially dependent on HER2. These results suggest that the combination of trastuzumab plus RAD001 simultaneously suppresses both PI3K and mTOR signaling, which, in turn, contributes to their synergistic inhibition of cell proliferation.

We further evaluated the ability of RAD001 to overcome trastuzumab-resistant growth in HR5 and HR6 cell lines, which were derived from BT474 xenografts with acquired trastuzumab resistance *in vivo* (15). Compared with parental, trastuzumab-sensitive BT474 cells, both resistant lines exhibited higher basal levels of pS6, which were not altered by trastuzumab treatment. Treatment with RAD001, but not trastuzumab, significantly inhibited HR5/HR6 cell growth (Fig. 5E and F), suggesting that mTOR inhibition may be an effective therapeutic strategy against trastuzumab-resistant breast cancers.

Because HER2-mediated AKT activation is partially suppressed by trastuzumab but RAD001 treatment increased pAKT (Fig. 5B), we assessed whether direct inhibition of AKT could synergize with mTOR inhibition to suppress cell proliferation. Indeed, treatment with RAD001 or an AKT1/2 inhibitor (AKTi) significantly inhibited cell proliferation, and the combination was more effective than either agent alone (Fig. 5C; all $P <$ 0.01). Whereas AKTi only modestly decreased pS6, RAD001 completely blocked it (Fig. 5D). RAD001 increased pAKT and pMAPK, and cotreatment with AKTi partially abrogated pAKT but further increased pMAPK.

The above data imply that trastuzumab is unable to inhibit mTOR. mTOR inhibition results in AKT activation, and counteracting this activation with trastuzumab or the

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AKT inhibitor improves response to treatment. These findings collectively suggest that mTOR signaling promotes resistance to HER2 inhibitors. To test this, we used small interfering RNA to knockdown *TSC2* expression, thus derepressing Rheb to activate mTOR (Fig. 6A and B). The EGFR/HER2 dual kinase inhibitor lapatinib potently inhibits PI3K signaling in HER2$^+$ cancer cell lines (41). Whereas lapatinib treatment effectively decreased pHER2, pHER3, pAKT, pMAPK, and pS6 levels, pS6 remained higher in siTSC2 cells compared with siCtl, indicating HER2-independent activation of mTOR. RAD001 treatment completely inhibited pS6, and the combination of lapatinib plus RAD001

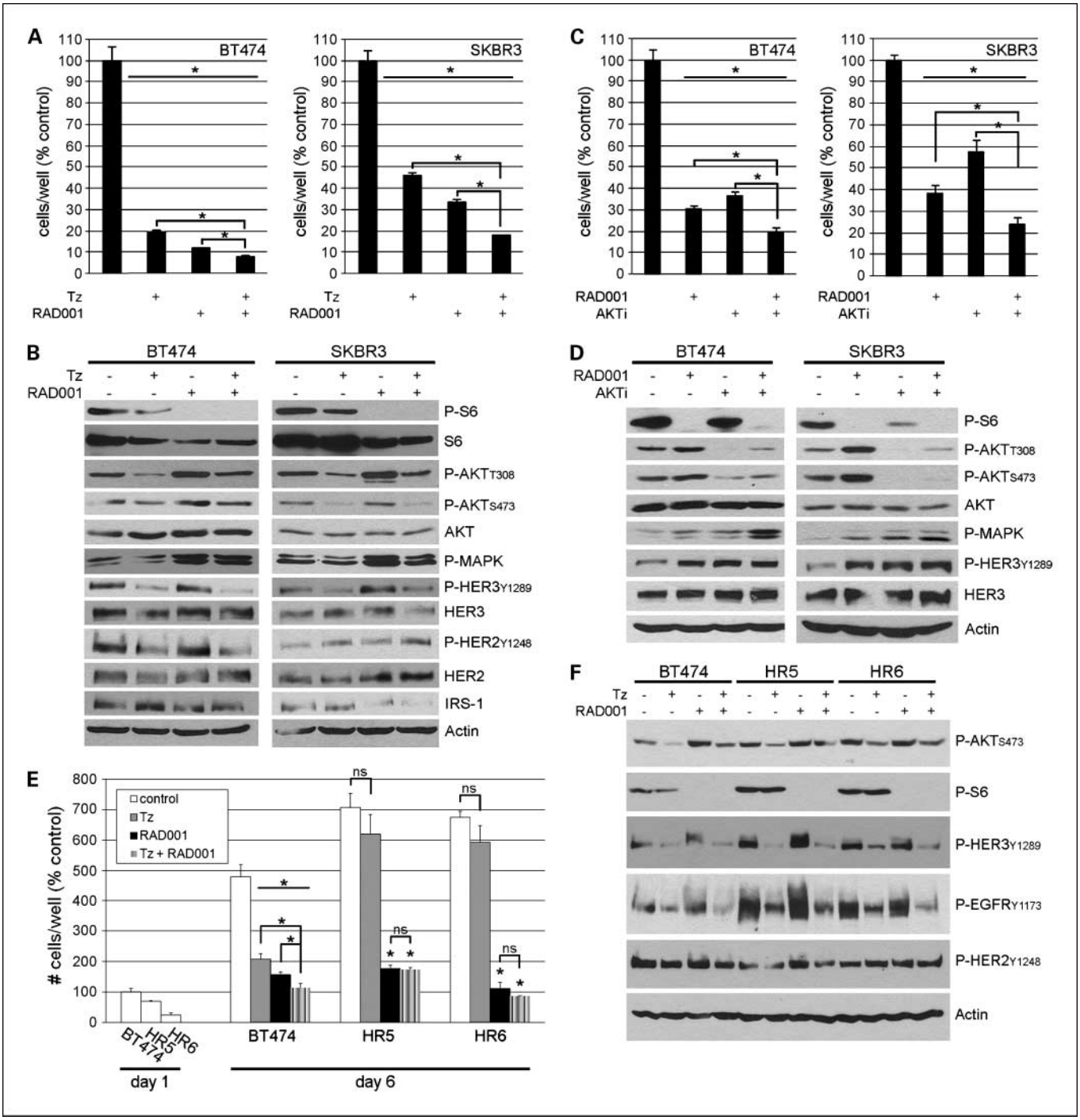

Fig. 5. RAD001 synergizes with trastuzumab to inhibit HER2$^+$ breast cancer cell growth. *A,* BT474 and SKBR3 cells were treated with or without trastuzumab, RAD001, or the combination in their respective growth medium. Cells were counted after 6 to 7 d [mean ± SD of triplicates (% control)]. *, $P < 0.005$, *t* test. *B,* immunoblot analysis of cells treated with or without trastuzumab and RAD001 under serum-free conditions for 24 h. Blots were probed with indicated antibodies. *C,* BT474 and SKBR3 cells were treated with or without RAD001, AKTi, or both. Cells were analyzed as in *A*. *, $P < 0.01$. *D,* immunoblot analysis of cells treated with or without RAD001 and AKTi under serum-free conditions for 24 h. *E,* BT474, HR5, and HR6 cells were treated as in *A* and counted after 1 or 6 d. *, $P < 0.05$. *F,* immunoblot analysis of cells treated as in *B*.

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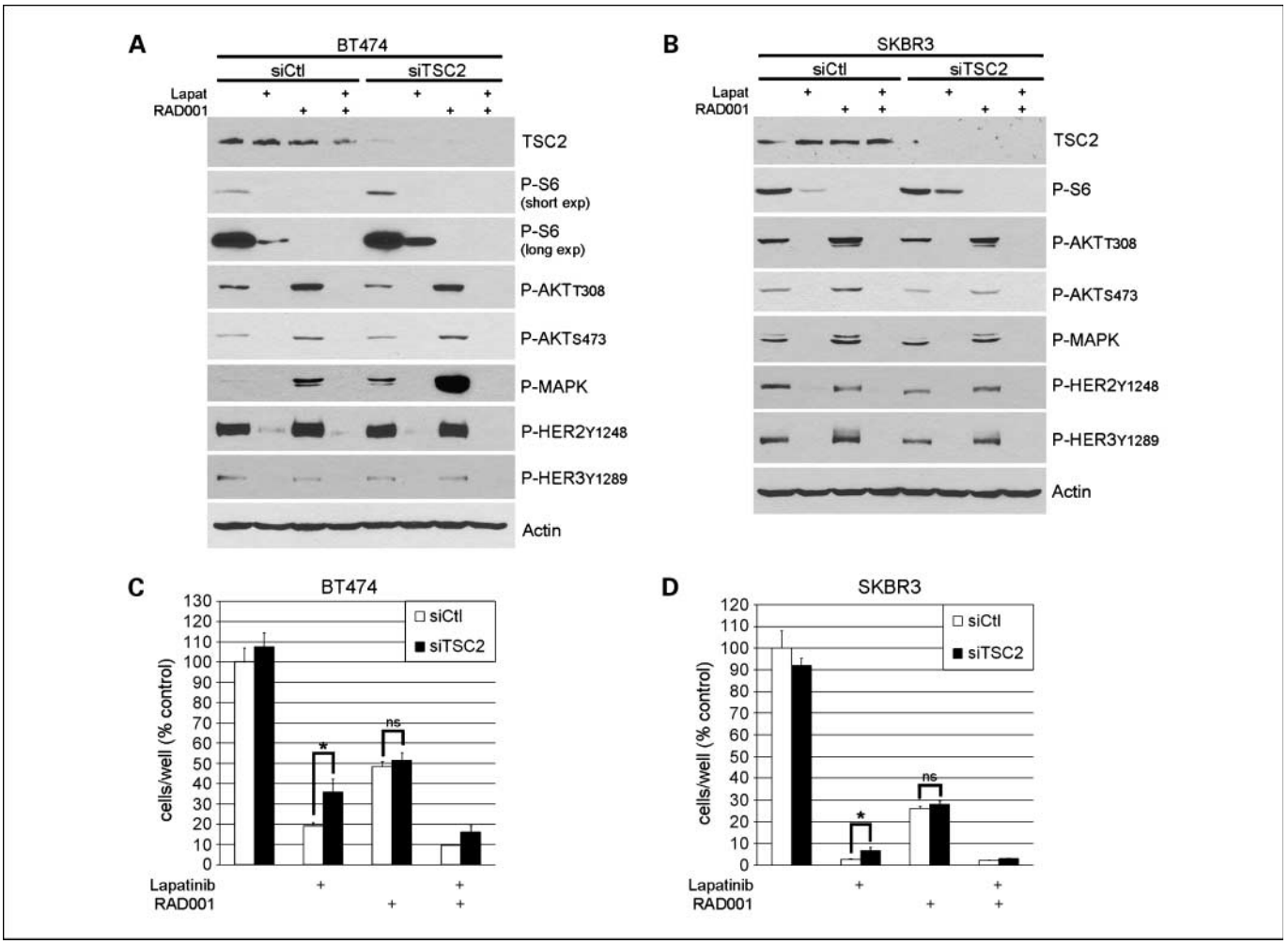

Fig. 6. Constitutive mTOR signaling attenuates response to lapatinib in HER2$^+$ breast cancer cells. *A* and *B*, immunoblot analysis of BT474 (*A*) and SKBR3 (*B*) cells transfected with small interfering RNA against *TSC2* or control (*siCtl*) and then treated with or without lapatinib, RAD001, or both under serum-free conditions for 24 h. Blots were probed with indicated antibodies. *C* and *D*, proliferation assays of cells treated as in *A* and *B* in growth medium. Cells were counted after 6 to 7 d [mean ± SD of triplicates (% control)]. *, $P < 0.05$, *t* test.

completely blocked both pS6 and pAKT. We found that *TSC2* knockdown attenuated lapatinib-induced growth inhibition (Fig. 6C and D), further implying an association between the decoupling of mTOR from HER2 upstream and the relative resistance to lapatinib. The shift in the degree of response to lapatinib conferred by *TSC2* knockdown was abrogated by RAD001 treatment, further implying that the increased growth of siTSC2 cells in the presence of the HER2 inhibitor was due to mTOR signaling. Notably, trastuzumab, lapatinib, and AKTi decreased pAKT (Figs. 5B and D and 6A and B) but did not completely block pS6, suggesting that blocking mTOR by inhibiting upstream signaling components may be less effective than inhibiting mTOR directly. These results collectively suggest that blockade of mTOR is required for the full antitumor effect of HER2 inhibitors.

Discussion

Herein, we show the complete regression of HER2-overexpressing tumors using the combination of trastuzumab and rapamycin. Treatment with the combination promptly induced tumor cell death within 1.5 days as detected by noninvasive imaging. These findings support the clinical exploration of combined trastuzumab and mTOR inhibitor therapies for the treatment of HER2$^+$ breast cancer and the use of noninvasive imaging approaches that detect tumor cell apoptosis (like Annexin-V imaging) to detect early response to therapy.

The antitumor mechanism(s) of trastuzumab action in patients with HER2$^+$ tumors remains unclear. Trastuzumab is thought to mediate antibody-dependent cellular cytotoxicity by binding Fcγ receptor III (10, 42). One week of neoadjuvant trastuzumab therapy increased apoptosis in HER2$^+$ human breast cancers with no significant effect on Ki-67 immunoreactivity (9). We found previously that 2 to 3 weeks of trastuzumab treatment also increased apoptosis in MMTV/HER2 tumors, but cell proliferation was unaffected (31). Herein, the combination of trastuzumab plus rapamycin significantly increased NIR700-Annexin-V tumor uptake as assessed by noninvasive imaging at 40 h after a single dose, suggesting rapid induction of cell death. In contrast, trastuzumab induces a

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cytostatic effect on cultured HER2$^+$ cancer cells without inducing apoptosis (Fig. 5A; Supplementary Fig. S4; ref. 11).

Whereas trastuzumab treatment did not alter MMTV/HER2 tumor cell proliferation *in vivo*, rapamycin treatment drastically decreased both pS6 levels and Ki-67 immunoreactivity after only 3 days (Fig. 3). Mosley et al. observed similar effects of rapamycin on mammary tumors in MMTV/Neu transgenic female mice, which express the rat homologue of *HER2* (43). mTOR is a critical signaling hub in the PI3K/AKT pathway. Consistent with this, rapamycin inhibits the growth of tumor cells with oncogenic mutations in *AKT* or deletion of the tumor suppressor *PTEN* (reviewed in ref. 44). We therefore speculate that mTOR inhibition blocks cell proliferation to potentiate trastuzumab effects. Interestingly, the potential immunosuppressive effects of rapamycin (45), if they occurred in our model, did not block the antitumor effect of trastuzumab. Therefore, if trastuzumab used an immune mechanism to induce tumor regression, this action was not appreciably affected by rapamycin. Additionally, rapamycin can have antiangiogenic effects on tumor vasculature and decrease tumor blood vessel permeability (23). Although we found that the combination of trastuzumab plus rapamycin induced significant tumor regression after 0.5 weeks (Fig. 2), we did not observe changes in tumor vasculature after 3 or 6 days of single or combined drug treatments (as assessed by H&E staining and CD31 immunohistochemistry). Our results are in agreement with a previous report on rapamycin treatment of mice bearing MMTV/Neu tumors (43).

Inhibition of mTOR has been shown to upregulate PI3K signaling to activate the AKT (39) and Ras/MAPK (40) pathways through derepression of feedback loops. Similarly, we observed RAD001-induced increases in pAKT and pMAPK in BT474 and SKBR3 cells *in vitro*, which may be related to an increase in pHER3 (Figs. 5 and 6). The reason that these effects were not observed in MMTV/HER2 tumors on rapamycin treatment will require further study (Fig. 3C; Supplementary Fig. S3). Whereas O'Reilly et al. found that inhibition of mTOR increased pAKT in MCF-7 and DU-145 cancer cells via an insulin-like growth factor-I receptor/IRS-1–dependent mechanism (39), we observed RAD001-induced upregulation of pHER3 but not IRS-1 in BT474 and SKBR3 cells (Fig. 5). Because trastuzumab inhibited the RAD001-induced increases in pHER3 and pAKT, we deduce that mTOR inhibition increased PI3K signaling via HER2-activated HER3. We speculate that cells upregulate intact mechanisms of PI3K activation when confronted with mTOR inhibition [via HER3 in HER2$^+$ BT474 and SKBR3 cells (Fig. 5) and via IRS-1 in HER2-low MCF-7 and DU-145 cells (39)].

mTOR inhibitors have been shown to synergize with receptor tyrosine kinase inhibitors. Treatment of cancer cells with the EGFR inhibitor erlotinib or an insulin-like growth factor-I receptor inhibitor blocked rapamycin-induced increases in pAKT, and drug combinations were more effective than single-agent treatments at inhibiting cell growth (46). RAD001 combined with the EGFR inhibitor gefitinib more effectively slowed the growth of human GEO colon cancer xenografts in nude mice than either drug alone (47). Similarly, we showed that mTOR inhibitors combined with either trastuzumab or an AKT inhibitor were more effective than any single agent. Trastuzumab plus RAD001 simultaneously suppressed PI3K and mTOR signaling as shown by reduced pAKT and pS6 levels (Fig. 5). In contrast, lapatinib plus RAD001 was only slightly more effective than lapatinib alone in BT474 cells and not in SKBR3 cells (Fig. 6). This may be related to the ability of lapatinib to potently inhibit pAKT and, in turn, significantly reduce mTOR activity. These effects were partially abrogated by *TSC2* knockdown, which conferred HER2-independent activation of mTOR. These findings collectively highlight the need to completely block both PI3K/AKT and mTOR for maximal action of anti-HER2 therapies.

We found that trastuzumab plus rapamycin induced complete regression of all MMTV/HER2 tumors (Fig. 2). In contrast, Lu et al. reported that treatment of severe combined immunodeficiency mice bearing BT474 xenografts with trastuzumab, RAD001, or the combination did not significantly affect tumor growth, although the combination showed an inhibitory trend (21). However, Lu et al. used suboptimal doses of 0.5 mg/kg trastuzumab twice weekly [effective doses in mice are 10-30 mg/kg two times a week (27, 48)]. Our conflicting observations may be attributable to differences between experimental systems, including the drugs, doses, routes of administration, tumor types, and immune competence of each animal model. For example, although BT474 xenografts in nude mice are initially sensitive to trastuzumab treatment, we found that tumors became resistant after 5 weeks (15). Given the emerging importance of the immunologic role of trastuzumab in its antitumor action (10), we cannot rule out that the synergistic effects of trastuzumab and rapamycin seen in the MMTV/HER2 model depend on an intact host immune system.

HER2$^+$ human breast tumors showed increased caspase-3 activation by immunohistochemistry after 1 week of trastuzumab therapy (9). Obtaining patient tumor tissue through serial biopsy is invasive and clinically impractical to routinely assess treatment response. Alternatively, noninvasive molecular imaging biomarkers such as Annexin-V binding and [^{18}F]FDG uptake could be valuable for clinical prediction of treatment response. We showed previously that NIR700-Annexin-V imaging correlated with tumor cell apoptosis and predicted outcome following trastuzumab therapy in MMTV/HER2 tumor-bearing mice (31). Because treatment with the combination of trastuzumab plus rapamycin induced significant tumor regression after 0.5 weeks (Fig. 2), we analyzed early induction of cell death by Annexin-V imaging after a single treatment in each of the arms. Only the combination significantly increased tumor Annexin-V uptake compared with controls (Fig. 4A). Because all tumors treated with the combination ultimately regressed completely, Annexin-V imaging was strongly predictive of outcome in this model. We also observed a trend toward decreased [^{18}F]FDG uptake after 1 week of combination treatment. Immunohistochemical analysis of cleaved caspase-3 and H&E staining after 3 and 6 days of treatment showed widespread areas of necrosis in all groups, precluding quantification of apoptotic cells. The detection of changes in cell death by conventional immunohistochemistry may be further complicated by heterogeneity between tumors, which can be circumvented by serial, noninvasive imaging of the same subjects pretreatment and post-treatment (Fig. 4). Our data support further exploration of the clinical potential of Annexin-V and [^{18}F]FDG imaging as noninvasive tools to assess early response to therapy.

In conclusion, our findings support the ongoing clinical evaluation of drug combinations simultaneously targeting

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both HER2 and mTOR. Early results from a phase I trial in patients with trastuzumab-resistant breast cancer showed that the combination of trastuzumab, RAD001, and paclitaxel induced a partial response in 5 of 7 patients, minor regression in 1 patient, stable disease in another, and prevented progression of disease in 11 of 13 patients who continued treatment (24). In another phase I trial testing the combination of trastuzumab, RAD001, and vinorelbine in 22 heavily pretreated breast cancer patients, 1 had complete response, 2 had partial response, 15 had stable disease, and 4 had progressive disease, yielding a clinical benefit rate (complete response + partial response + stable disease >24 weeks) of 55% (25). Because mTOR is a critical PI3K pathway effector and mutational activation of the PI3K pathway has been clinically associated with trastuzumab resistance (13, 14), our findings suggest that combined treatment with an mTOR inhibitor should be an effective therapeutic strategy to combat trastuzumab-resistant, $HER2^+$ cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest are disclosed.

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