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Preclinical Testing of Clinically Applicable Strategies for Overcoming Trastuzumab Resistance Caused by PTEN Deficiency

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Abstract Purpose: We have previously shown that PTEN loss confers trastuzumab resistance in ErbB2-overexpressing breast cancer using cell culture, xenograft models, and patient samples. This is a critical clinical problem because trastuzumab is used in a variety of therapeutic regimens, and at the current time, there are no established clinical strategies to overcome trastuzumab resistance. Here, we did preclinical studies on the efficacy of clinically applicable inhibitors of the Akt/mammalian target of rapamycin (mTOR) pathway to restore trastuzumab sensitivity to PTEN-deficient cells.

Experimental Design: Cell culture and xenograft models were used to test a panel of clinically applicable, small-molecule inhibitors of the Akt/mTOR signal transduction pathway, a critical pathway downstream of ErbB2, and identify compounds with the ability to restore trastuzumab sensitivity to PTEN-deficient cells.

Results: When trastuzumab was combined with the Akt inhibitor triciribine, breast cancer cell growth was inhibited and apoptosis was induced. In a xenograft model, combination therapy with trastuzumab and triciribine dramatically inhibited tumor growth. The combination of trastuzumab and the mTOR inhibitor RAD001 also slowed breast cancer cell growth *in vitro* and *in vivo*.

Conclusions: Combining trastuzumab with inhibitors of the Akt/mTOR pathway is a clinically applicable strategy and combinations of trastuzumab with triciribine or RAD001 are promising regimens for rescue of trastuzumab resistance caused by PTEN loss.

Breast cancer patients whose tumors overexpress the ErbB2 receptor tyrosine kinase have poor overall survival and shorter time to relapse (1). ErbB2 (HER2, Neu) activates the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway, which promotes proliferation and invasion and inhibits apoptosis. Trastuzumab (Herceptin; Genentech) is a humanized monoclonal antibody that targets

ErbB2. When given with adjuvant chemotherapy, trastuzumab significantly improves disease-free survival following surgical removal of ErbB2-positive breast tumors (2, 3). However, the overall response to trastuzumab is limited. Less than 30% of patients with ErbB2-positive tumors have a durable response to trastuzumab as a single agent and 2% to 5% will suffer from clinical cardiac dysfunction as a side effect (4, 5). We previously investigated the mechanisms of trastuzumab resistance and discovered an important novel mechanism. Trastuzumab treatment increased PTEN phosphatase activity and PTEN was required for the antitumor activity of trastuzumab (6). Moreover, PTEN loss predicted poor clinical response to trastuzumab-based chemotherapy in patients (6). Because PTEN opposes the actions of PI3K, we proposed a strategy to overcome trastuzumab resistance using PI3K inhibitors. We showed a synergistic inhibition of tumor growth following combination treatment of trastuzumab and wortmannin or LY294002 *in vitro* and *in vivo* (6). However, these drugs have poor clinical potential and are unlikely to become available to clinical oncologists. Both act nonspecifically on other PI3K family members (7). Wortmannin has a short half-life (8) and LY294002 causes an untoward patchy dermatitis in animal models (9). Thus, they have not been used in clinical trials despite antitumor activities *in vitro* and *in vivo* (10). In the current study, we focused on compounds with clinical potential, limiting our study to inhibitors that were either in clinical trials, under development for clinical trials, or previously tested in patients. We obtained six small-molecule inhibitors of the PI3K pathway and examined their preclinical

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efficacy in combination with trastuzumab for treating PTEN-deficient breast tumors. We identified two compounds with the ability to rescue trastuzumab sensitivity in PTEN-deficient cells.

Materials and Methods

Cell lines and cell cultures. The tumorigenic BT474.m1 subline was a gift from Dr. Dajun Yang (Georgetown University, Washington, DC). It was maintained in DMEM/Ham's F-12 (1:1) with 8% to 10% fetal bovine serum.

Antibodies and reagents. Trastuzumab was a gift from Genentech. RAD001 (everolimus) was a gift from Novartis. QLT0267 and KP 372-1 were gifts from QLT, Inc. Triciribine [6-amino-4-methyl-8-(β -D-ribofuranosyl)-4H,8H-pyrrolo[4,3,2-de]pyrimido[4,5-c]pyridazine] was purchased from Berry & Associates, Inc. Edelfosine was purchased from Calbiochem. A selective Akt inhibitor, 4ADPIB [4-amino-2-(3,4-dichloro-phenyl)-N-(1H-indazol-5-yl)-butyramide; U.S. patent 6,919340], was synthesized. PTEN antibodies were from Santa Cruz Biotechnology. β -Actin antibodies were from Sigma. All other antibodies were purchased from Cell Signaling Technology.

PTEN antisense and nonspecific oligonucleotide transient transfection. Antisense oligonucleotides specific for PTEN, control nonspecific oligonucleotides, and procedures for transfection have been described previously (6).

Cell proliferation assay. PTEN antisense/nonspecific-transfected BT474.m1 cells were plated (2,500/0.32 cm² well). Cells were treated with inhibitors of the Akt/mTOR pathway alone or in combination with trastuzumab as described for 5 days, and viable cells were measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay using the CellTiter96 AQ nonradioactive cell proliferation assay kits according to the manufacturer's protocol (Promega; ref. 6). Treated cells were compared with control DMSO-treated BT474.m1 cells to calculate percentage of growth inhibition.

APO-BrdU terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. The PTEN antisense- and nonspecific-transfected BT474.m1 cells were plated in six-well plates (4×10^5 to 6×10^5 per well). Twenty-four hours after plating, the cells were treated as indicated for 72 h with trastuzumab, triciribine, and/or RAD001. The floating and adherent cells were collected, labeled, and stained using the APO-BrdU terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay kit (Phoenix Flow Systems) according to the manufacturer's protocol. Data were collected and analyzed using a FACScan flow cytometer and CellQuest Pro 4.02 software (Becton Dickinson). At least 10,000 events were examined.

SDS-PAGE and immunoblot analysis. Cells transfected with PTEN antisense/nonspecific oligonucleotides were treated as indicated. Immunoblotting was done as described previously (6).

Xenograft human tumor model in severe combined immunodeficient mice. Female, 6-week-old, severe combined immunodeficient mice were from Taconic Farms. Tumor xenografts were done as described previously (6). When the xenograft tumors reached the average size of 100 to 150 mm³, the mice were divided into six groups, each with seven mice and an even distribution of tumor sizes, and treated as follows. PTEN antisense (30 μ g) oligonucleotides were given to each mouse weekly via intratumor injection. One week after PTEN antisense oligonucleotide administration was initiated, drug treatment began. Trastuzumab was given at a dose of 0.5 mg/kg twice weekly in 200 μ L saline through intratumor injection at multiple sites. Triciribine was given at a dose of 0.5 mg/kg/d in 200 μ L 20% DMSO saline solution through i.p. injection. RAD001 was given via gavage at a dose of 1 μ g/kg in 500 μ L 5% glucose water twice weekly. Twenty percent DMSO saline solution (200 μ L/d) was given through i.p. injection. The tumors were measured twice weekly with calipers and the volume of the tumors was calculated as follows: volume = length \times width² / 2.

Statistical analysis. One-way ANOVA was done using GraphPad Prism 3.0 for Windows (GraphPad Software).

Results

Triciribine and RAD001 potentiate growth inhibition by trastuzumab in PTEN-deficient cells. To find a strategy to overcome trastuzumab resistance, particularly resistance caused by PTEN loss, we tested six different small-molecule inhibitors, which directly or indirectly targeted the PI3K/Akt/mTOR signal transduction pathway, a major pathway activated by overexpression of ErbB2 and the loss of PTEN. Our goal was to identify compounds that would exhibit synergistic effects with trastuzumab, preferably at a low dose of the compound to minimize toxicity. The drugs we chose targeted Akt, mTOR, and integrin-linked kinase (Table 1). BT474.m1 cells are a tumorigenic subline of the BT474 breast cancer cell line and express high levels of ErbB2. When PTEN levels are decreased by transfection with PTEN antisense oligonucleotides, BT474.m1 breast cancer cells become more resistant to the antiproliferative effects of trastuzumab than cells with normal levels of PTEN and provide a good experimental model for breast cancers in which trastuzumab resistance is caused by PTEN loss (Fig. 1A and B; ref. 6). Nonspecific oligonucleotides were transfected as controls. Treatment with PTEN antisense oligonucleotides effectively lowered PTEN levels (Fig. 3A and B). Neither PTEN antisense nor nonspecific control oligonucleotides altered ErbB2 levels in the cells (Supplementary Fig. S1; data not shown).

We treated PTEN antisense- and nonspecific-transfected BT474.m1 cells with each of the six compounds or trastuzumab alone and in combination for 5 days and evaluated cell proliferation compared with DMSO-treated control. Using growth inhibition as a biological end point, we compared the ability of each drug to exhibit cooperative effects with trastuzumab using doses of drug that resulted in approximately 20% to 40% growth inhibition when given alone (Table 1).

Almost all of the compounds displayed growth-inhibitory effects, particularly at high concentrations and in cells with intact PTEN (Table 1; Supplementary Figs. S2 and S3). However, two of the compounds, triciribine and RAD001, markedly enhanced growth inhibition in the PTEN antisense cells when combined with trastuzumab compared with trastuzumab or either compound alone (Table 1). Triciribine (also called API-2), a compound that inhibits Akt activation, potentiated growth inhibition by trastuzumab over a 20-fold concentration range (Fig. 1A; data not shown). The mTOR inhibitor RAD001 (everolimus) increased growth inhibition by trastuzumab when RAD001 was given at low doses (<1 nmol/L; Fig. 1B). Strikingly, triciribine and RAD001 were able to cooperate with trastuzumab to inhibit cell growth at similar levels in the PTEN antisense and nonspecific cells (Fig. 1A and B). In essence, triciribine and RAD001 were able to restore trastuzumab sensitivity to PTEN-deficient cells. Triciribine and RAD001 were also effective as single agents, both in PTEN antisense and nonspecific cells, at doses greater than 5 μ mol/L and 1.5 nmol/L for triciribine and RAD001, respectively (Fig. 1A and B).

A third compound, the integrin-linked kinase inhibitor QLT0267, potentiated growth inhibition by trastuzumab within a narrow dose range (~5-15 μ mol/L; Table 1;

Table 1. Panel of Akt/mTOR inhibitors

Drug	Target	% Growth inhibition in PTEN antisense BT474.m1 cells			Trastuzumab + drug significantly better than either alone?
		Trastuzumab only	Drug only	Trastuzumab + drug	
Triciribine	Akt	9.5 ± 4.2	19.2 ± 9.2	36.6 ± 3.3	Yes ($P < 0.001$)
KP 372-1	Akt	0 ± 4.1	36 ± 2.1	38.8 ± 3.2	No
4ADPIB	Akt	0.9 ± 9.7	23.7 ± 2.4	32.2 ± 3.4	No
Edelfosine	Akt	2.2 ± 9.6	41.8 ± 1.2	51.9 ± 2.3	No
RAD001	mTOR	10.9 ± 4.9	19.3 ± 2.5	33.7 ± 1.4	Yes ($P < 0.05$)
QLT0267	ILK	20 ± 6.8	30.3 ± 8.4	44.8 ± 6.9	Yes ($P < 0.05$)

NOTE: Growth inhibition was assessed in PTEN antisense-transfected BT474.m1 cells. Each compound was tested individually at multiple doses (see Supplementary Figs. S2 and S3). Doses shown are as follows: triciribine, 1 $\mu\text{mol/L}$; RAD001, 0.2 nmol/L ; QLT0267, 10 $\mu\text{mol/L}$; KP 372-1, 0.05 $\mu\text{mol/L}$; 4ADPIB, 5 $\mu\text{mol/L}$; edelfosine, 7.5 $\mu\text{mol/L}$; and trastuzumab, 2 $\mu\text{g/mL}$. The SD in the percentage growth inhibition is indicated. Results shown are the combined data from two to three experiments with triplicates of each treatment within each experiment. Abbreviation: ILK, integrin-linked kinase.

Supplementary Fig. S3). Because the dose range in which QLT0267 exhibited cooperative effects with trastuzumab was narrow, we did not investigate this compound further. At concentrations $>20 \mu\text{mol/L}$, QLT0267 had no cooperative effect with trastuzumab but significantly inhibited cell growth as a single agent (Supplementary Fig. S3).

Induction of apoptosis following combination treatment. To assess if growth inhibition was accompanied by apoptosis, we treated PTEN antisense- and nonspecific-transfected BT474.m1 cells with triciribine, RAD001, and trastuzumab, alone or combined, and quantified the levels of apoptosis (Fig. 2). RAD001 did not significantly induce apoptosis alone or in combination

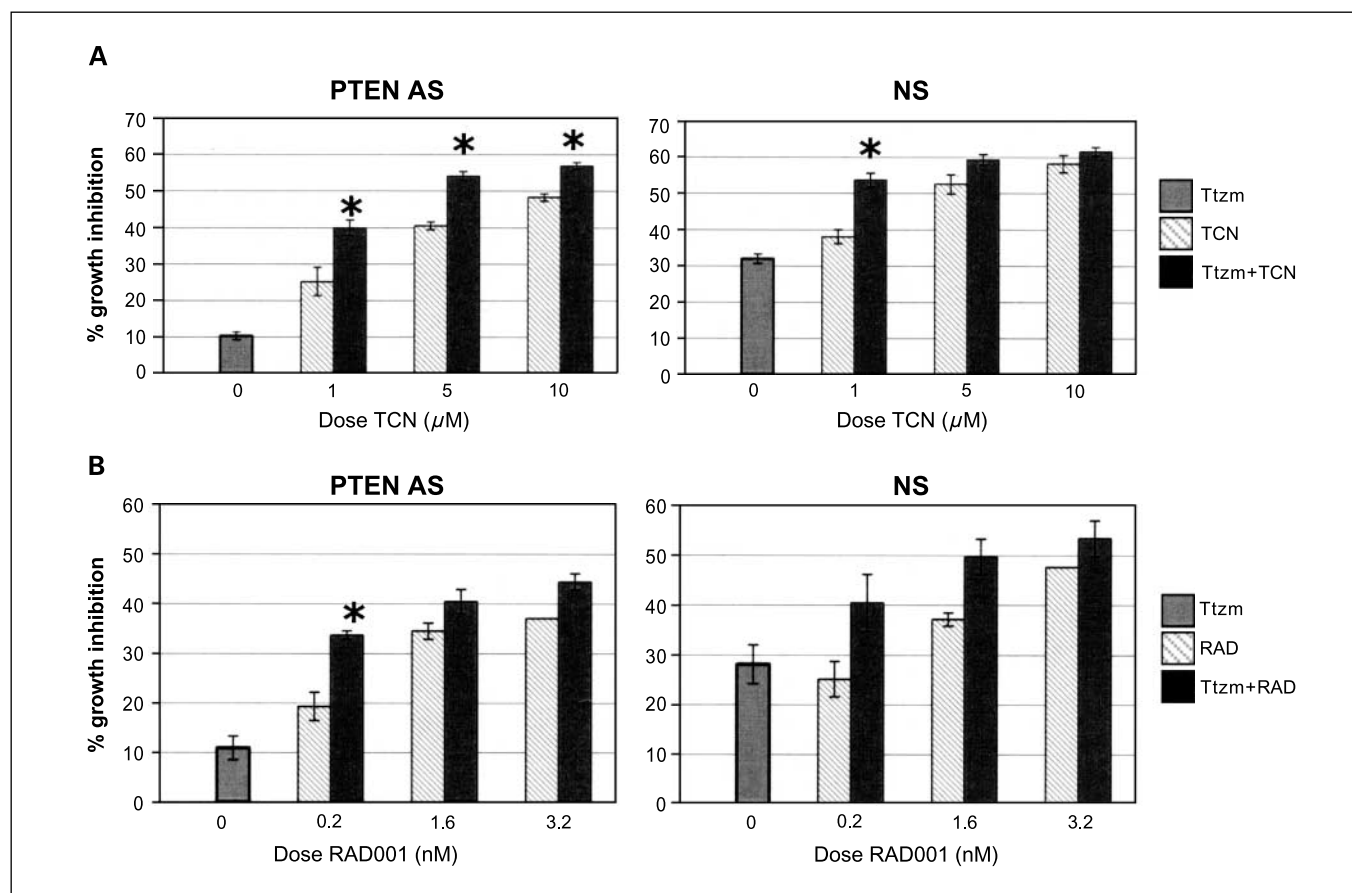


Fig. 1. Growth inhibition by the combination of trastuzumab and Akt/mTOR pathway inhibitors. PTEN antisense or nonspecific oligonucleotide-transfected BT474.m1 cells were treated with inhibitors of the Akt/mTOR pathway alone or in combination with trastuzumab, and the relative cell growth was assessed. *A*, tricycline (*TCN*) inhibits cell growth in combination with trastuzumab (*Tzm*). BT474.m1 cells were transfected with PTEN antisense (*AS*) oligonucleotide or nonspecific (*NS*) oligonucleotide, treated with trastuzumab and tricycline, alone and in combination at multiple doses of tricycline, and assayed for growth inhibition. Trastuzumab was given at a single concentration. *B*, RAD001 (*RAD*) inhibits cell growth in combination with trastuzumab. BT474.m1 cells were transfected with PTEN antisense oligonucleotide or nonspecific oligonucleotide, treated with trastuzumab and RAD001, alone and in combination at multiple doses of RAD001, and assayed for growth inhibition. Asterisk, significant difference in growth inhibition following combination treatment compared with either trastuzumab or tricycline/RAD001 alone. $P < 0.05$ was considered significant. Columns, mean; bars, SE.

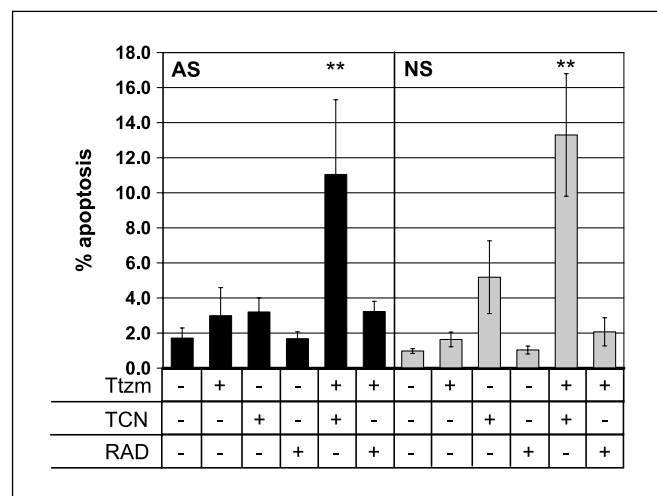


Fig. 2. Synergistic effects on apoptosis. Twenty-four hours after plating, PTEN antisense- and nonspecific-transfected BT474.m1 cells were treated as indicated with trastuzumab, tricitribine, and/or RAD001 at the following concentrations: trastuzumab, 2 μg/mL; tricitribine, 2.5 μmol/L; and RAD001, 0.4 nmol/L. APO-BrdU terminal deoxynucleotidyl transferase – mediated dUTP nick end labeling assays were done to assess apoptosis. The experiment was done thrice. Columns, mean apoptosis; bars, SD. Trastuzumab + tricitribine treatment significantly induced apoptosis ($P < 0.01$) compared with all other treatments.

with trastuzumab. Although the number of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive apoptotic cells increased slightly following treatment with trastuzumab or tricitribine alone, this increase was not statistically significant. However, the combination of tricitribine and trastuzumab significantly induced apoptosis compared with all other treatments in both PTEN antisense- and nonspecific-transfected cells (Fig. 2).

Inhibition of downstream signaling molecules. Next, we did immunoblot analysis to verify that tricitribine and RAD001 blocked activation of Akt and mTOR, important signaling molecules activated by ErbB2 and the targets of tricitribine and RAD001, respectively. Phosphorylation of Akt on Thr³⁰⁸ and Ser⁴⁷³ was analyzed as an indicator of Akt activity, and mTOR activity was assessed by the phosphorylation of p70S6K (70-kDa ribosomal protein S6 kinase), a mTOR target. After tricitribine treatment, phosphorylation of Akt on both sites was substantially decreased (Fig. 3A). In PTEN-deficient cells, the levels of Akt phosphorylation following tricitribine and trastuzumab combination treatment were similar to those seen in cells with intact PTEN (Fig. 3A, lanes 4 and 8). Thus, tricitribine overcame the adverse effects of PTEN loss by effectively blocking Akt activation.

RAD001 dramatically blocked phosphorylation of p70S6K (Fig. 3B). However, RAD001 combined with trastuzumab did not lower p70S6K phosphorylation beyond that seen with RAD001 alone (Fig. 3B). A feedback loop has been recently identified, which results in Akt phosphorylation and activation following treatment with mTOR inhibitors, such as RAD001 (11). We also observed feedback activation of Akt by RAD001 and combination therapy with trastuzumab and RAD001 eliminated Akt phosphorylation by this feedback loop (Fig. 3B, lane 3 versus lane 4), consistent with the notion that Akt activation following mTOR inhibition is dependent on upstream receptor tyrosine kinases (11). In summary, both drugs inhibited their predicted target kinases and combination treat-

ment had a larger inhibitory effect on the Akt/mTOR signaling pathway than any single agent even in PTEN-deficient cells.

Inhibition of tumor growth in a severe combined immunodeficient mice xenograft model. The above biological and molecular data were very promising; however, *in vivo* studies provide the most stringent test for therapeutic efficacy. Therefore, we tested tricitribine and RAD001 *in vivo*. BT474.m1 cell xenografts were injected into the mammary fat pad of 6-week-old severe combined immunodeficient mice. After tumors formed, the mice received PTEN antisense weekly via intratumor injection. This protocol effectively models PTEN-deficient tumors *in vivo* (6). The mice were randomized into treatment groups receiving tricitribine, RAD001, trastuzumab, or DMSO alone or in combination. After treatment, the growth patterns of the tumors treated with DMSO, trastuzumab, RAD001, or tricitribine alone were similar (Fig. 4A and B). Growth of the tumors was not inhibited and the mice were euthanized after 3 weeks due to large tumor burdens. In contrast, combination treatment with tricitribine and trastuzumab dramatically and significantly inhibited tumor growth (Fig. 4A). Many of the tumors actually decreased in size and four of seven mice had

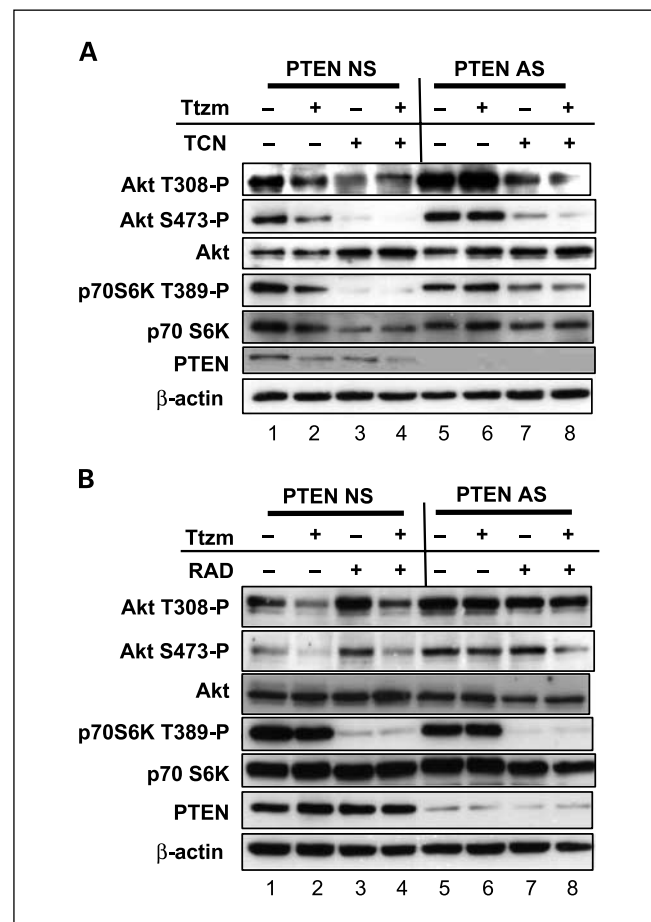


Fig. 3. Inhibition of Akt and p70S6K activity. To evaluate the effects of these drugs on the Akt/mTOR pathway, PTEN antisense and nonspecific oligonucleotides were transfected into BT474.m1 cells. Two days later, the cells were treated for 2 h with trastuzumab and tricitribine (A) or trastuzumab and RAD001 (B). Total cell lysates were collected, separated by SDS-PAGE, and immunoblotted as indicated. The concentration of trastuzumab was 2 μg/mL, tricitribine was 2.5 μmol/L, and RAD001 was 0.4 nmol/L. The experiments were repeated at least twice to insure that results were reproducible.

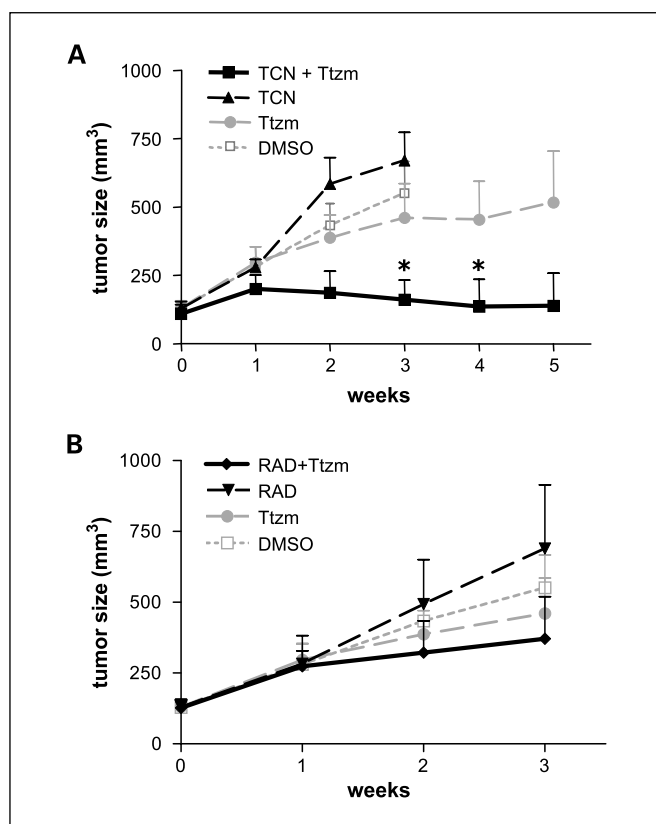


Fig. 4. Combination treatments inhibited tumor growth in a severe combined immunodeficient mice xenograft model. Severe combined immunodeficient mice received BT474.m1 breast cancer cell xenografts in mammary fat pad. The xenografts grew for 3 wk to generate tumors with an average size of 100 to 150 mm³. PTEN antisense oligonucleotides, trastuzumab, tricyclic nucleoside (A), and RAD001 (B) were given as described in Materials and Methods. The tumors were measured twice weekly with calipers and tumor size was averaged for each treatment group. Points, mean; bars, SE. Asterisk, significant difference in growth inhibition following combination treatment compared with either trastuzumab, tricyclic nucleoside, or DMSO alone. $P < 0.05$ was considered significant.

no palpable tumors after 5 weeks of treatment (data not shown). Following treatment with RAD001 and trastuzumab, tumor growth was relatively slower compared with RAD001 or trastuzumab alone (Fig. 4B). Thus, combining trastuzumab with tricyclic nucleoside or RAD001 effectively inhibited ErbB2-overexpressing, PTEN-deficient human breast cancer xenografts *in vivo*.

Discussion

Because PTEN loss is one important mechanism underlying trastuzumab resistance in ErbB2-overexpressing breast cancers, we hypothesized that targeting molecules downstream of ErbB2, PI3K, and PTEN could overcome trastuzumab resistance. After testing six currently available small-molecule inhibitors that target the Akt/mTOR pathway, we identified two, tricyclic nucleoside and RAD001, which restored trastuzumab sensitivity *in vitro* and *in vivo* to breast cancer cells that were previously trastuzumab resistant due to PTEN loss.

In addition to our strategy of combining clinically applicable PI3K/Akt pathway inhibitors and trastuzumab, several other strategies have also been proposed to overcome trastuzumab resistance based on preclinical findings (reviewed recently in

ref. 12). These include combining trastuzumab with other ErbB2-targeting antibodies, such as 2C4 (Omnitarg), or with tyrosine kinase inhibitors that block multiple members of the epidermal growth factor receptor/ErbB family, such as lapatinib (13–17); combining trastuzumab with anti-insulin-like growth factor-I receptor antibodies or insulin-like growth factor-I receptor kinase inhibitors (18, 19); and combining trastuzumab with proteasome inhibitors, such as bortezomib, which increase both PTEN and p27 expression levels (20, 21). All of these proposed strategies are supported by convincing preclinical data, and clinical trials are clearly necessary to deduce how these strategies may improve patient outcome. The potential benefits of combining trastuzumab with tricyclic nucleoside and RAD001 are discussed below.

Tricyclic nucleoside was first synthesized in 1971 (22) and identified as an antineoplastic agent (10). Phase I clinical trials on tricyclic nucleoside proved that its safety and side effects were dose dependent (10, 23, 24). However, as a single drug in phase II trials, tricyclic nucleoside failed to show efficacy against advanced breast, colon, and lung cancer even at very high doses (23, 24). A few years ago, tricyclic nucleoside was found to inhibit Akt2 activation and have antitumor activity as a single agent against tumors with activated Akt (25). Thus, phase I trials of tricyclic nucleoside have been initiated for tumors with activated Akt using much lower doses of tricyclic nucleoside than those previously used that caused hepatotoxicity (10). In our study, tricyclic nucleoside restored trastuzumab sensitivity in PTEN-deficient breast tumor cells. This new combination was cytotoxic and quite effective in our preclinical experiments. The effect may be specific to tricyclic nucleoside, as several Akt inhibitors (KP 372-1, 4ADPIB, and edelfosine) did not potentiate growth inhibition by trastuzumab. Whether this difference may be attributed to the magnitude, kinetics, or mechanism of Akt inhibition by tricyclic nucleoside or to an additional off-target activity of tricyclic nucleoside is unknown. Furthermore, our dose titration data suggest that tricyclic nucleoside may be beneficial at low doses in rationally designed combination therapies. A renaissance of the 30-year-old drug may be justified.

The mTOR inhibitor RAD001 is an orally bioavailable rapamycin derivative (26). Although, our data showed only an incremental improvement in tumor inhibition by RAD001 in combination with trastuzumab *in vivo*, this may reflect the model system, pharmacodynamics, or metabolic differences between mice and humans. Therefore, it still holds promise as a therapeutic agent because of its low toxicity and its effects at even nanomolar concentrations. Numerous phase I and II clinical trials are ongoing with RAD001 (26). RAD001 is not cytotoxic but has been found to chemosensitize p53-intact tumor cells to DNA damage-induced apoptosis through inhibition of p21 translation (27). Trastuzumab does not cause DNA damage. Thus, the combination of RAD001 and trastuzumab might be more effective *in vivo* if a cytotoxic, DNA-damaging agent was added to the cocktail or if the dose of RAD001 was increased.

In our experiments, RAD001 treatment activated Akt. Other investigators have recently reported this feedback regulation of Akt by rapamycin and its derivatives as well (11, 28, 29). This feedback activation of Akt was PI3K and insulin-like growth factor-I receptor dependent in MCF-7 and MDA-MB-468 breast cancer cells, and inhibition of insulin-like growth factor-I receptor enhanced the antitumor effects of rapamycin in those cells (11). In our experiments, combination therapy with

trastuzumab and RAD001 eliminated Akt activation following mTOR inhibition and may have enhanced the antitumor effects of RAD001. Determining the molecules downstream of ErbB2 that mediate this feedback loop is clearly an area for future investigation. Taken together, the findings suggest that mTOR inhibitors may be most effective as antineoplastic agents when combined with an inhibitor of an appropriate upstream receptor tyrosine kinase.

In conclusion, we found that combining trastuzumab with tricitriline or RAD001 may be a clinically applicable strategy to overcome trastuzumab resistance, particularly that caused by PTEN loss. Trastuzumab resistance is a clinically devastating

problem. These studies suggest a rational improvement to trastuzumab-based therapy, which could directly affect the clinical management of breast cancer patients in general and particularly those with PTEN-deficient tumors (~40%; ref. 30). Clinical trials are clearly warranted to further these studies and have been initiated.

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