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P27^{kip1} Down-Regulation Is Associated with Trastuzumab Resistance in Breast Cancer Cells

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ABSTRACT

Trastuzumab (Herceptin) is a recombinant humanized monoclonal antibody directed against HER-2. The objective response rate to trastuzumab monotherapy is 12–34% for a median duration of 9 months, by which point most patients become resistant to treatment. We created two trastuzumab-resistant (TR) pools from the SKBR3 HER-2-overexpressing breast cancer cell line to study the mechanisms by which breast cancer cells escape trastuzumab-mediated growth inhibition. Both pools maintained *her-2* gene amplification and protein overexpression. Resistant cells demonstrated a higher S-phase fraction by flow cytometry and a faster doubling time of 24–36 h compared with 72 h for parental cells. The cyclin-dependent kinase inhibitor p27^{kip1} was decreased in TR cells, and cyclin-dependent kinase 2 activity was increased. Importantly, exogenous addition of p27^{kip1} increased trastuzumab sensitivity. Additionally, resistant cells displayed heightened sensitivity to the proteasome inhibitor MG132, which induced p27^{kip1} expression. Thus, we propose that trastuzumab resistance may be associated with decreased p27^{kip1} levels and may be susceptible to treatments that induce p27^{kip1} expression.

INTRODUCTION

The *her-2* (*erb*B2/*neu*) gene is amplified and/or overexpressed in approximately 20–30% of invasive breast carcinomas and is associated with increased metastatic potential and decreased overall survival (1). Trastuzumab (Herceptin; Genentech, South San Francisco, CA) is a recombinant humanized monoclonal antibody directed against the extracellular domain of the HER-2 tyrosine kinase receptor (2). Clinical studies established that trastuzumab is active against HER-2-overexpressing metastatic breast cancers (3, 4), leading to its approval in 1998 by the United States Food and Drug Administration. However, the objective response rates to trastuzumab monotherapy are actually quite low, ranging from 12% to 34% for a median duration of 9 months (4). Currently trastuzumab is administered in combination with chemotherapies such as paclitaxel (5, 6) or docetaxel (7), which increase response rates, time to disease progression, and overall survival compared with trastuzumab monotherapy.

Although the mechanisms by which trastuzumab induces regression of HER-2-overexpressing tumors are incompletely defined, several molecular and cellular effects have been observed *in vitro* (reviewed in Ref. 8). Trastuzumab and the murine monoclonal antibody 4D5, from which trastuzumab is derived, induce HER-2 receptor internalization and degradation in a dose-dependent manner in the BT474 and SKBR3 HER-2-overexpressing breast cancer cell lines (9, 10). Cells treated with trastuzumab undergo arrest during the G_1 phase of the cell cycle, with a concomitant reduction in proliferation due in part to induction of the cyclin-dependent kinase (cdk) inhibitor p27^{kip1} and increased formation of p27^{kip1}-cdk2 complexes (10–12).

Trastuzumab clearly offers clinical benefit to a subset of HER-2-positive breast cancers. However, the majority of patients who achieve an initial response generally acquire resistance within 1 year (13). Elucidating mechanisms by which tumors escape the cytotoxic properties of trastuzumab is critical to improving the survival of metastatic breast cancer patients whose tumors overexpress HER-2. In this study we found that trastuzumab-resistant (TR) breast cancer cells derived from the SKBR3 HER-2-overexpressing line have decreased p27^{kip1} levels in association with increased cdk2 activity and increased proliferation rate. Furthermore, we demonstrate that ectopic expression of p27^{kip1} increases trastuzumab sensitivity of resistant cells and that resistant cells display heightened sensitivity to pharmacological induction of p27^{kip1}. Our findings suggest that a subset of TR breast cancer cells may be susceptible to treatments that induce p27^{kip1} expression.

MATERIALS AND METHODS

Materials. Trastuzumab (Genentech) was dissolved in sterile water at 20 mg/ml. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2*H*-tetrazolium CellTiter 96 Aqueous One Solution proliferation reagent (Promega, Madison, WI) was used in accordance with manufacturer guidelines. MG132 (Calbiochem, EMD Biosciences, Inc., San Diego, CA) was dissolved as a 10 mM stock in DMSO.

Cell Culture. SKBR3 breast cancer cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 10% FCS. TR SKBR3 pools were developed as described previously (14) by continuously exposing cells to trastuzumab (4 μg/ml for pool 1 and 8 μg/ml for pool 2) for 3 months, at which point cells regained morphology similar to the parental line. Cells per plate were then pooled together and tested for dose response to trastuzumab as described below. Pools are now maintained in 4 μg/ml trastuzumab, a concentration at which parental cells are not viable. For serum starvation experiments, cells were maintained overnight in OPTIMEM I reduced serum medium (Life Technologies, Inc., Gaithersburg, MD).

Dose-Response Studies and Growth Curves. SKBR3 parental cells and TR pools were seeded at 5×10^4 cells/well in 12-well dishes. After 24 h cells were treated in triplicate with 2-fold serial dilutions of trastuzumab at doses ranging from 1 μg/ml to 32 μg/ml. Cells were trypsinized after 5 days, stained with trypan blue dye, and viable cells were counted by microscopic examination. For MG132 studies, cells were exposed to 2-fold concentrations ranging from 100 nM to 800 nM for 48 h. The results of trypan blue assays were confirmed by exposing parallel cultures to the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2*H*-tetrazolium reagent and measuring absorbance in a microplate reader as directed by the manufacturer. All of the experiments were performed in triplicate and repeated at least three times. Growth inhibition for all of the experiments is expressed as the percentage of viable cells compared with untreated cultures. For growth curves, SKBR3 parental cells and TR pools were seeded at 5×10^4 cells/well in 12-well dishes. After 24, 48, or 72 h cells were trypsinized, stained with trypan blue dye, and counted by microscopic examination.

Fluorescent *in Situ* Hybridization. Cytospin slides of parental and resistant cells were prepared. The PathVysion HER-2 DNA Probe kit (Vysis, Inc., Downers Grove, IL) was used as directed by the manufacturer. Briefly, a DNA probe that spans the entire *her-2* gene labeled in SpectrumOrange and a *cep-17* probe (specific for the α satellite DNA located at the centromere of chromo-

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some 17, 17p11.1-q11.1) labeled in SpectrumGreen were hybridized onto slides. Inclusion of the *cep-17* probe allows for the relative copy number of the *her-2* gene to be determined. Twenty nuclei were assessed for *her-2* and *cep-17* copy number, and the numbers were averaged for each gene. The ratio of average *her-2* to average *cep-17* copy number was then calculated. A normal average *her-2*:*cep-17* ratio is generally defined as 2; a ratio >2 indicates gene amplification.

Cell Cycle Analysis. Untreated parental SKBR3 cells, parental cells treated with 4 μg/ml trastuzumab for 72 h, and TR pools maintained in 4 μg/ml trastuzumab were fixed overnight in 70% ethanol and resuspended in propidium iodide (50 μg/ml) supplemented with RNase A (1 μg/ml). DNA content was measured using a FACScan cytometer (Becton Dickinson, Franklin Lakes, NJ).

Immunoblotting. Protein lysates were obtained using 1% NP40 lysis buffer [150 mM NaCl, 50 mM Tris (pH 8.0), and 1% NP40] and immunoblotted (50 μg) for p27kip1 (monoclonal SX53G8; DAKO, Carpinteria, CA), HER-2 (Ab-3; Oncogene Research Products, EMD Biosciences, Inc., San Diego, CA), or β-actin (Santa Cruz Biotechnology). Each primary antibody was used at a 1:1000 dilution in 5% nonfat milk in PBS-Tween. Secondary antibodies were chosen according to the species of origin of the primary antibody and detected using enhanced chemiluminescence (Amersham-Pharmacia Biotech, Piscataway, NJ).

Immunoprecipitation and Kinase Assays. For immunoprecipitation-immunoblot analyses, parental and resistant cells were lysed with 1% NP40 buffer. Total protein extracts (200 μg) were precleared with protein G-agarose for 1 h, and cdk2 was immunoprecipitated (polyclonal-agarose conjugate; Santa Cruz Biotechnology) overnight, washed with 1% NP40 buffer, and immunoblotted for cdk2 (monoclonal; Santa Cruz Biotechnology) and p27kip1 (monoclonal SX53G8; DAKO). Incubating lysates with normal rabbit IgG overnight produced immunoprecipitation controls. Because the nuclear protein histone H1 is a substrate of phosphorylation by the cyclin E/cdk2 complex, the kinase activity of the complex can be evaluated by the detection of radiolabeled histone H1 protein (histone H1 kinase assay). In the current study, to measure the phosphorylation activity of p27-associated cdk2, histone H1 kinase assay was performed. Cdk2 was immunoprecipitated from total protein extracts (200 μg) overnight and incubated with [32P]dATP, ATP, and histone H1 (Sigma) in kinase buffer. Samples were analyzed by SDS-PAGE and autoradiography, and relative cdk2 kinase activity was measured by quantitating [32P]histone H1 using the NIH Image program. Experiments were performed in duplicate.

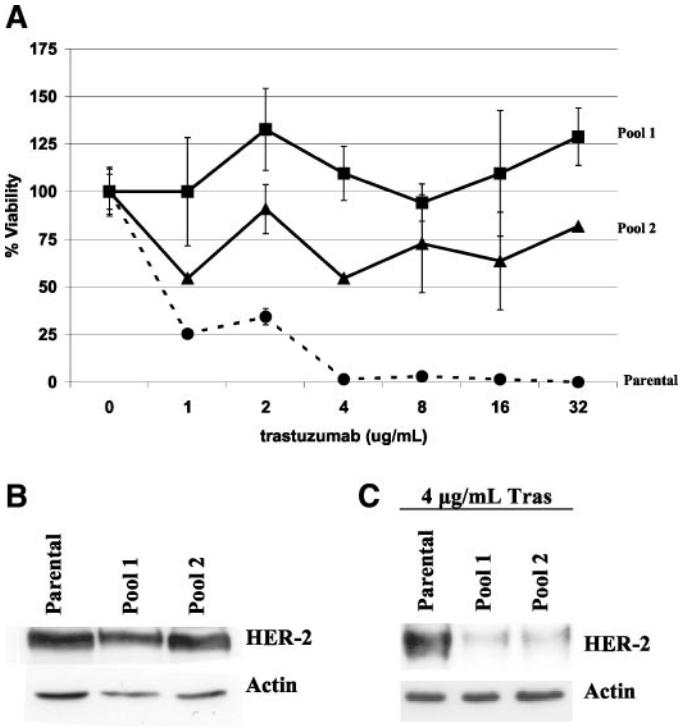

Fig. 1. Development of HER-2-overexpressing trastuzumab-resistant SKBR3-derived pools. *A*, SKBR3 parental cells (···· with ●) and two pools of SKBR3 cells cultured for 3 months in 4 μg/ml (pool 1; ——— with ■) or 8 μg/ml (pool 2; ——— with ▲) of trastuzumab were treated in triplicate with 2-fold serial dilutions of trastuzumab at doses ranging from 1 μg/ml to 32 μg/ml. After 5 days cells were trypsinized and counted by trypan blue exclusion. Results are shown as the percentage of viable cells compared with untreated control cultures for each cell line. All experiments were repeated at least three times. *B*, SKBR3 parental cells and resistant pools (in the absence of trastuzumab) were lysed for protein and immunoblotted (50 μg) for HER-2 and β-actin. *C*, SKBR3 parental cells and resistant pools were maintained in trastuzumab (4 μg/ml), lysed for protein, and immunoblotted (50 μg) for HER-2 and β-actin; *bars*, ±SD.

Table 1 *FISH*[a] *analysis of her-2 gene copy number in SKBR3 parental and TR cells*

Gene copy numbers for *her-2* and *cep-17* were determined for parental and resistant pools using the PathVysion HER-2 DNA Probe kKit (Vysis, Inc.) Twenty nuclei were counted for *her-2* and *cep-17*, which were visualized by hybridizing with specific DNA probes each linked to a different fluorophore. Counts for each gene were averaged, and the ratio of average *her-2* to average *cep-17* copy number was calculated.

Cell	Parental	Pool 1	Pool 2
Ratio of average *her-2* to average *cep-17* gene copy number[b]	3.26	3.70	3.53

[a] FISH, fluorescence *in situ* hybridization.
[b] A *her-2*:*cep-17* ratio >2 indicates *her-2* gene amplification.

Transfection Assays. Parental and resistant cells were transiently transfected with 25 ng or 100 ng of pCMV-FLAG-p27kip1 (provided by Dr. Mong-Hong Lee, M.D. Anderson Cancer Center) or 100 ng of an empty pCMV vector as a control for 48 h using Lipofectamine (Invitrogen). At that time, cells were either lysed for protein and immunoblotted for p27kip1 (monoclonal SX53G8, DAKO) or else incubated with trastuzumab (1, 5, or 10 μg/ml) for 72 h. Trastuzumab-treated p27kip1-transfected and trastuzumab-treated control vector-transfected cells were counted by trypan blue exclusion assay. Cell viability is reported as a percentage of control cells transfected with the pCMV-FLAG-p27kip1 or the empty pCMV vector but not treated with trastuzumab.

RESULTS

Development of TR SKBR3 Pools. HER-2-overexpressing SKBR3 breast cancer cells were continuously exposed to trastuzumab for 3 months, at which point cells regained morphology similar to the parental line. Two different doses of trastuzumab (4 μg/ml for pool 1 and 8 μg/ml for pool 2) were used in separate cultures to ensure that cells were exposed to a high enough dose to kill all of the sensitive cells. Cells per plate were then pooled together. Sensitivity to trastuzumab was determined by treating pools with 2-fold serial dilutions of the drug and performing trypan blue exclusion assays after 5 days. Growth inhibition is expressed as the percentage of viable cells compared with untreated cultures (Fig. 1*A*). A dose of 4 μg/ml trastuzumab was lethal to parental cells, but the majority of pool 1 and 2 cells remained viable. Thus, these pools demonstrated resistance to trastuzumab and are now maintained in 4 μg/ml trastuzumab. Trastuzumab resistance was also confirmed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2*H*-tetrazolium colorimetric assay (Promega; data not shown). To ensure that cells were specifically resistant to trastuzumab, we treated resistant and parental cells with an anti-epidermal growth factor receptor (EGFR) antibody (data not shown). Resistant and parental cells were similarly sensitive to EGFR blockade, suggesting that there is not cross-resistance to another antibody, but that the resistance is specific for trastuzumab.

To eliminate the possibility that we had selected a population of cells that do not possess *her-2* gene amplification, fluorescent *in situ* hybridization was performed for both *her-2* and *cep-17* using the PathVysion HER-2 DNA Probe kit. The ratio of the average *her-2* gene copy number to the average *cep-17* gene copy number was similar among all three of the samples (Table 1), demonstrating that both TR pools possess *her-2* amplification at the same level as parental cells. Immunoblotting additionally demonstrated that resist-

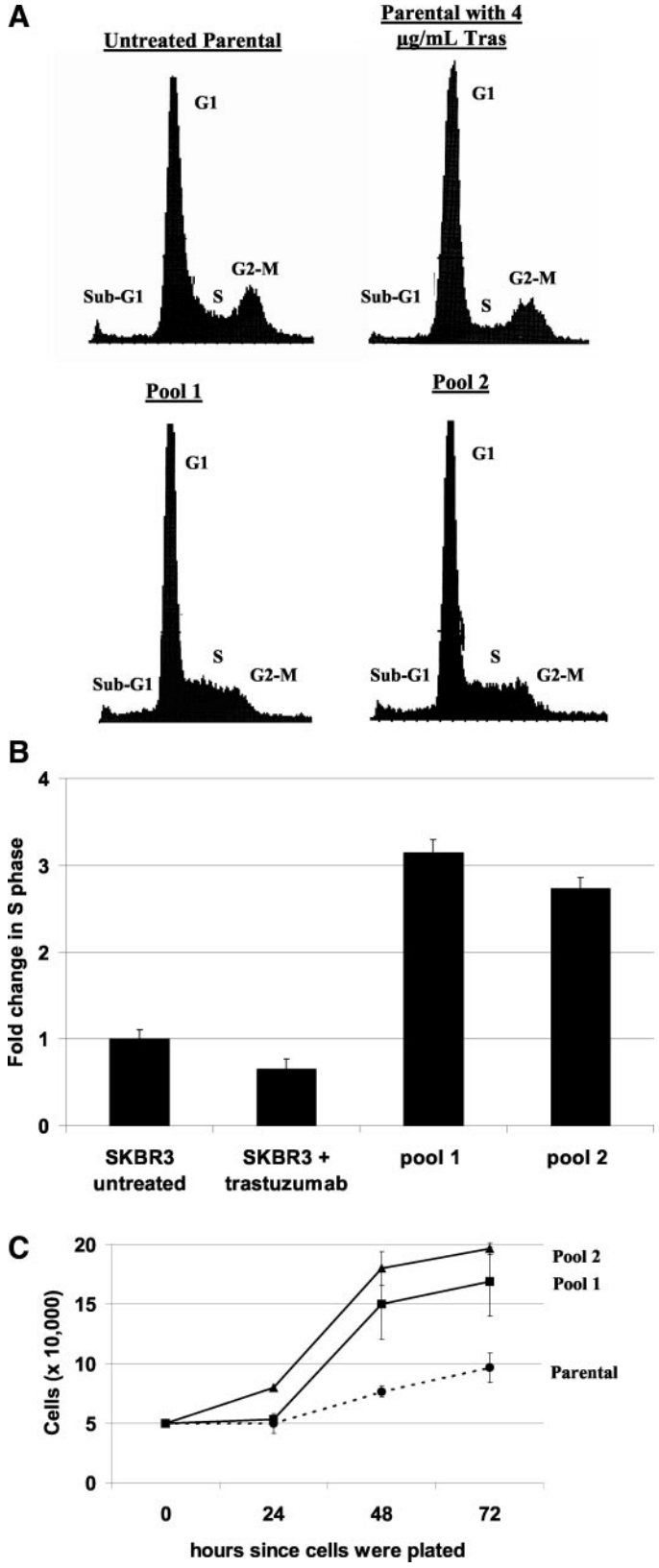

Fig. 2. Trastuzumab-resistant pools display increased proliferation. *A*, duplicate cultures of untreated SKBR3 parental cells, parental cells treated with 4 μg/ml trastuzumab for 72 h, and trastuzumab-resistant pools 1 and 2 maintained in 4 μg/ml trastuzumab were fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry. Representative cell cycle profiles are shown for each cell line. *B*, the fold change in the percentage of S-phase cells relative to untreated parental cells is shown on the *Y* axis. Cell cycle analyses were repeated at least three times. *C*, SKBR3 parental cells (···· with ●) and trastuzumab-resistant pools (pool 1, ——— with ■ and pool 2, ——— with ▲) were seeded in triplicate at 5×10^4 cells/well in 12-well dishes. After 24, 48, or 72 h cells were trypsinized, stained with trypan blue dye, and counted by microscopic examination. Growth curves are plotted as the number of cells (×10,000) *versus* the number of hours since cells were plated; *bars*, ±SD.

Table 2 *Cell cycle percentages for SKBR3 parental and trastuzumab-resistant cells*

Untreated SKBR3 parental cells, parental cells treated with 4 μg/ml Tras[a] for 72 h, and Tras-resistant pools 1 and 2 maintained at 4 μg/ml Tras were fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry. The percentage of cells in each cell cycle phase is shown.

Cell	Sub-G_1	G_1	S	G_2-M
Parental	5.48 ± 0.16	58.84 ± 3.22	10.72 ± 1.08	25.64 ± 0.49
Parental + Tras	5.14 ± 0.04	63.9 ± 0.02	6.99 ± 1.25	25.04 ± 1.27
Pool 1	8.34 ± 0.51	59.87 ± 0.09	33.69 ± 1.63	6.44 ± 1.55
Pool 2	7.48 ± 0.78	59.05 ± 3.26	29.33 ± 1.31	11.62 ± 1.95

[a] Tras, trastuzumab.

ant pools that have been maintained in the absence of trastuzumab for 96 h express HER-2 at levels equivalent to parental cells (Fig. 1*B*). Additionally, HER-2 levels were down-regulated when the resistant pools were maintained in trastuzumab (Fig. 1*C*), suggesting that the molecular mechanisms contributing to resistance lie downstream of the receptor.

TR Cells Have an Increased Proliferation Rate. Cell cycle analysis of untreated parental cells, parental cells treated with 4 μg/ml trastuzumab for 72 h, and TR pools maintained in 4 μg/ml trastuzumab was performed by flow cytometry. Representative cell cycle profiles are shown for each cell line (Fig. 2*A*). Trastuzumab produced a 1.5-fold decrease in the percentage of proliferating parental cells (Fig. 2*B*) with a modest increase in G_1-phase cells (Table 2). The percentage of G_1 cells for each pool was similar to the untreated parental cells. However, the S-phase fraction in the TR pools was three times higher than that of untreated parental cells. Concomitant with increased proliferation was a 3-fold decrease in G2-M cells *versus* untreated parental cells. Growth curves (Fig. 2*C*) additionally demonstrated that the doubling time for TR pools is 2–3-fold faster than for parental cells, as TR pools double in 24–36 h compared with 72 h for the parental cells. These results indicate that TR SKBR3-derived cells have an increased S-phase fraction and reduced doubling time, and confirm that these pools are resistant to trastuzumab-mediated inhibition of proliferation.

TR Cells Express Reduced Levels of p27^{kip1}. The growth-inhibitory properties of trastuzumab depend in part on effects on the cdk-inhibiting protein p27^{kip1}. Trastuzumab increases the half-life of p27^{kip1} by decreasing cyclin E/cdk2-mediated phosphorylation of p27^{kip1} and blocking subsequent ubiquitin-dependent degradation (15). Trastuzumab also increases association between p27^{kip1} and cdk2 complexes resulting in G_1 arrest (12). Importantly, small interfering RNA that reduced p27^{kip1} expression levels also blocked trastuzumab-mediated growth arrest in SKBR3 cells (15). Thus, we examined expression levels of p27^{kip1} in our TR SKBR3 model. Parental cells treated with 4 μg/ml trastuzumab over a 72 h time course demonstrated induction of the p27^{kip1}protein (Fig. 3*A*). Importantly, both of the TR pools expressed reduced levels of p27^{kip1} in comparison with untreated parental cells, with pool 2 showing the greatest reduction. To ensure that the reduction in p27^{kip1} was not merely a reflection of the increase in S phase, cell proliferation was blocked by incubating overnight in reduced serum medium. Immunoblotting serum-starved cells for p27^{kip1} confirmed that TR cells express lower levels of p27^{kip1} than parental cells (Fig. 3*B*).

Cdk2 Kinase Activity Is Elevated in TR Cells. The p27^{kip1} protein binds and inhibits cdk2 complexes. Thus, we examined the interaction between p27^{kip1} and cdk2 and measured cdk2 kinase activity in the TR pools. Cdk2 was immunoprecipitated from parental

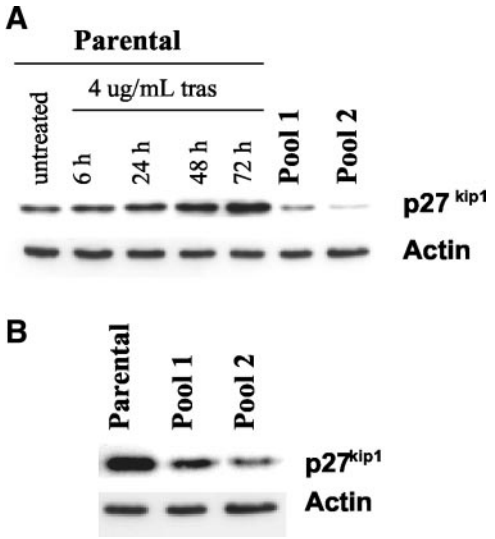

Fig. 3. Trastuzumab-resistant cells express reduced levels of $p27^{kip1}$. *A*, SKBR3 parental cells untreated or treated with 4 μg/ml trastuzumab (*tras*) for 6, 24, 48, or 72 h, and pool 1 and 2 cells maintained at 4 μg/ml trastuzumab were lysed for protein. Total protein (50 μg) was immunoblotted for $p27^{kip1}$ and β-actin. *B*, parental and resistant cells were incubated in reduced serum medium overnight. Total protein (50 μg) was immunoblotted for $p27^{kip1}$ and β-actin.

and resistant cells and immunoblotted for $p27^{kip1}$ (Fig. 4*A*). Incubating lysates with normal rabbit IgG produced immunoprecipitation controls. Levels of cdk2 were quantitated and normalized, and the ratio of $p27^{kip1}$ to cdk2 was then determined. In comparison with parental cells, pools 1 and 2 displayed ~30% less $p27^{kip1}$ bound to cdk2. To determine whether this correlated with decreased inhibition of cdk2 activity, immunoprecipitation–cdk2 radioactive kinase assays were performed, using histone H1 as a substrate (Fig. 4*B*). Pool 1 displayed a very modest 1.7-fold increase in phosphorylated histone H1, and pool 2 showed a 2.9-fold elevated cdk2 kinase activity *versus* parental cells. These results were consistent with $p27^{kip1}$expression data, which showed reduced levels in both pools, but a greater reduction in pool 2. Thus, both TR pools harbor elevated cdk2 kinase activity in association with reduced $p27^{kip1}$levels, with pool 2 showing the greatest difference *versus* parental cells.

Expression of $p27^{kip1}$ Restores Trastuzumab Sensitivity to TR Pools. To determine whether reduced $p27^{kip1}$ contributes to trastuzumab resistance in our cell model, a mammalian expression vector containing a FLAG-$p27^{kip1}$ construct was transiently transfected into parental and resistant cells. A representative immunoblot of parental cells transfected with 25 ng or 100 ng of CMV-FLAG-$p27^{kip1}$ or with 100 ng of the empty vector control for 48 h demonstrated that FLAG-$p27^{kip1}$, which migrates higher than endogenous $p27^{kip1}$, is expressed efficiently in cells receiving pCMV-FLAG-$p27^{kip1}$ (Fig. 5*A*). Immunoblotting also indicated that 25 ng and 100 ng of CMV-FLAG-$p27^{kip1}$ encompass the level of endogenous $p27^{kip1}$ found in parental cells. Cells transfected with 100 ng control vector or 25 ng or 100 ng of pCMV-FLAG-$p27^{kip1}$ for 48 h were then treated with 1, 5, or 10 μg/ml trastuzumab for 72 h, and counted by trypan blue exclusion (Fig. 5*B*). Expression of $p27^{kip1}$ increased the sensitivity of parental and resistant cells to trastuzumab. Parental cells transfected with 25 ng FLAG-$p27^{kip1}$ were similarly sensitive to trastuzumab as control transfectants, probably because these cells already express relatively high levels of endogenous $p27^{kip1}$, at least compared with the resistant cells. FLAG-$p27^{kip1}$ at 100 ng concentration increased the sensitivity of parental cells. The trastuzumab sensitivity of both TR pools 1 and 2 dramatically increased when $p27^{kip1}$ was transfected at either 25 ng or 100 ng. These data suggest that reduced levels of $p27^{kip1}$ may be a major mechanism of trastuzumab resistance in these cells. However, it is not likely to be the only mechanism. The slightly different responses of pool 1 *versus* pool 2 support the concept that mechanisms contributing to trastuzumab resistance are likely to vary among different cancer cell populations but that reduced $p27^{kip1}$ expression may be one important mechanism.

TR Pools Demonstrate Increased Sensitivity to Pharmacological Induction of $p27^{kip1}$. The peptide aldehyde MG132 (Cbz-leu-leu-leucinal) inhibits proteasome-mediated degradation of multiple cell cycle regulating proteins including $p27^{kip1}$ (16). Parental and resistant pools were treated with MG132 for 48 h and counted by trypan blue exclusion (Fig. 6*A*). Both pools demonstrated increased sensitivity to MG132 relative to parental cells, with ~50% of resistant cells viable at 400 nM MG132 *versus* ~90% of parental cells. This dose of MG132 induces $p27^{kip1}$ expression to a similar degree in resistant and parental cells (Fig. 6*B*). Hence, resistant cells appear to be more sensitive to induction of $p27^{kip1}$ *versus* parental cells. These results support the finding that $p27^{kip1}$ expression inhibits the survival of these TR cells.

DISCUSSION

In the clinical setting, trastuzumab achieves an objective response rate of up to 34% for 9 months when administered as a first-line agent in HER-2-overexpressing metastatic breast cancers (4). The remaining cases do not demonstrate tumor regression (17). Furthermore, tumors that initially respond to trastuzumab begin to progress again within 1 year (4). We have created TR cells to determine potential mechanisms by which tumors escape trastuzumab-mediated growth inhibition and to identify novel agents that may overcome this resistance.

Trastuzumab-mediated growth inhibition requires expression of $p27^{kip1}$, as small interfering RNA against $p27^{kip1}$ blocked growth arrest of SKBR3 cells exposed to trastuzumab (15). Thus, we examined whether reduced $p27^{kip1}$ expression contributed to trastuzumab resistance in our SKBR3 cell model. We demonstrated here that SKBR3 TR cells have an increased S-phase fraction associated with

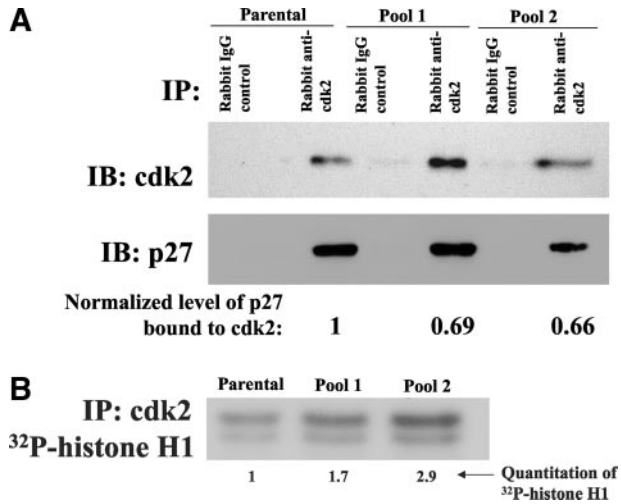

Fig. 4. Trastuzumab-resistant cells exhibit elevated cyclin-dependent kinase (*cdk*)2 activity. SKBR3 parental and resistant cells were lysed for protein. *A*, total protein lysates (200 μg) were precleared and immunoprecipitated overnight with an anti-Cdk2 rabbit antibody or a rabbit IgG control. After washing immunoprecipitates were immunoblotted with an anti-cdk2 monoclonal or an anti-$p27^{kip1}$ monoclonal antibody. Level of cdk2 was quantitated and normalized between samples; then, the ratio of p27 to cdk2 was determined per sample. *B*, cdk2 was immunoprecipitated from total protein extracts (200 μg) overnight and incubated with [^{32}P]dATP, ATP, and histone H1 in kinase buffer. Samples were analyzed by SDS-PAGE and autoradiography, and relative cdk2 kinase activity was measured by quantitating [^{32}P]histone H1 using the NIH Image program.

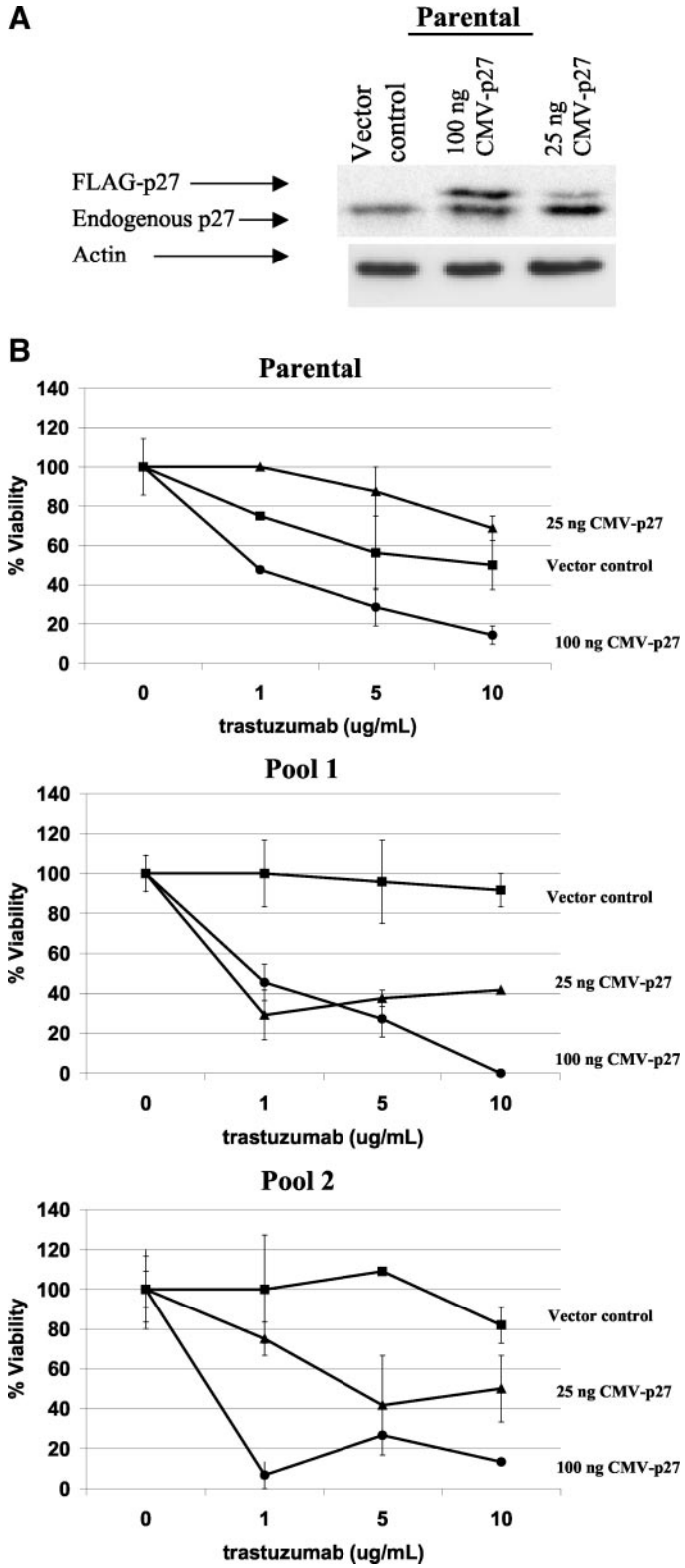

Fig. 5. $p27^{kip1}$ restores trastuzumab sensitivity to trastuzumab-resistant pools. *A*, parental, pool 1, and pool 2 cells were seeded at 5×10^4, and transiently transfected with 25 ng or 100 ng of pCMV-FLAG- $p27^{kip1}$ or 100 ng empty pCMV vector control for 48 h. Cells were lysed for protein and immunoblotted (25 μg) for $p27^{kip1}$ and β-actin. A representative immunoblot of transfected parental cells is shown. Transfected $p27^{kip1}$ migrates higher than endogenous $p27^{kip1}$ due to FLAG tag conjugate. *B*, cells transfected for 48 h were then treated with 1, 5, or 10 μg/ml trastuzumab and counted by trypan blue exclusion after 72 h. Cell viability is presented as the percentage of viable cells *versus* untreated cells that were transfected with the empty pCMV vector control or pCMV-FLAG- $p27^{kip1}$; *bars*, ±SD.

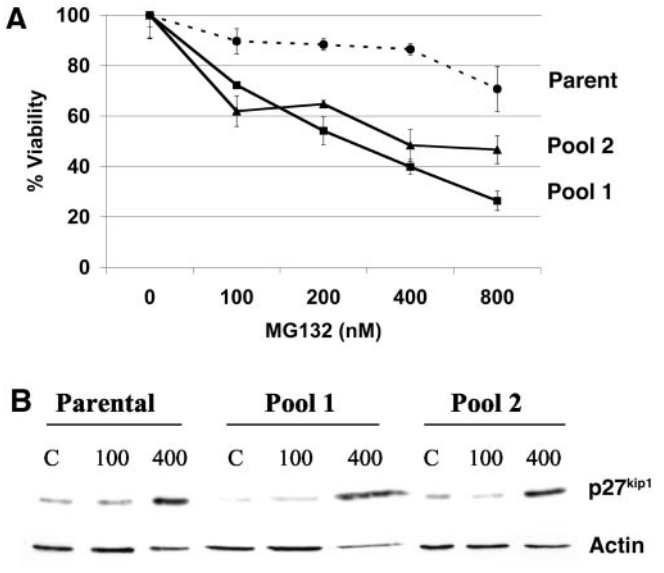

Fig. 6. Trastuzumab-resistant (TR) pools are sensitive to pharmacological induction of $p27^{kip1}$ by the proteasome inhibitor MG132. *A*, parental and TR pools were treated in triplicate with 2-fold serial dilutions of MG132 at doses ranging from 100 nM to 800 nM. After 48 h cells were trypsinized and counted by trypan blue exclusion. Results are shown as the percentage of viable cells compared with untreated control cultures for each cell line. All experiments were repeated at least three times. *B*, parental and TR pools were treated with DMSO as a control (*C*) or MG132 at 100 nM or 400 nM for 48 h, at which point cells were lysed for protein and immunoblotted for $p27^{kip1}$ and actin; *bars*, ±SD.

reduced $p27^{kip1}$ levels and increased cdk2 kinase activity relative to parental cells. Expression of $p27^{kip1}$ restored trastuzumab sensitivity in the resistant cells, suggesting that $p27^{kip1}$ contributes to trastuzumab resistance. Sensitivity of TR pools to trastuzumab was restored close to parental levels upon transfection with $p27^{kip1}$. Additionally, both pools responded similarly to the proteasome inhibitor MG132, which induces $p27^{kip1}$ expression, exhibiting increased sensitivity *versus* parental cells.

$p27^{kip1}$ is a distal downstream effector of multiple converging growth factor receptor pathways including EGFR, HER-2, and IGF-IR. Hence, down-regulation of $p27^{kip1}$ in our resistant cells is possibly a reflection of aberrant signaling from one of these receptor pathways or from a middle signaling molecule such as phosphatidylinositol 3′-kinase or mitogen-activated protein kinase. Studies are ongoing to evaluate these possibilities. Nonetheless, the finding of reduced $p27^{kip1}$ levels in these cells provides a possible marker of trastuzumab resistance and a putative therapeutic target.

Because the TR pools used in this study were derived from one cell line, it is likely that multiple mechanisms of resistance exist and that these may vary among cell lines. Additionally, mechanisms contributing to acquired *versus* inherent resistance may differ. The pools developed during this study may harbor resistance acquired over time or may represent a selected subpopulation of cells with inherent resistance. Interestingly, discontinuation of trastuzumab treatment for 4 months did not restore sensitivity to the TR pools, suggesting that the aberrant molecular mechanisms that contribute to resistance in these pools are either inherent or are acquired and maintained long-term. Additionally, the resistant cells used in this study are pools of multiple clones and, thus, represent multiple genetic or molecular alterations. Another approach to our research question would have been to use clones, which represent one isolated alteration. However, because pools represent the alterations that are represented in the majority of cells, we chose to use this approach. The drawback to our approach is that pools may contain cells with various degrees of resistance.

An important consideration is whether cells developed resistance specifically to trastuzumab or whether cross-resistance to other antibodies exists. Thus, we examined the response of resistant pools to an anti-EGFR antibody (data not shown). Resistant and parental cells were similarly sensitive to EGFR blockade, suggesting that there is not cross-resistance, but that resistance is specific for trastuzumab. These results open up the possibility that other HER family-targeted antibodies or kinase inhibitors may be effective treatments against cancers that have progressed while on trastuzumab. Experiments addressing this possibility are ongoing.

Our results suggest that a subset of TR breast cancers express reduced p27^{kip1} levels with increased cdk2 activity. These findings support p27^{kip1} as a potential therapeutic target in TR breast cancers. The p27^{kip1} protein is degraded via ubiquitin-dependent mechanisms (18). Thus, the potential efficacy of proteasome inhibitors, which are known to induce p27^{kip1} expression, is being tested against the TR pools. In addition, these resistant cells may demonstrate sensitivity to cdk2 inhibiting drugs, as their levels of endogenous cdk2 kinase activity are slightly elevated. In conclusion, our data suggest that p27^{kip1} may be a relevant therapeutic target in TR breast cancers and support the development and testing of treatments that induce p27^{kip1} expression, such as proteasome inhibitors, or therapies that block cdk2 activity in these cancers.

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REFERENCES

1. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987;235:177–82.
2. Carter P, Presta L, Gorman CM, et al. Humanization of an anti-p185her2 antibody for human cancer therapy. Proc Natl Acad Sci USA 1992;89:4285–9.
3. Baselga J, Tripathy D, Mendelsohn J, et al. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. J Clin Oncol 1996;14:737–44.
4. Cobleigh MA, Vogel CL, Tripathy D, et al. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER-2 overexpressing metastatic breast cancer that has progresssed after chemotherapy for metastatic disease. J Clin Oncol 1999;17:2639–48.
5. Seidman AD, Fornier M, Esteva FJ, et al. Weekly trastuzumab and paclitaxel therapy for metastatic breast cancer with analysis of efficacy by HER2 immunophenotype and gene amplification. J Clin Oncol 2001;19:2587–95.
6. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N. Engl J Med 2001;344:783–92.
7. Esteva FJ, Valero V, Booser D, et al. Phase II study of weekly docetaxel and trastuzumab for patients with HER-2-overexpressing metastatic breast cancer. J Clin Oncol 2002;20:1800–8.
8. Nahta R, Esteva FJ. HER-2-targeted therapy: lessons learned and future directions. Clin Cancer Res 2003;9:5078–84.
9. Baselga J, Albanell J, Molina MA, Arribas J. Mechanism of action of trastuzumab and scientific update. Semin Oncol 2001;28:4–11.
10. Sliwkowski MX, Lofgren JA, Lewis GD, Hotaling TE, Fendly BM, Fox JA. Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin). Semin Oncol 1999;26(Suppl 12):60–70.
11. Lane HA, Beuvink I, Motoyama AB, Daly JM, Neve RM, Hynes NE. ErbB2 potentiates breast tumor proliferation through modulation of p27(Kip1)-Cdk2 complex formation: receptor overexpression does not determine growth dependency. Mol Cell Biol 2000;20:3210–23.
12. Lane HA, Motoyama AB, Beuvink I, Hynes NE. Modulation of p27/Cdk2 complex formation through 4D5-mediated inhibition of HER2 receptor signaling. Ann Oncol 2001;12:21–2.
13. Albanell J, Baselga J. Unraveling resistance to trastuzumab (Herceptin): Insulin-like growth factor-I receptor, a new suspect. J Natl Cancer Inst 2001;93:1830–2.
14. Nahta R, Esteva FJ. In vitro effects of trastuzumab and vinorelbine in trastuzumab-resistant breast cancer cells. Cancer Chemother Pharmacol 2004;53:186–90.
15. Le XF, Claret FX, Lammayot A, et al. The role of cyclin-dependent kinase inhibitor p27Kip1 in anti-HER2 antibody-induced G1 cell cycle arrest and tumor growth inhibition. J Biol Chem 2003;278:23441–50.
16. Lee DH, Goldberg AL. Proteasome inhibitors: valuable new tools for cell biologists. Trends Cell Biol 1998;8:397–403.
17. Cardoso F, Piccart MJ, Durbecq V, Di Leo A. Resistance to trastuzumab: a necessary evil or a temporary challenge? Clin Breast Cancer 2002;3:247–57.
18. Pagano M, Tam SW, Theodoras AM, et al. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science 1995;269:682–5.