

Neoadjuvant Trastuzumab Induces Apoptosis in Primary Breast Cancers

Syed K. Mohsin, Heidi L. Weiss, M. Carolina Gutierrez, Gary C. Chamness, Rachel Schiff, Michael P. DiGiovanna, Chun-Xia Wang, Susan G. Hilsenbeck, C. Kent Osborne, D. Craig Allred, Richard Elledge, and Jenny C. Chang

From the Breast Center at Baylor College of Medicine/The Methodist Hospital; Department of Pathology, Baylor College of Medicine, Houston, TX; and Department of Internal Medicine, Yale Cancer Center, New Haven, CT.

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Terms in blue are defined in the glossary, found at the end of this issue and online at www.jco.org.

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Address reprint requests to Jenny C. Chang, MD, Breast Center at Baylor College of Medicine/The Methodist Hospital, 1 Baylor Plaza, MS 600, Houston, TX 77030; e-mail: jcchang@breastcenter.tmc.edu.

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A B S T R A C T

Purpose

Greater understanding of the cellular response in trastuzumab-treated patients will provide insight into the clinical management of patients.

Patients and Methods

We performed a neoadjuvant trial in 35 patients with locally advanced HER-2/neu overexpressing breast cancers who received weekly trastuzumab given as a single agent for the first 3 weeks, followed by a combination of trastuzumab and docetaxel for 12 weeks before surgery. Sequential core biopsies were taken at baseline and within weeks 1 and 3 after the first dose of trastuzumab. Clinical response to trastuzumab was assessed by tumor measurements on day 22 before chemotherapy. Core biopsies were assessed by immunohistochemistry for cell cycle and proliferation (Ki67, p27, phosphorylated [p]-MAPK), apoptosis and survival (apoptotic index, p-Akt), epidermal growth factor receptor, and total and p-HER-2.

Results

There was early tumor regression with a median decrease of -20.0% (range, 0% to 60.4%) after only 3 weeks of trastuzumab, and eight patients (23%) had a partial response. Consistent with the clinical regressions, apoptosis was significantly induced (median increase from 3.5% to 4.7% ; $P = .006$) within week 1, a 35% increase above baseline. No significant change in epidermal growth factor receptor score was observed in week 1, without changes in total or p-HER-2 expression. Tumors with high baseline Ki67 were less likely to respond ($P = .02$).

Conclusion

In primary breast cancers, trastuzumab substantially induces apoptosis, providing a molecular explanation for both its therapeutic efficacy and its successful combination with cytotoxic chemotherapy.

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INTRODUCTION

In the United States, over 200,000 women will be diagnosed with breast cancer every year, and approximately one third will eventually die of the disease. Tamoxifen, which targets the estrogen receptor, is the single most active systemic agent in reducing tumor recurrences and mortality in early breast cancer.^{1,2} Using this successful paradigm, there has been a continued search for other biologic therapies against

other targets, like HER-2.^{3,4} Based on the association between poor prognosis and HER-2 overexpression, antibodies that specifically target HER-2 were developed for their therapeutic efficacy. Trastuzumab (Herceptin; Genentech, South San Francisco, CA) is a recombinant, DNA-derived, humanized, monoclonal antibody that selectively binds with high affinity to the extracellular domain of HER-2. The current clinical indication for the use of trastuzumab

is in the treatment of metastatic breast cancer patients with HER-2 overexpressing tumors.⁵

Pivotal multicenter efficacy trials in metastatic breast cancer patients whose tumors had HER-2 overexpression showed unequivocally improved response rates and survival that was almost twice as long in patients who received trastuzumab in addition to chemotherapy.⁵ However, the reported incidence of cardiac dysfunction in this study was as high as 28% in patients on trastuzumab plus anthracyclines.⁶ Furthermore, although trastuzumab is highly efficacious, still only a minority of metastatic patients with HER-2 overexpressing breast cancers respond to trastuzumab monotherapy. In a multicenter phase II study, 222 women with HER-2 overexpression (defined as at least one third of the tumor cells showing membranous HER-2 staining), only 18% of the patients responded to trastuzumab.⁷ The majority of these patients had tumors that were also gene amplified for HER-2 as measured by **fluorescent in situ hybridization**, and yet they were resistant to therapy. Hence, in addition to protein overexpression and gene amplification of HER-2, there is a need to find additional predictive markers of response, in order to select patients who would benefit from this treatment while sparing others the adverse effects and expense.

Defining the molecular mechanisms of the cellular response to trastuzumab may provide insight for the optimal clinical use of this receptor-targeted strategy, as well as for the mechanisms of cellular resistance to HER-2 blockade. For example, if the main mechanism of action involves **cell cycle** arrest or downregulation of signaling pathways regulating cell proliferation alone, then prolonged therapy is indicated. However, if trastuzumab acts by induction of **apoptosis**, then shorter treatment durations should be studied in clinical trials. To date, only in vitro data exists on the mechanism of action of trastuzumab. These laboratory model systems can only approximate the complex interplay between tumor and host responses.

Therefore, to prospectively address this issue, we initiated a study to collect serial samples of human breast cancer for molecular analyses in patients undergoing neoadjuvant trastuzumab monotherapy. Our objectives were first to identify predictive markers by correlating pretreatment biomarkers with clinical response to trastuzumab at 3 weeks, and second, to explore its mechanism of action by measuring serial changes in these biomarkers after treatment. Several molecular pathways were selected based on in vitro data on their potential to mediate growth factor-induced changes in tumor growth. These included cell cycle and proliferation (**Ki67**, **p27**, **phosphorylated [p]-MAPK**), apoptosis and survival pathways (**apoptotic index**, **p-Akt**), **epidermal growth factor receptor (EGFR)**, and total and **p-HER-2** expression.

PATIENTS AND METHODS

Clinical Study

From September 1999 to June 2003, patients with HER-2 overexpressing (defined as HercepTest [DakoCytomation, Carpinteria, CA] score of 3+, or by other methods where > one third of cells had complete membrane staining, or semiquantitative immunohistochemical score of ≥ 5 by Allred scoring system,⁸ or HER-2 amplified by fluorescent in situ hybridization) breast cancer (primary cancers > 4 cm, or with clinically evident axillary metastases), with or without gross metastatic disease, were considered for a phase II study with neoadjuvant trastuzumab. This study was approved by the institutional review board of Baylor College of Medicine (Houston, TX). In brief, the inclusion criteria were (1) age greater than 18 years and a diagnosis of breast cancer confirmed by core needle biopsy, (2) postmenopausal, or premenopausal with appropriate contraception, (3) Eastern Cooperative Oncology Group performance status ≥ 0 , and (4) adequate liver and kidney function tests (within 1.5 times the institution's upper limit of normal). Exclusion criteria included (1) severe underlying chronic illness or disease, and (2) prior systemic treatments or treatment with other chemotherapeutic drugs while on study.

Clinical staging and size of the primary tumor were recorded at the start of treatment and after 3 weeks of trastuzumab monotherapy. Tumor size (product of the two largest perpendicular diameters) measured before and after 3 weeks of neoadjuvant trastuzumab was used to compute the percentage of residual disease.

Core biopsies of the primary cancers were undertaken before administration of single-agent trastuzumab as neoadjuvant treatment. Trastuzumab was administered initially as an intravenous loading dose of 4 mg/m². Core biopsies were then performed 24 hours after the completion of the first trastuzumab infusion (day 1). Core biopsies were also performed just before each subsequent weekly infusion of trastuzumab (2 mg/m²) on days 8, 15, and 22. In order to account for missing serial biopsies at all four time points, biopsies from days 1 or 8 were grouped and considered as within 1 week of therapy, and biopsies from days 15 or 22 were considered as within 3 weeks of therapy. Although the present study focuses on the first 3 weeks, on day 22, trastuzumab therapy was followed by docetaxel (Taxotere, 100 mg/m²; Sanofi-Aventis Inc, Bridgewater, NJ) chemotherapy. Docetaxel was given every 3 weeks for a total of four cycles together with weekly trastuzumab. Following completion of neoadjuvant treatment, primary surgery was undertaken if the tumor was operable. After recovery from surgery, standard adjuvant therapy with or without radiation was administered. Weekly trastuzumab (2 mg/m²) was then recommenced 1 month after completion of adjuvant chemotherapy for 1 year, or until evidence of disease progression or unacceptable toxicity. The treatment schedule is shown in Figure 1.

Laboratory Methods

All core needle biopsies were immediately placed in 10% neutral buffered formalin, fixed for 4 to 6 hours, and processed to paraffin blocks. One hematoxylin and eosin stained slide from each block was examined microscopically to confirm the presence of tumor before biomarker evaluation. Immunohistochemistry was performed on 4-micron-thick deparaffinized sections on plus-coated slides (Fisher Scientific, Houston, TX). All antibodies except anti-HER-2 required antigen retrieval (AR) in a pressure cooker. The details of the antibodies, clone, dilution, source, and AR conditions are provided in Table 1.

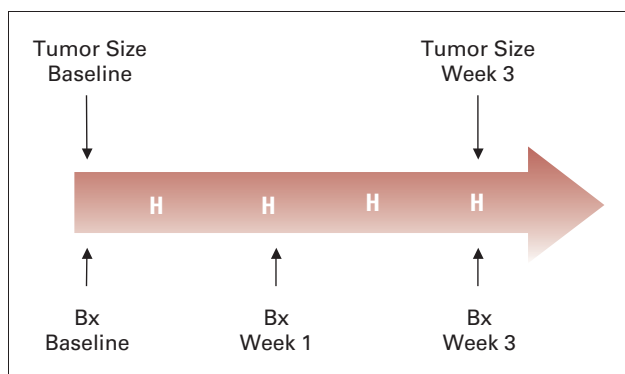


Fig 1. Schema of the clinical study of neoadjuvant trastuzumab. Bx, biopsy.

Briefly, after AR, endogenous peroxidase and biotin were blocked with 3% hydrogen peroxide and the Avidin-Biotin blocking kit (Vector, CA), respectively. This was followed by incubation with primary antibody (Table 1) for 60 minutes at room temperature, appropriate secondary antibody (DakoCytomation), labeled streptavidin-horseradish-peroxidase (DakoCytomation), DAB+ chromogen (DakoCytomation), and 0.2% osmium tetroxide (Sigma Chemicals, St Louis, MO), followed by counterstaining with light hematoxylin. Appropriate positive controls for each antibody and negative controls using species-matched immunoglobulin to replace the primary antibody were run with each batch. For the immunostaining of p-HER-2, slides were first baked in a 60°C oven overnight. After deparaffinization and rehydration, the tissue sections were subjected to target retrieval by incubating slides at 95°C to 98°C for 20 minutes using DAKO Target Retrieval Solution (S1699; DAKO Corporation, Carpinteria, CA). The DAKO Catalyzed Signal Amplification Kit (K1500) was used for the staining, and the staining procedure was performed using a DAKO Autostainer Universal Staining System. In brief, the tissue sections were first incubated with peroxidase block for 5 minutes. After a buffer rinse and buffer bath step, the sections were then blocked with Avidin and Biotin Block (X0590) for 10 minutes, respectively. After another buffer rinse and buffer bath step, sections were blocked with protein block for 5 minutes first, then incubated with antibody specific for p-HER2 (Tyr-1248; *c-erbB2/HER-2/neu* phospho-specific Ab-18; NeoMarkers Fremont, CA) for 30 minutes. The antibody was diluted in DAKO Antibody Diluent with Background Reducing Components (S3022) to a final concentration of 2.5 mg/mL. The remainder of the staining steps followed the DAKO Catalyzed Signal Amplification kit standard protocol starting with incubating tissue sections with streptavidin-biotin complex. The wash buffer solution used for the staining is triethanolamine-buffered saline (S3306). Representative examples of immunohistochemistry staining are shown in Figure 2.

EGFR, HER-2, p-Akt, p-MAPK, and p27 were scored using the Allred scoring system, which uses a scale of 0 to 8 based on both proportion and intensity of immunostaining in the tumor cells.⁸ These slides were scored by estimating the proportion and average intensity of positive tumor cells (proportion score: 0, none; 1, <1/100, 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 2/3; 5, > 2/3; intensity score: 0, none; 1, weak; 2, intermediate; 3, strong).⁸ HER-2 phosphorylated staining was scored by two investigators (S.M. and M.G.) using a three-point system similar to the intensity score of the Allred scoring system. Ki-67 staining for proliferation index and cleaved caspase 3 staining for apoptotic index were scored by point counting of at least 500 cancer cells, and results were presented as percent-positive tumor cells. All the slides were scored by a breast cancer pathologist (S.M.) without knowledge of the clinical outcome.

Statistical Methods

Descriptive statistics were calculated to summarize patient characteristics, tumor size, and biomarker levels from pre- and post-therapy biopsies. Paired comparisons of tumor size from the initial to day 22 measurements were performed using the Wilcoxon signed ranks test. Median tumor decrease percentage was then used to categorize patients into responders and nonresponders. Initial biomarker levels were compared between resistant and sensitive tumors using the Wilcoxon rank-sum test. Simultaneous analysis of the biomarkers that were significantly predictive of tumor response from the univariate analysis was performed using multivariate logistic regression.

The change in biomarker levels due to trastuzumab therapy was evaluated by calculating the difference in each biomarker from initial biopsy and the repeat samples obtained within 1 week and between 2 and 3 weeks of therapy. In order to account for missing serial biopsies at all four time points, biopsies from days 1 or 8 were grouped and considered as within 1 week of therapy, and biopsies from days 15 or 22 were considered as within 3 weeks of therapy. Nonparametric paired comparisons were employed to define any statistically significant changes in biomarker levels. Finally, the correlations among the various initial biomarker levels were assessed by calculating Spearman's correlation coefficients between biomarker scores.

RESULTS

Clinical Characteristics and Responses to Neoadjuvant Trastuzumab in Treatment-Naïve Patients

The clinical characteristics of the 35 patients enrolled on this phase II neoadjuvant study are included in Table 2. At presentation, the median tumor size was large at

Table 1. Primary Antibodies and Antigen Retrieval Conditions for Immunohistochemistry

Antibody	Clone	Dilution	Source	AR Solution
EGFR	31G7	1:25	Zymed	Ficlin
HER-2 (total)	Tab250/CB11	1:400/200	Zymed/Novocastra	No AR
Ki-67	MIB-1	1:200	Dakocytomation	Tris-HCl pH 9.0
Cleaved caspase-3	Polyclonal	1:50	Cell Signaling	Tris-HCl pH 9.0
AKT-phos	Polyclonal	1:80	Cell Signaling	Tris-EDTA pH 8.0
ERK1/2-phos	Polyclonal	1:100	Cell Signaling	Tris-EDTA pH 8.0
p27	SX53G8	1:200	Dakocytomation	Tris-HCl pH 9.0

Abbreviation: AR, antigen retrieval; EGFR, epidermal growth factor receptor; phos, phosphorylated.

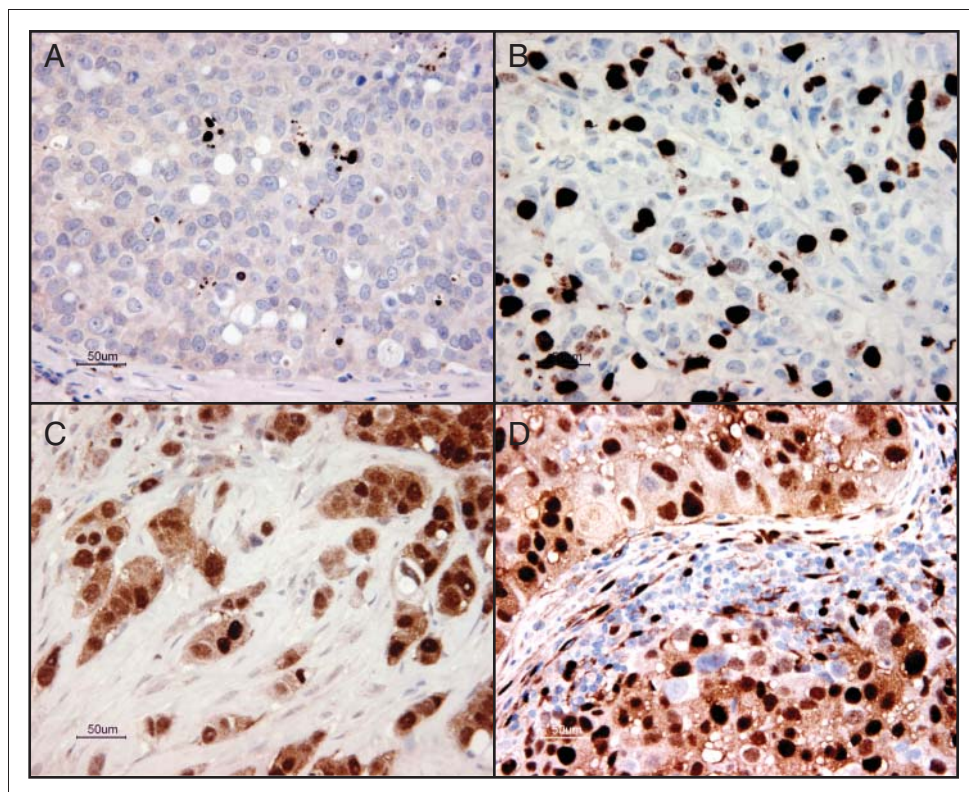


Fig 2. Representative examples of immunohistochemistry staining for (A) cleaved caspase 3; (B) Ki67; (C) phosphorylated (p) -Akt; and (D) p-MAPK (400 \times).

10 \times 10 cm² (range, 4 \times 4 cm² to 25 \times 25 cm²). We did not expect measurable evidence of tumor regression with this short treatment duration. However, regression in the product of bidimensional tumor measurements with a median decrease of -20.0% (range, 0% to 78.6%; $P = .0001$) was observed in primary tumors after only 3 weeks of single-agent trastuzumab. Most surprisingly, partial response ($\geq 50\%$ tumor reduction) was observed in 23% (eight of 35 patients). Minor response ($> 10\%$, $< 50\%$ tumor decrease) was seen in 40% (14 of 35 patients), and stable disease, which includes those with minor responses ($< 50\%$ decrease, $< 25\%$ increase in tumor size), in 77% (27 of 35 patients). No tumors increased in size during this 3-week period. Using the median percentage decrease in tumor size, we defined responders as those with 20% reduction in tumor size or more, and nonresponders as those with less than 20% tumor reduction.

Serial Changes in Biomarkers With Trastuzumab Treatment

Survival pathways (p-Akt, cleaved caspase 3). Trastuzumab does not induce apoptosis in cell culture. Consistent with the observed clinical tumor regression after 3 weeks of trastuzumab, in this in vivo study, the apoptosis index (cleaved caspase 3) was significantly induced with a median increase from 3.5% to 4.7%

($P = .006$) within 1 week after trastuzumab therapy (Fig 3; Table 3), representing a 35% increase in apoptosis above baseline. Current thinking suggests reductions in activated Akt, a major signaling molecule in the cell survival pathways, should lead to induction of apoptosis. There was no significant change in expression of either nuclear or cytoplasmic p-Akt with trastuzumab treatment. However, in responders, there was a nonsignificant decrease in nuclear p-Akt, while no change was observed in nonresponders (-1.0 v 0.0 ; $P =$ not significant).

Cell cycle and proliferation markers (Ki67, p27, cytoplasmic and nuclear p-MAPK). Contrary to in vitro data, trastuzumab did not decrease cell cycle proliferation (Ki67) in clinical tumors at either 1 or 3 weeks (Table 3). p27, the cell cycle inhibitor, was not increased with trastuzumab treatment. A small decrease in cytoplasmic p-MAPK was noted 3 weeks after exposure to trastuzumab ($P = .05$; Fig 4; Table 3). These data suggest that trastuzumab exerts some of its effects by decreasing p-MAPK, but no change in Ki67 or p27 was observed.

Changes in signal transduction (EGFR and HER-2). Semiquantitative median measurements of total and p-HER-2 did not change significantly with trastuzumab therapy (Table 3). Trastuzumab in this study did not downregulate membrane HER-2, as expected from in vitro data. Decrease in p-HER-2 would be difficult to detect, as most tumors do not express p-HER-2.

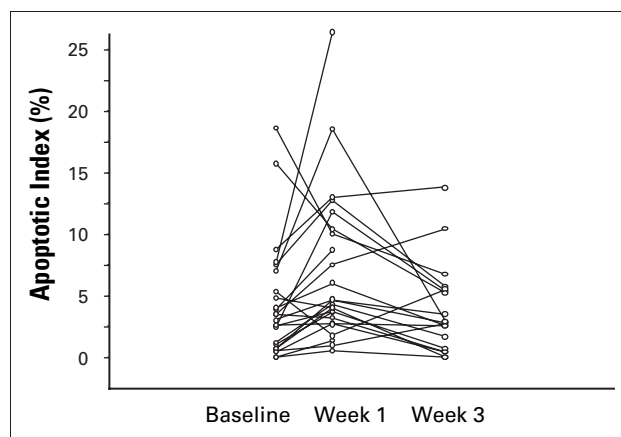
Table 2. Patient and Tumor Characteristics of Treatment-Naïve Patients on Neoadjuvant Trastuzumab Monotherapy (N = 35)

	No. of Patients	%
Age, years		
Median	53.5	
Range	33-69	
No. with inflammatory breast cancers	11	31
Tumor size at baseline, cm ²		
Median	10×10	
Range	4×4–25×25	
Decrease in tumor size, %		
Median	20.0	
Range	0-78.6	
Clinical response		
Partial response	8	23
Stable disease	27	77
Progressive disease	0	0
Score of biomarkers at baseline (n = 30)		
Ki67		
Median	59.5	
Range	4-92	
p27		
Median	6	
Range	0-8	
p-MAPK		
Nuclear		
Median	3	
Range	0-7	
Cytoplasmic		
Median	4	
Range	0-7	
Apoptotic index		
Median	3	
Range	0-18.6	
p-Akt		
Nuclear		
Median	5.5	
Range	0-8	
Cytoplasmic		
Median	5.0	
Range	0-8	
Total HER-2		
Median	7	
Range	0-8	
p-HER-2		
Median	0	
Range	0-3	
EGFR		
Median	0	
Range	0-8	

Abbreviations: p-MAPK, phosphorylated mitogen-activated protein kinase; p-Akt, phosphorylated Akt; p-HER-2, phosphorylated HER-2; EGFR, epidermal growth factor receptor.

Semiquantitative median measurement of EGFR, on the other hand, showed significant increase in week 1 (Table 3; Fig 5), from a median score of 0 to 3 ($P = .04$).

Pretreatment predictive markers of response to trastuzumab. Considering the next biomarkers in the pretreatment specimens that may be predictive indicators of response and resistance, a panel involved in a variety of cellular pathways was examined. Expression of these factors was then correlated with response to treatment (Fig 6; Table 4). By univariate analysis, high proliferation as assessed by Ki67 staining was significantly associated with poor response ($P = .02$). Although not statistically significant, higher apoptotic index was associated with resis-

**Fig 3.** Increase in apoptosis within 1 week of trastuzumab treatment.

tance to trastuzumab (3.8% v 2.5%; $P = .08$). The expression of other biomarkers was not significantly correlated with response to treatment. From this preliminary data, it is likely that rapidly proliferating tumors express a molecular phenotype, which appears to be less sensitive to trastuzumab.

Correlation between expression of different biomarkers at baseline. A significant positive correlation was observed between baseline levels of apoptosis, p-Akt, and p-MAPK. A significant positive correlation was also noted between p-HER-2 and total HER-2, apoptosis, and p-Akt, while a negative correlation was observed between p-HER-2 and p27. p-Akt was significantly positively correlated with Ki67, p-MAPK, and total HER-2 expression (Table 5).

DISCUSSION

This is the first neoadjuvant trastuzumab monotherapy study in patients with HER-2 overexpressing breast cancers. The clinical efficacy with rapid and substantial tumor reductions in some patients presenting with large initial tumors indicates that the monoclonal antibody can be safely administered at least for several weeks as a single agent without fear of tumor progression. When the study was first started, the uncertainty of the clinical efficacy of neoadjuvant single-agent trastuzumab limited its duration to 3 weeks. It seems likely that the true response rate would have been higher if therapy was continued for several months. The presenting tumors at diagnosis were large in this study, and tumor regressions of at least 20% following only 3 weeks of the monoclonal antibody were observed. The rapid response seen in this study also suggests that primary HER-2 overexpressing tumors may be much more sensitive to trastuzumab than metastatic lesions in which the response rate after months of treatment is approximately 30%.⁹ Longer treatment durations of HER-2 targeted therapy given as a single agent should be investigated in clinical trials with careful monitoring of patients to assess its activity in this setting.

Table 3. Changes in Expression of Biomarkers With Trastuzumab at 1 and 3 Weeks

Biomarker	Median Score*	P†
Apoptotic Index‡		
Baseline v 1 week (n = 25)	3.5% v 4.7%	.006
Baseline v 3 weeks (n = 23)	2.6% v 2.5%	.50
Nuclear p-Akt		
Baseline v 1 week (n = 27)	6.0 v 6.0	.93
Baseline v 3 weeks (n = 24)	6.0 v 6.0	.88
Cytoplasmic p-Akt		
Baseline v 1 week (n = 27)	5.0 v 5.0	.48
Baseline v 3 weeks (n = 24)	5.0 v 5.0	.47
Ki67‡		
Baseline v 1 week (n = 27)	52% v 59%	.68
Baseline v 3 weeks (n = 25)	61% v 73%	.66
p27		
Baseline v 1 week (n = 26)	6.0 v 6.0	.12
Baseline v 3 weeks (n = 24)	6.0 v 6.0	.44
Cytoplasmic p-MAPK		
Baseline v 1 week (n = 25)	4.0 v 4.0	.95
Baseline v 3 weeks (n = 24)	4.0 v 3.0	.05
Nuclear p-MAPK		
Baseline v 1 week (n = 25)	4.0 v 4.0	.81
Baseline v 3 weeks (n = 24)	3.5 v 3.0	.13
HER-2		
Baseline v 1 week (n = 27)	7.0 v 7.0	.42
Baseline v 3 weeks (n = 24)	7.0 v 7.0	.21
p-HER-2		
Baseline v 1 week (n = 18)	0 v 0	.99
Baseline v 3 weeks (n = 19)	0 v 0	.44
EGFR		
Baseline v 1 week (n = 25)	0 v 3.0	.04
Baseline v 3 weeks (n = 22)	0 v 0	.22

Abbreviations: p-Akt, phosphorylated Akt; p-MAPK, phosphorylated mitogen-activated protein kinase; p-HER-2, phosphorylated HER-2; EGFR, epidermal growth factor receptor.
 *Allred semi-quantitative immunohistochemical score is based on the sum of the proportion and intensity scores.
 †Comparison of median with baseline based on Wilcoxon signed-rank test.
 ‡Apoptotic index and Ki67 were assessed as direct percentage of positive cells.

Consistent with these clinical observations of tumor shrinkage, this is the first study to suggest that trastuzumab results in tumor regression by induction of apoptosis. Inhibition of PI3K/Akt by trastuzumab has been reported in cultured cells.¹⁰ This suggested a possible mechanism of action of trastuzumab by inducing apoptosis through inhibition of the PI3K/Akt pathway and the activation of cell death pathways. However, trastuzumab does not induce apoptosis in cultured cells, and attempts to demonstrate cell death induced by trastuzumab on BT-474 have been unsuccessful.¹⁰ Our data suggest that trastuzumab does induce apoptosis to similar degree as cytotoxic chemotherapy.¹¹ Trastuzumab also resulted in a nonsignificant decrease in nuclear p-Akt in sensitive tumors, further supporting the suggestion that Akt survival pathway is important in its mechanism of action. Since reductions in p-Akt induced by trastuzumab would be expected to precede apoptosis, it is possible that the peak reduction occurred very early, before the first repeat biopsy, and was therefore not detected in this study. Overall, trastuzumab appears to affect survival pathways, resulting in decrease in nuclear p-Akt, especially in responders, and the induction of ap-

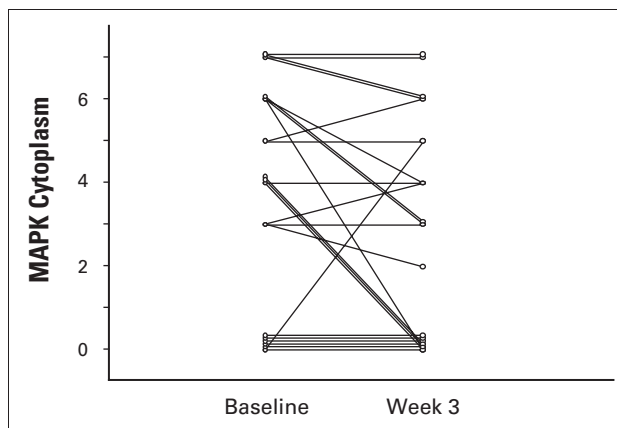


Fig 4. Decrease in cytoplasmic phosphorylated-MAPK in sequential biopsies with trastuzumab.

optosis. Recent evidence in support of this mechanism shows that PTEN, the lipid phosphatase and tension homologue, not only antagonizes tumorigenesis but also sensitizes breast cancers to trastuzumab.¹² PTEN normally opposes PI3K/Akt signaling, and trastuzumab stabilizes PTEN on binding to HER-2, and downregulates Akt signaling.¹² Our in vivo result with trastuzumab further confirms that trastuzumab's main mechanism is by inhibiting PI3K/Akt pathways, and increasing apoptosis.

In addition, trastuzumab contains a human Fc fragment and thus may be capable of eliciting an immunologic response in a human host. Laboratory evidence also suggests that trastuzumab may act through an immunologic mechanism unrelated to HER-2 activity, and may be mediated by antibody-dependent cellular toxicity.¹³ This degree of tumor shrinkage may, in part, be related to this mechanism of action, but was not studied in this report.

Because the mechanism of therapeutic action has not been clarified, trastuzumab is currently administered indefinitely in patients with metastatic disease, while 1 or 2 years of therapy is being tested in clinical adjuvant studies. These

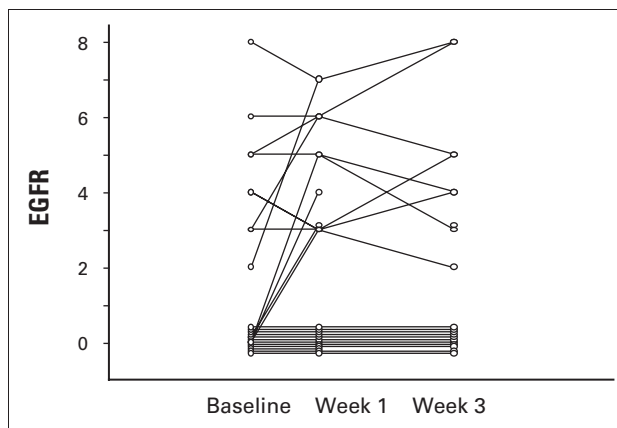


Fig 5. Increase in epidermal growth factor receptor (EGFR) in sequential biopsies with trastuzumab.

Table 4. Predictive Biomarkers of Response to Trastuzumab in Initial Biopsy

	Median IHC Score*		P
	Responders (n = 16)	Nonresponders (n = 14)	
Ki67†	49%	74%	.02
p27	6.0	4.5	.28
p-MAPK			
Nuclear	4.0	3.0	.64
Cytoplasmic	4.0	4.0	.53
Apoptotic index†	2.5%	3.8%	.08
p-Akt			
Nuclear	4.5	6.0	.44
Cytoplasmic	4.0	5.0	.43
HER-2	7.0	7.0	.83
EGFR	0	0	.98
ER	0	0	.27
p-HER-2	0	0	.91

Abbreviations: p-MAPK, phosphorylated mitogen-activated protein kinase; p-Akt, phosphorylated Akt; EGFR, epidermal growth factor receptor; ER, estrogen receptor; p-HER-2, phosphorylated HER-2.
 *Allred semi-quantitative immunohistochemical score is based on the sum of the proportion and intensity scores.
 †Apoptotic index and Ki67 were assessed as direct percent of positive cells.

recommendations are empirical. The demonstration that trastuzumab may work by cytotoxic rather than cytostatic mechanisms justifies investigation of shorter treatment durations in metastatic clinical trials. These results suggest that trastuzumab is as effective as cytotoxic chemotherapy in the induction of apoptosis. Most chemotherapy agents induce apoptosis in 1% to 2% of cells within 24 hours after exposure, data similar to ours with trastuzumab.¹¹ The induction of apoptotic pathways by trastuzumab also explains its additive or synergistic activity when it is combined with chemotherapy in breast cancer. Resistance to chemotherapy-induced cell death may be overcome by blocking a common antiapoptotic pathway with trastuzumab. However, unlike trastuzumab, neoadjuvant hormonal studies evaluating apoptosis have been conflicting, and most studies have shown that hormonal agents like tamoxifen primarily

Table 5. Significant Correlations Between Biomarkers From Initial Biopsy

Biomarkers	Spearman Correlation Coefficient	P
Apoptotic index		
p-Akt (n = 29)	0.57	.001
p-MAPK (n = 28)	0.46	.014
p-HER-2		
Apoptotic index (n = 20)	0.54	.013
HER-2 (n = 21)	0.45	.042
p-Akt (n = 21)	0.58	.006
p27 (n = 21)	-0.45	.039
p-Akt		
p-MAPK (n = 29)	0.39	.038
HER-2 (n = 30)	0.43	.018
Ki-67 (n = 30)	0.36	.047
p-MAPK		
HER-2 (n = 29)	0.78	< .0001

Abbreviations: p-Akt, phosphorylated Akt; p-MAPK, phosphorylated mitogen-activated protein kinase; p-HER-2, phosphorylated HER-2.

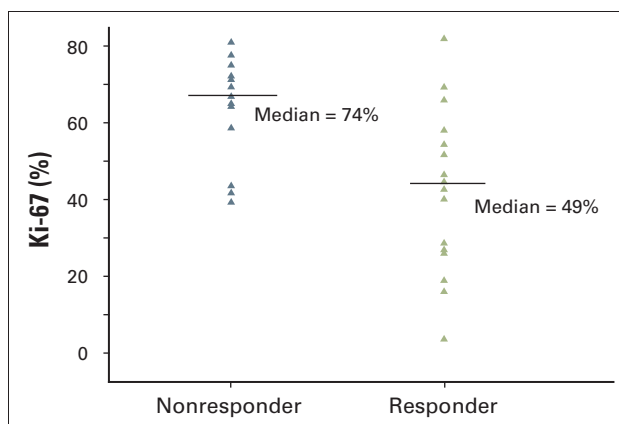


Fig 6. High baseline Ki67 is associated with resistance to trastuzumab.

affect proliferation.¹⁴ This may, in part, explain the antagonism between chemotherapy and hormonal agents, while the combination of chemotherapy and trastuzumab are at least additive if not synergistic.

EGFR and HER-2 signaling activate pathways regulating cell-cycle progression and cell proliferation. In the laboratory, blockade of these receptors by monoclonal antibodies results in inhibition of cell proliferation,¹⁵⁻¹⁷ upregulation of the cell cycle inhibitor p27, thereby producing cell cycle arrest at G₁.¹⁸⁻²¹ These in vitro data indicate that interference with this cell cycle progression by trastuzumab should lead to a decrease in proliferation as measured by Ki67 index, accompanied by an increase in the cell-cycle inhibitor p27, and a decrease in p-MAPK. Our results did not show a significant change in cell proliferation as measured by Ki67 or an expected increase in p27. A decrease in p-MAPK at week 3 was observed. These data suggest that trastuzumab does exert an effect on cell-cycle kinetics, but the primary mode of action is by induction of apoptosis. It is possible that a decrease in the rate of cell proliferation did occur but was not reflected in Ki67 measurements, which primarily assess the cycling (non-G₀) cell population and not cell cycle progression per se.²² In addition, decreases in cell proliferation may be observed if trastuzumab was continued beyond 3 weeks. Nevertheless, our results suggest that in human breast cancers, trastuzumab appears to primarily affect cell survival and has less effect on cell-cycle kinetics, at least after a short duration of treatment.

In this study, we did not observe a decrease in total or p-HER-2 with trastuzumab exposure, in contrast to in vitro data where trastuzumab receptor-mediated endocytosis with downregulation of HER-2 levels has been identified.²³ However, most tumors do not express activated p-HER-2, and therefore any decrease in expression would not be detected by immunohistochemistry. Interestingly, we found an increase in EGFR expression with trastuzumab treatment. This may reflect a compensatory

mechanism within the HER family of tyrosine kinase receptors. Speculatively, the increase in EGFR may be an adaptive mechanism to circumvent a primarily HER-2 blockage with trastuzumab, which may have less effect on EGFR and HER-3, thereby increasing in EGFR/HER-3 or EGFR homodimers. If so, like the increase in EGFR, a similar increase in HER-3 should be observed. If this is shown in future studies, then a more total blockade of the HER family of tyrosine kinases with combining HER-2 targeting agents may be a superior therapeutic strategy than trastuzumab alone.²⁴

To our knowledge, this is the first study to demonstrate that tumors resistant to trastuzumab are more rapidly proliferating. This is in direct contrast to results with chemotherapy, where the literature indicates rapidly proliferating tumors are sensitive to chemotherapy, not resistant.²⁵ This may partly explain the observed clinical synergism between trastuzumab and chemotherapy, as these treatments may affect different tumor populations. However, inhibition of cell survival pathways, rendering the cells more susceptible to chemotherapy-induced apoptosis, may also play a role.

A significant positive correlation was observed between baseline levels of apoptosis and p-Akt, contrary to current dogma where an inverse relationship is expected. This most likely reflects the complex interplay and feedback mechanisms between survival factors and apoptotic pathways in tumors growing in vivo and not in cell culture. We did see significant correlation between HER-2 and apoptosis, while a negative correlation was observed between HER-2 and p27.

In conclusion, contrary to in vitro data, this prospective in vivo study demonstrates that trastuzumab induces apoptosis but does not affect cell proliferation as measured by Ki67 in the primary breast cancers of women receiving neoadjuvant treatment. This data suggests that trastuzumab would not likely antagonize the effects of chemotherapy on a cell kinetic basis, which might be of concern with other growth factor inhibitors,²⁶ but would act coordinately to induce cell death. In addition, since trastuzumab results in tumor cell death, shorter treatment durations rather than indefinite long-term treatment should be investigated in metastatic patients. In addition, increase in EGFR expression with trastuzumab treatment suggests that compensatory mechanisms exist within the HER network of tyrosine kinase receptors, and combination HER-2 targeting agents should be investigated in clinical studies.

Authors' Disclosures of Potential Conflicts of Interest

The following authors or their immediate family members have indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. Research Funding: Jenny C. Chang, Genentech. For a detailed description of this category, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and Disclosures of Potential Conflicts of Interest found in Information for Contributors in the front of each issue.

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