Rational Combinations of Trastuzumab With Chemotherapeutic Drugs Used in the Treatment of Breast Cancer

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Background: **Trastuzumab, a humanized anti-HER2 antibody, increases the clinical benefit of first-line chemotherapy in patients with metastatic breast cancers that overexpress HER2. We characterized interactions between trastuzumab and chemotherapeutic agents commonly used in the treatment of breast cancer.** ***Methods:*** **Multiple drug effect/combination index isobologram analysis was used to study the efficacy of chemotherapeutic drug plus trastuzumab combinations tested against four HER2-overexpressing breast cancer cell lines (SK-BR-3, BT-474, MDA-MB-361, and MDA-MB-453). Combination index values were derived from parameters of the median effect plots, and statistical tests were used to determine whether the mean combination index values at multiple effect levels were statistically significantly different from a combination index value of 1.0. Values less than 1.0 indicate synergistic interactions, values greater than 1.0 indicate antagonistic interactions, and values equal to 1.0 indicate additive interactions.** ***Results:*** **At a wide range of clinically achievable drug concentrations, synergistic interactions were observed in all four breast cancer cell lines for trastuzumab plus carboplatin (mean combination index values ranged from 0.32 [*P*<.001] to 0.53 [*P*<.001]), 4-hydroxycyclophosphamide (mean combination index values ranged from 0.38 [*P*<.001] to 0.73 [*P* = .010]), docetaxel (mean combination index values ranged from 0.30 [*P*<.001] to 0.62 [*P*<.001]), and vinorelbine (mean combination index values ranged from 0.24 [*P*<.001] to 0.78 [*P*<.034]). Additive interactions were observed in all four cell lines with trastuzumab plus doxorubicin, epirubicin, and paclitaxel. Interactions between trastuzumab and gemcitabine were synergistic at low concentrations of gemcitabine and antagonistic at high concentrations. A synergistic interaction was observed with a three-drug combination of docetaxel plus carboplatin plus trastuzumab in SK-BR-3 cells (mean combination index value = 0.09; *P*<.001).** ***Conclusion:*** **Consistent synergistic interactions of trastuzumab plus carboplatin, 4-hydroxycyclophosphamide, docetaxel, or vinorelbine across a wide range of clinically relevant concentrations in HER2-overexpressing breast cancer cells indicate that these are rational combinations to test in human clinical trials. [J Natl Cancer Inst 2004;96:739–49]**

The traditional approach for the introduction of new agents into cancer therapy has been to add the new drug to accepted and/or established treatment regimens. This approach, although sometimes effective, is empiric and frequently does not take advantage of, much less optimize, the potential positive molecular interactions between the drugs being used. Indeed, the majority of current clinical studies investigate the sequencing and scheduling of drugs rather than determining the optimal molecular interactions between the agents. With the potential introduction of a large number of new molecularly targeted drugs in oncology, it becomes increasingly important to understand the effects of these molecular interactions.

One type of new molecularly targeted agent is specific anti–growth factor receptor antibodies. Such antibodies have been shown to potentiate the cytotoxic effects of certain DNA-damaging chemotherapeutic agents or ionizing radiation in tumor cell lines and xenografts that overexpress particular growth factor receptors *(1–5).* For example, anti–epidermal growth factor receptor (EGFR) antibodies potentiate the DNA-damaging cytotoxic effects of cisplatin against epidermoid carcinoma cells *(1).* The mechanism of this synergy involves attenuation of DNA repair activity after the antibodies have bound to extracellular epitopes of the EGFR or to HER2 *(3,4).* This mechanism indicates that there is an interaction between cell surface growth factor receptor signal transduction pathways and DNA repair processes *(3,4).* For the humanized monoclonal anti-HER2 antibody trastuzumab, the magnitude of the increased cytotoxic effects of chemotherapeutic agents are logarithmic, with mathematical computations demonstrating formal synergy between a number of anticancer drugs and trastuzumab when used in combination against cancers that overexpress HER2 protein *(6,7).* The synergistic effect of trastuzumab and chemotherapeutic agents on cancer cells is both dose and schedule dependent, and the synergy is specific for cancer cells that overexpress HER2 *(6).* Although the precise biochemical events responsible for the apparent connection between growth factor signal transduction and DNA repair processes have yet to be clarified, there is strong preclinical (*in vitro* and *in vivo*) and clinical evidence for this interaction *(2–8).*

In this study, we investigated and characterized the interactions between trastuzumab and nine chemotherapeutic drugs that

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DOI: 10.1093/jnci/djh131

represent six different classes of cytotoxic agents commonly used in breast cancer treatment. In addition, we expand our previous work on trastuzumab synergy *(6,7)* by extending observations to multiple HER2-overexpressing target cell lines to ensure that the observed results are not cell line–specific phenomena, but rather something generic to trastuzumab in HER2-overexpressing cells.

Materials and Methods

Cell Lines and Cell Culture

Human breast carcinoma cell lines that constitutively overexpress HER2 because of amplification of the HER2 gene (SK-BR-3, BT-474, MDA-MB-361, and MDA-MB-453) were obtained from American Type Culture Collection (ATCC, Manassas, VA). All cell lines were derived independently from different patients. Parental MCF-7 cells, which lack HER2 overexpression, were obtained from ATCC. Stable HER2-transfected MCF-7 (MCF-7/HER2) cells were established previously using a retroviral expression vector containing the full-length cDNA of the human HER2/neu gene *(9,10)*. All cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 m*M* glutamine, and 1% penicillin G–streptomycin–fungizone solution (Irvine Scientific, Santa Ana, CA).

Quantitative Enzyme-Linked Immunosorbent Assay Detection of HER2 Protein in HER2-Overexpressing Breast Cancer Cell Lines

To determine HER2 protein levels in cell lines, cell pellets from confluent breast cancer cell cultures harvested by disaggregation in a solution of trypsin and EDTA were washed three times in phosphate-buffered saline (PBS) and stored at −70 °C until use. The frozen cell pellets were pulverized with a microdismembrator (Braun-Melsungen, Melsungen, Germany) for 30 seconds at maximum power. The resulting powder was suspended in Tris-buffered saline (TBS; pH 8.5) containing 1% Triton X-100 nonionic detergent. The suspension was incubated at 4 °C for 12 hours with gentle agitation and centrifuged at 100 000*g* for 1 hour; the supernatant was collected and stored at −70 °C. HER2 protein levels were measured in the supernatant by using an enzyme-linked immunosorbent assay (ELISA; Oncogene Research Products, Cambridge, MA) with a murine monoclonal anti-human HER2 capture antibody and a rabbit anti-human HER2 detection antibody, according the manufacturer's recommended protocol. Recombinant p105 HER2 ectodomain was used as a positive control, and PBS was used as a negative control. Results are expressed in femtomoles per microgram of protein. Data shown represent the mean of three replicates, with each experiment repeated three times.

Multiple Drug Effect Analysis of Trastuzumab in Combination With Cytotoxic Chemotherapeutic Agents in HER2-Overexpressing Breast Cancer Cell Lines

Nine cytotoxic drugs representing six different classes of cytotoxic chemotherapeutic agents were analyzed, including the platinum analog carboplatin (Bristol Laboratories, Princeton, NJ); the anthracycline antibiotics doxorubicin (Gensia Laboratories, Irvine, CA) and epirubicin (Pharmacia & Upjohn, Erlangen, Germany); the alkylating agent 4-hydroxycyclophosphamide (Asta Medica, Frankfurt, Germany); the taxanes docetaxel (Aventis Pharmaceuticals, Collegeville, PA) and paclitaxel (Mead Johnson/Bristol-Myers Squibb, Princeton, NJ); the vinca alkaloid vinorelbine (GlaxoSmithKline, Research Triangle Park, NC); and the antimetabolites gemcitabine (Eli Lilly, Indianapolis, IN) and furtulon (Hoffman-La Roche, Basel, Switzerland). Each of the drugs chosen has reported clinical activity against human breast cancer *(11–17)*.

Aliquots of 3×10^3 to 5×10^3 SK-BR-3, BT-474, MDA-MB-361, or MDA-MB-453 cells were plated in 96-well plates. After 24 hours, experimental media containing either excipient control, trastuzumab (Genentech, South San Francisco, CA), chemotherapeutic agent, or the combination of trastuzumab plus the chemotherapeutic agent were added to appropriate wells, and serial twofold dilutions were made to span clinically relevant concentration ranges (i.e., concentrations sufficient to inhibit growth of control cells by 20%–90% [$\approx IC_{20}$–IC_{90}]). After the cells were incubated for 72–120 hours, the media were removed, the plates were washed with PBS, and the cells were stained with 0.5% *N*-hexamethylpararosaniline (crystal violet) in methanol. To each well, 0.1 mL of Sorenson's buffer (0.025 *M* sodium citrate, 0.025 *M* citric acid in 50% ethanol) was added to solubilize the stain, and the plates were analyzed in an ELISA plate reader at 540 nm. Absorbance at this wavelength is proportional to the number of cells *(18–20)*. Assays were performed in triplicate. The results are expressed as percent cell growth relative to growth of control cells.

Statistical Analyses

For each assay, the log of the fractional growth inhibition was plotted against the log of the drug concentration, and the Pearson correlation coefficient (r) from the linear regression curve was calculated. To ensure quality control, all r values were required to be greater than .85 for the data to be subjected to multiple drug effect analysis. Multiple drug effect analysis was performed using computer software from Biosoft (Cambridge, U.K.) as described *(6,21,22)*. Details of this methodology have been published previously *(6,21,22)*. Briefly, the $\log[(1/f) - 1]$, where f is cell survival, was plotted against log(drug concentration). From the resulting median effect lines, the x-intercept (log EC_{50}) and slope m were calculated for each drug. These parameters were then used to calculate doses of the component drugs (and combinations) required to produce various cytotoxicity levels according to equation 1. For each level of cytotoxicity, combination index values were then calculated according to equation 2, in which $(D)_1$ and $(D)_2$ are the concentrations of the combination required to produce survival f; $(Df)_1$ and $(Df)_2$ are the concentrations of the component drugs required to produce f.

$$\text{Dose}_1 = \text{Dose } EC_{50}[(1 - f)/f]^{1/m}. \quad [1]$$

$$\text{Combination index} = (D)_1/(Df)_1 + (D)_2/(Df)_2 + \alpha(D)_1(D)_2/(Df)_1(Df)_2. \quad [2]$$

The combination indices were calculated on the basis of the conservative assumption of mutually nonexclusive drug interactions. Two-sided statistical tests were then used (1-group, two-tailed t test, with degrees of freedom = number of concentrations tested – 1) to determine whether mean combination index values resulting from separate experiments at multiple effect levels were statistically significantly different from a combina-

tion index of 1. In this analysis, synergy is defined as combination index values statistically significantly less than 1.0, antagonism as combination index values statistically significantly greater than 1.0, and additivity as combination index values not statistically significantly different from 1.0.

Measurement of Unscheduled DNA Synthesis After Treatment With Carboplatin Plus Anti-HER2 Antibody

Unscheduled DNA synthesis, i.e., any DNA synthesis that occurs while the cell is in a phase of growth or arrest other than S phase, is a measure of DNA repair. For these experiments, we used only SK-BR-3 cells because we have previously published data on DNA repair after exposure to DNA-damaging agents and trastuzumab in other cells lines *(3,5)*, the results of which have been confirmed by others *(2,4)*. Unscheduled DNA synthesis was determined as described *(3)*. Subconfluent monolayers of SK-BR-3 cells were pre-incubated in the presence or absence of anti-HER2 antibody (200 μg/mL) in arginine-deficient, reduced-serum (0.5%) medium for 5 hours and incubated with hydroxyurea (5 m*M*) for 1 hour to induce cell cycle arrest. Cells were then treated with carboplatin (34 μ*M*) for 1 hour and incubated with [^{3}H]thymidine (10 μCi/mL; ICN Biochemicals, Irvine, CA) and hydroxyurea for 3 hours. Cells were harvested and [^{3}H]thymidine incorporation into DNA was determined by liquid scintillation counting. Experiments were performed in triplicate, in which carboplatin-induced DNA damage/repair served as a positive control and isotype-matched immunoglobulin G1 (IgG1) antibody served as the negative control. Results are expressed as a percentage of repair relative to the negative control.

Clonogenic Colony Count Assay

SK-BR-3 cells (5×10^3 cells per 100-mm^3 dish) were plated in complete medium, cultured overnight, and treated with experimental media containing excipient control, anti-HER2 antibody (12.5 μg/mL), carboplatin (1.56 μg/mL), or a combination of both. Colony formation was assessed after 7 days. Colonies were washed in PBS, fixed in 95% ethanol, and stained with hematoxylin for 15 minutes. The dishes were rinsed with PBS, and cell clusters containing 20 or more cells were scored as a colony.

Efficacy of Docetaxel Plus Trastuzumab in an Athymic Mouse Model of HER2-Overexpressing Xenografts

The HER2-overexpressing cell lines do not spontaneously form xenografts in athymic mice, with the exception of the MCF-7/HER2 cells. We therefore used only these MCF-7/HER2 cells for our *in vivo* studies.

Care of the mice was in accordance with institutional animal committee guidelines. Mice were maintained and handled under aseptic conditions, and animals were allowed access to food and water *ad libitum*. Female athymic mice (21.0–30.1 g) aged 4–6 weeks from an outbred strain (CD1 nu/nu; Charles River, Cambridge, MA) were primed for 6 days with 17β-estradiol pellets injected subcutaneously (1.7 mg of estradiol per mouse; Innovative Research of America, Sarasota, FL). MCF-7/HER2 breast cancer cells (which form xenografts in athymic mice) were injected subcutaneously (2.2×10^7 cells per mouse) in the flank. A period of 7 days elapsed to allow formation of tumor nodules (mean xenograft volume = 47.4 ± 3.6 mm^3). Mice were then stratified into treatment groups with one tumor per mouse on the basis of their weight and tumor volume at the start of the experiment, such that the starting weight and tumor volume in each group were uniform. Mice (nine per group) were treated via intraperitoneal injection of 1) vehicle control (200 μL of PBS), 2) docetaxel (17.5 mg/kg in 200 μL of PBS), 3) trastuzumab (5 mg/kg in 200 μL of PBS), or 4) docetaxel plus trastuzumab (17.5 mg/kg and 5 mg/kg, respectively, in 200 μL of PBS). Tumor nodules were monitored twice weekly for 43 days by serial micrometer measurements made by a single observer (M. Beryt), with tumor volumes calculated as length × width × depth. Differences in xenograft volume between groups were analyzed by single-factor analysis of variance of the log-transformed tumor volume data *(5,6,10)*.

For *in vivo* experiments with trastuzumab plus capecitabine, athymic mice bearing MCF-7/HER2 xenografts were assigned to control or treatment groups of 10 mice each, with the exception of one control group of nine mice. Treatment with experimental or control agents began 8 days after xenograft inoculation, at which time xenograft volumes measured ≈50–100 mm^3. Tumor volumes, calculated as length × width × depth, were monitored weekly by a single observer using serial micrometer measurements. Capecitabine and its vehicle control (gum acacia) were administered via oral gavage 5 days per week for 4 weeks. Trastuzumab and its vehicle control were administered via intraperitoneal injection 1 day per week for 4 weeks. The dosing schedule for trastuzumab was designed to achieve target serum trough concentrations of greater than or equal to 20 μg/mL. Human myeloma IgG1 (Calbiochem-Novabiochem, La Jolla, CA) served as the negative control antibody for these experiments and was administered at the same dose and dose interval as trastuzumab. Differences in xenograft volumes between groups were analyzed by single-factor analysis of variance of the log-transformed tumor volume data.

Results

Overexpression of HER2 in Target Cell Lines

Multiple drug effect analysis was performed using four HER2-overexpressing cell lines *(23,24)*. Relative HER2 expression in the cell lines was quantified by ELISA *(25)*. Levels of HER2 overexpression were higher in SK-BR-3 and BT-474 cells than in MDA-MB-361 and MDA-MB-453 cells, in agreement with previously reported data *(24)* (Table 1).

Multiple Drug Effect/Combination Index Analysis of Trastuzumab in Combination With Cytotoxic Chemotherapeutic Agents Against Four Different HER2-Overexpressing Breast Cancer Cell Lines *In Vitro*

To determine the nature of the interaction between trastuzumab and various chemotherapeutic agents, we used the multiple drug effect analysis method of Chou and Talalay *(21)*, which quantitatively describes the interaction between two or more drugs *(6,22)*. The chemotherapeutic drugs, concentration ranges used, and trastuzumab-to-drug molar ratios are shown in Table 2. To provide a reference, Table 2 also contains the peak plasma concentrations achievable for these agents in humans.

Table 1. Amplification and overexpression of HER2 in breast carcinoma cell lines

Cell line	HER-2/neu expression, fmol/mg protein (95% CI)*	HER2 gene copy per cell†	HER2 gene copy per 17cen†
SK-BR-3	9156 (6478 to 11 834)	43	8
BT-474	12 256 (10 595 to 13 917)	47	11
MDA-MB-361	4043 (3598 to 4488)	11	3
MDA-MB-453	3526 (2608 to 4445)	11	2.5
MCF-7	297 (268 to 326)	2.5	1.0
MDF-7/HER2	4771 (4315 to 5227)	ND	ND

*HER2 protein levels were measured by a quantitative enzyme-linked immunosorbent assay. Results reflect the mean values and 95% confidence intervals (CIs) of triplicate measurements from three separate assays.

†Absolute and relative *(24)* HER2 copy number was assessed by fluorescence *in situ* hybridization. 17cen = chromosome 17 centromere probe signals; ND = not done.

Interaction Between Carboplatin and Trastuzumab

We previously reported a synergistic cytotoxic interaction between cisplatin and the anti-HER2 monoclonal antibodies 4D5 and trastuzumab *(5,6)*. Because of the differences between cisplatin and carboplatin *(11,26–34)*, it was important to assess whether anti-HER2 antibodies act synergistically with carboplatin. Combination index values were consistently less than 1.0 for all four cell lines. Mean combination index values ranged from 0.32 (95% confidence interval [CI] = 0.06 to 0.58; $P<.001$) in BT-474 cells to 0.53 (95% CI = 0.41 to 0.65; $P<.001$) in MDA-MB-361 cells, indicating a synergistic interaction against all four cell lines (Fig. 1; Table 3). There also appeared to be evidence of increased synergy in cells with higher quantitative HER2 overexpression (Fig. 1).

We next measured the effect of carboplatin alone or with trastuzumab on unscheduled DNA synthesis, a measure of DNA repair activity. Unscheduled DNA synthesis markedly increased in SK-BR-3 cells treated with carboplatin but not in cells treated with trastuzumab alone (Fig. 2, A). This latter result was expected because trastuzumab does not induce DNA damage *(3,5)* and has no effect on DNA repair activity. However, unscheduled DNA synthesis in SK-BR-3 cells incubated with trastuzumab for 5 hours and then with carboplatin was markedly reduced compared with SK-BR-3 cells incubated with carboplatin alone (Fig. 2, A), similar to results previously reported for cisplatin when given in combination with trastuzumab *(3,5)*.

We next examined whether the observed attenuation of DNA repair (i.e., unscheduled DNA synthesis) was associated with a coordinate increase in cytotoxicity. SK-BR-3 cells treated with the combination of trastuzumab plus carboplatin formed statistically significantly ($P<.001$) fewer colonies than SK-BR-3 cells treated with carboplatin or trastuzumab alone (Fig. 2, B).

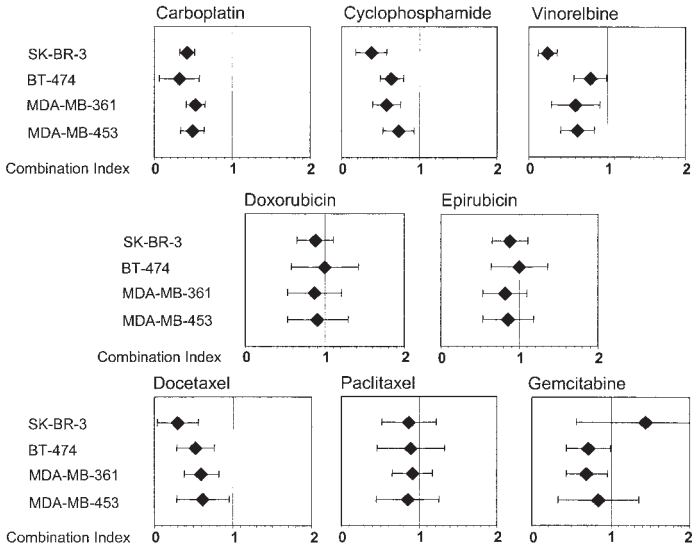

Fig. 1. Mean combination index values for chemotherapeutic drug–trastuzumab combinations in four different human breast cancer cell lines. **Error bars** indicates the 95% confidence intervals of the mean value. Mean is derived from three replicates spanning clinically relevant concentration ranges sufficient to inhibit growth of control cells by 20%–90%. Combination index values were derived from parameters of the median effect plots, and statistical tests were used to determine whether the combination index values at multiple effect levels (IC_{20}–IC_{90}) were statistically significantly different from combination index values equal to 1. Values that are statistically significantly less than 1 indicate synergistic interactions. Values that are statistically significantly greater than 1 indicate antagonistic interactions. Values equal to (or not statistically significantly different from) 1 indicate additive interactions.

Interaction Between 4-Hydroxycyclophosphamide and Trastuzumab

Trastuzumab plus cyclophosphamide increased antitumor efficacy *in vivo* against HER2-overexpresing breast carcinoma xenografts *(6)*. Because cyclophosphamide is metabolized *in vivo* to the active species 4-hydroxycyclophosphamide, we evaluated the interaction between 4-hydroxycyclophosphamide and trastuzumab. *In vitro*, the two agents had a synergistic interaction against all four cell lines, with mean combination index values ranging from 0.38 (95% CI = 0.18 to 0.58; $P<.001$) in

Table 2. Chemotherapeutic drugs, concentration ranges, and trastuzumab plus drug molar ratios and published peak plasma concentrations achievable for these agents in humans

Chemotherapeutic agent	Drug concentration range*	Trastuzumab-to-drug ratio	Plasma peak drug concentration in humans	Reference
4-hydroxycyclophosphamide	0.35–90 μ*M*	1.5×10^{-4}	128 μ*M*	*(72)*
Carboplatin	0.5–135 μ*M*	1.0×10^{-4}	100 μ*M*	*(73)*
Docetaxel	0.06–14.5 n*M*	0.9	3.4 μ*M*	*(74,80)*
Paclitaxel	0.06–14.6 n*M*	0.9	3.19 μ*M*	*(75)*
Vinorelbine	0.017–4.6 n*M*	2.9	0.6–1.0 μ*M*	*(76,77)*
Epirubicin	0.003–0.86 μ*M*	1.5×10^{-2}	1.7 μ*M*	*(78)*
Doxorubicin	0.003–0.86 μ*M*	1.5×10^{-2}	5.6 μ*M*	*(79)*
Gemcitabine	0.2–424 n*M*	1.0	100 μ*M*	*(43)*
Trastuzumab	0.05–432 n*M*		0.816 μ*M*	*(8)*

*The range chosen reflects the concentrations needed to span the effective dose–response curve for each drug (from ≈IC_{20} to ≈IC_{90}).

Table 3. Mean combination index values for chemotherapy drug plus trastuzumab combinations *in vitro*

Chemotherapeutic agent	SK-BR-3		BT-474		MDA-MB-361		MDA-MB-453	
	Combination index (95% CI)	*P* value*	Combination index (95% CI)	*P* value*	Combination index (95% CI)	*P* value*	Combination index (95% CI)	*P* value*
4-hydroxycyclophosphamide	0.38 (0.18 to 0.58)	<.001	0.64 (0.49 to 0.79)	<.001	0.58 (0.40 to 0.76)	.004	0.73 (0.53 to 0.93)	.010
Carboplatin	0.42 (0.32 to 0.52)	<.001	0.32 (0.06 to 0.58)	<.001	0.53 (0.41 to 0.65)	<.001	0.49 (0.34 to 0.64)	<.001
Vinorelbine	0.24 (0.12 to 0.36)	<.001	0.78 (0.57 to 0.99)	.034	0.59 (0.28 to 0.90)	.009	0.62 (0.41 to 0.83)	<.001
Docetaxel	0.30 (0.04 to 0.56)	<.001	0.52 (0.28 to 0.76)	<.001	0.60 (0.38 to 0.82)	.001	0.62 (0.29 to 0.95)	<.001
Paclitaxel	0.87 (0.52 to 1.22)	.381	0.89 (0.46 to 1.32)	.416	0.91 (0.65 to 1.17)	.460	0.85 (0.45 to 1.25)	.427
Doxorubicin	0.88 (0.65 to 1.11)	.284	1.00 (0.58 to 1.42)	.929	0.87 (0.53 to 1.21)	.456	0.91 (0.53 to 1.29)	.634
Epirubicin	0.88 (0.65 to 1.11)	.297	1.00 (0.64 to 1.36)	.981	0.82 (0.54 to 1.10)	.060	0.86 (0.54 to 1.18)	.365
Gemcitabine	1.44 (0.56 to 2.32)	.311	0.71 (0.43 to 0.99)	.039	0.69 (0.43 to 0.95)	.011	0.84 (0.33 to 1.35)	.530

**P* values indicate level of statistical significance compared with a combination index value of 1.0. The mean combination index value, resulting from separate experiments at multiple effect levels, that is statistically significantly less than 1.0 indicates synergy, a value that is statistically significantly greater than 1.0 indicates antagonism, and a value that is not statistically significantly different from 1.0 indicates additivity. CI = confidence interval.

SK-BR-3 cells to 0.73 (95% CI = 0.53 to 0.93; $P = .010$) in MDA-MB-453 cells (Fig. 1; Table 3).

Interactions Between Anthracycline Antibiotics and Trastuzumab

We evaluated the interactions between doxorubicin and trastuzumab and between epirubicin and trastuzumab. *In vitro*, both anthracyclines had additive interactions against all four cell lines when used in combination with trastuzumab (Fig. 1; Table 3). The observed interactions for epirubicin were similar to those of doxorubicin in all four HER2-overexpressing cell lines (Fig. 1; Table 3).

Interaction Between Taxanes and Trastuzumab

We next tested the interaction between taxanes and trastuzumab. The combination of trastuzumab with docetaxel against the four HER2-overexpressing breast cancer cell lines resulted in combination index values ranging from 0.30 (95% CI = 0.04 to 0.56; $P<.001$) in SK-BR-3 cells to 0.62 (95% CI = 0.29 to 0.95; $P<.001$) in MDA-MB-453 cells (Fig. 1; Table 3), indicating synergy for this combination. Cell lines with higher HER2 levels (SK-BR-3 and BT-474) had lower combination index values than cell lines with lower HER2 levels, indicating enhanced synergy against tumor cells with higher HER2 overexpression. By contrast, the combination of trastuzumab with paclitaxel resulted in an additive interaction against the four HER2-overexpressing cell lines, with mean combination index values ranging from 0.85 (95% CI = 0.45 to 1.25; $P = .427$) in MDA-MB-453 cells to 0.91 (95% CI = 0.65 to 1.17; $P = .460$) in MDA-MB-361 cells (Fig. 1; Table 3).

Given the synergistic interaction we observed between trastuzumab and docetaxel *in vitro*, we evaluated the potential antitumor activity of this combination *in vivo*. In preliminary experiments, we determined the dose, route of administration, and schedule for the docetaxel and trastuzumab combination and selected a docetaxel concentration that was administered at a dose similar to that published previously *(35)*. Tumor volumes in mice that received trastuzumab alone were statistically significantly ($P<.05$) smaller than tumor volumes in mice that received a control antibody (Fig. 3). Tumor volumes in mice that received docetaxel alone were also statistically significantly ($P<.05$) smaller than tumor volumes in mice that received a control antibody or trastuzumab alone (Fig. 3). Among all treatment groups, mice that received the combined treatment of trastuzumab plus docetaxel had the largest reduction in tumor volume (Fig. 3). The difference between tumor volumes in mice that received the combination treatment and those that received the control antibody or trastuzumab alone was statistically significant for the duration of the experiment ($P<.05$). The difference between tumor volumes in mice that received docetaxel alone and those that received the combination treatment was statistically significant on days 17 ($P = .032$) through 21 ($P = .05$). Moreover, there were more complete responses by day 27 in mice that received the combination treatment (four of nine) than in mice that received docetaxel alone (one of nine) or

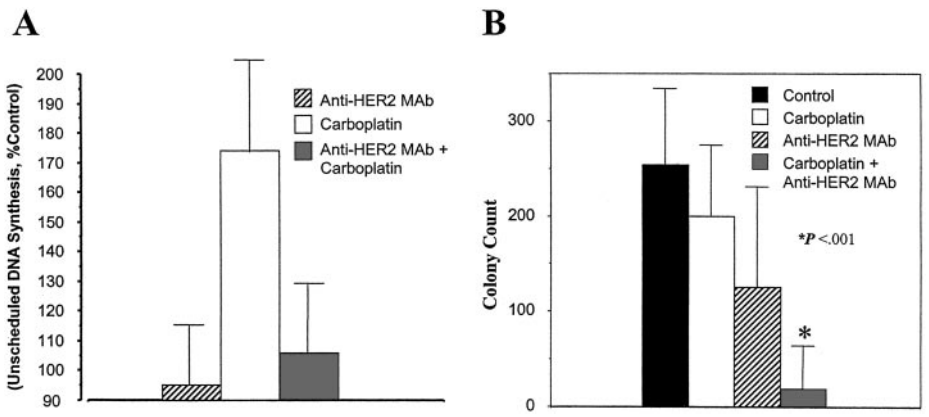

Fig. 2. Repair activity in and proliferation of SK-BR-3 human breast cancer cells in response to anti-HER2 antibodies (trastuzumab), carboplatin, the combination of anti-HER2 antibodies and carboplatin, or to control medium. **A**) DNA repair activity was determined using an unscheduled DNA synthesis assay as described *(3)*. Data are reported as a percentage of that in control-treated cells (i.e., cells treated with drug diluent). **Bars** represent mean (with upper 95% confidence interval) values of three replicates. **B**) Proliferation was determined using a clonogenic assay. SK-BR-3 cells were treated with carboplatin at 1.56 μg/mL, anti-HER2 antibody 4D5 at 12.5 μg/mL, or the combination. Control SK-BR-3 cells were treated with culture medium. Colonies of at least 20 cells were counted after 7 days. **Bars** represent mean (with upper 95% confidence interval) values of three replicates. The number of colonies in cells treated with the combination was statistically significantly less than the number of colonies in the control cells.

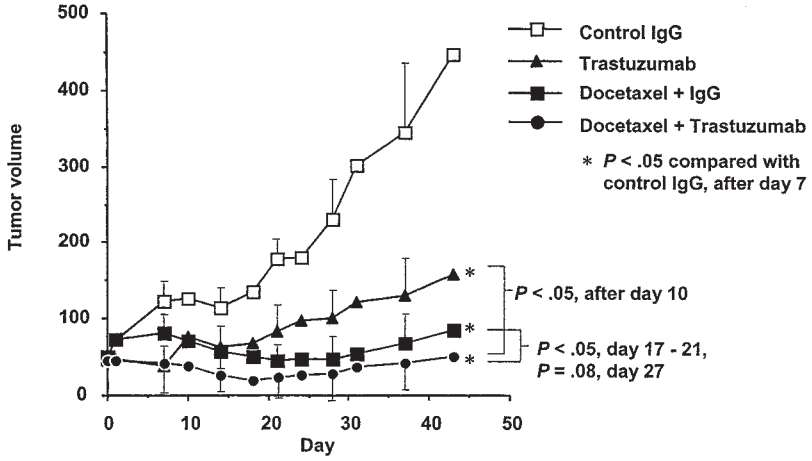

Fig. 3. Effect of anti-HER2 monoclonal antibody trastuzumab alone or in combination with docetaxel on volume of MCF-7/HER2-transfected breast cancer xenografts in athymic mice. Tumor volume was measured with calipers twice weekly. Mice were treated with the anti-HER2 monoclonal antibody trastuzumab alone, docetaxel plus the control immunoglobulin G (IgG) antibody, the combination of docetaxel plus trastuzumab, or the control IgG antibody. Each group contained nine mice, and each mouse had one tumor. Statistical significance of the differences was analyzed by single-factor analysis of variance of the log-transformed tumor volume data.

trastuzumab alone (two of nine). In addition, the time to a complete response was statistically significantly shorter in mice that received the combination treatment than in mice that received the other treatments ($P<.003$) (data not shown). All of the complete responses were sustained until day 58 (data not shown), and necropsy revealed no evidence of tumor at the termination of the experiment. Thus, these results, which are consistent with those observed *in vitro* from the combination index methodology, indicate that the combination of docetaxel plus trastuzumab has better antitumor efficacy *in vivo* than either agent alone.

Interaction Between Vinorelbine and Trastuzumab

Vinorelbine is a semisynthetic vinca alkaloid derivative of vinblastine, which has clinical activity in patients with advanced breast cancer *(14,36–38).* We previously reported an additive interaction between vinblastine and trastuzumab against SK-BR-3 cells *in vitro* and MCF-7/HER2 xenografts *in vivo (6).* The interaction between vinorelbine and trastuzumab appeared to be synergistic against all four HER2-overexpressing cell lines (Fig. 1; Table 3), with mean combination index values ranging from 0.24 (95% CI = 0.12 to 0.36; $P<.001$) in SK-BR-3 cells to 0.78 (95% CI = 0.57 to 0.99; $P = .034$) in BT-474 cells. These results suggest the potential for greater efficacy with this combination than with either agent alone and are consistent with clinical reports *(39–41).*

Interaction Between Gemcitabine and Trastuzumab

Gemcitabine is a deoxycytidine analog antimetabolite with broad clinical activity against a variety of cancers, including breast cancer *(15,42).* The interaction between the combination of gemcitabine, at concentrations well below the plasma peak concentration of 100 μM achievable in humans *(43),* and trastuzumab was concentration dependent, ranging from synergistic at low concentrations of gemcitabine (i.e., ≤106 nmol) to additive or antagonistic at high concentrations of gemcitabine (≥212 nmol) (IC_{80}–IC_{90}) (Fig. 4). The mean combination index values were 1.44 (95% CI = 0.56 to 2.32; $P = .311$) in SK-BR-3 cells, 0.71 (95% CI = 0.43 to 0.99; $P = .039$) in BT-474 cells, 0.69 (95% CI = 0.43 to 0.95; $P = .011$) in MD-MBA-361 cells, and 0.84 (95% CI = 0.33 to 1.35; $P = .530$) in MD-MBA-453 cells (Fig. 1). However, the mean combination index values do not appear to precisely reflect the concentration dependence of the gemcitabine interactions. The concentration dependence of the *in vitro* interaction between trastuzumab and gemcitabine makes it difficult to predict the precise interaction between the two drugs

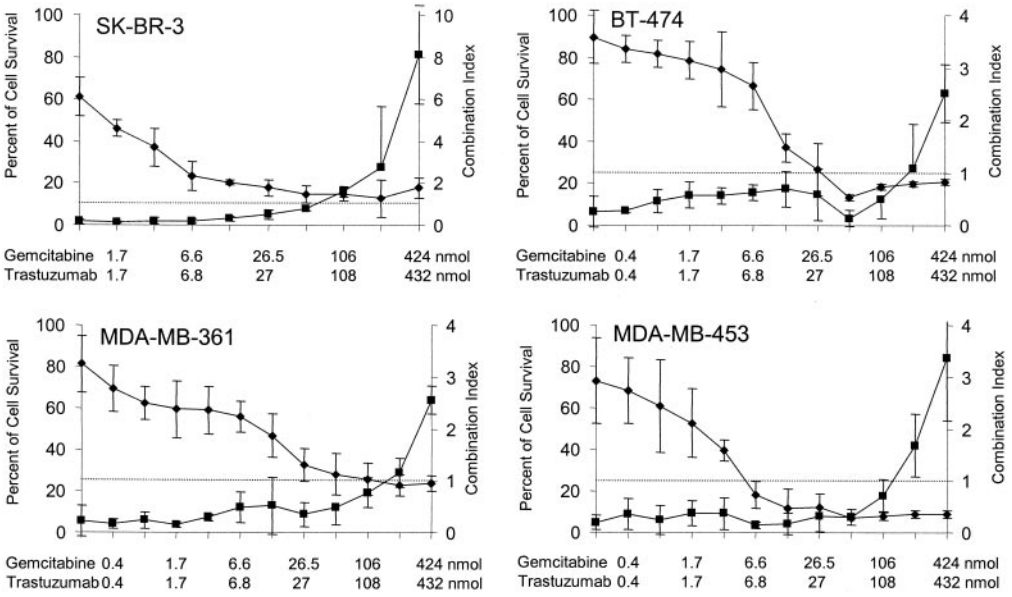

Fig. 4. Combination index values and percent cell survival of SK-BR-3, BT-474, MDA-MB-361, and MDA-MB-453 human breast cancer cells treated with gemcitabine in combination with trastuzumab at multiple effect levels. Cell survival was determined using the crystal violet assay, and data are expressed as mean combination index values ± 95% confidence intervals (■) and as fraction of surviving cells (♦). Gemcitabine, in combination with trastuzumab, had synergistic effects at lower concentration ranges (<106 nmol in SK-BR-3 cells, and <212 nmol in BT-474, MDA-MB-361, and MDA-MB-453 cells), and antagonistic effects at higher concentrations. **Dotted line** identifies a combination index of 1.

in vivo, where the drug concentrations vary according to their pharmacokinetic characteristics and intra-tumoral distribution.

Interaction Between Fluoropyrimidines and Trastuzumab

We previously reported an antagonistic relationship between the antimetabolite 5-fluorouracil, a fluoropyrimidine, and trastuzumab against HER2-overexpressing SK-BR-3 breast carcinoma cells *(6).* Capecitabine is an orally administered fluoropyrimidine carbamate prodrug of 5′-deoxy-5-fluorouridine (5′-DFUR or furtulon) which, *in vivo*, is ultimately converted to 5-fluorouracil. Hepatic carboxylesterase hydrolyzes much of the compound to 5′-deoxy-5-fluorocytidine (5′-DFCR). Cytidine deaminase, an enzyme found in most tissues, including tumors, subsequently converts 5′-DFCR to furtulon. The enzyme thymidine phosphorylase then hydrolyzes 5′-DFUR to the active drug 5-fluorouracil. *In vitro*, the interaction between furtulon, in concentrations of up to 0.26 μ*M*, and trastuzumab against SK-BR-3 cells resulted in mean combination index values of 1.64 (95% CI = 1.41 to 1.87) and 2.11 (95% CI = 1.76 to 2.46) in two separate experiments, indicating that the interaction was antagonistic ($P<.05$). *In vivo*, tumor volumes from mice treated with combinations of capecitabine at two different doses (134.6 or 538.5 mg/kg/day administered by oral gavage for 30 days) plus trastuzumab (0.2 or 0.4 mg/kg/wk administered intraperitoneally for 31 days) were not statistically significantly smaller than those from mice treated with trastuzumab alone ($P>.05$, data not shown). Thus, these data are consistent with *in vitro* observations demonstrating antagonism between either 5-fluorouracil or furtulon and trastuzumab against HER2-overexpressing KPL-4 and BT-474 cells *(44)*, although we could not independently confirm the additive *in vivo* interaction between capecitabine and trastuzumab previously reported in breast cancer xenografts *(44).*

Triple Drug Regimen Containing Trastuzumab

Having identified several individual cytotoxic agents that, when used in combination with trastuzumab, have synergistic cytotoxic effects against HER2-overexpressing cell lines or xenografts, we wanted to test whether combinations of agents would further increase cytotoxicity. Therefore, we investigated the combination of carboplatin, docetaxel, and trastuzumab using the multiple drug effect/combination index model. This combination of synergistic agents yielded strong synergistic interactions across a wide concentration range. The mean combination index value for this three-drug combination was 0.09 (95% CI = 0.06 to 0.12; $P<.001$) (Table 4), suggesting that this drug combination is highly synergistic *in vitro.* The clinical activity of these three drugs as single agents against breast cancers is well established *(11,12,45);* therefore, their use in combination, given the magnitude of observed synergy between these agents *in vitro*, plus the potential absence of an anthracycline antibiotic in this combination, suggest that this combination is potentially useful for clinical evaluation.

Discussion

Using the median effect equation of Chou and the combination index equation of Chou and Talalay, quantitation of synergism or antagonism at different concentration and effect levels allows one to select the best pair, or even triplet, of drugs to combine for potentially maximal antitumor efficacy *(21,22).* Such analyses have previously been shown to be useful for rational clinical protocol designs *(21,22,46,47).* This method of analysis has been useful in identifying combinations of anticancer agents *(46,48,49)*, combinations of anti-HIV agents, and immunosuppressants for organ transplantation, and in purging leukemia cells for autologous bone marrow transplantation *(47,50,51).*

This preclinical study reports quantitative interactions between trastuzumab and different chemotherapeutic drugs representing six classes of cytotoxic anticancer drugs. Importantly, these interactions were tested and confirmed using four different HER2-overexpressing breast cancer cell lines, which we have independently characterized for quantitative HER2 overexpression, indicating that these phenomena are not cell line specific (Table 3).

Four potentially important conclusions can be drawn from this study. First, there was a synergistic interaction between carboplatin and trastuzumab in all four cell lines. It was important to assess whether the reported synergy between anti-HER2 antibodies and cisplatin *(6)* also occurs with carboplatin because there are several differences between these platinum salts. Carboplatin is hydrolyzed more slowly than cisplatin, which delays the onset of maximal DNA cross-linking *(26).* Moreover, the molar concentration of carboplatin required for DNA–DNA intra-strand cross-links and DNA–protein cross-links is less than that of cisplatin *(27).* Clinical differences between these platinum salts are also apparent, with carboplatin having a longer serum half-life and less nephrotoxicity, neurotoxicity, and gastrointestinal toxicity than cisplatin *(28).* Carboplatin has reported clinical activity against breast cancer, primarily when used as first-line therapy or in high-dose combination regimens *(11,29–34).* The mechanism for synergy between anti-HER2 antibody

Table 4. Actual experimental values for the combination index as a function of fractional inhibition of SK-BR-3 cell proliferation by a mixture of carboplatin plus docetaxel plus trastuzumab

Drug concentrations	Combination index values					*r* value for median effect line
	ED11*	ED22	ED39	ED72	ED96	
Carboplatin (0.5–135 μ*M*) plus docetaxel (0.06–14.5 n*M*) plus trastuzumab (0.15–80 n*M*)	0.055	0.087	0.079	0.094	0.118	0.991
Combined effect	Synergy	Synergy	Synergy	Synergy	Synergy	

*All combination index values are statistically significantly less than 1.0 ($P<.001$), which indicates a synergistic interaction. Pearson correlation coefficient *r* values for median effect lines of each single-agent dose–effect curve: carboplatin $r = 0.986$, docetaxel $r = 0.960$, and trastuzumab $r = 0.853$. ED = effective dose required to achieve the percent growth inhibition indicated.

and carboplatin—an attenuation of DNA repair activity—was identical to that described for cisplatin.

Second, there was a synergistic interaction between the alkylating agent 4-hydroxycyclophosphamide and trastuzumab. The activity of this combination was similar to that reported for thiotepa *(6).* Cyclophosphamide is the most widely used alkylating agent in clinical settings; therefore, it is important to show experimentally that there is consistency with the interaction between trastuzumab and both of these alkylating agents (i.e., the observation is not unique to thiotepa).

Third, there was an additive interaction between the anthracycline antibiotics (doxorubicin and epirubicin) and trastuzumab. The combination of trastuzumab with doxorubicin has been shown to have an additive interaction both *in vitro* and *in vivo (6,7).* In a randomized, controlled trial in which patients with breast cancer were randomly assigned to receive doxorubicin-based chemotherapy alone or in combination with trastuzumab, patients receiving the combination therapy had an increased response rate and improved time to tumor progression and survival *(52).* However, the results were associated with an increase in doxorubicin-associated cardiotoxicity, which could not have been predicted from preclinical models because trastuzumab does not bind to murine myocardial HER2 receptors *(52–54).* To avoid the potentially increased risk of anthracycline-associated cardiotoxicity seen with the doxorubicin–trastuzumab combination, the use of less cardiotoxic anthracyclines in combination with trastuzumab has been proposed *(52).* Epirubicin, an epimer of doxorubicin that has clinical activity against breast cancer *(13),* is less cardiotoxic than doxorubicin in both human and animal models of myocyte function *(55,56).* Whether the use of epirubicin will reduce cardiotoxicity is the subject of ongoing clinical trials *(57).*

Fourth, we did not anticipate the results of the interactions between trastuzumab and taxanes and vinca alkaloids. We observed lower combination indices (denoting greater synergy) for the docetaxel plus trastuzumab combination than for the paclitaxel plus trastuzumab combination in all four cell lines. Moreover, *in vivo,* the combination of docetaxel plus trastuzumab had statistically significantly increased antitumor efficacy against MCF-7/HER2-overexpressing xenografts compared with either single agent alone, and there was an increase in durable complete responses in mice treated with docetaxel plus trastuzumab compared with that in mice treated with either agent alone. This result was not observed previously in our model that used mice treated with paclitaxel plus trastuzumab *(6).* The mechanism behind the unique interaction between trastuzumab and docetaxel has yet to be defined, but at least five differences between paclitaxel and docetaxel might explain the observed interaction. First, docetaxel has more potent cytotoxic antitumor effects than paclitaxel on an eqimolar basis *(58);* second, docetaxel achieves higher intracellular concentrations with less cellular efflux of the drug *(59);* third, docetaxel has a higher affinity for microtubules than paclitaxel does *(60,61);* fourth, co-incubation of docetaxel with trastuzumab results in increased apoptosis in SK-BR-3 cells compared with that caused by equimolar concentrations of paclitaxel with trastuzumab *(62);* and fifth, docetaxel is associated with increased phosphorylation of Bcl-2, leading to increased apoptosis at lower concentrations of docetaxel than of paclitaxel *(63).* Any one or more of these mechanisms may account for the observed differences between docetaxel and paclitaxel in the *in vitro* interaction with trastuzumab.

These data suggest differences between the taxane compounds that merit further clinical exploration to test the hypothesis that docetaxel may have superior activity to paclitaxel, particularly in patients receiving trastuzumab. To this end, the combination of docetaxel and trastuzumab has recently been evaluated in clinical phase II studies *(64–66).* The preliminary results have been encouraging in that the combination of docetaxel and trastuzumab demonstrated high levels of activity when used as first- or second-line treatment for HER2-positive metastatic breast cancer *(64–66).* In contrast to our observed distinction between paclitaxel and docetaxel with regard to interaction with trastuzumab, Merlin et al. *(67)* reported internally conflicting data concerning *in vitro* interactions between trastuzumab and taxanes: the combination of trastuzumab and either docetaxel or paclitaxel had additive effects in HER2-overexpressing SK-BR-3 cells but synergistic effects in MCF-7 cells, which lack HER2 gene amplification and do not overexpress HER2 protein *(10,25).*

Methodologic considerations may help explain the differences between our observation of synergy between docetaxel and trastuzumab for four breast cancer cell lines and previous studies *(67).* Correct application of the multiple drug effect analysis methodology requires that a dose–effect relationship exist for each agent tested and for each combination of drugs *(21,22).* However, trastuzumab (at physiologically relevant concentrations) has no growth-inhibiting effects on cell lines such as MCF-7 *(6,68).* Because no dose–effect relationship exists between trastuzumab concentration and cell growth inhibition in MCF-7 cells, the median effect principle, on which multiple drug effect analysis is based, does not apply *(21).* Our analysis also differs from that of Merlin et al. *(67)* in several ways: 1) we calculated combination index values only for actual observed experimental dose levels instead of using interpolated combination index values, 2) we analyzed our data with the conservative assumption of mutually nonexclusive drug interactions, 3) we used the same trastuzumab plus cytotoxic drug concentration ratios for both docetaxel and paclitaxel, 4) we used four independent HER2-overexpressing breast cancer cell lines to avoid cell line–specific observations, 5) we tested drug interactions over a wide concentration range (i.e., concentrations sufficient to inhibit cell growth by 20% to 90% [IC_{20}–IC_{90}]), and 6) we demonstrated that the *in vitro* observations are supported by and consistent with *in vivo* models. Using this approach, we were able to identify distinct differences between the drug interactions of trastuzumab with either docetaxel or paclitaxel that were reproducible across all four HER2-overexpressing cell lines.

Similarly, our data suggest that the interaction between trastuzumab and different vinca alkaloids is specific to each drug, which may merit further clinical evaluation. In particular, the interaction for the combination of vinorelbine plus trastuzumab was synergistic in all four HER2-overexpressing cell lines, whereas the interaction for the combination of vinblastine plus trastuzumab was only additive *(6).* Recently, data from phase II clinical trials that included the combination of vinorelbine plus trastuzumab verify the clinical activity of this regimen *(39–41).*

We also found that the interaction between gemcitabine and trastuzumab is concentration dependent. At lower gemcitabine concentrations, the combination was synergistic in all four cell lines, yet at higher concentrations (i.e., higher than the IC_{80}–IC_{90}

level), the combination ranged from additive to antagonistic in all four cell lines (Fig. 4). The high variability of the interaction between gemcitabine and trastuzumab at these concentrations, all of which are below the clinically relevant plasma peak concentration, make it difficult to predict how effective the combination may be in a clinical setting *(69)*.

For two of the drugs tested (carboplatin and docetaxel), there was evidence of an association between the extent of synergy with trastuzumab and the level of HER2 overexpression in the target cells, suggesting that the mechanism of synergy is HER2 dependent for these drugs (Fig. 1). A similar association was observed with vinorelbine and 4-hydroxycyclophosphamide in three of the four cell lines analyzed. Such findings are consistent with our previous findings *(6)*, which established that synergy for trastuzumab plus cytotoxic drug combinations is specific for HER2-overexpressing tumor cells and is not seen in cells with normal HER2 expression levels.

This study is the first, to our knowledge, to report an analysis of a three-drug interaction involving trastuzumab. We found that the three-way interaction between trastuzumab, carboplatin, and docetaxel was highly synergistic, even at very low drug concentrations. The high degree of synergy observed with this combination suggests that it should be very active clinically. An additional advantage of such a combination is that it might help reduce the risk of cardiotoxicity observed with the anthracycline antibiotic drugs. Each of these three agents has clinically significant activity in first-line treatment of advanced breast cancer when used alone *(11,12,45)*, and the combination of taxanes with platinum salts has been shown to have activity in the disease *(31,32)*. It is logical to conclude that the addition of trastuzumab to taxane-plus-platinum combinations, with the attendant synergistic interaction between each of these drugs and the antibody, may result in improved therapeutic efficacy. This question is currently the subject of several completed *(70)* and ongoing *(71)* clinical trials.

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NOTES

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Manuscript received August 11, 2003; revised March 16, 2004; accepted April 7, 2004.