Inhibitory effects of combinations of HER-2/*neu* antibody and chemotherapeutic agents used for treatment of human breast cancers

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Previous studies have demonstrated a synergistic interaction between rhuMAb HER2 and the cytotoxic drug cisplatin in human breast and ovarian cancer cells. To define the nature of the interaction between rhuMAb HER2 and other classes of cytotoxic drugs, we applied multiple drug effect/combination index (CI) isobologram analysis to a variety of chemotherapeutic drug/rhuMAb HER2 combinations *in vitro*. Synergistic interactions at clinically relevant drug concentrations were observed for rhuMAb HER2 in combination with cisplatin (CI = 0.48, P = 0.003), thiotepa (CI = 0.67, P = 0.0008), and etoposide (CI = 0.54, P = 0.0003). Additive cytotoxic effects were observed with rhuMAb HER2 plus doxorubicin (CI = 1.16, P = 0.13), paclitaxel (CI = 0.91, P = 0.21), methotrexate (CI = 1.15, P = 0.28), and vinblastine (CI = 1.09, P = 0.26). One drug, 5-fluorouracil, was found to be antagonistic with rhuMAb HER2 *in vitro* (CI = 2.87, P = 0.0001). *In vivo* drug/rhuMAb HER2 studies were conducted with HER-2/*neu*-transfected, MCF7 human breast cancer xenografts in athymic mice. Combinations of rhuMAb HER2 plus cyclophosphamide, doxorubicin, paclitaxel, methotrexate, etoposide, and vinblastine *in vivo* resulted in a significant reduction in xenograft volume compared to chemotherapy alone (P < 0.05). Xenografts treated with rhuMAb HER2 plus 5-fluorouracil were not significantly different from 5-fluorouracil alone controls consistent with the subadditive effects observed with this combination *in vitro*. The synergistic interaction of rhuMAb HER2 with alkylating agents, platinum analogs and topoisomerase II inhibitors, as well as the additive interaction with taxanes, anthracyclines and some antimetabolites in HER-2/*neu*-overexpressing breast cancer cells demonstrates that these are rational combinations to test in human clinical trials.

Keywords: HER-2/*neu* (c-*erb*B-2); chemotherapy; breast cancer; multiple drug effects analysis; synergy

Introduction

Overexpression of p185[HER-2/neu], resulting from amplification of the HER-2/*neu* gene, is associated with poor clinical outcome in 25–30% of carcinomas of the breast (Slamon *et al.*, 1987), as well as in other human malignancies (Semba *et al.*, 1985; Slamon *et al.*, 1989; Berchuck *et al.*, 1991; Yonemura *et al.*, 1991; Hetzel *et al.*, 1992; Lukes *et al.*, 1994; Press *et al.*, 1994; Saffari *et al.*, 1995). The murine monoclonal antibody 4D5 has specificity for a juxtamembrane epitope in the extracellular domain (ECD) of the p185[HER-2/neu] protein (Fendly *et al.*, 1990) and is capable of eliciting an antiproliferative effect against murine cells transformed by HER-2/*neu* as well as human malignant cell lines and xenografts overexpressing this oncogene (Chazin *et al.*, 1992). Importantly, this growth inhibitory effect is specific for cells with HER-2/*neu* overexpression and does not occur with cells expressing normal amounts of the protein (Hudziak *et al.*, 1989; Chazin *et al.*, 1992). A recombinant, humanized form of 4D5 (rhuMAb HER2) has been generated by inserting the complementary-determining regions (CDRs) of 4D5 into the framework of a consensus human IgG₁ (Carter *et al.*, 1992). When compared to murine 4D5, rhuMAb HER2 exhibits a stronger binding affinity for p185[HER-2/neu] but has similar specific antiproliferative activity against HER-2/*neu*-overexpressing cell lines and xenografts.

To determine how best to use this antibody both as a single agent and in combination with established cancer therapeutics, we undertook a series of studies to evaluate its inhibitory effects in preclinical models *in vitro* and *in vivo*. These studies were based on a previous report of enhanced activity of cisplatin (CDDP) when used in combination with antibodies directed against the epidermal growth factor receptor (EGFR) (Aboud-Pirak *et al.*, 1988). Initial studies showed that when used in combination with the drug CDDP, 4D5, rhuMAb HER2, as well as other anti-HER-2/*neu* antibodies, potentiate cytotoxicity of the chemotherapeutic by decreasing DNA repair activity following CDDP-induced DNA damage (Hancock *et al.*, 1991; Pietras *et al.*, 1994). This effect, termed receptor enhanced chemosensitivity (REC), specifically targets HER-2/*neu*-overexpressing cells and has no effect on cells or tissues expressing physiologic levels of the gene. The interaction between 4D5 and CDDP in inhibiting HER-2/*neu*-overexpressing cell lines has been shown to be synergistic resulting in a two-log increase in CDDP-induced cytotoxicity as well as pathologic complete remissions in experimental animals bearing HER-2/*neu*-overexpressing human breast cancer xenografts (Pietras *et al.*, 1994).

Synergy, as it applies to drug-drug interactions, is defined as a combination of two or more drugs which achieves a therapeutic effect greater than that expected by the simple addition of the effects of the component drugs. Such synergistic interactions between drugs may

improve therapeutic results in cancer treatment if the synergism is specific for tumor cells (Chou and Talalay, 1984). Moreover, analysis of the nature of the interaction between two drugs (synergism, addition, or antagonism) may yield insight into the biochemical mechanisms of interaction of the drugs. For example, two drugs targeting the same enzyme or biochemical pathway may compete with one another resulting in an antagonistic interaction, whereas two drugs targeting completely independent pathways may be additive, and one drug which potentiates the action of another may result in therapeutic synergy.

In order to characterize the effects of combinations of rhuMAb HER2 cytotoxic chemotherapeutic drugs commonly used in breast cancer therapy, we utilized the median-effect/combination-index isobologram method of multiple drug effect analysis. With this methodology, combination index (CI) values are calculated for different dose-effect levels based on parameters derived from median-effect plots of the chemotherapeutic drugs alone, rhuMAb HER2 alone, and the combination of the two at fixed molar ratios. CI values <1 indicate synergy, CI = 1 indicates addition, and CI > 1 denotes antagonism (Chou and Talalay, 1984). We performed this analysis with rhuMAb HER2 in combination with eight drugs representing seven different classes of cytotoxic chemotherapeutics *in vitro*. Assays were performed *in vitro* for drug/rhuMAb HER2 combinations at clinically relevant drug/antibody concentrations using a cytotoxicity endpoint employing SK-BR-3 human breast cancer cells which contain HER-2/*neu* gene amplification/overexpression. In addition, to circumvent the possibility that any observed interaction might be unique to an individual cell line or to a specific method of analysis, parallel studies were conducted *in vivo* with the same rhuMAb HER2/drug combinations. HER-2/*neu*-transfected MCF7 human breast carcinoma xenografts which, in contrast to SK-BR-3 cells are tumorigenic in athymic mice, served as the tumor target for the *in vivo* studies. Using this model we also investigated the effect of various chemotherapeutic drugs on the pharmacokinetics of rhuMAb HER2 in a subset of mice receiving either rhuMAb HER2 alone or rhuMAb HER-2 plus cytotoxic drug. Finally, we sought to assess the effect of xenograft size (i.e. tumor burden) on rhuMAb HER2 serum concentrations.

Results

Multiple drug effect analysis of rhuMAb HER2 in combination with cytotoxic chemotherapy drugs on SK-BR-3 breast carcinoma cells *in vitro*

To extend the observations on anti-HER2 monoclonal antibodies in combination with CDDP, and to conduct a comprehensive survey of rhuMAb HER2 in combination with other classes of cytotoxic chemotherapeutic drugs available for clinical use, rhuMAb HER2 was analysed in combination with seven different drug classes. Representative drugs included: the anthracycline antibiotic, doxorubicin (DOX); the taxane drug, paclitaxel (TAX); a topoisomerase II inhibitor etoposide (VP-16); a platinum analog cisplatin (CDDP); a vinca alkaloid vinblastine (VBL); the alkylating agents, thiotepa (TSPA) for *in vitro* experiments and cyclophosphamide (CPA) for *in vivo* experiments; and the antimetabolite drugs methotrexate (MTX) and 5-fluorouracil (5-FU).

In this analysis, dose response curves were constructed for each drug alone, rhuMAb HER2 alone, and the combinations at fixed molar ratios defined as the ratio of the two agents at their maximally effective dose. A representative example of the multiple drug effect analyses performed for all of the chemotherapeutic agent/rhuMAb HER2 combinations is shown for the alkylating agent TSPA (Figure 1 and Table 1). In this analysis Fa and Fu are the fractions of SK-BR-3 cells affected or unaffected, respectively, by the dose (D) of either agent (drug or antibody). DM is the dose required to produce the median effect (analogous to the IC_{50}), and m is the Hill coefficient used to determine whether the dose effect relationships follow sigmoidal dose-response curves (Hill, 1913). Linear regression correlation coefficients (r-values) of the median effect plots (Table 1) reflect that the dose-effect relationships for TSPA, rhuMAb HER2, and the combination, con-
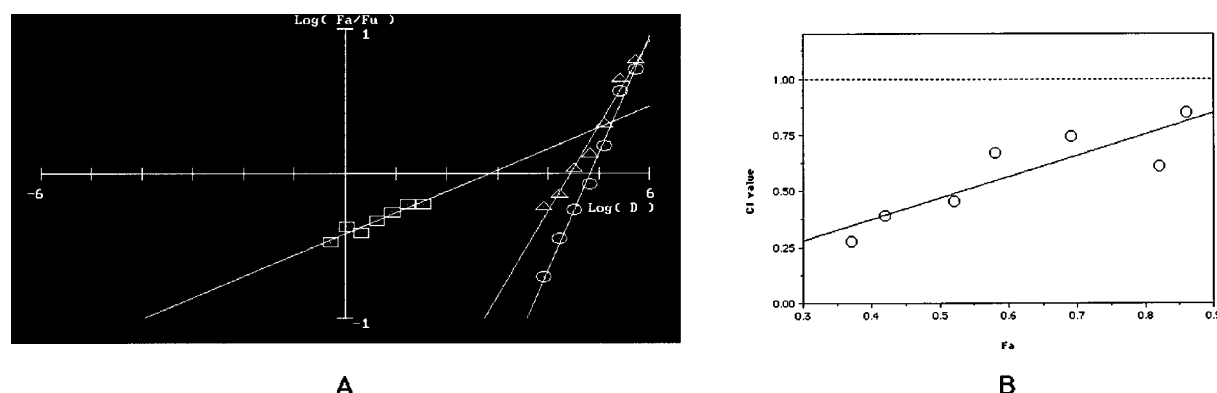

Figure 1 (a) Multiple drug effect plot of TSPA, rhuMAb HER2 and the combination where Fa = the fraction of SK-BR-3 cells affected by the drugs, Fu = the fraction of cells unaffected, and D = drug dose. (b) Combination Index values for TSPA in combination with rhuMAb HER2 at multiple effect levels. CI values <1 indicate synergy

form to the principle of mass action (in general, r-values >0.9 confirm the validity of this methodology) (Chou and Talalay, 1984). CI values for the combination of TSPA and rhuMAb HER2 were significantly less than 1.0 across all combination doses tested ($P = 0.0008$) indicating a synergistic interaction (Figure 1b). A summary of the data from the same analysis applied to each of the eight cytotoxic drug/rhuMAb HER2 combinations tested (Table 2) demonstrates that CDDP, TSPA, and VP-16 exhibit synergistic therapeutic interactions (CI $<$ 1; $P < 0.001$) with rhuMAb HER2 across a wide range ($\sim 0.2-0.8$) of Fa values. Additive interactions (CI $=$ 1) were observed for TAX, DOX, MTX, and VBL in combination with rhuMAb HER2, while only one drug, 5-FU, was found to exhibit an antagonistic (CI $>$ 1; $P = 0.0001$) interaction (Table 2).

P185$^{HER-2/neu}$ expression and tyrosine phosphorylation following exposure to cytotoxic agents

Previous work has demonstrated that exposure of several cancer cell lines to the anthracycline DOX results in an increase in expression of the EGFR and/or its ligand TGF-α (Zuckier and Tritton, 1983; Hanauske *et al.*, 1987; Baselga *et al.*, 1992, 1993). This phenomenon has been proposed to explain the synergistic cytotoxic effects of DOX used in combination with anti-EGFR monoclonal antibodies (Baselga *et al.*, 1992). To test whether p185$^{HER-2/neu}$ expression is similarly altered by DOX, protein expression levels were measured at various times following DOX exposure (Figure 2a). These studies demonstrate that following exposure to DOX, p185$^{HER-2/neu}$ expression levels in SK-BR-3 breast carcinoma cells are unaltered, unlike the reported effects of DOX on EGFR expression in A431 cells (Baselga *et al.*, 1992). We next considered the possibility that cytotoxic drugs may impact p185$^{HER-2/neu}$ functional activity rather than expression levels. We therefore determined the effect of the various cytotoxic drugs on heregulin B-1 and 4D5-induced tyrosine phosphorylation of p185$^{HER-2/neu}$
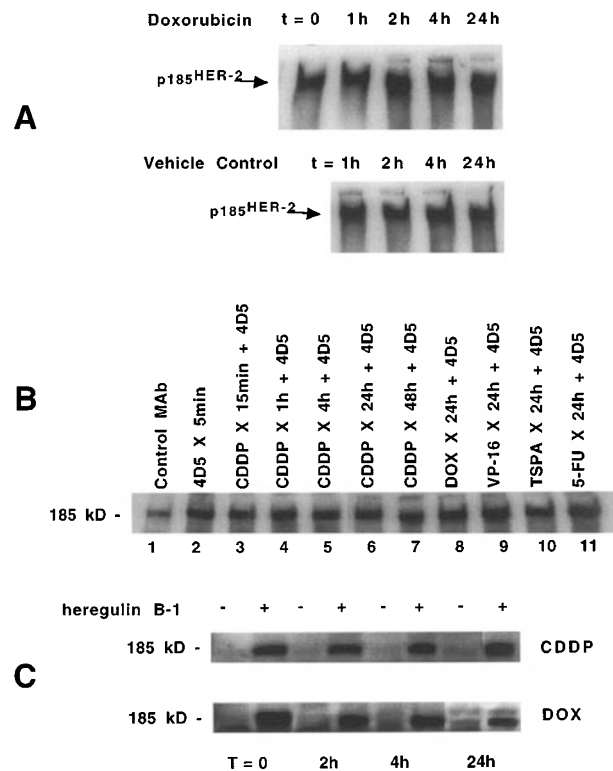

Figure 2 (**a**) Expression of p185$^{HER-2/neu}$ in SK-BR-3 cells following exposure to DOX at the IC$_{30}$ (30 nM) concentration for the times indicated. (**b**) MAb 4D5-induced tyrosine phosphorylation of p185$^{HER-2/neu}$ in SK-BR-3 cells following exposure to chemotherapeutic agents at the IC$_{30}$ concentration at the indicated time points. 4D5-associated tyrosine phosphorylation (lane 2) was observed under all of the chemotherapy conditions tested (lanes 3–11) compared to control (lane 1). (**c**) Heregulin-induced p185$^{HER-2/neu}$ tyrosine phosphorylation in MCF7 cells following exposure to chemotherapeutic drugs at the IC$_{30}$ concentration. These data demonstrate that p185$^{HER-2/neu}$ expression and phosphorylation state are unaltered by prior exposure to the chemotherapeutic agents tested

Table 1 Calculated values for the Combination Index as a function of fractional inhibition of SK-BR-3 cell proliferation by a mixture of TSPA and rhuMAb HER2

| Drug | Combination Index Values | | | | | Parameters | | |
	ED30	ED40	ED50	ED60	ED70	Dm	m	r
TSPA						66.2 μM	0.81	0.99
rhuMAb HER2						675.0 nM	0.15	0.96
TSPA + rhuMAb HER2	0.52	0.37	0.41	0.49	0.60	27.1 μM	0.59	0.99
Diagnosis of combined effect	Synergy	Synergy	Synergy	Synergy	Synergy			

Table 2 Mean combination index values for chemotherapeutic drug/rhuMAb HER2 combinations *in vitro*

Drug	rhuMAb HER2/drug molar ratio	Drug Dose Range (μM)	Combination Index (Mean $\pm$ s.e.m.)	P value	Interaction
TSPA	6.4×10^{-5}	$8.25 - 1.06 \times 10^{3}$	0.67 ± 0.12	0.0008	Synergy
CDDP	4.0×10^{-4}	$6.5 \times 10^{-1} - 1.7 \times 10^{2}$	0.56 ± 0.15	0.001	Synergy
VP-16	9.9×10^{-4}	$2.6 \times 10^{-1} - 6.8 \times 10^{1}$	0.54 ± 0.15	0.0003	Synergy
DOX	9.8×10^{-3}	$2.7 \times 10^{-2} - 6.9$	1.16 ± 0.18	0.13	Addition
TAX	1.4×10^{-1}	$1.8 \times 10^{-3} - 5.0 \times 10^{-1}$	0.91 ± 0.23	0.21	Addition
MTX	3.3×10^{-1}	$8.0 \times 10^{-4} - 2.0 \times 10^{-1}$	1.36 ± 0.17	0.21	Addition
VBL	1.7	$1.6 \times 10^{-4} - 3.9 \times 10^{-2}$	1.09 ± 0.19	0.26	Addition
5-FU	8.8×10^{-5}	$3.0 - 7.65 \times 10^{2}$	2.87 ± 0.51	0.0001	Antagonism

P values indicate level of significance compared to CI $=$ 1.0

(Yarden, 1990; Holmes *et al.*, 1992). MCF7 or SK-BR-3 breast carcinoma cells were treated with cytotoxic drugs, then allowed to incubate with heregulin (10 nM), or 4D5 (12.5 μg/ml). Protein lysates were then analysed by anti-phosphotyrosine immunoblot. These studies demonstrate an increase in p185$^{HER-2/neu}$ tyrosine phosphorylation following incubation with 4D5 compared to a non-specific isotype control antibody (Figure 2b, lanes 1 and 2). Prior exposure of the cells to the three drugs which were found to be synergistic with anti-HER-2/*neu* antibody (CDDP, TSPA, and VP-16) had no effect on 4D5-induced p185 tyrosine phosphorylation (Figure 2b, lanes 3–7 and lanes 9 and 10). Similarly, neither DOX which is additive, nor 5-FU which is antagonistic, had effects on 4D5-induced p185 tyrosine phosphorylation (Figure 2b, lanes 8 and 11). In addition, when heregulin B-1 is used to activate p185$^{HER-2/neu}$ kinase, preincubation of MCF7 breast carcinoma cells with CDDP or DOX had no effect on heregulin-induced p185$^{HER-2/neu}$ tyrosine phosphorylation (Figure 2c). Preincubation of MCF7 cells with TSPA, VP-16, TAX, MTX, VBL, or 5-FU likewise had no effect on heregulin-induced p185$^{HER-2/neu}$ tyrosine phosphorylation (data not shown). Taken together

these data demonstrate that none of the synergistic, additive, or antagonistic effects of chemotherapeutic drugs with anti-HER-2/*neu* antibody can be explained on the basis of either chemotherapy-induced alteration of p185$^{HER-2/neu}$ protein expression levels or its phosphorylation.

Anti-HER-2/neu *antibodies alter cell cycle distribution of HER-2/neu-overexpressing human breast cancer cells*

The cytotoxic effects of antimetabolite drugs are cell cycle dependent (Tannock, 1978). To identify a possible mechanism for the antagonism of 5-FU with rhuMAb HER2 we investigated the effects of murine 4D5 and rhuMAb HER2 on cell cycle distribution of exponentially growing SK-BR-3 and MCF7 cells *in vitro* (Figures 3 and 4). Both the murine 4D5 and rhuMAb HER2 antibodies reduce the percentage of cells undergoing S phase as well as increase the percentage of cells in G0/G1, and these effects are dose-dependent with the maximal antiproliferative activity occurring at antibody concentrations between 1 and 10 μg/ml (Figure 4). There was no significant difference in the magnitude of decrease in S phase
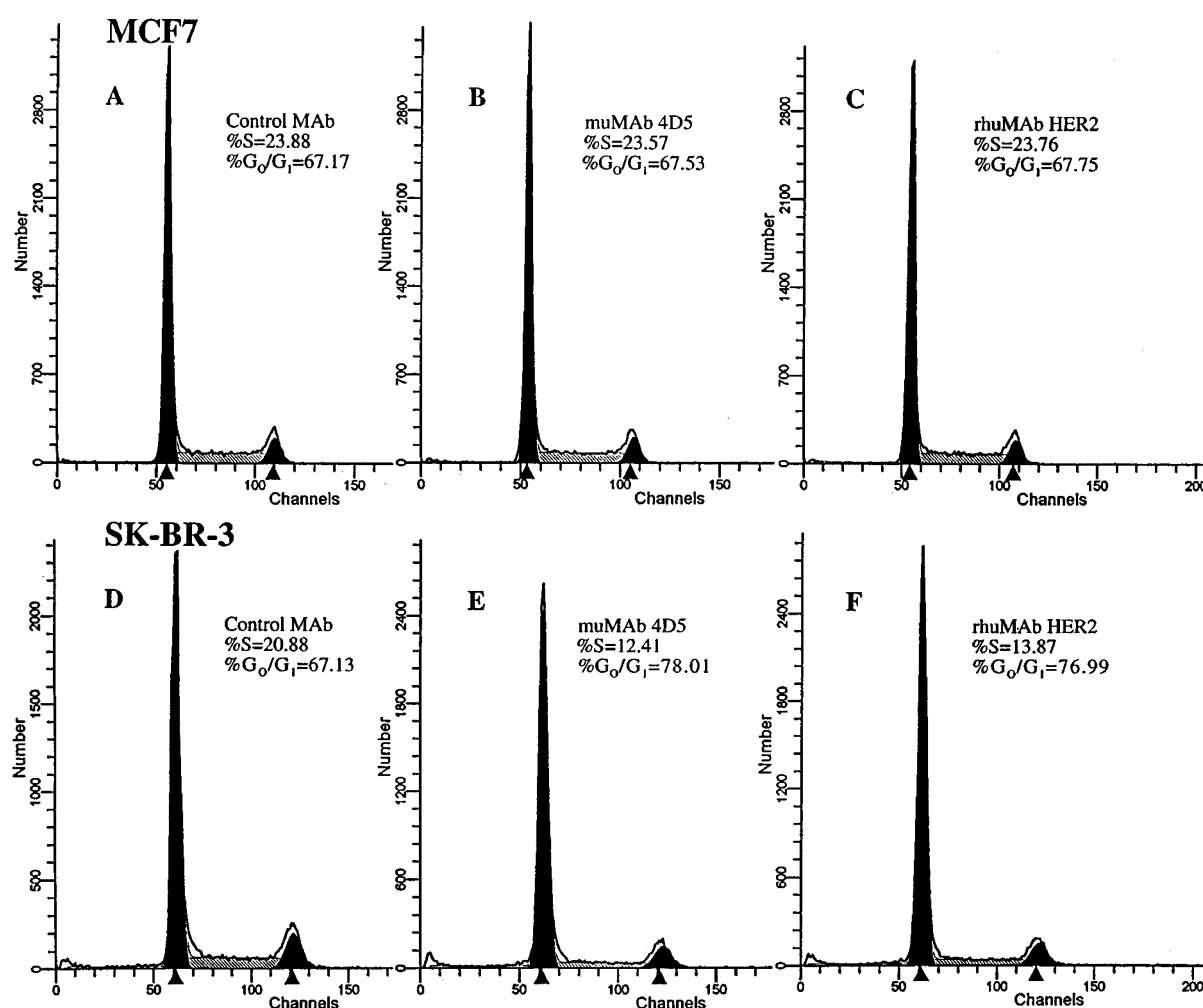

Figure 3 DNA fluorescence flow cytometry histograms of propidium iodide-stained nuclei obtained from MCF7 (**a**–**c**) and SK-BR-3 (**d**–**f**) breast carcinoma cells following treatment with control antibody 6E10, murine anti-p185$^{HER-2/neu}$ antibody 4D5, or humanized anti-p185$^{HER-2/neu}$ antibody (rhuMAb HER2) at a dose of 1 μg/ml for 72 h. These data demonstrate a significant reduction in the fraction of breast carcinoma cells undergoing S phase following treatment with anti-HER-2 antibodies 4D5 and rhuMAb HER2. This effect is specific for cells with HER-2/*neu*-overexpression (SK-BR-3 cells)
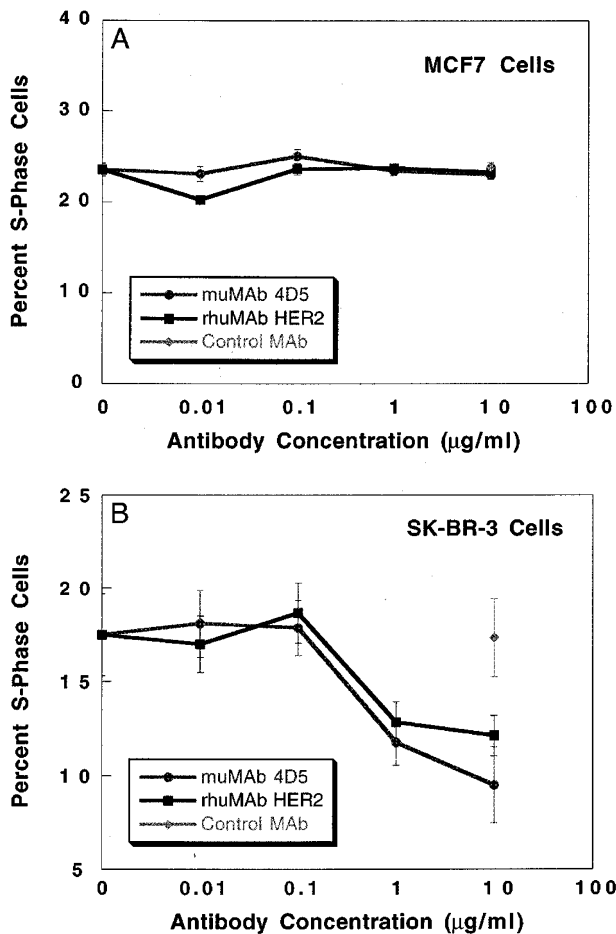
Figure 4 Effect of anti-p185$^{HER-2/neu}$ MAb dose on cell cycle distribution of breast cells without (**a**) and with (**b**) HER-2/*neu* overexpression

fraction of SK-BR-3 cells comparing 4D5 and rhuMAb HER2 indicating the humanization of the murine antibody did not adversely impact its antiproliferative activity. The lack of any effect on cell cycle distribution of MCF7 cells demonstrates the specificity of these antibodies for cells with HER-2/*neu* overexpression. These data suggest that a decrease in the percentage of SK-BR-3 cells in S phase may result in a decreased sensitivity to 5-FU. An antagonistic interaction for the combination of rhuMAb HER2 with the antimetabolite MTX was not observed. The lack of antagonism between MTX and rhuMAb HER2 *in vitro* may be due to the longer incubation period required for MTX (120 vs 72 h) to elicit cytotoxicity in the assay used for the multiple drug effect analysis, and the fact that MTX exerts cytotoxic effects in other phases of the cell cycle in addition to S phase (Buick, 1994).

Effect of rhuMAb HER2 in combination with multiple chemotherapeutic drugs on growth of HER-2/neu-transfected MCF7 breast xenografts in vivo

To further evaluate the potential therapeutic effects of rhuMAb HER2/chemotherapy combinations and to extend our observations beyond a single cell line and preclinical model, a series of *in vivo* studies were performed using human breast cancer xenografts in

athymic mice. All of the doses, routes of administration, and dose intervals for the various cytotoxic drugs and rhuMAb HER2 were based on independent dose finding experiments for this specific strain, age, weight, and sex of athymic mouse. The cytotoxic drug doses used were at or near the maximum tolerated doses previously reported in the literature (Giovanella *et al.*, 1977; Boven and Winograd, 1991).

For the alkylating agent cyclophosphamide CPA, combination with rhuMAb HER2 resulted in a significant reduction ($P < 0.05$) in day 21 xenograft volume compared to either agent alone (Figure 5a). The combination of the anthracycline antibiotic DOX plus rhuMAb HER2 also significantly reduced MCF7/HER-2 xenograft volume compared to either single agent alone (Figure 5b). The combination of the taxane compound TAX plus rhuMAb HER2, which demonstrated an additive interaction *in vitro*, resulted in a significant reduction in day 20 xenograft volume compared to treatment with TAX alone (Figure 5c). However, the difference between rhuMAb HER2 alone and rhuMAb HER2 plus TAX did not reach statistical significance. This is likely due to the relatively small sample size in each group and the fact that the dose of rhuMAb HER2 in this particular analysis (10 mg/kg I.P. twice weekly) yielded a marked reduction in xenograft growth even when used as a single agent.

The following four rhuMAb HER2/drug combinations were studied in a single *in vivo* experiment. For this experiment, a 'rational dose' (RD) or rhuMAb HER2 was chosen as new information became available based on comparative pharmacokinetic studies from both humans and athymic mice. RD is the dose of a given drug which can reproduce a serum level in experimental animals similar to that observed in human subjects (Inaba *et al.*, 1988). The RD for rhuMAb HER2 resulted in a lower cumulative rhuMAb HER2 dose (16 mg/kg vs 30–50 mg/kg) during the 21 day observation period for this experiment compared to the three *in vivo* studies reported above. With this approach, a significant reduction in day 21 xenograft volume was observed for the topoisomerase II inhibitor VP-16 when used in combination with rhuMAb HER2 compared to either agent alone (Figure 6a). The combination of the microtubule inhibitor VBL with rhuMAb HER2 also significantly reduced MCF7/HER-2 xenograft volume compared to treatment with VBL alone or single agent rhuMAb HER2 (Figure 6b). For the antimetabolite class of cytotoxic chemotherapeutics, two drugs with clinical activity against breast cancer were chosen for combination studies. Treatment with MTX, which targets dihydrofolate reductase, plus rhuMAb HER2 resulted in a significant reduction in day 21 MCF7/HER-2 xenograft volume when compared to either MTX alone or rhuMAb HER2 alone (Figure 6c). Finally, the antimetabolite drug 5-FU, which targets thymidylate synthetase, and which was found to be antagonistic when combined with rhuMAb HER2 *in vitro*, did not yield a significant reduction in xenograft volume when compared to 5-FU alone *in vivo* (Figure 6d). Although the combination of rhuMAb HER2 plus 5-FU was superior to rhuMAb HER2 alone in this experiment ($P < 0.05$), the 5-FU dose used had sufficient anti-tumor efficacy as a single agent such
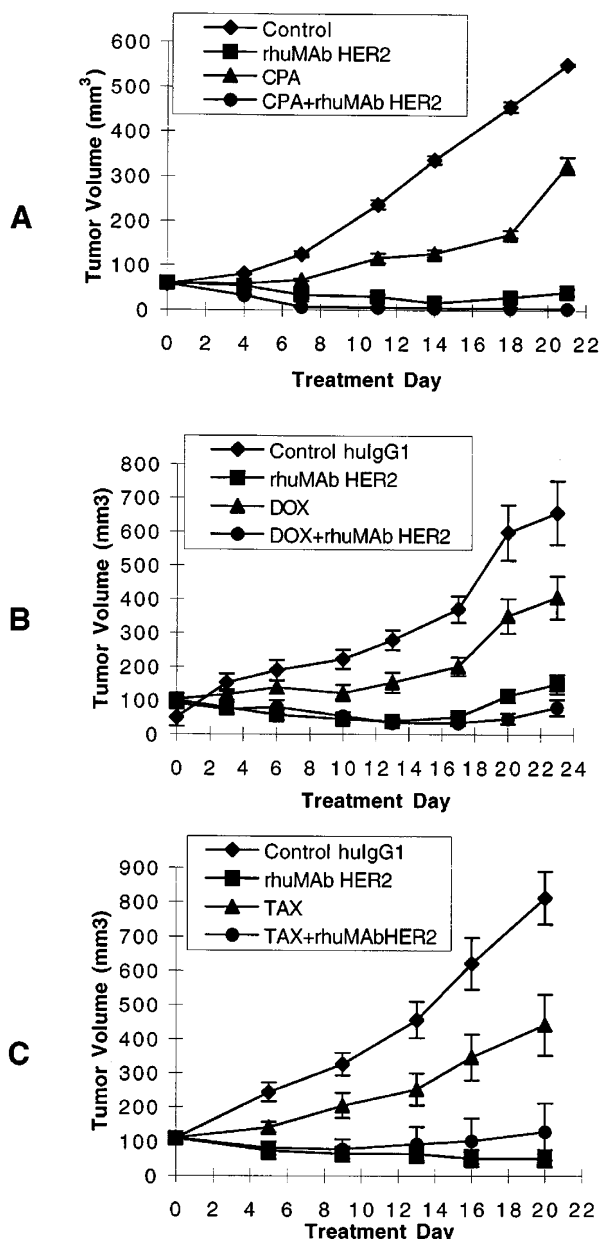
Figure 5 Combination treatment of MCF7/HER-2 breast carcinoma xenografts in athymic mice with rhuMAb HER2 plus chemotherapeutic agents CPA (**a**), DOX (**b**), and TAX (**c**). For each drug, significant reduction in xenograft volume was observed for rhuMAb HER2/drug combinations compared to drug alone controls ($P < 0.05$)

that it was not possible to resolve potential differences between 5-FU alone and the combination with the sample sizes chosen (10 mice/group).

Correlation between rhuMAb HER-2 serum concentration and MCF7/HER-2 xenograft volume

To investigate the relationship between rhuMAb HER2 concentration and xenograft size, trough rhuMAb HER2 serum concentration was measured in a subset of mice on day 64 following extended rhuMAb HER2 treatment at the RD (8 mg/kg loading dose and eight weekly i.p. injections of 4 mg/kg) (Figure 7). A significant inverse correlation (Spearman Rank Corre-

lation rho $= -0.543$; $P = 0.0067$) between trough rhuMAb HER2 concentration and xenograft volume was observed, suggesting that the MCF7/HER-2 xenograft size significantly affects rhuMAb HER2 pharmacology. Furthermore, this effect is independent of serum shed HER-2/*neu* ECD concentration as this molecule was undetectable in any of the murine serum samples analysed (data not shown).

To determine if chemotherapeutic drugs have an effect on rhuMAb HER2 pharmacology, day 64 trough serum rhuMAb HER2 concentrations were analysed by treatment group in a subset of mice used for the *in vivo* studies. Controlling for xenograft size, there was no significant difference in rhuMAb HER2 trough concentration between any of the treatment groups in Figure 7 (data not shown).

Discussion

The protein products of transforming oncogenes have been a target for anti-cancer drug development since the initial discovery of these genes, however there is only one currently approved drug specifically targeting these proteins in clinical use. Identification of the HER-2/*neu* gene alteration and its association with aggressive forms of human breast cancer has resulted in its successful therapeutic targeting (Slamon *et al.*, 1987, 1989; Baselga *et al.*, 1996; Pegram *et al.*, 1998). The interaction of anti-HER-2/*neu* antibodies with p185^HER-2/neu results in receptor tyrosine phosphoryla-tion. (Yarden, 1990), downregulation of receptor expression (Park *et al.*, 1992), internalization of the antibody-receptor complex (Maier *et al.*, 1991), and a decrease in the association of p185^HER-2/neu with its heterodimeric partners HER-3 and/or HER-4 (Reese *et al.*, 1996; Klapper *et al.*, 1997). These events are accompanied by a number of biological effects including most importantly a decrease in cell prolifera-tion (Rodriguez *et al.*, 1993), alteration of cell cycle distribution, and a marked decrease in the ability of the cell to excise and repair DNA damage induced by platinum analogs (Pietras *et al.*, 1994; Arteaga *et al.*, 1994). This enhanced cytotoxic activity is specific for malignant cell lines or xenografts with HER-2/*neu* receptor overexpression since anti-HER-2/*neu* antibo-dies have no such effect on cell lines with physiologic HER-2/*neu* expression levels (Hancock *et al.*, 1991; Pietras *et al.*, 1994). Interaction between the p185^HER-2/neu signaling pathway and CDDP-DNA repair mechanisms has been confirmed using tyrosine kinase inhibitors to block p185^HER-2/neu receptor phosphorylation which inhibits antibody induced attenuation of repair of platinum-DNA adducts (Arteaga *et al.*, 1994). Moreover, reversal of CDDP resistance is possible through transfection and over-expression of HER-2/*neu* cDNA followed by incuba-tion with anti-HER-2/*neu* antibody (Pietras *et al.*, 1994). As a result of this work, studies demonstrating the clinical efficacy of the combination of an anti-HER-2/*neu* antibody plus CDDP were conducted in breast cancer patients with HER-2-overexpressing breast cancers who previously exhibited clinical drug resistance to cytotoxic therapy (Pegram *et al.*, 1998).

To test whether this receptor enhanced chemosensi-tivity mechanism could be observed with other classes
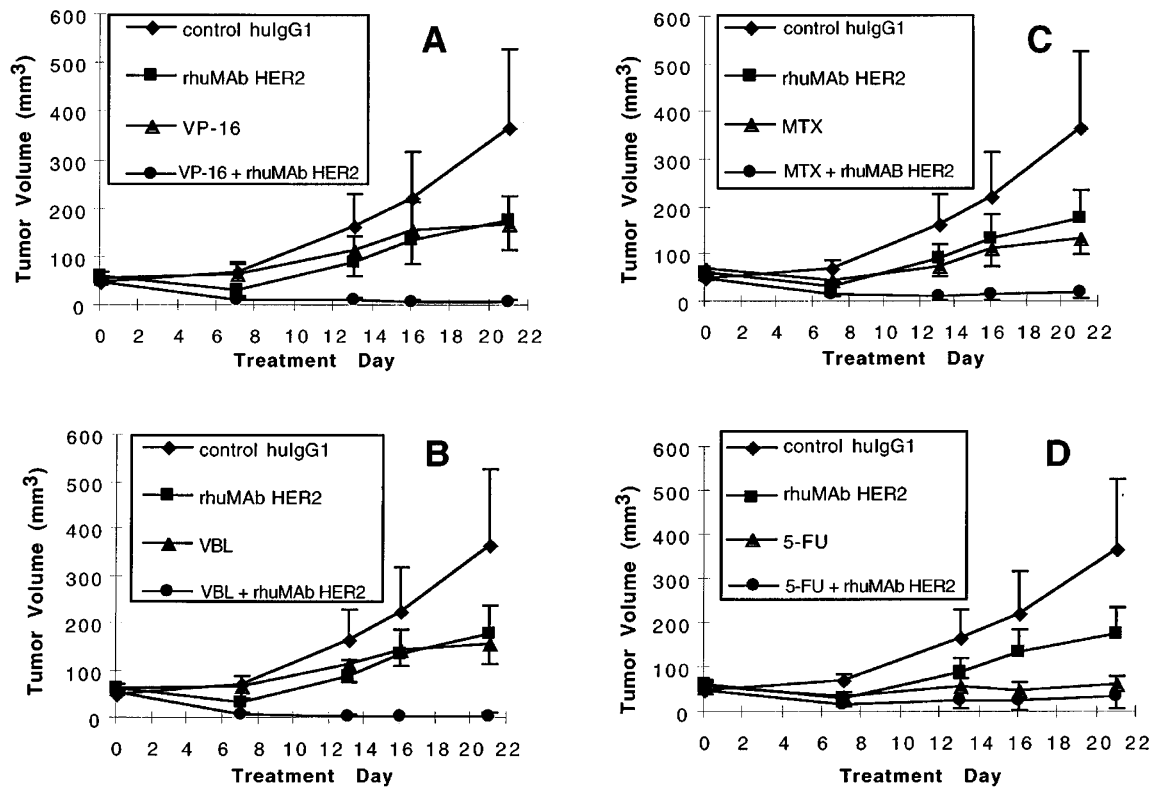
Figure 6 Treatment of MCF7/HER2 xenografts with rhuMAb HER2 in combination with VP-16 (**a**), VBL (**b**), MTX (**c**), and 5-FU (**d**). Combination drug/rhuMAb HER2 treatment resulted in a significant reduction in xenograft volume compared to drug alone, or rhuMAb HER2 alone, controls ($P < 0.05$) for each of the drugs indicated with the exception of 5-FU
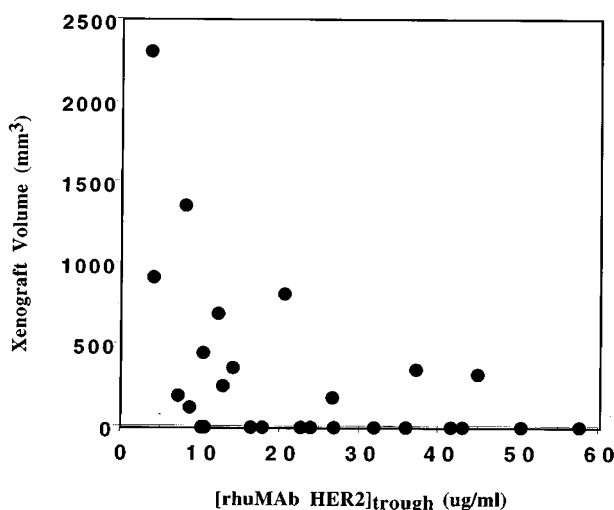

Figure 7 Inverse relationship between MCF7/HER-2 xenograft volume and trough rhuMAb HER2 concentration in murine serum (Spearman Rank Correlation rho $= -0.543$; $P = 0.0067$). These data suggest that binding of rhuMAb HER2 to HER-2/*neu*-overexpressing xenografts reduces serum rhuMAb HER2 concentrations

of cytotoxic chemotherapeutic agents, we performed a series of studies evaluating combinations of cytotoxic agents with rhuMAb HER2 testing seven classes of chemotherapeutics in common clinical use. All concentration ranges of cytotoxic drugs and rhuMAb HER2 tested in these studies were conducted at serum

concentrations achieved in humans (Pegram *et al.*, 1997, 1998). Data from the multiple drug effect analysis methodology are useful, not only in establishing hypotheses as to the mechanism of action of multi-drug combinations, but can also provide insight as to how two drugs should be administered temporally to gain the maximum therapeutic effect. For example, two drugs which are synergistic might best be administered together whereas two antagonistic drugs would be most effective if given sequentially. Data from the current study demonstrate that the platinum compound CDDP, the alkylating agent TSPA, and the topoisomerase II inhibitor VP-16 are synergistic in combination with rhuMAb HER2 in treating HER-2/*neu*-overexpressing SK-BR-3 breast carcinoma cells *in vitro*. These results suggest the possibility of an interaction between the HER-2/*neu* signaling pathway and intracellular DNA repair mechanisms involved with repair of DNA damage resulting from these specific DNA damaging agents. Other potential mechanisms might also explain the synergy observed between rhuMAb HER2 and these agents, including the possibility that rhuMAb HER2 could impact the cellular pharmacology of the drugs resulting in an increase in their cytotoxic activity. An argument against this hypothesis is the fact that the anti-HER-2/*neu* antibody has no effect on the net cellular incorporation of ^{14}C-labeled carboplatin (Pietras *et al.*, 1994) or [^{14}C]-doxorubicin in target cells (Pegram *et al.*, 1992). Another possible mechanism for the observed synergy with rhuMAb HER2 is an effect of cytotoxic drugs on the expression level and/or kinase

activity of p185[HER-2/neu]. An analogous mechanism has been postulated for the EGFR where low doses of DOX appear to increase receptor expression enhancing the antiproliferative activity of anti-EGFR antibody (Zuckier and Tritton, 1983; Hanauske *et al*., 1987; Baselga *et al*., 1992, 1993). The current data demonstrate no change in p185[HER-2/neu] expression levels or in HER-2/*neu* receptor tyrosine phosphorylation following exposure to cytotoxic drugs, suggesting that unlike the EGFR, this mechanism is not operative for the HER-2/*neu* receptor.

Most of the rhuMAb HER2/drug combinations evaluated in this study demonstrate additive rather than synergistic interactions suggesting that the majority of observed antiproliferative effects of rhuMAb HER2 plus cytotoxic drugs are due to a mechanism of action involving each agent acting independently. It is interesting to note that the mechanisms of action of many of the drugs demonstrating additivity do not involve direct DNA damage, but rather disruption of microtubule polymerization/depolymerization (taxanes and vinca alkaloids) or inhibition of DNA synthesis (antimetabolites). This observation is consistent with the hypothesis that the synergy between cytotoxic drugs and rhuMAb HER2 involves an interaction between the HER-2/*neu* signaling and DNA repair pathways. Subsequent to our initial demonstration of the additive effects of rhuMAb HER2 with TAX (Hsu *et al*., 1997), studies confirming this additive interaction were published (Baselga *et al*., 1998). The antimetabolite drug 5-FU is the only drug which demonstrated antagonism when used in combination with rhuMAb HER2 *in vitro*. We have not yet defined the mechanism of this interaction, but it may be the result of alterations in cell cycle distribution caused by rhuMAb HER2 as seen in the current data. It could also be the result of intracellular pharmacological effects, alteration of the enzymatic activity responsible for conversion of 5-FU to 5-fluorodeoxyuridine monophosphate, or an impact on the level of the target enzyme thymidylate synthetase. Further work is needed to explore these possibilities.

The multiple drug effect model is not easily applied to analysis of *in vivo* studies since such analyses, with the number of drugs reported in this study, would require at least 600 athymic mice (assuming five mice per group, five data points for each dose response curve, and three dose response curves – for each drug alone, and in combination with rhuMAb HER2). Consequently we used a more conventional approach for analysis of the *in vivo* data (i.e. single factor ANOVA at fixed time points following treatment of mice with optimal drug or rhuMAb HER2 doses). The cytotoxic drug doses chosen for these experiments are at or near the MTD reported in the literature for each of the cytotoxic drugs. The rhuMAb HER2 doses and schedules were designed to achieve target serum concentrations of $\geqslant 10-20$ μg/ml in mice bearing HER-2/*neu*-overexpressing xenografts of $50-500$ mm³ in size. This antibody concentration is associated with our previously published maximal antiproliferative effect *in vitro* (De Santes *et al*., 1992). With this *in vivo* approach, we demonstrated significantly superior anti-tumor efficacy of rhuMAb HER2 in combination with CPA, DOX, MTX, TAX, VP-16, and VBL when compared to effects of each chemotherapeutic drug

alone. These results are consistent with the *in vitro* data which demonstrate that rhuMAb HER2 is either additive or synergistic with each of these drugs. For the drug 5-FU, which was antagonistic with rhuMAb HER2 *in vitro*, the same combination *in vivo* was superior to rhuMAb HER2 alone but not to 5-FU alone. Although this could be secondary to an antagonistic effect, it is also possible that the sample sizes in each treatment group were not sufficient to discriminate between 5-FU alone and the combination, especially in light of the fact that single agent 5-FU had a marked effect on xenograft volume in this model. It is important to note that in the analysis of the combination studies *in vivo*, rhuMAb HER2 had no deleterious effect on chemotherapeutic drug efficacy. Additionally, we did not observe any overt increase in toxicity, as determined by measurement of animal weights, observations of activity level, and overall survival, in mice treated with rhuMAb HER2/chemotherapy combinations.

Previous analysis of rhuMAb HER2 pharmacokinetics in human subjects demonstrate an inverse association between serum concentrations of rhuMAb HER2 and the shed HER-2/*neu* ECD (Pegram *et al*., 1998). One mechanism which may explain this observation is the direct binding of rhuMAb HER2 to shed HER-2/*neu* ECD in the circulation resulting in a more rapid clearance of the resulting antigen/antibody complex by the reticuloendothelial system. Another potential mechanism is that high serum shed HER-2/*neu* ECD may be a marker of increased tumor burden, resulting in an inverse association between rhuMAb HER2 concentration and shed HER-2/*neu* ECD due to increased binding and turnover of rhuMAb HER2 directly by tumor cells. In the MCF7/HER-2 xenograft model, we measured rhuMAb HER2 trough concentration, shed HER-2/*neu* ECD, and tumor volume concurrently. These data demonstrate a significant inverse relationship between rhuMAb HER2 trough concentration and xenograft volume. This relationship is independent of serum shed HER-2/*neu* ECD since no serum shed HER-2/*neu* ECD could be detected using a sensitive ELISA assay (Sias *et al*., 1990) in this model. These data demonstrate that tumor burden alone in the absence of shed HER-2/*neu* ECD is sufficient to affect rhuMAb HER2 pharmacokinetics. In addition the current data demonstrate that prior treatment with the drugs MTX, 5-FU, VP-16, and VBL *in vivo* had no effect on rhuMAb HER2 trough levels in murine serum. Consistent with this is the published data showing concomitant administration of the drug CDDP had no impact on mean pharmacokinetic parameters of rhuMAb HER2 in a phase II clinical trial of CDDP plus rhuMAb HER2 in 39 patients with advanced breast cancer (Pegram *et al*., 1998). Taken together, these data suggest that the cytotoxic chemotherapeutic drugs evaluated have no effect on rhuMAb HER2 pharmacokinetics *in vivo*.

It is now generally accepted that identification of molecular alterations which play a role in the pathogenesis of specific human malignancies will lead to the development of targeted therapeutics which should be more effective and less toxic than currently available agents. Activation of HER-2/*neu* resulting from gene amplification in human breast cancer is one of what is hoped to be a number of molecular targets

for future drug design in this disease as well as other human cancers. Studies leading to a greater understanding of the biological consequences of HER-2/*neu*-directed therapies should allow the integration of this molecularly-targeted approach with currently available cancer treatments. The additive or synergistic therapeutic interaction between rhuMAb HER2 and a number of chemotherapeutic drugs suggests that such combinations could be successfully exploited in future human clinical trials.

Materials and methods

Multiple drug effect analysis of rhuMAb HER2 in combination with cytotoxic chemotherapeutic agents against HER-2/neu-overexpressing SK-BR-3 breast carcinoma cells in vitro

Aliquots of 5×10^3 SK-BR-3 cells were plated in 96-well microdilution plates. Following cell adherence (24 h), experimental media containing either rhuMAb HER2 (Genentech, Inc. South San Francisco, CA, USA) or control media was added to appropriate wells. After incubation for 24 h, chemotherapeutic agent or control solution was added to triplicate wells and serial twofold dilutions were performed to span the dose range ($\sim EC_{10} - EC_{90}$) suitable for the dose-effect analysis for rhuMAb HER2 and each of the cytotoxic drugs. The dose ranges for rhuMAb HER2 and each drug tested in these experiments are listed in Table 2. We have previously shown that these doses are relevant to drug/rhuMAb HER2 concentrations achievable in human subjects (Pegram *et al.*, 1997, 1998). Eight cytotoxic drugs representative of seven different classes of cytotoxic chemotherapeutic agents were analysed including: platinum analogs – cisplatin (CDDP; Bristol Laboratories, Princeton, NJ, USA); anthracycline antibiotics – doxorubicin (DOX; Cetus Corporation, Emeryville, CA, USA); alkylating agents – thiotepa (TSPA; Lederle Laboratories, Pearl River, NY, USA); taxanes – paclitaxel (TAX; Mead Johnson, Princeton, NJ, USA); vinca alkaloids – vinblastine (VBL; Eli Lilly Co., Indianapolis, IN, USA); topoisomerase II inhibitors – etoposide (VP-16; Bristol Laboratories, Princeton, NJ, USA); and antimetabolites – 5-fluorouracil (5-FU; Solo Park Laboratories, Inc., Elk Grove Village, IL, USA) and methotrexate (MTX; Immunex Corporation, Seattle, WA, USA).

Following incubation for 72 h (120 h for MTX) plates were washed with PBS and stained with 0.5% N-Hexamethylpararosaniline (crystal violet) in methanol. Sorenson's buffer (0.025 M sodium citrate, 0.025 M citric acid in 50% ethanol) 0.1 ml was added to each well, and the plates were analysed in an ELISA plate reader at 540 nm wavelength. Absorbance at this wavelength correlates with cell survival (Flick and Gifford, 1984; Gillies *et al.*, 1986; Reile *et al.*, 1990). Absorbance values from control wells in each plate were compared statistically to ensure even loading of cells from plate to plate for each experiment. Multiple drug effect analysis was performed using computer software (Biosoft, Cambridge, UK). Details of this methodology have been published previously (Chou and Talalay, 1984; Bible and Kaufmann, 1997). Briefly, the $\log[(1/f) - 1]$ was plotted against log (drug dose) (Figure 1). 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 (a). For each level of cytotoxicity, combination index (CI) values were then calculated according to equation (b) where $(D)_1$ 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.

$$\text{Dose}_1 = \text{Dose IC}_{50}[(1 \quad \text{f})/\text{f}]^{1/m} \qquad \text{(a)}$$

$$\text{CI} = (D)_1/(Df)_1 + (D)_2/(Df)_2 + \alpha(D)_1(D)_2/(Df)_1(Df)_2 \quad \text{(b)}$$

The CIs were calculated based on the conservative assumption of mutually nonexclusive drug interactions ($\alpha = 1$), i.e. cytotoxic drugs have mechanisms of action unique from rhuMAb HER2. Statistical tests were then applied (student *t*-test) to determine if the mean CI values resulting from separate experiments at multiple effect levels were significantly different from CI = 1.

Western blot analysis

MCF7 and SK-BR-3 cells were allowed to incubate with cytotoxic drugs at the IC_{30} concentration for the times indicated in Figure 2. Following drug exposure, cells were allowed to incubate with monoclonal anti-HER-2 antibody 4D5 (12.5 μg/ml) for 5 min at 37°C or recombinant heregulin B-1 (10 nM) for 15 min at 37°C or control solutions. Cells were then washed in PBS and lysed at 4°C in 20 mM Tris pH 8.0, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenyl-methyl-sulfonylfluoride, leupeptin 1 μg/ml and aprotinin 1 μg/ml. Insoluble material was cleared by centrifugation and protein was quantitated using BCA (Pierce Biochemicals, Rockford, IL, USA), resolved by SDS–PAGE, and transferred to immobilon-P (Millipore, Bedford, MA, USA). P185$^{\text{HER-2/neu}}$ protein expression was detected using anti-c-*neu* (Oncogene Science, Uniondale, NY, USA); and anti-phosphotyrosine immunoblotting was performed using monoclonal antibody PY20 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell cycle analysis

SK-BR-3 or MCF7 breast cancer cells were plated at a density of 2×10^6/dish in 60×15-mm culture dishes and allowed to adhere overnight. Monolayers were washed with PBS and allowed to incubate with media containing anti-HER-2 or control antibodies at concentrations of 0.01 – 10 μg/ml. Following 72 h incubation, cells were trypsinized, washed with PBS, fixed in ice-cold methanol, and stored at -20°C. Fixed cells were then washed twice with PBS and allowed to incubate with RNAse 100 μg/ml (Worthington Biochemical) for 30 min at 37°C. Following centrifugation, nuclei were subjected to propidium iodide 50 μg/ml (Molecular Probes, Inc.) staining in PBS. Samples were analysed by flow cytometry (Epics Elite, Coulter Corporation) using Modfit LT software (Verity Software House).

Analysis of rhuMAb HER2 in combination with cytotoxic chemotherapeutic drugs against HER-2/neu-overexpressing breast carcinoma xenografts in vivo

HER-2/*neu*-transfected MCF7 cells which express high levels of p185$^{\text{HER-2/neu}}$ and form xenografts in athymic mice were injected subcutaneously (s.q.) at $\sim 1.0 \times 10^7$ cells/tumor in the mid-back region of 4 – 6-week-old, female, CD-1 (*nu/nu*), athymic mice (Charles River Laboratories, Wilmington, MA, USA). Prior to cell injection, all mice were primed with 17β-estradiol (Innovative Research of America, Sarasota, FL, USA) applied s.q. (1.7 mg estradiol/pellet) to promote tumor growth. Tumor volumes, calculated as the product of length, width, and depth, were monitored twice weekly by serial micrometer measurements by a single observer. Five to ten animals were randomly assigned to each treatment group.

Statistical tests were performed (single-factor ANOVA) to assure uniformity in starting tumor volumes between treatment and control groups at the beginning of each experiment. All drugs, with the exception of VP-16 which was administered s.q., were administered by intraperitoneal (i.p.) injection. The dosage of chemotherapeutic agents tested were as follows: DOX (5 mg/kg, day 1), MTX (2 mg/kg, days 1 – 5), VP-16 (20 mg/kg, days 1 – 3), 5-FU (16 mg/kg, days 1 – 4), VBL (0.8 mg/kg, days 1 and 2), cyclophosphamide (CPA; 80 mg/kg, days 0, 4 and 8) and TAX (15 mg/kg, days 1 – 3). These doses were based on independent dose-finding experiments conducted in our laboratory and were near the maximum-tolerated dose for this specific age and strain of female athymic mice. To assure accurate dosing, drug doses were individualized based upon animal weights determined immediately prior to each injection. Treatment with control antibody, cytotoxic drug, rhuMAb HER2, or the combination was initiated 9 – 14 days status post xenograft inoculation at which time xenograft volumes measured $\sim 50 – 100$ mm³. Differences in day 21 xenograft volumes between groups were analysed by single-factor ANOVA of the log transformed tumor volume data. Three dosing schedules of rhuMAb HER2 were used for these experiments. All dosing schedules were designed to achieve target serum concentrations of $\geqslant 10 – 20$ μg/ml during the time chemotherapeutics agents were administered. For the *in vivo* experiments with MTX, VP-16, 5-FU, and VBL, the loading dose of rhuMAb HER2 was 8 mg/kg, and the weekly maintenance dose was 4 mg/kg. For the experiments with DOX and CPA, the dose of rhuMAb HER2 was 10 mg/kg, days 0, 4, and 8. And for the *in vivo* experiment with TAX, the rhuMAb HER2 dose was 10 mg/kg twice per week. Human myeloma IgG$_1$ (Calbiochem-Novabiochem, La Jolla, CA, USA) served as the control antibody for these experiments and was administered at the same dose and dose interval as rhuMAb HER2.

Measurement of rhuMAb HER2 in murine serum

The trough concentration of rhuMAb HER2 in mouse serum was measured using an ELISA with the extracellular domain (ECD) of p185$^{HER-2/neu}$ as the coat antigen. In this format, 100 μl of p185$^{HER-2/neu}$ (Genentech, Inc.) was added to MaxiSorp 96-well microtiter plates (Nunc, Roskilde,

Denmark) at 1 mg/ml in 0.05 M sodium carbonate, pH 9.6. After overnight incubation at 2 – 8°C, plates were washed three times with ELISA wash buffer (PBS containing 0.05% Tween-20) using a Biotek EL304 platewasher (Biotek Instruments, Inc., Winooski, VT, USA). Plates were then blocked with 200 μl/well of ELISA diluent (PBS containing 0.5% BSA, 0.05% Tween-20, and 0.05% Proclin300, pH 7.2) for 1 – 2 h at ambient temperature with agitation. After blocking, plates were washed again three times with ELISA wash buffer. Subsequently, 100 μl of standards, samples, or controls were added to duplicate wells and allowed to incubate for 1 h at ambient temperature. After incubation, the plates were washed six times in ELISA wash buffer, and 100 μl of PBS, pH 7.2, containing 2.2 mmol orthophenylene diamine (OPD), (Sigma Chemical Co.) and 0.012% (vol/vol) hydrogen peroxide (H$_2$O$_2$; Sigma Chemical Co.) were added to each well. When color had fully developed, the reaction was quenched with 100 μl/well of 4.5 molar sulfuric acid. Absorbence values at 492 nm minus 405 nm reference absorbence were measured using an automatic plate reader (Molecular Devices, Palo Alto, CA, USA). A 4-parameter curve fit program was used to generate the standard curve, from which sample and control concentrations were interpolated (SOFTmax). The standard curve range for the assay was 1.56 – 100 mg/ml.

Detection of p185$^{HER-2/neu}$ extracellular domain in murine serum

The method for detection of shed HER-2/*neu* extracellular domain (ECD) levels in serum has been described in detail elsewhere (Sias *et al.*, 1990). Briefly, the ELISA employs pairs of anti-HER-2/*neu* monoclonal antibodies (Genentech, Inc.) which recognize mutually exclusive determinants of the extracellular domain of p185$^{HER-2/neu}$. Wells were coated overnight at 4°C with MAb 7F3 which does not compete with rhuMAb HER2 ECD binding. Assay standards (recombinant, p185$^{HER-2/neu}$ ECD) and murine serum samples were added to appropriate wells and allowed to incubate for 2 h. Following a wash step, secondary antibody was added (MAb 4D5 to detect free shed HER-2 ECD, and MAb 2C4 to detect total shed HER-2 ECD) for 2 h. The bound conjugate is detected with OPD substrate and the resulting absorbence is measured at 490 nm wavelength. The range of the assay is 8.3 – 1800 ng/ml.

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