Oncogene (1999) 18, 2241–2251

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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$^{HER\text{-}2/neu}$, 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$^{HER\text{-}2/neu}$ 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 IgG$_1$ (Carter *et al.*, 1992). When compared to murine 4D5, rhuMAb HER2 exhibits a stronger binding affinity for p185$^{HER\text{-}2/neu}$ 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

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Received 13 May 1998; revised 27 October 1998; accepted 27 October 1998

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-

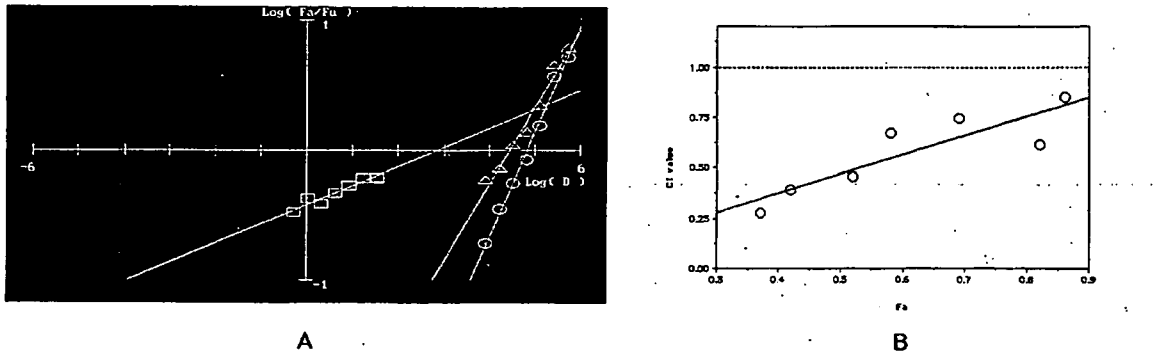

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Figure 1 (a) Multiple drug effect plot of TSPA, rhuMAb HER2 and the combination where Fa = the fraction of SK-BR-3 cells affected by the drugs, Fu = the fraction of cells unaffected, and D = drug dose. (b) Combination Index values for TSPA in combination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

$P185^{HER\text{-}2/neu}$ 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 $p185^{HER\text{-}2/neu}$ 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, $p185^{HER\text{-}2/neu}$ 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 $p185^{HER\text{-}2/neu}$ 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 $p185^{HER\text{-}2/neu}$

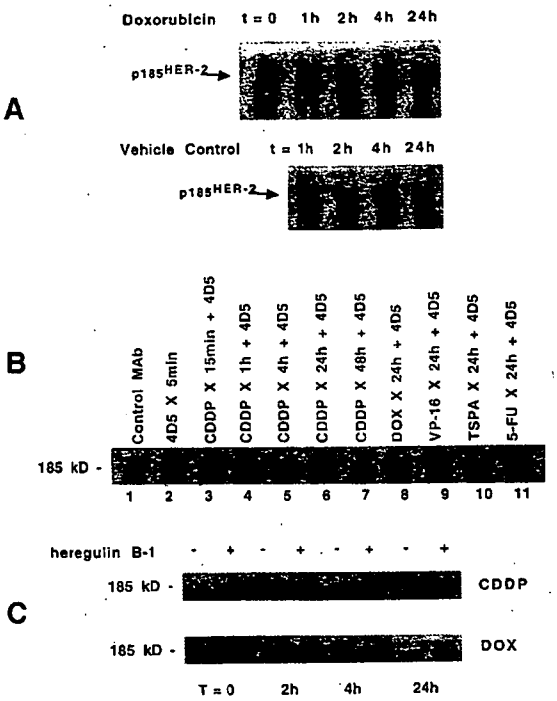

Figure 2 (a) Expression of $p185^{HER\text{-}2/neu}$ in SK-BR-3 cells following exposure to DOX at the IC_{30} (30 nM) concentration for the times indicated. (b) MAb 4D5-induced tyrosine phosphorylation of $p185^{HER\text{-}2/neu}$ in SK-BR-3 cells following exposure to chemotherapeutic agents at the 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 $p185^{HER\text{-}2/neu}$ tyrosine phosphorylation in MCF7 cells following exposure to chemotherapeutic drugs at the IC_{30} concentration. These data demonstrate that $p185^{HER\text{-}2/neu}$ 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

	Combination Index Values					*Parameters*		
Drug	*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±s.e.m.)*	*P value*	*Interaction*
TSPA	6.4×10^{-5}	$8.25-1.06\times10^{3}$	0.67±0.12	0.0008	Synergy
CDDP	4.0×10^{-4}	$6.5\times10^{-1}-1.7\times10^{2}$	0.56±0.15	0.001	Synergy
VP-16	9.9×10^{-4}	$2.6\times10^{-1}-6.8\times10^{1}$	0.54±0.15	0.0003	Synergy
DOX	9.8×10^{-3}	$2.7\times10^{-2}-6.9$	1.16±0.18	0.13	Addition
TAX	1.4×10^{-1}	$1.8\times10^{-3}-5.0\times10^{-1}$	0.91±0.23	0.21	Addition
MTX	3.3×10^{-1}	$8.0\times10^{-4}-2.0\times10^{-1}$	1.36±0.17	0.21	Addition
VBL	1.7	$1.6\times10^{-4}-3.9\times10^{-2}$	1.09±0.19	0.26	Addition
5-FU	8.8×10^{-5}	$3.0-7.65\times10^{2}$	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 $p185^{HER\text{-}2/neu}$ 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 $p185^{HER\text{-}2/neu}$ kinase, preincubation of MCF7 breast carcinoma cells with CDDP or DOX had no effect on heregulin-induced $p185^{HER\text{-}2/neu}$ 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 $p185^{HER\text{-}2/neu}$ 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 $p185^{HER\text{-}2/neu}$ 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

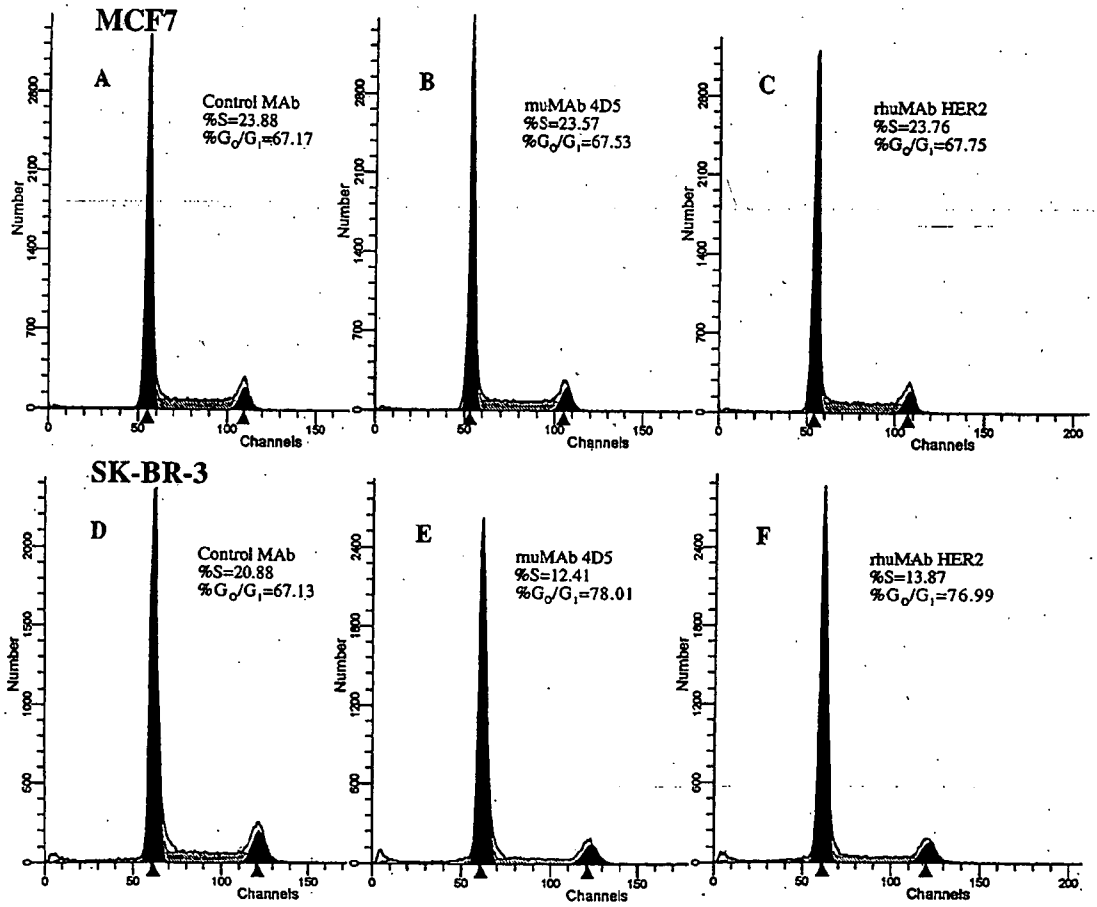

Figure 3 DNA fluorescence flow cytometry histograms of propidium iodide-stained nuclei obtained from MCF7 (a–c) and SK-BR-3 (d–f) breast carcinoma cells following treatment with control antibody 6E10, murine anti-$p185^{HER\text{-}2/neu}$ antibody 4D5, or humanized anti-$p185^{HER\text{-}2/neu}$ antibody (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)

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