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Recombinant Humanized Anti-HER2 Antibody (Herceptin™) Enhances the Antitumor Activity of Paclitaxel and Doxorubicin against HER2/*neu* Overexpressing Human Breast Cancer Xenografts[1]

Jose Baselga,[2] Larry Norton, Joan Albanell, Young-Mee Kim, and John Mendelsohn[3]

Laboratory of Receptor Biology and Department of Medicine [J. B., Y-M. K., J. M.] and Breast Cancer Medicine Service [J. B., L. N.], Memorial Sloan-Kettering Cancer Center, New York, New York 10021; and Medical Oncology Service, Vall d'Hebron University Hospital, 08035 Barcelona, Spain [J. B., J. A.]

ABSTRACT

Recombinant humanized anti-HER2 antibody, rhuMAb HER2, inhibits the growth of breast cancer cells overexpressing HER2 and has clinical activity. We explored in preclinical models its capacity to enhance the tumoricidal effects of paclitaxel and doxorubicin. In cultures of naturally HER2-overexpressing cancer cells, rhuMAb HER2 inhibited growth and enhanced the cytotoxic effects of paclitaxel. Treatment of well established BT-474 breast cancer xenografts overexpressing HER2 in athymic mice with rhuMAb HER2 resulted in a dose-dependent antitumor activity. In combination studies, treatment with paclitaxel and rhuMAb HER2 or doxorubicin and rhuMAb HER2 resulted in greater inhibition of growth than that observed with any agent alone. The combination of paclitaxel and rhuMAb HER2 resulted in the highest tumor growth inhibition and had a significantly superior complete tumor regression rate when compared with either paclitaxel or rhuMAb HER2 alone. Clinical trials that are built on these results are under way.

INTRODUCTION

The HER2 gene (also known as neu and as *c-erbB-2*) encodes a 185-kDa transmembrane tyrosine/kinase receptor, designated $p185^{HER2}$, that has partial homology with the other members of the EGFR[4] family (1–3). HER2 is overexpressed in 25–30% of breast cancers and predicts for a worse prognosis as measured by lower overall survival and disease free survival (4–6). Antibodies directed at $p185^{HER2}$ can inhibit the growth of tumor xenografts and transformed cells that express high levels of this receptor (7–10). The murine MAb 4D5, directed against the extracellular domain of $p185^{HER2}$, is a potent inhibitor of growth of human breast cancer cells that overexpress HER2 (11). However, murine antibodies are limited clinically because they are immunogenic. To facilitate clinical investigation, MAb 4D5 was humanized by inserting the complementary determining regions of MAb 4D5 into the framework of a consensus human immunoglobulin G_1 (12). The resulting recombinant humanized anti-$p185^{HER2}$ monoclonal antibody, rhuMAb HER2 (Herceptin), has a higher affinity for $p185^{HER2}$ (K_D=0.1 nM) than the murine MAb 4D5, and has a cytostatic growth inhibitory effect against breast cancer cells overexpressing HER2 (12, 13). RhuMAb HER2 was found to be safe and to have dose-dependent pharmacokinetics in clinical phase I studies. The proof-of-principle of HER2 as a therapeutic target for anticancer therapy was recently established in patients with HER2-overexpressing metastatic breast cancer. Weekly administration of rhuMAb HER2 induced tumor responses and the combined rate of clinical response and disease stabilization was half of the evaluable patients (14).

One way to optimize the clinical role of anti-HER2 MAbs might be to administer them in combination with chemotherapy. Previous studies with anti-HER2 antibodies have shown enhancement of the antitumor activity of cisplatin (7, 15). It has been postulated that the mechanism for this interaction is the interference of anti-HER2 antibodies with repair of cisplatin-induced DNA-damage (15, 16). Paclitaxel and doxorubicin are two of the most active chemotherapeutic agents for the treatment of patients with breast cancer (17). Thus, finding enhanced antitumor activity of these drugs when combined with anti-HER2 MAbs would have distinct clinical implications for breast cancer therapy. We had previously observed that MAbs C225 and 528 directed at the EGFR, a member of the same tyrosine kinase receptor family, markedly enhanced the antitumor activity of doxorubicin and paclitaxel against cancer cells overexpressing the EGFR (18, 19). Taking these results into consideration, we decided to conduct the present studies with rhuMAb HER2 in combination with paclitaxel or doxorubicin. We have observed enhanced and concentration-dependent inhibition of growth in cultures of human cancer cell lines overexpressing HER2 treated with rhuMAb HER2 plus paclitaxel, and striking antitumor effects in breast carcinoma xenografts, resulting in the cure of well established tumors. RhuMAb HER2 also enhanced, but to a lesser extent, the *in vivo* antitumor effects of doxorubicin.

MATERIALS AND METHODS

Compounds. RhuMAb HER2 and rhu IgG1 were provided by Genentech Inc. (South San Francisco, CA). Paclitaxel was from the Bristol Myers-Squibb Company (Princeton, NJ), and doxorubicin was from Adria Laboratories (Columbus, OH).

Cell Lines. Human breast adenocarcinoma cell lines BT-474, SK-BR-3, and MCF7/HER2 and the human ovarian carcinoma cell line SK-OV-3 were chosen for the present series of studies. BT-474, SK-BR-3, and SK-OV-3 cells were obtained from the American Type Culture Collection (Manassas, VA). The levels of HER2 expression in these cells relative to the normal mammary epithelial cell line 184 are: BT-474, 25-fold increase; SK-BR-3, 33-fold increase; and SK-OV-3, 16.7-fold increase (11). MCF7/HER2–18 cells were a gift of Dr. C. C. Benz (University of California, San Francisco, CA). These cells are a subclone of MCF7 cells that have been transfected with a full length HER2 cDNA coding region, and have a 45-fold increased expression of HER2 (20).

Cell Culture and Monolayer Growth Assay. BT-474 cells were maintained in 1:1 DMEM/Ham's (v/v) supplemented with 10% FCS, 300 mg/l L-glutamine, and 10 mg/ml human insulin. SK-BR-3 and SK-OV-3 cells were cultured in DMEM/Hams's (v/v) with 10% FCS. MCF7/HER2 cells were cultured in DMEM/H16 medium (1 g/l glucose), with 10% FCS, 100 units/ml penicillin, 100 units/ml streptomycin, and 400 μg/ml G-418. All cells were grown at 37°C and 5% CO_2. For monolayer growth assays, cells were distributed into 6-well plates (Falcon 3046, Lincoln Park, NJ) at 10.000 cells/well. On the next day, cells were changed to medium containing 0.5% FCS for 18 h, and then treatment was added. Paclitaxel was added to appropriate wells, with

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[2] To whom requests for reprints should be addressed, at Medical Oncology Service, Vall d'Hebron University Hospital, Paseo Vall d'Hebrón 119–129, Barcelona 08035, Spain. Phone: 011-34-93-2746077; Fax: 011-34-93-2746059; E-mail: baselga@hg.vhebron.es.

[3] Present address: U. T. M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030.

[4] The abbreviations used are: EGFR, epidermal growth factor receptor; MAb, monoclonal antibody; rhuMAb HER2, recombinant humanized MAb HER2.

or without rhuMAb HER2, at concentrations indicated in "Results." Paclitaxel was removed after 1 h by washing the cells, followed by the addition of cell culture medium and rhuMAb HER2. The medium and MAb were replenished every 2–3 days. After 5 days, cells were harvested by trypsinization and counted with a Coulter counter.

Soft Agar Colony Forming Assay. For soft agar assays, a bottom layer of 1 ml of the corresponding culture media containing 0.7% agar (DIFCO Laboratories, Detroit, MI) and 10% FCS was prepared in 35-mm 6-well plates (Falcon 3046). After the bottom layer was solidified, 20.000 cells/well were added in 1.5 ml culture media containing the sample, 0.35% agar, and 10% FCS. RhuMAb HER2 and paclitaxel were added at the concentrations specified in "Results" and the figures. Triplicates were performed for every condition. Cells were incubated 11–14 days at 37°C in 5% CO_2 atmosphere. Colonies with more than 25 cells were then counted manually.

Assay of Tumor Growth in Athymic Nude Mice. Female BALB/c nude mice, 6–8 weeks of age, were used. These mice were bred and maintained in the animal facility at Memorial Sloan-Kettering Cancer Center as described previously (18). BT-474 cells were selected because they express high levels of HER2, have a high level of basal phosphorylation of the receptor, and are growth-inhibited by anti-HER2 MAbs (11, 21). BALB/c nude mice received implants of slow release estrogen pellets (0.72 mg 17β-estradiol; Innovative Research of America, Toledo, OH) and, on the following day, with 1×10^7 BT-474 cells s.c. In our initial studies, we observed significant discrepancies between the rate of tumor take and the tumor size among animals. To optimize the model and enhance tumorigenicity, a large and rapidly growing tumor was removed from one of the mice and these cells were subcultured and expanded. These cells retained both the level of HER2 expression and their response to rhuMAb HER2 when compared with control cells (data not shown), and they were used in all of the experiments described in this study.

Tumors were measured every 3–4 days with vernier calipers. Tumor volume was calculated by the formula: $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2. When tumors reached a mean size of 0.2–0.3 cm^3, the animals were divided into groups with comparable tumor size and treated as described in the text and figures. Briefly, for rhuMAb HER2 treatment, mice received the antibody in PBS, at a dose range of 0.1–30 mg/kg i.p. twice a week. Paclitaxel was given by slow retro-orbital i.v. injection in a solution of normal saline with 8% Cremophor EL and 8% ethanol at a dose range of 5–10 mg/kg on days 1 and 4 (two doses total). This dose schedule was suggested by Dr. Jackie Plowman (National Cancer Institute, Bethesda, MD) and confirmed in our experimental model. Doxorubicin was given i.p. in distilled water at the indicated dose schedules as described previously by us (18). The mice were followed for the observation of xenograft growth rate, body weight changes, and life span.

Statistical Analysis. Rates of complete tumor regression among different treatment groups were compared using the Pearson χ^2 test, and statistical significance of differences in tumor growth among the different treatment groups was determined by the Mann-Whitney *U* test using SPSS 6.1 software. Two-sided *P*s are given at a 95% significance level.

RESULTS

Additive Inhibition of Growth by rhuMAb HER2 and Paclitaxel in Monolayer Cultures. To characterize the antiproliferative effects of rhuMAb HER2 plus paclitaxel in monolayer cultures, BT-474 cells were treated with increasing concentrations of these compounds. Treatment with rhuMAb HER2 (3–30 nM) continuously for 5 days produced a concentration-dependent inhibition of BT-474 proliferation. Exposure of cells to paclitaxel for 1 h (2–50 nM) also resulted in a concentration-dependent inhibition of cell proliferation (data not shown). We then proceeded to combination experiments. RhuMAb HER2 showed an additive and concentration-dependent effect on the growth inhibition induced by paclitaxel. The enhancement of the growth inhibition seen with paclitaxel plus rhuMAb HER2, *versus* paclitaxel alone, ranged from 41–82% at the doses tested (data not shown).

Additive Inhibition of Anchorage-independent Growth by rhuMAb HER2 and Paclitaxel. A series of assays were conducted to characterize the combined effects of rhuMAb HER2 and paclitaxel in soft agar, a more stringent test of mitogenic capacity because several cycles of cell division are required to form a detectable colony. The experiments were conducted in a series of cancer cell lines expressing high levels of HER2 receptors to validate the data obtained with BT-474 cells. RhuMAb HER2 produced a concentration-dependent inhibition of the clonogenic growth of breast cancer cells BT-474 (rhuMAb HER2 dose range, 0.5–2.5 nM) and SK-BR-3 (rhuMAb HER2 dose range, 0.1–10 nM) and also inhibited, but to a lesser degree, the growth of ovarian cancer cells SK-OV-3 (rhuMAb HER2 dose range, 10–100 nM; data not shown). Clonogenic assays of these cell lines after 1-h exposure to increasing concentrations of paclitaxel (dose range, 0.25–900 μM) also showed growth inhibition in a concentration-dependent manner. On the basis of the response data from these experiments, combined treatment assays with increasing concentrations of rhuMAb HER2 and paclitaxel were performed. As shown in Fig. 1, the cotreatment with rhuMAb HER2 and paclitaxel resulted in an additive inhibition of the growth of these three cell lines with endogenous HER2 overexpression. The magnitude of the rhuMAb HER2-mediated enhancement of the antitumor effects of paclitaxel was up to 67% in BT-474 cells, 50% in SK-BR-3 cells, and 32% in SK-OV-3 cells (Fig. 1, *A–C*; for each cell line, only data for the rhuMAb HER2 dose that produced the highest increase in paclitaxel cytotoxicity are shown). In contrast, the growth of the MCF7 cell line transfected with HER2, which has been reported to be resistant *in vitro* to the antiproliferative effects of MAbs directed against the HER2 receptor (20), was minimally affected by rhuMAb HER2 (2.5–100 nM). Cotreatment with this antibody and paclitaxel did not increase the growth suppressive effects of paclitaxel in these cells (Fig. 1*D*).

Effects of rhuMAb HER2 upon Well Established Tumor Xenografts. We conducted animal experiments to determine the efficacy of rhuMAb HER2 in nude mice bearing BT-474 xenografts. In a first set of 39 animals, rhuMAb HER2 was given at doses ranging from 1–30 mg/kg twice a week for 4 weeks. At least nine animals were treated in each group. The control group was treated with a nonspecific rhu IgG MAb at a dose of 30 mg/kg i.p. twice a week, which was the same as the highest dose level of rhuMAb HER2. Treatment was started when xenografts reached a mean size of 0.3 cm^3 (day 7). Marked antitumor activity was observed at all dose levels. Complete tumor eradication was seen in 3 of 10 mice treated with rhuMAb HER2 at 30 mg/kg, in 5 of 10 mice treated at 10 mg/kg, and in 3 of 8 mice treated at 1 mg/kg (Fig 2*A*). Antibody administration was nontoxic, as assayed by animal survival and weight loss.

To better define whether there was a dose-response relationship with rhuMAb HER2 treatment, a second animal experiment was conducted using lower doses of antibody. In this experiment rhuMAb HER2 was administered at doses of 0.1, 0.3, and to 1 mg/kg, given i.p. twice a week for 5 weeks. The total number of mice was 24, allocated into different treatment groups of at least 5 animals/group (Fig. 2*B*). The control group was treated with the nonspecific rhu IgG at a dose of 1 m/kg i.p. Treatment was started when tumors reached a mean size of 0.2 cm^3 (day 10). In this experiment, a dose-dependent antitumor activity was observed (Fig. 2*B*). Doses of 0.1, 0.3, and 1 mg/kg resulted in an average inhibition of tumor growth at 5 weeks of 25, 40, and 80%, respectively, as compared with those mice treated with control antibody. No animal toxicity was observed. A dose of rhuMAb HER2 of 0.3 mg/kg, that modestly inhibited the growth of the BT-474 xenografts, was then chosen for the subsequent combination treatment studies.

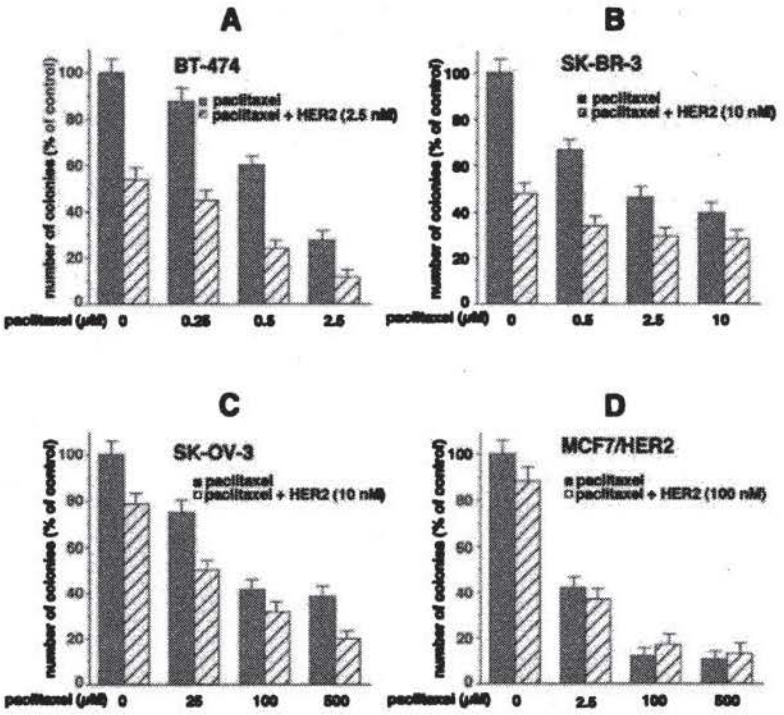

Fig. 1. Cytotoxicity of paclitaxel in combination with rhuMAb HER2 (HER2) in soft agar cultures of BT-474 (*A*), SK-BR-3 (*B*), SK-OV-3 (*C*), and MCF7/HER2 (*D*) cells. Paclitaxel was added for 1 h in the continuous presence or absence of rhuMAb HER2. Cytotoxicity was enhanced in rhuMAb HER2-sensitive cells (BT-474, SK-BR-3, and SK-OV-3 cells, *A-C*), but not in the rhuMAb HER2-resistant MCF7/HER2 cells (*D*). Results represent the mean + SE of triplicate readings.

Effects of rhuMAb HER2 Combined with Paclitaxel or Doxorubicin upon Well Established Tumor Xenografts. We explored next the effects of paclitaxel or doxorubicin plus rhuMAb HER2 in a series of experiments with well established BT-474 xenografts in nude mice.

First, we studied whether rhuMAb HER2 could enhance the antitumor activity of equipotent doses of paclitaxel or doxorubicin (Fig 3*A*). We chose a doxorubicin dose of 10 mg/kg body weight, because we had previously determined it to be a dose killing 10% of the animals (18). The dose of paclitaxel was 10 mg/kg i.v. on day 1 and day 4. This dose was nontoxic, but had antitumor activity similar to the dose of doxorubicin used in preliminary experiments (data not shown). A modest schedule of rhuMAb HER2 of 0.3 mg/kg i.p. twice a week for 5 weeks was chosen to prevent tumor regressions attributable to antibody alone. Control animals were treated with the nonspecific rhu MAb IgG at 0.3 mg/kg i.p. twice a week for 5 weeks. Fifty-one animals were allocated into the different treatment groups after tumors reached an average volume of 0.2 cm^3 (day 11). At least seven animals were treated in each group. Treatment groups consisted of: control MAb; rhuMAb HER2; paclitaxel plus control MAb; paclitaxel plus rhuMAb HER2; doxorubicin plus control MAb; and doxorubicin plus rhuMAb HER2 (Fig. 3*A*). In this experiment, the growth inhibition resulting from the single modality therapies was similar; average tumor volume at 5 weeks was reduced by 36% with rhuMAb HER2 (P = 0.2), 27% with doxorubicin (P = 0.38), and 35% with paclitaxel (P = 0.3). Combined therapy with rhuMAb HER2 plus doxorubicin inhibited growth by 70% *versus* control treated mice (P = 0.04), but it was not statistically superior than doxorubicin alone (P = 0.16) or rhuMAb HER2 alone (P = 0.59). The enhancement of antitumor activity was more profound with the combination of rhuMAb HER2 plus paclitaxel, resulting in growth inhibition of 93% (P = 0.006). In addition, growth inhibition at 5

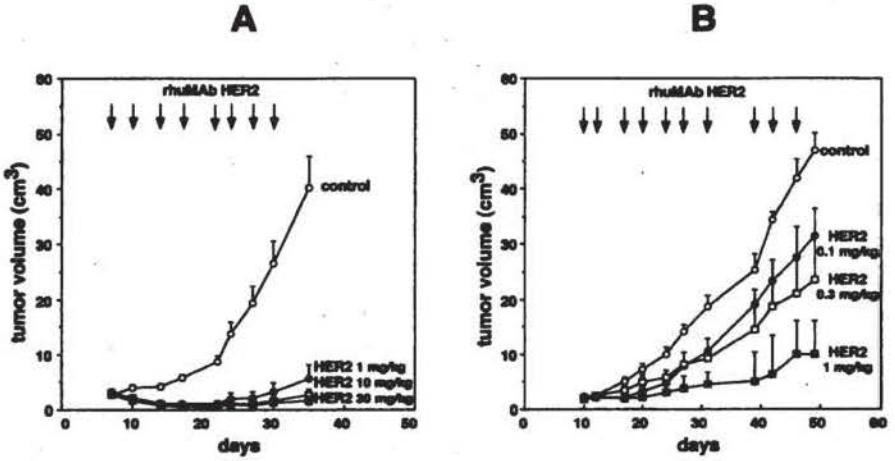

Fig. 2. Activity of rhuMAb HER2 (HER2) against well established BT-474 tumor xenografts in athymic mice in two separate experiments. *A*, rhuMAb HER2 was given i.p. twice a week for 4 weeks at doses of 1, 10, and 30 mg/kg. The control group was treated with a nonspecific rhuMAb IgG at a dose of 30 mg/kg. RhuMAb HER2 at doses equal to or greater than 1 mg/kg markedly suppressed the growth of BT-474 xenografts. *B*, in this experiment lower doses of rhuMAB HER2 were used to define whether rhuMAB HER2 had a dose-response relationship. RhuMAb HER2 was given i.p. twice a week for 5 weeks at doses of 0.1, 0.3, and 1 mg/kg. The control group was treated with nonspecific rhuMAb IgG at a dose of 1 mg/kg. At these dose levels, rhuMAb HER2 induced a dose-dependent inhibition of growth of the BT-474 xenografts. Results are given as mean tumor volume + SE. *Arrows* show days on which treatment was administered.

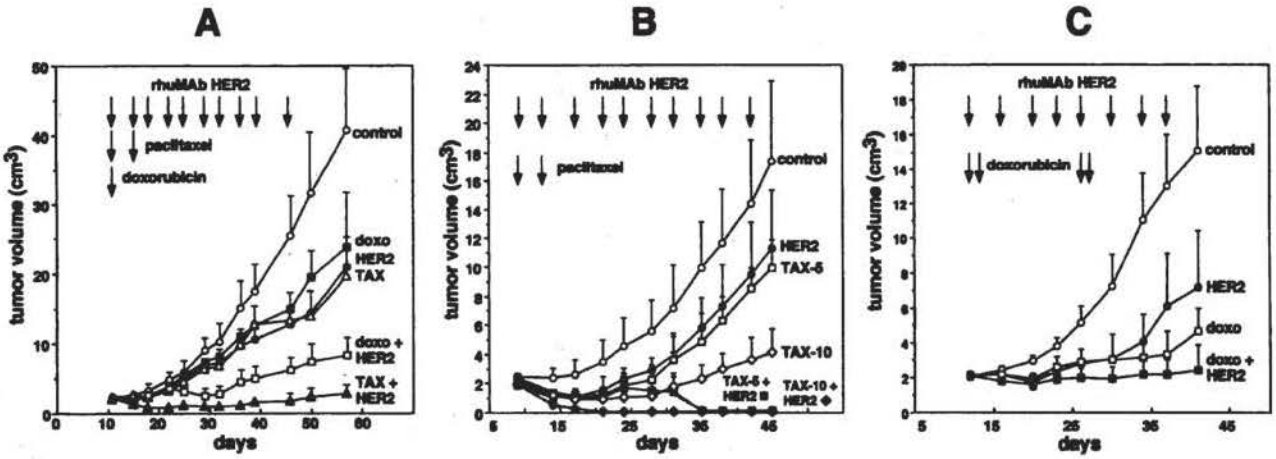

Fig. 3. *A*, antitumor activity of rhuMAb HER2 (HER2) in combination with paclitaxel (TAX) or doxorubicin (doxo) against well established BT-474 tumor xenografts in athymic mice. The control group was treated with the control rhuMAb IgG, 0.3 mg/kg twice weekly i.p. RhuMAb HER2 was given i.p. twice a week for 5 weeks at a dose of 0.3 mg/kg. Paclitaxel was given i.v. at a dose of 10 mg/kg on days 1 and 4. Doxorubicin was administered i.p. at a dose of 10 mg/kg body weight on day 1. Doxorubicin and paclitaxel, given each in combination with the control antibody, resulted in an equipotent, but modest, antitumor activity. The combined treatment with rhuMAb HER2 plus either paclitaxel or doxorubicin resulted in a marked enhancement of the antitumor effects of both chemotherapeutic agents, with greater inhibition of tumor growth in the group of animals treated with paclitaxel and rhuMAb HER2. *B*, antitumor activity of rhuMAb HER2 (HER2) in combination with two dose levels of paclitaxel (TAX) against well established BT-474 tumor xenografts in athymic mice. The control group was treated with the nonspecific rhuMAb IgG 0.3 mg/kg twice weekly i.p. RhuMAb HER2 was given at a dose of 0.3 mg/kg i.p. twice a week for 5 weeks and paclitaxel was given i.v. at two dose levels: 5 and 10 mg/kg on days 1 and 4. Treatment with rhuMAb HER2 resulted in a modest inhibition of growth. Treatment with paclitaxel resulted in a dose-dependent inhibition of growth with greater inhibition of growth at the 10 mg/kg dose level than at the 5 mg/kg dose level. RhuMAb HER2 plus paclitaxel resulted in a striking inhibition of growth regardless of the dose of paclitaxel. *C*, antitumor activity of rhuMAb HER2 (HER2) in combination with repeated doxorubicin (doxo) administration. The control group was treated with PBS. RhuMAb HER2 was given i.p. twice a week for 4 weeks at doses of 0.3 mg/kg, and doxorubicin was given i.p. at a dose of 3.75 mg/kg body weight (days 1 and 2) and repeated on days 14 and 15. RhuMAb HER2 enhanced the antitumor activity of doxorubicin although the combined therapy was not statistically superior than doxorubicin alone or rhuMAb HER2 alone (see text). Results are given as mean tumor volume + SE. *Arrows* show days on which treatment was administered.

weeks was significantly superior in the group treated with rhuMAb HER2 plus paclitaxel *versus* paclitaxel alone ($P = 0.016$), but not *versus* rhuMAb HER2 alone ($P = 0.4$). The significance of all these findings was statistically confirmed at earlier (3 weeks) and latter (8 weeks, which was the time when overall follow-up ended) analyzed time points. RhuMAb HER2 did not increase the toxicity of paclitaxel or doxorubicin in mice as determined by animal survival and weight loss (data not shown).

The above experiment, showing the enhancement of antitumor activity when paclitaxel was given in combination with rhuMAB HER2, was performed with only one dose level of paclitaxel. Therefore, we could not exclude the possibility that the results were restricted to the paclitaxel dose level used. To determine whether the enhanced antitumor effects against xenografts were dose-dependent, as was observed in *in vitro* experiments, a follow-up experiment was conducted with two dose levels of paclitaxel (Fig. 3*B*). On day 10 when tumors reached a mean size of 0.2 cm^3, 50 animals were allocated into treatment groups consisting of at least 8 animals. RhuMAb HER2 dose was unchanged at 0.3 mg/kg twice a week i.p. for 5 weeks and paclitaxel was given at 5 mg/kg and 10 mg/kg i.v. given on days 1 and 4, either alone or in combination with rhuMAb HER2 (Fig 3B). The average tumor volume at 5 weeks, as compared with the control mice treated with rhu IgG, was reduced by 42% with rhuMAb HER2 alone ($P = 0.4$), by 51% with paclitaxel 5 mg/kg × 2 ($P = 0.7$), and by 77% with paclitaxel 10 mg/kg × 2 ($P = 0.3$). In mice treated with rhuMAb HER2 plus paclitaxel (5 or 10 mg/kg × 2) the growth of the xenografts was strikingly affected. Average tumor volume was reduced by more than 98% ($P = 0.04$ for paclitaxel 5 mg/kg × 2 plus rhuMAb HER2 *versus* control; $P = 0.01$ for paclitaxel 10 mg/kg × 2 plus rhuMAb HER2 *versus* control) and resulted in the eradication of well established xenografts in five of eight mice (paclitaxel 5 mg/kg × 2) and in seven of eight mice (paclitaxel 10 mg/kg × 2). In this experiment, growth inhibition effects at 5 weeks resulting from combined treatment with paclitaxel 10 mg/kg × 2 plus rhuMAb HER2 was superior to rhuMAb HER2 alone ($P = 0.02$) and paclitaxel 10 mg/kg × 2 alone (0.02). The significance of these findings was statistically confirmed at earlier (3 weeks) and latter (7 weeks, which was the time when overall follow-up ended) analyzed time points. Combined paclitaxel 5 mg/kg × 2 plus rhuMAbHER2 was also significantly superior than rhuMAb HER2 alone ($P = 0.02$; *versus* paclitaxel 5 mg/kg × 2 alone, $P = 0.08$). In seven mice whose tumors were completely eradicated upon treatment with rhuMAb HER2 plus paclitaxel at both doses and who were followed for 90 days after cell inoculation, no evidence of tumor regrowth was observed. In another two mice with small tumors after therapy and that were followed for 90 days, tumor size was stabilized in one and minimal regrowth was observed in the other.

As seen in Fig. 3*A*, the combination of doxorubicin and rhuMAb HER2 seemed to be less active than paclitaxel and rhuMAb HER2. An attempt was made to improve the antitumor activity of this combination by changing the schedule of doxorubicin administration (Fig. 3*C*). In the prior experiment, the maximally tolerated single dose of doxorubicin (10 mg/kg body weight) had been administered. Here, we opted for two successive administrations of doxorubicin over a 2-week period to increase the total dose of doxorubicin without causing prohibitive toxicity. The schedule used was 3.75 mg/kg body weight given on treatment days 1 and 2, with repeat doxorubicin administration on treatment days 14 and 15 when animals had recovered from the first doxorubicin administration. Fifty-nine mice bearing well established BT-474 tumor xenografts were allocated into treatment groups of at least 13 animals each (Fig. 3*C*). Treatment was started when tumors reached a mean size of 0.2 cm^3. The combined therapy resulted in an enhanced antitumor effect, as shown by an average reduction of tumor volume at 5 weeks by 84% ($P = 0.0008$) as compared with a reduction of 54% with rhuMAb HER2 alone ($P = 0.01$) and 75% with doxorubicin alone ($P = 0.016$) *versus* control-treated mice. However, combined doxorubicin plus rhuMAb HER2 was not statistically better than doxorubicin alone ($P = 0.4$) or

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