The Trifunctional Antibody Ertumaxomab Destroys Tumor Cells That Express Low Levels of Human Epidermal Growth Factor Receptor 2

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Abstract

Human epidermal growth factor receptor 2 (HER2/neu) is an important target for the treatment of the breast cancers in which it is overexpressed. However, no approved anti-HER2/neu therapy is available for the majority of breast cancer patients, who express HER2/neu at low levels (with scores of 1+ or 2+/fluorescence in situ hybridization–negative). The trifunctional antibody ertumaxomab targets HER2/neu, CD3, and activating Fcγ receptors. In presence of ertumaxomab, tri-cell complexes consisting of tumor cells, T cells, and accessory cells form to cause tumor cell lysis. In a phase I trial with metastatic breast cancer patients, ertumaxomab could be applied safely and resulted in radiographically confirmed clinical responses. In this study, we compare ertumaxomab- and trastuzumab-mediated killing of cancer cell lines that express HER2/neu at low and high levels. Under optimal conditions for trastuzumab-mediated destruction of HER2/neu-overexpressing cells, ertumaxomab was able to mediate the elimination of tumor cell lines that express HER2/neu at low levels (1+). Ertumaxomab-mediated activity was accompanied by a Th1-based cytokine release, a unique mode of action of trifunctional antibodies. Competitive binding studies with trastuzumab and 520C9 mapped the binding site of ertumaxomab to the extracellular regions II and III of the HER2/neu ectodomain. This site is distinct from the binding site of trastuzumab, so that HER2/neu-expressing tumor cells can be eliminated by ertumaxomab in the presence of high amounts of trastuzumab. The ability of ertumaxomab to induce cytotoxicity against various tumor cell lines, including those with low HER2/neu antigen density, may provide a novel therapeutic option for breast cancer patients who are not eligible for trastuzumab treatment.

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Introduction

The proto-oncogene HER2 codes for the human epidermal growth factor receptor 2 (HER2/neu), which is overexpressed in 20% to 30% of breast cancer patients (1–3). HER2/neu overexpression is usually based on gene amplification. It is correlated with a poor prognosis, reducing progression-free outcomes and overall survival (4, 5). HER2/neu is an important target for antibody-mediated therapy in breast cancer patients.

The humanized monoclonal anti-HER2/neu antibody trastuzumab inhibits growth of tumor cell lines that strongly express the HER2/neu antigen (6). Several clinical studies have shown the greatest benefit from trastuzumab treatment (7, 8) among women with metastatic breast cancers (scored 3+ or 2+ by immunohistochemistry), and with HER2 gene amplifications (confirmed by fluorescent in situ hybridization; FISH). Based on these results, assessment of the HER2/neu status is absolutely required for all breast cancer patients who may be considered for trastuzumab therapy. However, trastuzumab therapy cannot be offered to the majority of breast cancer patients who have low levels of HER2/neu expression (scored 1+ and 2+) and negative FISH results.

Ertumaxomab is a new member of a family of trifunctional bsicpecific antibodies (anti-HER2/neu × anti-CD3). Kiewe and colleagues (9) presented the first promising clinical data on the safety and efficacy of ertumaxomab in the treatment of metastatic breast cancer patients with different HER2/neu expression levels. Such as BiUII and catumaxomab, which target EpCAM instead of HER2/neu, ertumaxomab evokes a concerted interaction of different immune cell types directed against the tumor (10, 11). Such as BiUII, ertumaxomab might simultaneously recruit and activate FcγRI and FcγRIII-positive accessory cells (i.e., monocytes, macrophages, natural killer cells, and dendritic cells) through its unique isotype combination (mouse IgG2a and rat IgG2b), leading to the phagocytosis of the tumor cells (12). The importance of the mouse/rat hybrid Fc region in the process of immunization has been shown in immunocompetent mouse tumor models, using the trifunctional surrogate antibody BiLu (anti-human EpCAM x anti-murine CD3; refs. 13, 14). As evidenced by Riesenberg and colleagues (15), perforin-mediated cytotoxicity may contribute to the antitumor response. Taken together, these data suggest that ertumaxomab may induce the formation of a tri-cell-complex consisting of HER2/neu+ tumor cells, CD3+ T cells, and FcγR+ accessory cells, leading to efficient elimination of tumor cells.

In this study, we report on the high efficacy of the trifunctional antibody ertumaxomab to specifically eradicate different HER2/neu-positive tumor cell lines, accompanied by the activation of a Th1-type cytokine pattern. In contrast to the monospecific humanized antibody trastuzumab, ertumaxomab destroys tumor cell lines with high and also with low HER2/neu expression. In addition, we are able to show that trastuzumab and ertumaxomab recognize different epitopes on Her2/neu. The possible clinical implications of these findings are discussed.

Materials and Methods

Antibodies, effector cells, and target cell lines. We used freshly harvested peripheral blood mononuclear cells (PBMC) from healthy donors as effector cells. The cells were purified by density centrifugation through Ficoll Histopaque (PAN Biotech) at 897 × g, 15 min, 20°C. They were washed twice with PBS without Mg2+ or Ca2+ (PAN Biotech), and
centrifuged at 458 g, for 10 min, at 20°C. The supernatant was removed, and the pellet was resuspended in 20 mL RPMI 1640, supplemented with 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 1 mmol/L nonessential amino acids, and 10% FCS (PAN Biotech). Cell number and viability were determined using a Neubauer counting chamber after trypan blue staining (Sigma-Aldrich). The breast cancer cell lines SK-BR-3 (ATCC HTB-30) and BT-20 (ATCC HTB-19), the human ileocaecal adenocarcinoma cell line HCT-8 (ATCC CCL-244), and the lung cancer cell line SK-LU-1 (ATCC HTB-57) served as target cells in cytotoxicity assays with PBMC effector cells, followed by 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) cell proliferation assessment (16). The trifunctional antibody ertumaxomab with anti-HER2/neu/anti-CD3 antigen specificities was manufactured by TRION Pharma. The HER2/neu-specific monoclonal mouse antibodies 2502A and 520C9 (ATCC HB-8696; ref. 17) were produced by TRION Research. 2502A constitutes the HER2/neu binding arm of ertumaxomab. The humanized IgG1 antibody trastuzumab (Roche) also recognizes the HER2/neu protein.

Quantitative determination of the cell surface antigen HER2/neu. HER2/neu antigen expression on the surface of tumor target cell lines was quantified using DAKO QIFIKIT (DAKO Cytomation), according to the manufacturer’s protocol. HER2/neu-specific 2502A was used as the primary antibody for HER2/neu detection and quantification. HER2/neu antigen quantity is indicated as specific antibody-binding capacity units after subtraction of isotype control (mouse IgG2a) background.

FISH analysis. Target cell lines (e.g., SK-BR-3 or HCT-8) were spun down on cytopsin slides, and pretreated with pepsin (Sigma-Aldrich) for 5 min at 37°C. Subsequently, cells were fixed with formaldehyde (Sigma-Aldrich) and dehydrated in alcohol (70–100%). Probe hybridization was then performed with SPEC HER2/CEN 17 (Zytovision), overnight at 37°C. The SPEC HER2/CEN 17 Dual Color Probe is a mixture of an orange fluorochrome directly labeled to the centromeric region of chromosome 17 (D17Z1), and a green fluorochrome directly labeled SPEC HER2 probe, specific for the HER2 gene at 17q12. After counterstaining with 4,6-diamidino-2-phenylindole (Zytovision), slides were evaluated at ×100 oil magnification, applying a computerized image analysis (MDS; Applied Imaging).

Fluorescence-activated cell sorter binding competition analysis. Highly positive HER2/neu SK-BR-3 cells were preincubated with varying concentrations of either 520C9 (10,000–100 ng/mL) or trastuzumab (10,000–100 ng/mL) for 10 min, followed by addition of a constant concentration of ertumaxomab (1,000 ng/mL), and further incubation for 45 min. After a washing step, ertumaxomab cell binding was detected by fluorescence-activated cell sorting (FACS) with an anti-rat IgG (H+L) FITC-labeled secondary detection antibody (Dianova).

Cytotoxicity assay. Effector cells (2 × 10⁵; PBMC) and target cells (SK-BR-3, HCT-8, BT-20, or SK-LU-1) at varying E/T ratios were coincubated in flat-bottomed 96-well plates (Greiner) with either trastuzumab or ertumaxomab and combinations of different antibodies concentrations as indicated. PBMCs coincubated with tumor cells alone (allogeneic setting) were used as controls. After 4 d, PBMCs were washed and proliferation of tumor cells was analyzed using the XTT Cell Proliferation kit II, as described by the manufacturer (Roche). Absorbance was measured in a Versamax microplate reader (Molecular Devices), and raw data were analyzed in Excel XP (Microsoft). Percent cytotoxicity was calculated as follows: [absorbance allogeneic reaction – absorbance sample]/absorbance allogeneic reaction × 100. All tests were performed in duplicate, and experiments were repeated with PBMC from three different healthy donors. It should be noted that the value of

Figure 1. HER2/neu-expressing profiles of used cell lines: A, SK-BR-3, (B) HCT-8, (C) BT-20, and (D) SK-LU-1. FACS, fluorescence activated cell sorter histogram, FL1-H = 2502A + secondary detection antibody rat anti-mouse IgG H+L FITC, mouse IgG2a isotype control Me361 (TRION Research) + secondary detection antibody rat anti-mouse IgG H+L FITC; MFI, mean fluorescence intensity; SABC, specific antigen binding capacity as determined by DAKO QIFIKIT.
allogeneic cytotoxicity in the absence of respective antibodies in each sample was always below 13%. In addition, by using these specific assay conditions all antibody entities such as trastuzumab or ertumaxomab completely failed to inhibit growth of respective tumor target cells (HCT-8) or showed only minimal cytotoxic activity (SK-BR-3 cells) in the absence of PBMCs (data not shown). The HER2/neu specificity of ertumaxomab was analyzed by preincubation of the PBMC effector and HCT-8 target cells with excess amounts of the anti-HER2/neu blocking antibody 2502A (200, 500, or 2,000 ng/mL) for 15 min, followed by addition of different concentrations of ertumaxomab (0.001–100 ng/mL).

**Measurement of cytokines.** Ertumaxomab- or trastuzumab-induced cytokine release was determined by culturing PBMCs from healthy donors with target cells (HCT-8) for 24 h in 96 flat-bottomed well plates. Ertumaxomab (100–0.001 ng/mL) or trastuzumab (5,000–0.001 ng/mL) were added at different concentrations. After 24 h, the supernatants were collected and frozen at −20 °C. Cytokines were measured with the human Th1/Th2 cytoketric bead array kit (BD Biosciences) comprising interleukin (IL)-2, IL-6, IFN-γ, and tumor necrosis factor-α (TNF-α; data not shown for TNF-α). Data acquisition and analysis were performed using a FACS Calibur with the cytometric bead array software (BD Biosciences).

**Analysis of cytokine-induced cytotoxicity.** Cytokine-induced killing of the target cell line HCT-8 was analyzed by transferring the supernatants of cytokotoxicity assay samples to 3 × 104 freshly plated tumor cells (HCT-8). Tumor cell proliferation was analyzed using the XTT Cell Proliferation kit II (Roche). Extinction was measured in a Versamax microplate reader (Molecular Devices). Raw data were analyzed in Excel XP (Microsoft). All experiments were performed in duplicates.

### Results

**Quantification of HER2/neu expression on target cells.** To evaluate the influence of the HER2/neu expression level on ertumaxomab- and trastuzumab-mediated cytotoxicity, the amount of HER2/neu antigen on the surface of different human cancer cell lines (SK-BR-3/breast, BT-20/breast, HCT-8/colon, and SK-LU-1/lung) was determined. As expected, SK-BR-3 cells that are scored 3+ (17, 18) stained intensively with anti-HER2/neu monoclonal antibody (mAb) 2502A. Consistent with the intensive staining, the DAKO QIFI test displayed a high specific antigen binding capacity (Fig. 1A). The HCT-8 cells showed a significantly weaker HER2/neu surface density, as did the other cell lines tested (BT-20 and SK-LU-1). Subsequently, the FACS analyses were completed by FISH. Thereby, SK-BR-3 cells revealed amplification of the HER2/neu gene locus, whereas HCT-8, BT-20, and SK-LU-1 cells did not (Fig. 1A, B, C, and D). In summary, the target cell lines HCT-8, BT-20, and SK-LU-1 showed low levels of HER2/neu expression (1+), whereas SK-BR-3 cells scored 3+ for HER2/neu, in agreement with the DAKO HercepTest classification.

**Cytotoxicity of ertumaxomab and trastuzumab against tumor cells with a high HER2/neu expression profile.** Trastuzumab is well-known to promote tumor cell death in tumor cells with a high HER2/neu expression profile. HER2/neu-overexpressing cell lines such as SK-BR-3 (17, 18). We compared the ability of ertumaxomab to inhibit SK-BR-3 growth with trastuzumab at effector (PBMC) to target ratios (E/T) of 20:1 and 5:1. Different antibody concentrations of trastuzumab (5,000–0.001 ng/mL) and ertumaxomab (100–0.001 ng/mL) were added to allogeneic cell samples. As shown in Fig. 2A, SK-BR-3 cells were completely lysed by both ertumaxomab and trastuzumab at an E/T ratio of 20:1. However, at suboptimal E/T conditions (5:1), trastuzumab-induced killing of SK-BR-3 cells declined to ~40%, even at high concentrations (5,000–1,000 ng/mL; Fig. 2B). In contrast, maximal target cell killing remained at ertumaxomab concentrations as low as 25 ng/mL. These results show the superior ability of ertumaxomab to lyse cells that express high levels of HER2/neu under unfavorable E/T ratios.

**Cytotoxicity of ertumaxomab and trastuzumab against tumor cells with a low HER2/neu expression profile.** The efficacious killing of SK-BR-3 tumor cells by ertumaxomab, even at low effector cell numbers, led to the hypothesis that ertumaxomab might also be able to eliminate tumor cells that express HER2/neu at low levels (1+). Based on this hypothesis, a cytotoxicity assay was established to analyze the antitumor efficacy of ertumaxomab, compared with trastuzumab, on cell lines that express low levels of HER2/neu: HCT-8, BT-20, and SK-LU-1. Each of the three cell lines, derived from different carcinomas (colon, breast, and lung), was completely killed in the presence of ertumaxomab in a dose range of 2–5 ng/mL. In contrast, trastuzumab entirely failed to exert any cytotoxic effects on HCT-8, BT-20, and SK-LU-1 cancer cells, even at high concentrations up to 5,000 ng/mL, and with E/T ratios of 20:1 (Fig. 3A, C, and D)—the conditions that are optimal for trastuzumab induced SK-BR-3 cell lysis (Fig. 2A). Interestingly, the cytotoxic antitumor potential of ertumaxomab remained comparable in these experiments, even at an unfavorable E/T ratio of 7:1 (Fig. 3B).

**Cytokines induced by ertumaxomab in the presence of HCT-8 target cells and PBMC.** As both CD3+ T cells and accessory immune cells are redirected by ertumaxomab to target cells, the interaction of these immune cell types can be assessed by detecting the relevant cytokines secreted into the supernatant of the
Doses of ertumaxomab ranging from 1 to 100 ng/mL were able to stimulate high levels of the pro-inflammatory cytokines [i.e., IL-6; IFN-\(\gamma\); TNF-\(\alpha\)] after 24 hours of incubation time (Fig. 4A and B). Of note, in the presence of accessory cells, T cells, and tumor cells, ertumaxomab also induced the production of IL-2 (Fig. 4C). A strong Th1 response is suggested by the high IFN-\(\gamma\) and IL-2 levels that were present in samples treated with ertumaxomab. Furthermore, IL-6 secretion was increased, indicating a proinflammatory response with the contributions of accessory cells (19). In contrast, trastuzumab merely stimulated IL-6 secretion, and at significantly lower levels (Fig. 4B). Interestingly, supernatant transfer experiments showed that all the cytokines induced by ertumaxomab did not have any effect on the growth of HCT-8 cells (Fig. 4A–C). These results strongly support the view that ertumaxomab-induced killing of HCT-8 cells is specific and not mediated by the cytokines per se and depends on physical contact of the redirected effector cells and HCT-8 cells.

Our results show that the release of proinflammatory cytokines and IL-2, which is stimulated by ertumaxomab, reflects the engagement of both Fc\(\gamma\) receptor+ accessory immune cells and CD3\(^+\) T-cells. The less pronounced IL-6 secretion induced by trastuzumab is probably evoked by binding to Fc\(\gamma\)R on accessory cells alone.

**Dependence of ertumaxomab-mediated lysis on HER2/neu binding.** To address the question of whether the provoked cytotoxic effects of ertumaxomab on HCT-8 target cells depend on the expression of the HER2/neu antigen, we initiated blocking experiments with an HER2/neu antibody. We preincubated the cytotoxicity samples with excess amounts of anti-HER2/neu antibody 2502A. In three independent experiments with effector cells from different donors, we observed a significant dose-dependent reduction of tumor cell killing at 2502A blocking concentrations between 200 and 2,000 ng/mL (Fig. 4D). These results show that direct binding of ertumaxomab to HER2/neu is mandatory for target cell destruction.

**Inhibition of ertumaxomab binding to HER2/neu by antibody 520C9.** To define the anti-HER2/neu binding region of ertumaxomab, competitive binding analysis of trastuzumab with mAb 520C9 were performed on SK-BR-3 cells. MAb 520C9 was able to inhibit ertumaxomab binding but trastuzumab was not. This result suggests that 520C9 and ertumaxomab recognize similar epitopes (Fig. 5A). Indeed, the HER2/neu binding site of the mAb 520C9 was previously mapped to the extracellular domain of HER2/neu (amino acid positions 243–370), covering parts of subdomains II and III (20). We conclude that trastuzumab and ertumaxomab have different binding epitopes on HER2/neu, which do not interfere with each other. Having shown that ertumaxomab and trastuzumab recognize different HER2/neu epitopes (Fig. 5A), we further asked whether ertumaxomab was able to induce the killing of target cells that express low levels of HER2/neu (e.g., HCT-8) in the respective cell growth media. Doses of ertumaxomab ranging from 1 to 100 ng/mL were able to stimulate high levels of the pro-inflammatory cytokines [i.e., IL-6; IFN-\(\gamma\); TNF-\(\alpha\)] after 24 hours of incubation time (Fig. 4A and B). Of note, in the presence of accessory cells, T cells, and tumor cells, ertumaxomab also induced the production of IL-2 (Fig. 4C). A strong Th1 response is suggested by the high IFN-\(\gamma\) and IL-2 levels that were present in samples treated with ertumaxomab. Furthermore, IL-6 secretion was increased, indicating a proinflammatory response with the contributions of accessory cells (19). In contrast, trastuzumab merely stimulated IL-6 secretion, and at significantly lower levels (Fig. 4B). Interestingly, supernatant transfer experiments showed that all the cytokines induced by ertumaxomab did not have any effect on the growth of HCT-8 cells (Fig. 4A–C). These results strongly support the view that ertumaxomab-induced killing of HCT-8 cells is specific and not mediated by the cytokines per se and depends on physical contact of the redirected effector cells and HCT-8 cells.

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presence of trastuzumab. Both antibodies, ertumaxomab and
trastuzumab, were used in combination in competitive cytotoxicity
assays (Fig. 5B). Effector and HCT-8 target cells were preincubated
with a constant concentration of trastuzumab (5,000 ng/mL),
followed by the addition of ertumaxomab (50 ng/mL). In this
setting, trastuzumab alone (5,000 ng/mL) failed again to lyse
HCT-8 target cells. Ertumaxomab-mediated maximal cell lysis was
present at concentration as low as 50 ng/mL. No difference was
observed in the killing efficacy of ertumaxomab between samples
with (100-fold excess) trastuzumab or without. The observation
that excess amounts of trastuzumab do not hamper the lysis of
target cells by ertumaxomab confirms that the two antibodies
recognize different HER2/neu epitopes (Fig. 5A).

Discussion

It is well-established that tumor therapy with the anti-HER2/neu
antibody trastuzumab achieves significant survival benefits in
patients with HER2/neu overexpressing metastatic breast cancer,
whether trastuzumab is used with or without chemotherapy as a
first-line therapy until disease progression (7, 8). Moreover, a
retrospective analysis of breast cancer patients enrolled during
clinical trials, with tumors scored 2+ or 3+ by immunohistochem-
istry, showed the most beneficial treatment effects in patients who
carried FISH-positive tumors. This retrospective analysis indicates
that breast cancer HER2/neu overexpression most frequently
correlates with gene amplification (22). Unfortunately, overexpres-
sion of the HER2/neu antigen is only detectable in 20% to 30% of
breast cancer patients (1, 2), leaving the majority with fewer
therapeutic options.

This study describes the cytotoxic capacity of the trifunctional
antibody ertumaxomab for tumor cell lines that express HER2/neu
at high and low levels, in the presence of immune effector cells
(PBMC) in vitro. Only slight differences could be assessed in the
killing efficiency of SK-BR-3 cells between trastuzumab and
erumaxomab, at a relatively high E/T ratio of 20:1. However, at
more unfavorable E/T ratios (7:1 or lower), probably resembling the
situation at the tumor site, only ertumaxomab was able to
efficiently lyse SK-BR-3 cells (Fig. 2). This observed superiority of
erumaxomab over trastuzumab is based on the particular mode of
action that all members of the trifunctional antibody family (e.g.,
erumaxomab, catumaxomab, Bi20/FBTA05, BiLu, and BiUII) have
in common. These therapeutic antibodies induce a simultaneous
recruitment and activation of T cells and accessory cells, leading to
the destruction of targeted tumor cells by different killer
mechanisms (9, 12, 13, 15, 23).

Figure 4. A-C, influence of ertumaxomab-induced cytokines IFN-γ, IL-6, and IL-2 on freshly plated HCT-8 cells (HER2 low, HER2 ampl -). Cytokine levels of supernatants of ertumaxomab or trastuzumab cytotoxicity experiments were analyzed after 24 h. Supernatants containing ertumaxomab- or trastuzumab-induced cytokines were transferred to freshly plated HCT-8 cells (1 × 104). HCT-8 growth (C) was measured after a 3-d incubation period with the XTT proliferation assay. As a control was a sample of HCT-8 cells incubated with RPMI1640 medium, which was set as a standard of 100% HCT-8 growth; points, mean. Ab, antibody.

D, HER2/neu specificity of ertumaxomab-induced cytotoxicity against HCT-8 cells: Cytotoxicity experiments were done in duplicates with 2 × 105 PBMC of 3 different healthy donors and an E/T ratio of 6:1. PBMC and target cells were preincubated with 200 ng/mL (●), 500 ng/mL (○), or 2,000 ng/mL (X) of the anti-HER2/neu antibody 2502A followed by addition of ertumaxomab (100–0.001 ng/mL). Ertumaxomab-induced cytotoxicity was compared with samples without preincubation of 2502A (■). Points, mean; bars, SD.
The unique mode of action of ertumaxomab led us to the hypothesis that tumor cells with low HER2/neu expression profiles, categorized as immunohistochemistry 1+ and FISH negative, might be potential targets for ertumaxomab therapy. To address this question, we chose three tumor cell lines originating from different tissues (breast, colon, and lung), each of which has a detectable low HER2/neu-expressing profile, identified by quantitative FACS measurements and FISH analysis. Cytotoxicity assays revealed that ertumaxomab has a strong killing activity against all three cell lines at concentrations above 5 ng/mL, and at E/T ratios of 20:1 or even lower (Fig. 3). Trastuzumab, which acts against tumor cells mainly by means of antibody-dependent cellular cytotoxicity (17, 24), without contributions from T cells, completely failed to inhibit growth of these three cell lines, even at high concentrations.

High quantities of IFN-γ, TNF-α (data not shown), and IL-6 were detected in the supernatants of mixed HCT-8/PBMC samples to which ertumaxomab had been added. IL-2 secretion was stimulated as well as IFN-γ, a strong indication of a Th1-type T-cell activation. The induction of these cytokines confirms findings described previously for other trifunctional antibodies (10, 25), and supports the view that their mode of action is mainly independent of the targeted antigen. The cytokines IFN-γ, TNF-α, and IL-6 were also released 3 or 6 hours after ertumaxomab infusion in a clinical trial with metastatic breast cancer patients, confirming the relevance of our in vitro results for the clinic (9).

Trastuzumab induced significant amounts of only IL-6, which is most likely attributable to accessory cells stimulated by Fcγ receptor engagement (Fig. 4B; ref. 26). These results further emphasize the substantial differences in the mechanisms of action between ertumaxomab and trastuzumab.

Because the transfer of ertumaxomab-treated mixed cell culture supernatants had no growth inhibition effect on freshly plated HCT-8 cells, the killing of this cell line was not induced by the detected mediator substances, such as TNF-α or IFN-γ. Although sufficient at low levels, the necessity of HER2/neu antigen expression for ertumaxomab-provoked tumor cell destruction...
was finally shown by HER2/neu blocking experiments. Preincubation of HCT-8 target cells with the parental bivalent anti-HER2/neu antibody 2502A inhibited ertumaxomab-induced killing up to 60%. This inhibition shows that physical contact of ertumaxomab with the tumor cell is required for its efficacy (Fig. 4D). This finding has been confirmed in vivo for the trispecific surrogate antibody BiLu, which has no effect on the growth of target antigen negative tumor cells, whereas it mediates full protection against antigen-positive tumor cells in mice (13).

An important result, with possible consequences for clinical use, was that ertumaxomab and trastuzumab recognize two different HER2/neu epitopes. The HER2/neu binding site for trastuzumab is located in the cysteine-rich extracellular subdomain IV (amino acids 529–627; ref. 21). Ertumaxomab binding is not competitive and must be distinct from that of trastuzumab. In consequence, patients that have already received trastuzumab and are refractory to trastuzumab treatment may benefit from ertumaxomab administration because no interference in binding or killing efficacy was observed in coinubcation experiments (Fig. 5B). Therefore, a subsequent or even simultaneous application of both antibodies could be reasonable. In conclusion, ertumaxomab may give new treatment opportunities for breast cancer patients with HER2/neu expression, independent of the expression profile that is currently under investigation in phase II clinical studies.

**Disclosure of Potential Conflicts of Interest**

H. Lindhofer: commercial research support, TRION Research GmbH; ownership interest and patents, TRION Pharma GmbH. The other authors disclosed no potential conflicts of interest.

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**References**


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