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

Michael Jäger,1 Alexandra Schoberth,1 Peter Rut,1 Jürgen Hess,2 and Horst Lindhofer1,2

TRION Research GmbH, Martinsried, Germany and TRION Pharma GmbH, Munich, Germany

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, tricell 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, only 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.


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 bispecific 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 direct–labeled CEN 17 probe, specific for the α satellite centromeric region of chromosome 17 (D17Z1), and a green fluorochrome direct–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 discarded by washing, 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 trastuzumab – absorbance sample)/(absorbance trastuzumab)] × 100.

**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; 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 (100–0.001 ng/mL).

**Results**

**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-bottom 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 cytometric 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.

**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, BT20/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. 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 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-γ; TNF-α (data not shown)]-after 24 hours of incubation time (Fig. 4 A and B). Of note, in the presence of accessory cells, T cells, and tumor cells, ertumaxomab also induced the production of IL-2 (Fig. 4 C). A strong Th1 response is suggested by the high IFN-γ 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. 4 B). 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. 4 A–C). These results show that direct binding of ertumaxomab to HER2/neu is mandatory for target cell destruction.

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 520C9. In three independent experiments with effector cells from different donors, we observed a significant dose-dependent reduction of tumor cell killing at 520C9 blocking concentrations between 200 and 2,000 ng/mL (Fig. 4 D). 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. 5 A). 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). This 520C9 site is distinct from the binding site of trastuzumab, which is located in region IV (21). We conclude that trastuzumab and ertumaxomab have different binding epitopes on HER2/neu, which do not interfere with each other.

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

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. 4 D). These results show that direct binding of ertumaxomab to HER2/neu is mandatory for target cell destruction.

**Figure 3.** Cytotoxicity against (A) HCT-8 cells (HER2 low, HER2 ampl−; E/T, 20:1); (B) HCT-8 cells (E/T, 7:1); (C) BT-20 cells (HER2 low, HER2 ampl−; E/T, 20:1) and (D) SK-LU-1 cells (HER2 low, HER2 ampl−; E/T, 20:1) mediated by trastuzumab (5,000–0.001 ng/mL) or ertumaxomab (100–0.001 ng/mL). Cytotoxicity experiments were performed thrice and samples measured in duplicates with 2 × 10^5 PBMC of 3 different healthy donors; sample: PBMC + HCT-8 + antibody; allogeneic reaction: PBMC + HCT-8 cells. Points, mean; bars, SD.
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 immunohistochemistry, 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, overexpression 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 ertumaxomab, 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 ertumaxomab over trastuzumab is based on the particular mode of action that all members of the trifunctional antibody family (e.g., ertumaxomab, catumaxomab, Bi20/FTB105, 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/C0). 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 × 10⁶). HCT-8 growth (%) 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 × 10⁴ 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

Figure 5. A, FACS competition binding: inhibition of ertumaxomab (ertu; 1 µg/mL) binding to SK-BR-3 cells in the presence of varying ratios of 520C9 or trastuzumab (trastu) compared with ertumaxomab (10:1, 1:1, 1:10); all experiments were performed thrice, as control, the binding capacity of ertumaxomab, 520C9, and trastuzumab to SK-BR-3 cells was analyzed; points, mean; bars, SD. B, cytotoxicity of ertumaxomab (50 ng/mL) induced against HCT-8 cells (HER2 low, HER2 ampl−) after preincubation with a 100-fold higher concentration of trastuzumab (5,000 ng/mL). As controls were samples with trastuzumab (5,000 ng/mL) and ertumaxomab (50 ng/mL) alone. Cytotoxicity experiments were done in duplicate with 2 × 10⁶ PBMC of 3 different healthy donors and a HCT-8 cell concentration of 1 × 10⁵ (E/T, 20:1); sample: PBMC + HCT-8 + antibody; allogeneic reaction: PBMC + HCT-8 cells. Points, mean; bars, SD.
was finally shown by HER2/neu blocking experiments. Preincuba-
tion 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 trisfunctional 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 with trastuzumab. It is competitive with mAb 520C9 (Fig. 5A), whose HER2/neu binding site was previously mapped to the extracellular HER2/neu subdomains II and III (amino acid positions 243–370; ref. 20). The HER2/neu binding epitope of ertumaxomab must be distinct from that of trastuzumab. In consequence, patients that have already received trastuzumab and are refractory to
traztuzumab treatment may benefit from ertumaxomab adminis-
tration 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.

Acknowledgments

Received 7/25/08; revised 1/28/09; accepted 3/25/09; published OnlineFirst 5/12/09.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Melanie Honcia, Annette Arbter, Melanie Goelden, and Kathrin Gasow for their expert technical assistance.

References

1. Owens MA, Horten BC, Da Silva MM. HER2 amplifi-
8. Cobleigh MA, Vogel CL, Tripathy D, et al. Multina-
16. Stangmaier M, Faltin M, Ruf P, Bodenhausen A, Schroder P, Lindhofer H. Bi20 (FBTA05), a novel trifunctional bispecific antibody (anti-CD20 x anti-
Cancer Research

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

Michael Jäger, Alexandra Schoberth, Peter Ruf, et al.

Cancer Res  Published OnlineFirst May 12, 2009.

Updated version  Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-08-2861

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.