Cancer Chemother Pharmacol (2010) 66:269–276
DOI 10.1007/s00280-009-1160-0

ORIGINAL ARTICLE

Preclinical study of prolonged administration of trastuzumab as combination therapy after disease progression during trastuzumab monotherapy

Kaori Fujimoto-Ouchi · Fumiko Sekiguchi · Kaname Yamamoto · Masatoshi Shirane · Yoriko Yamashita · Kazushige Mori

Received: 8 April 2009 / Accepted: 5 October 2009 / Published online: 11 November 2009

Abstract
Purpose The clinical relevance of prolonged trastuzumab administration in combination therapy beyond progressive disease (PD) has been suggested. Here, we examined whether trastuzumab treatment is effective in combination after failing to show antitumor activity as monotherapy in HER2-positive human breast cancer xenograft models.
Methods We established trastuzumab PD models with HER2-positive breast cancer xenograft models and compared the antitumor activity of trastuzumab in combination with a taxane versus monotherapy with a taxane in the models subsequent to tumor progression under trastuzumab monotherapy.
Results We established trastuzumab PD model using the HER2-positive human breast cancer line MDA-MB-361 and KPL-4 in in vivo. In these models, trastuzumab at the same dose as the initial treatment showed no significant antitumor activity at 3 weeks after start of treatment. Re-inoculated tumor tissues showing PD regained sensitivity to trastuzumab. In the trastuzumab PD models, the HER2 status of the tumor tissues did not decrease. Also, the pAKT level continued to decrease, as with the initial treatment, and IGF-1R was not found to be up-regulated. Instead, differences were observed in the gene-expression profiles of the tumor tissues showing PD. Trastuzumab in combination with G-CSF, which is expected to enhance antibody-dependent cellular cytotoxicity (ADCC), showed significant antitumor activity, even though the single agents alone showed no antitumor activity in the PD model. In the MDA-MB-361 trastuzumab PD model, the combination of trastuzumab with paclitaxel showed significantly more potent antitumor activity compared with paclitaxel or docetaxel monotherapy. In the KPL-4 trastuzumab PD model as well, trastuzumab showed significant antitumor activity in combination with taxanes or capecitabine after PD had developed in response to trastuzumab monotherapy.
Conclusion We established in vivo trastuzumab PD models, in which trastuzumab monotherapy ceases to have antitumor activity during the treatment. The mechanisms of PD with trastuzumab are considered to involve both reversible changes in the gene expression profiles in tumor tissues and a decrease of ADCC activity in the host. Our present results demonstrated that trastuzumab showed antitumor activity in combination with taxanes or capecitabine even though it showed no antitumor activity as a monotherapy, suggesting a clinical relevance of treatment with trastuzumab as a combination therapy beyond PD.

Keywords Trastuzumab · Progressive disease · Chemotherapy · Combination · ADCC · G-CSF

K. Fujimoto-Ouchi (✉) · F. Sekiguchi · K. Yamamoto · M. Shirane · Y. Yamashita · K. Mori
Product Research Department,
Chugai Pharmaceuticals Co., Ltd,
200 Kajiwara, Kamakura, Kanagawa 247-8530, Japan
e-mail: ohuchikor@chugai-pharm.co.jp

Introduction

HER2 overexpression is reportedly a factor of poor prognosis in clinical breast cancers [1], and thus treatments targeting HER2 are expected to show survival benefit. Trastuzumab (Herceptin®) is a humanized antibody specific to HER2, which is used in the first-line treatment for HER2-positive metastatic or early breast cancer. Clinical results have demonstrated that therapies combining trastuzumab with standard chemotherapies such as taxanes showed a survival benefit. However, the tumors of many of

these patients develop progressive disease (PD) during such therapies and often the treatment to which a patient has developed PD is discontinued and another treatment is prescribed. It has also been reported that the tumor tissues remain HER2-positive even after developing PD to trastuzumab therapies. This is in line with a recent finding that, in contrast to initial belief, trastuzumab does not down-regulate HER2 from the cell surface [2]. Taking into consideration that HER2 is a factor of poor prognosis, discontinuation of trastuzumab treatment is counterintuitive; and yet there is no adequate scientific rationale to continue the administration of trastuzumab. Here, we examined whether trastuzumab treatment should be prolonged as a combination therapy after showing no antitumor activity as monotherapy in xenograft models. In vitro lines resistant to trastuzumab have been established and their mechanisms of resistance have been reported to be associated with diminished inhibition of pAKT, up-regulation of IGF-1R, and up-regulation of PTEN [3–5]. The mechanism of trastuzumab action, however, includes antibody-dependent cellular cytotoxicity (ADCC) in addition to the direct inhibition of cell proliferation [6] and it has become clear that the in vivo effects of other anticancer drugs in combination therapy differ from the in vitro effects [6, 7]. To examine the mechanisms responsible for PD with trastuzumab, we attempted to establish in vivo PD models that would show all the mechanisms of trastuzumab activity.

Using these HER2-positive xenograft models, we investigated the combination therapies of trastuzumab with taxanes or capecitabine, standard treatments for clinical breast cancer, after PD with trastuzumab as a single agent [8–11] to demonstrate the clinical relevance of treatment with trastuzumab after PD.

Materials and methods

Chemicals

Trastuzumab was provided by F. Hoffman-La Roche (Nutley, NJ) as a freeze-dried powder and reconstituted with distilled water and diluted with saline. Human immunoglobulin G (IgG) was purchased from MP Biomedicals, Inc. (Aurora, OH, USA) and was reconstituted with distilled water and diluted with saline. Docetaxel was synthesized by Kanto Chemical Co., Inc. (Tokyo, Japan) as a fine powder and was dissolved in saline containing 2.5% (v/v) polysorbate 80 (Sigma-Aldrich Inc., St. Louis, MO, USA) and 2.5% (v/v) ethanol. Paclitaxel was purchased from Wako Pure Chemical Industries, Ltd (Tokyo, Japan) and was dissolved in saline containing 5% (v/v) cremophol EL (Sigma-Aldrich) and 5% (v/v) ethanol. Capecitabine was provided by F. Hoffman-La Roche as a bulk powder and dissolved in 40 mM citrate buffer (pH 6.0) containing 5% (w/v) gum arabic. G-CSF (genetical recombinant lenograstim) produced by Chugai Pharmaceuticals Co., Ltd) was diluted with 0.01% of polysorbate 20 in PBS.

Animals

Female 5-week-old BALB/c-nu/nu mice (CAnN.Cg-Foxn1 < nu >/CrlCrlj nu/nu) were obtained from Charles River Japan (Yokohama, Japan). All animals were allowed to acclimatize and recover from shipping-related stress for 1 week prior to the study. The health of the mice was monitored by daily observation. Chlorinated water and irradiated food were provided ad libitum, and the animals were kept in a controlled light–dark cycle (12–12 h). All animal experiments were performed in accordance with the Guidelines for the Accommodation and Care of Laboratory Animals promulgated by Chugai Pharmaceutical Research Center.

Tumors

The HER2-positive human inflammatory breast cancer cell line KPL-4 [12, 13] was kindly provided by Dr. J. Kurebayashi (Kawasaki Medical School, Kurashiki, Japan). KPL-4 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The HER2-positive human breast cancer cell line MDA-MB-361 was obtained from the American Type Culture Collection (Rockville, MD). An in vivo line of MDA-MB-361 was established in our laboratory and maintained in BALB/c-nu/nu mice after subcutaneous (sc) inoculation of tumor pieces. Both cell lines were confirmed to be HER2 positive by IHC and FISH [14] diagnostic methods.

Establishment of the in vivo trastuzumab PD model

A piece of MDA-MB-361 tumor tissue was inoculated subcutaneously into the right flank of each mouse. A suspension of KPL-4 cells (5×10^6 cells/mouse) was orthotopically transplanted into the second mammary fat pad of female BALB/c-nu/nu mice. Several weeks after tumor inoculation, mice were randomly allocated to control and treatment groups after verification of tumor formation. Administration of trastuzumab began when tumor volumes had reached 0.2–0.3 cm^3 (designated as day 1). Trastuzumab was administered intraperitoneally (ip) once a week (qw) for 3 weeks. To evaluate the antitumor activity and tolerability of the test agents, tumor volume and body weight were measured twice a week. The tumor volumes (V) were estimated from the equation $V = ab^2/2$, where a and b are tumor length and width, respectively. The growth ratio of tumor volume was calculated to be the ratio

of tumor volume on the evaluation day to that of the day of the previous measurement. The percentage of tumor growth inhibition (TGI%) was calculated as follows: TGI% = {1 − (tumor volume of treatment group on evaluation day − tumor volume of treatment group on day 1)/(tumor volume of control group at evaluation day − tumor volume of control group on day 1)} × 100. For the tumor re-inoculation experiments, MDA-MB-361 tumor tissue was resected after 3 weeks of trastuzumab treatment and 2-mm square pieces of tumor tissue were then inoculated into BALB/c-nu/nu mice by the same method as the initial tumor inoculation.

Quantification of pAKT and IGF-1R in tumor tissues

Tumor tissues and blood were sampled at the start of treatment (group a), after 3 weeks of trastuzumab treatment (group c), and at the time the tumor volume reached that of the trastuzumab-treated group (group b) to eliminate the effect of tumor volume on efficacy. The tumor tissues were pulverized in liquid nitrogen, lysed in cell extraction buffer (BioSource, Flynn, CA) for 30 min, and then homogenized with a potter homogenizer. The extracts were centrifuged at 150K×*g* for 10 min at 4°C. Aliquots of the clear lysates were dispensed and stored at −80°C until the pAKT and IGF-1R assays were carried out. ELISA of pAKT (BioSource) or IGF-1R (BioSource) was performed following the manufacturer's protocol.

Comprehensive analysis of gene expression in the tumor tissues

Tumor tissues were resected from xenograft models and immediately frozen in liquid nitrogen. Total RNA was extracted from the frozen tumor using Sepasol-RNA I (WAKO, Osaka, Japan) and was purified with an RNease column (Qiagen, Austin, TX). Total RNA (5 μg) was reverse transcribed to cDNA with a T7-(dT)24 primer. Biotin-labeled cRNA was first synthesized from cDNA using a MEGAscript In Vitro Transcript Kit (Ambion, Austin, TX), fragmented to an average size of 50–100 nucleotides by incubating at 95°C for 35 min in 40 mM Tris–acetate (pH 8.1) containing 100 mM potassium acetate and 30 mM magnesium acetate, and finally hybridized to murine GeneChip 430A 2.0 Array (Affymetrix, Santa Clara, CA). The hybridized cRNA probes were stained with streptavidin R-phycoerythrin Molecular Probes™ (Invitrogen, Carlsbad, CA) and then scanned with a confocal scanner (Affymetrix). The scanned data so obtained were normalized to correct for small differences in the levels of the cRNA probes and were processed for signal values using Affymetrix software (LIMS 5.0). We examined gene expression profiles in four tumor tissues from each group (control, human IgG-treated, and trastuzumab-treated). Signal intensities obtained from the GeneChip analysis were transformed to logarithmic values. We selected genes from the criteria of a minimum value >2 and more than two samples showing the present response in each group and a CV > 20% in all 12 samples from a data set of 54,613 arrays and then applied the values of the selected genes to Eisen's hierarchical clustering software (http://rana.stanford.edu/software).

Combination of trastuzumab with chemotherapy or G-CSF in the trastuzumab PD models

After the initial treatment with trastuzumab alone, mice were re-randomized and allocated to the control group, the trastuzumab group, the chemotherapy group, or the combination of trastuzumab with chemotherapy group. Each group was, respectively, treated with human IgG and vehicle of chemotherapy, trastuzumab and vehicle of chemotherapy, human IgG and chemotherapy, or trastuzumab and chemotherapy. Trastuzumab was administered ip qw for 3 weeks similar to the initial treatment. Docetaxel was administered intravenously (iv) once in 3 weeks (q3w). Paclitaxel was administered iv qw. Capecitabine was administered orally (po) once a day for 14 consecutive days. The maximum tolerated dose (MTD) was defined as half of the minimum toxic dose causing death (one mouse out of six mice, LD17) or resulting in more than 20% of body weight loss in a separate experiment. G-CSF was administered sc once a day for 6 days.

Statistical analysis

The Mann–Whitney *U* test was used to detect the statistical differences in tumor volume ($P < 0.05$) for the in vivo experiments and Student's *t* test was used ($P < 0.05$) for the in vitro experiments. The statistical analysis was carried out using an SAS preclinical package (SAS Institute, Inc., Tokyo, Japan).

Results

Establishing a trastuzumab PD model

The HER2-positive breast cancer cell line MDA-MB-361 was inoculated into BALB/c-nu/nu mice, and trastuzumab (30 mg/kg) was administered ip qw. Up to 1 week after the start of administration, trastuzumab showed an inhibitory effect on tumor growth in all of the animals but, as the treatment continued, a decrease in growth inhibition appeared in individual animals (Fig. 1a). The rate of tumor growth inhibition also decreased during the administration of trastuzumab in these animals, and they were considered to have

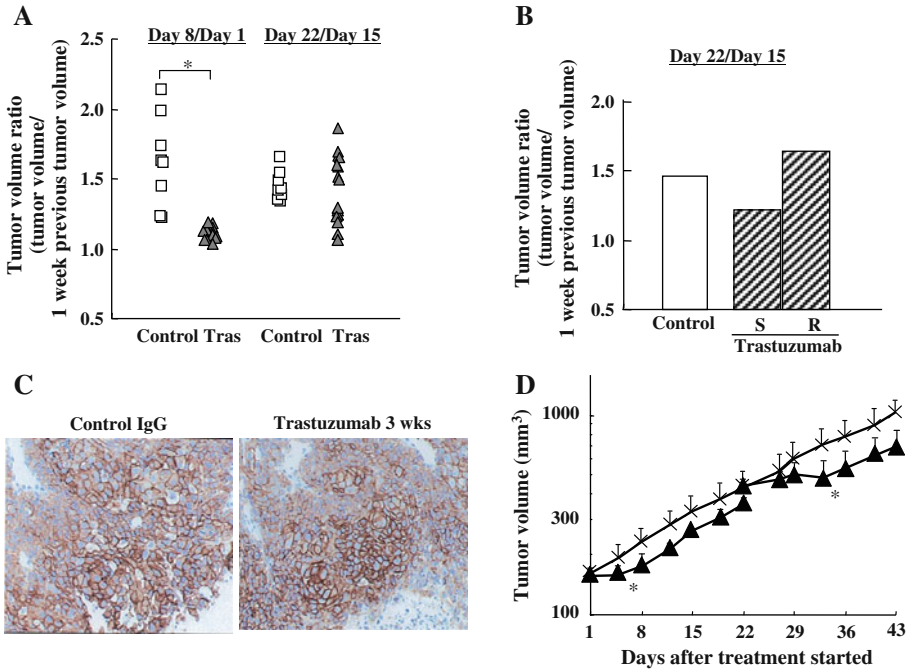

Fig. 1 Establishment of the trastuzumab progressive disease model of MDA-MB-361. **a** Appearance of the progressive tumor during 3 weeks of treatment with trastuzumab. Treatment with trastuzumab was started 52 days after the tumor inoculation. Mice were allocated to groups of 8 mice for control human IgG and 16 mice for trastuzumab treatment. Trastuzumab (30 mg/kg) and IgG (30 mg/kg) were administered ip qw for 3 weeks. Data points: IgG (*squares*), trastuzumab (*triangles*). *Symbols* represent the calculated ratio of the tumor volume versus the 1 week prior tumor volume. **b** Comparison of tumor volume ratio between PD tumor and tumor sensitive to trastuzumab treatment. Mean values are shown as *bars*. Control IgG treatment (*white bar*), trastuzumab treatment (*striped bar*), mean of tumor with a volume ratio lower than 1.4 (*S*), mean of tumor with a volume ratio higher than 1.4 (*R*). **c** Conservation of HER2 status beyond progressive disease after treatment with trastuzumab. Herceptest® (HER2 IHC) of the tumor tissues of MDA-MB-361. The method for IHC is described in the "Materials and methods". **d** Comparison of the antitumor activity of trastuzumab between large and small tumor tissues. Treatment with trastuzumab against small tumors or large ones was started 47 or 69 days after the tumor inoculation. Mice were randomly allocated to groups of six mice each. Trastuzumab (30 mg/kg) and IgG (30 mg/kg) were administered ip once a week for 3 weeks. Data points: control IgG (*cross marks*), trastuzumab (*closed triangles*)

become unresponsive to trastuzumab. The frequency of this condition was 8 out of 16 mice during the period ranging from 2 to 3 weeks after the start of administration. After 3 weeks of initial administration, the trastuzumab group showed less tumor growth inhibition compared with the group that had been switched to treatment with HuIgG. When divided by the tumor volume ratio on day 22 (1.4), the ratio of resistant tumors was significantly higher than that of tumors sensitive to trastuzumab (Fig. 1b). After 3 weeks of initial trastuzumab treatment, HER2 protein as determined by IHC had remained positive (Fig. 1c). In the investigation of the relationship between the size of the cancer tumor and its responsiveness to trastuzumab, we found an inhibitory effect on tumor growth when trastuzumab was administered to a tumor having the same volume as that of a tumor that had become unresponsive at 3 weeks after initial treatment (Fig. 1d). Based on our results, we regarded the group of individuals that had become unresponsive to trastuzumab monotherapy as trastuzumab PD models. A similar result was also observed in the KPL-4 model.

Changes in the tumor tissues of the trastuzumab PD model

To understand the mechanisms whereby tumor tissues become unresponsive to trastuzumab, MDA-MB-361 tumor tissue that had received the initial treatment with trastuzumab was re-inoculated into a different mouse. The re-inoculated tumor was found to be sensitive to be trastuzumab (Fig. 2a). Therefore, it is unlikely that the tumor tissue itself had been replaced with cells that had acquired resistance to trastuzumab, or that irreversible changes had occurred in the tumor tissues such as mutations of HER2-related signals. We confirmed that the sensitivity to trastuzumab monotherapy was similar in both the tumor before treatment (group a) and the control tumor tissue (group b) having the same volume as the tumor that had become unresponsive to trastuzumab. We then investigated the changes in the tumor tissues among groups sensitive to trastuzumab (groups a and b) and a refractory group (group c). The levels of pAKT in tumor were decreased in the trastuzumab PD tumors (group c), compared with the levels in the control groups a and b; and the levels of IGF-1R in

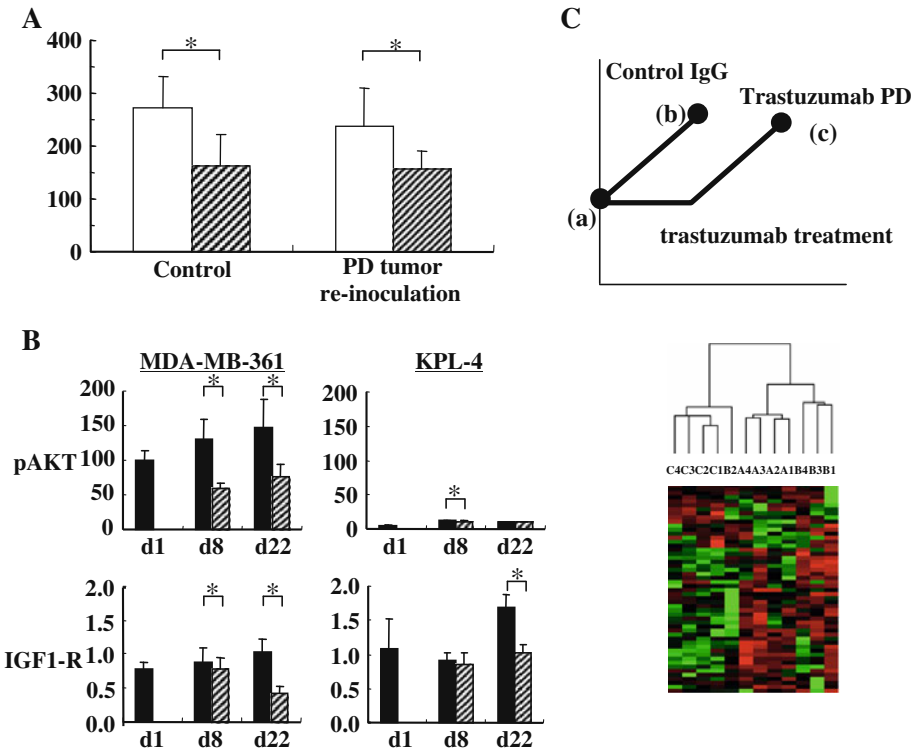

Fig. 2 Changes of HER2-related growth signals in tumor tissues beyond trastuzumab progressive disease. **a** Tumor volume of trastuzumab-treated mice re-inoculated with the MDA-MB-361 tumor grown under trastuzumab treatment on day 15. The original MDA-MB-361 (*left*). Treatment with trastuzumab for original tumors or re-inoculated ones was started 53 or 59 days after the tumor inoculation. Mice were randomly allocated to groups of six mice each. Trastuzumab (30 mg/kg) and IgG (30 mg/kg) were administered ip once a week for 3 weeks. Data *bars*: re-inoculated MDA-MB-361 (*left*). Control (*white*), and trastuzumab (*diagonal*). **b** Levels of pAKT and IGF1-R in the tumor tissues ($n = 6$), control group (*white*), and trastuzumab group (30 mg/kg/shot) (*diagonal*). **c** Comparison of the gene profiles between progressive disease tumors and control tumors. Mice were randomly allocated to groups of four mice: before treatment (*a*), control IgG (*b*), and trastuzumab treated (*c*). Trastuzumab or IgG were administered ip once a week for 3 weeks or 1 week. Tumor samples were collected on the 1st day (*a*), 8 days after (*b*), or 22 days after treatment (*c*)

PD tumors did not increase compared with the control groups a and b. Comprehensive GeneChip analyses (Fig. 2c) revealed differences in the gene expression of the (groups a, b, and c. Unsupervised hierarchical clustering analysis was carried out using 55 genes selected from microarray data according to the criteria described in "Materials and methods". Groups a, b, and c were clustered into each subgroup with the exception of one mouse. The gene expression patterns of groups a and b were relatively similar in comparison with group c, indicating that the gene expression profiles of tumor tissues were associated with trastuzumab sensitivity in MDA-MB-361.

Involvement of the immune system in the trastuzumab PD model

The mechanism of the activity of trastuzumab involves FCγR-mediated ADCC [6]. Based on a report that G-CSF enhances the appearance of FCγR in nude mice [15], we considered that G-CSF might enhance ADCC activity. After 3 weeks of trastuzumab monotherapy in the MDA-MB-361 model, mice were randomly reallocated and trastuzumab was administered in combination with G-CSF. The results showed that even though trastuzumab or G-CSF monotherapy showed no antitumor effect, tumor growth was significantly inhibited by the combination therapy (Fig. 3).

Study of combining taxane anticancer drugs in the trastuzumab PD model

After 3 weeks of trastuzumab monotherapy in the MDA-MB-361 model, mice were randomly reallocated and trastuzumab was administered in combination with either paclitaxel or docetaxel. The results showed that even though trastuzumab monotherapy showed no tumor inhibitory effect, the combination groups showed significant antitumor effects with paclitaxel (Fig. 4a) and with docetaxel (Fig. 4b). In the KPL-4 model, the same results were found (Table 1). We investigated the combination of trastuzumab with capecitabine in the KPL-4 trastuzumab PD model. Capecitabine combination therapy was significantly more

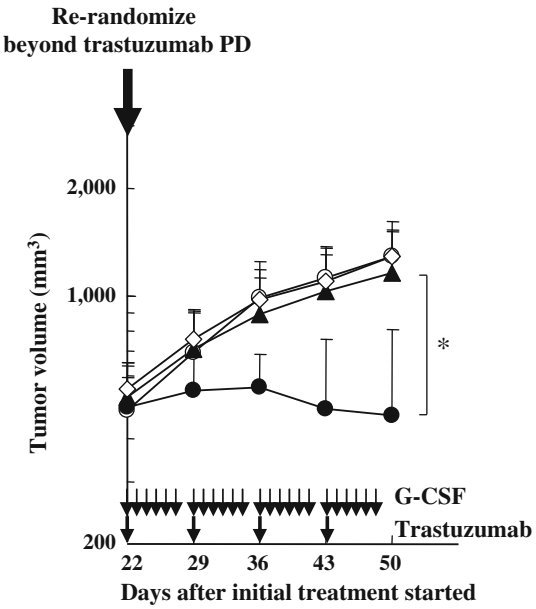

Fig. 3 Antitumor activity of combinations of trastuzumab with G-CSF in the MDA-MB-361 human breast cancer model. Combination treatment was started 54 days after the tumor inoculation and 3 weeks of treatment with trastuzumab. Mice were randomly allocated to groups of ten mice each. G-CSF (300 mg/kg, po) was administered six times a week for 4 weeks. Trastuzumab (30 mg/kg) and IgG (30 mg/kg) were administered ip once a week for 3 weeks. Data points: mean value + SD of tumor volume. Control IgG and vehicle (*diamonds*), trastuzumab and vehicle (*triangles*), control IgG and G-CSF (*open circles*), and combination with trastuzumab and G-CSF (*closed circles*)

effective than capecitabine alone, even in the PD model. Therefore, even if the tumor becomes unresponsive to trastuzumab monotherapy, an added effect can be gained by a regimen of trastuzumab in combination chemotherapy.

Discussion

Trastuzumab is an anticancer drug widely used for treating HER2-positive metastatic breast cancers. Recently, it has been suggested that prolonged administration of trastuzumab as a combination therapy beyond PD would be of clinical relevance. In the present study, we demonstrated in xenograft models that, even after showing a loss of antitumor activity as a monotherapy, trastuzumab in combination chemotherapy is an effective anticancer agent.

In the present study, we used in vivo murine xenograft models of HER2 + breast cancer as the experimental model. BT-474, KPL-4, and MDA-MB-361 have been reported to be natural HER2-overexpressing breast cancer xenograft models [12, 14, 16]. To eliminate the influence of the hormone receptors, the PgR-positive BT-474 model was not used and hormone receptor-negative KPL-4 and MDA-MB-361 models were used. Although in clinical practice trastuzumab is usually administered in combination with chemotherapies as the initial treatment, we established a trastuzumab monotherapy PD model to avoid the

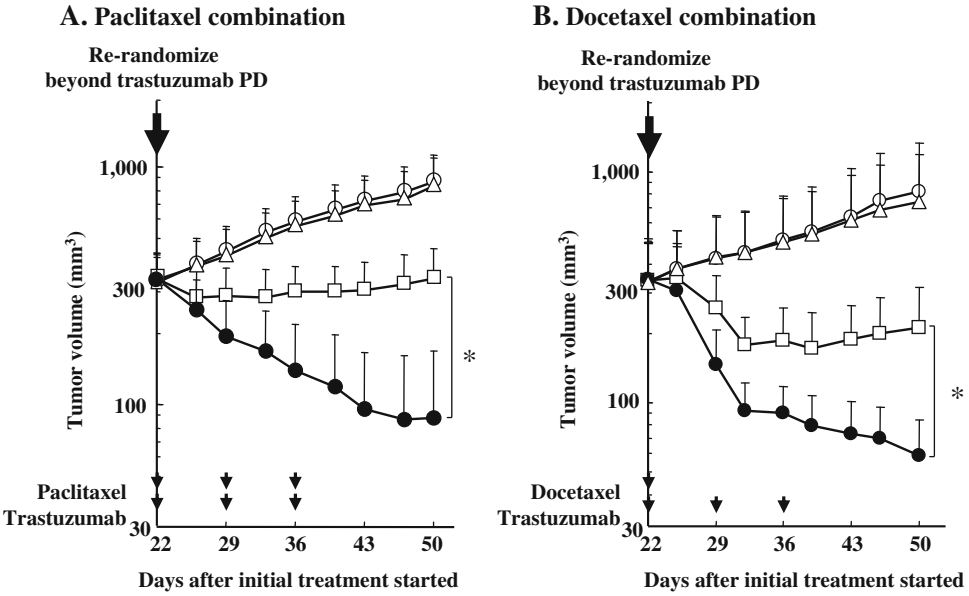

Fig. 4 Antitumor activity of combinations of trastuzumab with taxanes in the MDA-MB-361 human breast cancer model. Combination of trastuzumab with paclitaxel (**a**) or docetaxel (**b**). Previous treatments with trastuzumab were started 52 and 48 days after the tumor inoculation, respectively. After 3 weeks of treatment, mice were randomly allocated to groups of ten mice each. Paclitaxel (60 mg/kg, iv) was administered once in 3 weeks. Trastuzumab (30 mg/kg) and IgG (30 mg/kg) were administered ip once a week for 3 weeks. Data points: mean value + SD of tumor volume. Control IgG and vehicle (*circles*), trastuzumab and vehicle (*open triangles*), control IgG and taxane (*open squares*), and combination with trastuzumab and taxane (*closed squares*)

Table 1 Antitumor activity of three combinations of trastuzumab with capecitabine in the KPL-4 human breast cancer model

	n	Day 1	Day 22
Tumor volume at day 22 beyond trastuzumab progression disease			
Control	8	567 ± 73	879 ± 89
Trastuzumab	7	565 ± 52	836 ± 52
Capecitabine	8	545 ± 63	642 ± 86[a]
Combination	8	551 ± 61	531 ± 91[a,b]

Treatment was started 17 days after the tumor inoculation. After 3 weeks of treatment with trastuzumab, mice were randomly allocated to groups of eight mice each. Capecitabine (90 mg/kg, po), was administered consecutively once a day for 14 days. Trastuzumab (40 mg/kg) and IgG (40 mg/kg) were administered ip qw for 3 weeks. Data points: mean value ± SD of tumor volume

[a] Significantly different from the control group

[b] Significantly different from the capecitabine group

development of resistance to chemotherapy in this study and to clarify whether trastuzumab administration should be maintained in combination therapy after PD has developed with trastuzumab monotherapy.

Trastuzumab alone was initially effective in both of the HER2-positive models; however, 3 weeks after the start of administration, trastuzumab showed no significant antitumor activity in spite of the fact that HER2 continued to be overexpressed on the cell surface in the tumor tissues. Ritter et al. [17] also reported of HER2-positive expression remaining in their trastuzumab-resistant model. A possible mechanism for the development of resistance to trastuzumab is a weakening of antitumor activity by a reduced ability of trastuzumab to penetrate into tumors that have grown larger during the initial treatment. In the present study, however, trastuzumab initially showed antitumor activity in large tumors, thereby contradicting the explanation that large tumor volume as a mechanism of the PD that occurs after initial treatment.

Another possible mechanism for the development of resistance to trastuzumab in our preclinical models is the reversible change in the tumor tissue together with decreased ADCC activity in the host. Reversible changes in PD tumors were indicated by the fact that the antitumor activity of trastuzumab was restored when tumor tissue that had become resistant to trastuzumab monotherapy was re-inoculated into another mouse, suggesting that our model for in vivo PD was different from the resistant cell line established in vitro. In our models, PD with trastuzumab could not be explained by the selection of a population of resistant cells or an irreversible gene mutation. Furthermore, we observed neither attenuation of pAKT inhibition nor up-regulation of IGF-1R in tumor tissue, both of which have been reported as mechanisms of trastuzumab resistance [4]. The gene expression profiles in the tumors with PD, differed from the profiles in the tumor before PD had developed. Therefore, changes other than pAKT or IGF-1R might be involved in the PD mechanisms.

ADCC has been reported to play an important role in the antitumor activity of trastuzumab [4, 6, 18]. Therefore, an increase in ADCC activity would be expected to improve the efficacy of trastuzumab. G-CSF is used as a supportive treatment to improve neutropenia caused by chemotherapy in cancer patients, and it has been reported to increase FcγR expression in peripheral blood mononuclear cells [15, 19]. Because FcγR is important for ADCC, we examined the combination effect of G-CSF with trastuzumab. Although, neither G-CSF nor trastuzumab monotherapy alone showed any antitumor effects in this model, the combination of trastuzumab + G-CSF showed significantly stronger tumor growth inhibition than even the initial trastuzumab monotherapy. These results suggest that G-CSF restored ADCC activity and augmented the antitumor activity of trastuzumab in the PD model. G-CSF would be useful not only as supportive care in clinical but also to potentiate the antitumor activity of trastuzumab in combination therapy.

In the trastuzumab PD model, trastuzumab was compared with chemotherapy alone, even though trastuzumab showed no antitumor activity. Our results suggested that the trastuzumab combination therapy for HER-2 positive patients continued even after the development of PD. The mechanisms accounting for the combination effects of trastuzumab and chemotherapy treatments in this model are not yet clear. The antitumor activity of trastuzumab is possibly enhanced by ADCC activity because it has been reported that taxanes increase the TNF in macrophages and cancer cells by means of an inflammatory effect [20, 21]. Capecitabine has not been reported to augment ADCC activity, however, and thus further investigations are needed to clarify the mechanisms of the effects in combination with trastuzumab.

Our present results indicate that trastuzumab is able to potentiate the antitumor activity of taxanes or capecitabine even after it no longer shows antitumor activity as a monotherapy in xenograft models, thus suggesting a clinical relevance for cancer treatment with trastuzumab in combination therapy beyond PD.

References

1. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, Mcguire WL (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177–182
2. Austin CD, De Maziere AM, Pisacane PI et al (2004) Endocytosis and sorting of ErbB2 and the site of action of cancer therapeutics trastuzumab and geldanamycin. Mol Biol Cell 15:5268–5282
3. Yakes FM, Chinratanalab W, Ritter CA, King W, Seelig S, Arteaga CL (2002) Herceptin-induced inhibition of phosphatidylinositol-3 kinase and Akt Is required for antibody-mediated

effects on p27, cyclin D1, and antitumor action. Cancer Res 62:4132–4141
4. Nagata Y, Lan KH, Zhou X et al (2004) PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell 6:117–127
5. Nahta R, Yuan LX, Zhang B, Kobayashi R, Esteva FJ (2005) Insulin-like growth factor-I receptor/human epidermal growth factor receptor 2 heterodimerization contributes to trastuzumab resistance of breast cancer cells. Cancer Res 65:11118–11128
6. Clynes RA, Towers TL, Presta LG, Ravetch JV (2000) Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. Nat Med 6:443–446
7. Fujimoto-Ouchi K, Sekiguchi F, Tanaka Y (2002) Antitumor activity of combinations of anti-HER-2 antibody trastuzumab and oral fluoropyrimidines capecitabine/5′-dFUrd in human breast cancer models. Cancer Chemother Pharmacol 49:211–216
8. Seidman AD, Berry D, Cirrincione C et al (2008) Randomized phase III trial of weekly compared with every-3-weeks paclitaxel for metastatic breast cancer, with trastuzumab for all HER-2 overexpressors and random assignment to trastuzumab or not in HER-2 nonoverexpressors: final results of Cancer and Leukemia Group B protocol 9840. J Clin Oncol 26:1642–1649
9. Schaller G, Fuchs I, Gonsch T et al (2007) Phase II study of capecitabine plus trastuzumab in human epidermal growth factor receptor 2 overexpressing metastatic breast cancer pretreated with anthracyclines or taxanes. J Clin Oncol 25:3246–3250
10. Cobleigh MA, Vogel CL, Tripathy D et al (1999) Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J Clin Oncol 17:2639–2648
11. Marty M, Cognetti F, Maraninchi D et al (2005) Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer administered as first-line treatment: the M77001 study group. J Clin Oncol 23:4265–4274
12. Kurebayashi J, Otsuki T, Tang CK et al (1999) Isolation and characterization of a new human breast cancer cell line, KPL-4, expressing the Erb B family receptors and interleukin-6. Br J Cancer 79:707–717
13. Kunisue H, Kurebayashi J, Otsuki T et al (2000) Anti-HER2 antibody enhances the growth inhibitory effect of anti-oestrogen on breast cancer cells expressing both oestrogen receptors and HER2. Br J Cancer 82:46–51
14. Fujimoto-Ouchi K, Sekiguchi F, Yasuno H, Moriya Y, Mori K, Tanaka Y (2007) Antitumor activity of trastuzumab in combination with chemotherapy in human gastric cancer xenograft models. Cancer Chemother Pharmacol 59:795–805
15. Van Der Kolk LE, De Haas M, Grillo-Lopez AJ, JW Baars, Van Oers MH (2002) Analysis of CD20-dependent cellular cytotoxicity by G-CSF-stimulated neutrophils. Leukemia 16:693–699
16. Baselga J, Norton L, Albanell J, Kim YM, Mendelsohn J (1998) Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. Cancer Res 58:2825–2831
17. Ritter CA, Perez-Torres M, Rinehart C, Guix M, Dugger T, Engelman JA, Arteaga CL (2007) Human breast cancer cells selected for resistance to trastuzumab in vivo overexpress epidermal growth factor receptor and ErbB ligands and remain dependent on the ErbB receptor network. Clin Cancer Res 13(16):4909–4919
18. Barok M, Isola J, Palyi-Krekk Z et al (2007) Trastuzumab causes antibody-dependent cellular cytotoxicity-mediated growth inhibition of submacroscopic JIMT-1 breast cancer xenografts despite intrinsic drug resistance. Mol Cancer Ther 6:2065–2072
19. Kakinoki Y, Kubota H, Yamamoto Y (2004) CD64 surface expression on neutrophils and monocytes is significantly up-regulated after stimulation with granulocyte colony-stimulating factor during CHOP chemotherapy for patients with non-Hodgkin's lymphoma. Int J Hematol 79:55–62
20. Bogdan C, Ding A (1992) Taxol, a microtubule-stabilizing antineoplastic agent, induces expression of tumor necrosis factor alpha and interleukin-1 in macrophages. J Leukoc Biol 52:119–121
21. Sawada N, Ishikawa T, Fukase Y, Nishida M, Yoshikubo T, Ishitsuka H (1998) Induction of thymidine phosphorylase activity and enhancement of capecitabine efficacy by taxol/taxotere in human cancer xenografts. Clin Cancer Res 4:1013–1019