Glycoengineered Pichia produced anti-HER2 is comparable to trastuzumab in preclinical study

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Mammalian cell culture systems are used predominantly for the production of therapeutic monoclonal antibody (mAb) products. A number of alternative platforms, such as Pichia engineered with a humanized N-linked glycosylation pathway, have recently been developed for the production of mAbs. The glycosylation profiles of mAbs produced in glycoengineered Pichia are similar to those of mAbs produced in mammalian systems. This report presents for the first time the comprehensive characterization of an anti-human epidermal growth factor receptor 2 (HER2) mAb produced in glycoengineered Pichia, and a study comparing the anti-HER2 from Pichia, which had an amino acid sequence identical to trastuzumab, with trastuzumab. The comparative study covered a full spectrum of preclinical evaluation, including bioanalytical characterization, in vitro biological functions, in vivo anti-tumor efficacy and pharmacokinetics in both mice and non-human primates. Cell signaling and proliferation assays showed that anti-HER2 from Pichia had antagonist activities comparable to trastuzumab. However, Pichia-produced material showed a 5-fold increase in binding affinity to FcγRIIA and significantly enhanced antibody dependent cell-mediated cytotoxicity (ADCC) activity, presumably due to the lack of fucose on N-glycans. In a breast cancer xenograft mouse model, anti-HER2 was comparable to trastuzumab in tumor growth inhibition. Furthermore, comparable pharmacokinetic profiles were observed for anti-HER2 and trastuzumab in both mice and cynomolgus monkeys. We conclude that glycoengineered Pichia provides an alternative production platform for therapeutic mAbs and may be of particular interest for production of antibodies for which ADCC is part of the clinical mechanism of action.

Key words: glycoengineered Pichia, anti-HER2, trastuzumab, xenograft, PK, ADCC
and insertion of enzymes for pathways that produce human-like glycosylation structures, we have developed an expression system that retains all of the advantages of using yeast while permitting customization of glycosylation profiles of recombinant proteins. In addition, because yeast naturally lacks the post-translational mechanism to synthesize GDP-fucose and subsequently transfer it to N-linked oligosaccharides, glycoengineered *Pichia pastoris* is capable of producing antibodies lacking fucose at the asparagine site (N297) of human type N-linked glycans. Afucosylated antibodies have been shown to have enhanced antibody dependent cell-mediated cytotoxicity (ADCC) and, therefore, provide an opportunity for development of biobetter antibodies. For instance, we have demonstrated that afucosylated anti-CD20 antibody from glycoengineered Pichia has enhanced ADCC activity. This platform thus has great potential for production of antibodies whose efficacy is dependent on ADCC.

Trastuzumab (Herceptin) is an anti-human epidermal growth factor receptor 2 (HER2) IgG1 approved by both the US Food and Drug Administration and the European Medicines Agency for the treatment of breast cancers with high levels of HER2 expression (found in ~25% of breast cancer patients). The availability of trastuzumab has significantly improved the outcome of breast cancer patients and has borne out the great promise of molecular targeted mAb therapeutics; however, the clinical mechanism of action of trastuzumab is not entirely understood. In preclinical studies, multiple mechanisms of action have been shown to contribute to trastuzumab anti-tumor activity. The best-documented of these mechanisms are: (1) inhibition of cleavage and shedding of the extracellular domain of HER2, which prevents the formation of the active p95 transmembrane product; (2) inhibition of HER2-dependent intracellular signaling pathways important to survival/proliferation; (3) antibody-dependent receptor downregulation through endocytosis and degradation; (4) recruitment of immune effector cells via Fc mediated receptor interactions and ADCC.

Anti-HER2 mAb used in this study was produced in humanized *Pichia pastoris* and is therefore devoid of fucose on the N-linked human type of glycans in the Fc domain. The N-glycan composition is known to modulate the Fc effector functions of IgG1 antibodies. In general, the absence of fucose has been shown to increase the affinity for Fcγ receptor IIIa and to enhance FcyRIIa-mediated ADCC. The role of ADCC in trastuzumab efficacy is not entirely understood. Preclinical studies indicate that increasing the ADCC activity of trastuzumab can result in increased anti-tumor efficacy. Similarly, studies of clinical correlations between patient response, ex vivo ADCC activities and Fcγ receptor IIIa polymorphism have suggested a contribution by Fc effector functions to trastuzumab efficacy. However, a more recent analysis of FcγR polymorphisms in much larger patient cohorts found no significant correlations with response to trastuzumab in early and advanced breast cancer, casting doubt on the relevance of the preclinically observed enhanced ADCC activity.

Here, we report for the first time the preclinical characterization of anti-HER2 IgG1 with the same amino acid sequence as trastuzumab, but without core fucose at its glycans, that was produced in glycoengineered *Pichia pastoris*. We show that, compared to trastuzumab, the anti-HER2 mAb produced in glycoengineered *Pichia pastoris* (referred to herein as “anti-HER2 mAb”) has similar HER2 antagonist activity and anti-tumor efficacy and indistinguishable PK. Therefore, this study provides important validation of the glycoengineered Pichia platform for production of therapeutic antibodies.

### Results

Bioanalytical characterization of anti-HER2 antibody produced in glycoengineered *Pichia pastoris*. Glycoengineered Pichia strain YGLY8316 was chosen as the expression host for producing the anti-HER2 mAb. YGLY8316 is capable of transferring terminal β-1,4 galactose onto biantennary N-linked glycan, which yields antibody entirely devoid of fucose at its glycan structure. Anti-HER2 mAb produced in YGLY8316 was purified through affinity capture using protein A beads and further purified by ion exchange chromatography. Antibody purity by SDS-PAGE and its spectrum from size exclusion chromatography HPLC are shown in **Figure 1A and B** in comparison to trastuzumab. Purified anti-HER2 mAb was composed of more than 99% fully assembled antibody including double heavy and light chain, and the quality of the antibody profile was comparable to that of trastuzumab. Anti-HER2 mAb and trastuzumab were confirmed as having the same amino acid sequence by deconvoluted mass spectra of reduced and deglycosylated antibodies analyzed by a quadrupole time-of-flight (Q-TOF) mass spectrometer (**Fig. 1C**). The N-glycan composition of anti-HER2 mAb produced in YGLY8316 differed from that of trastuzumab primarily in the proportion of GlcNAc2Man3GlcNAc2 (G0), GlcNAc2Man3GlcNAc2Gal (G1) and GlcNAc2Man3GlcNAc2Gal2 (G2) and, more importantly, it was entirely devoid of fucose in the glycan structure (**Table 1**). On the other hand, trastuzumab contained largely fucosylated biantennary glycan including G0F, G1F and G2F, and more than 90% of glycans in trastuzumab were fucosylated while the Pichia produced anti-HER2 mAb was 100% afucosylated. Neither anti-HER2 mAb nor trastuzumab had detectable level of sialic acid content at their N-linked glycan. Anti-HER2 mAb also contained a small number of O-linked single mannose glycans (average of fewer than 3 per Ig molecule) but no O-linked glycans was detected in trastuzumab.

**HER2 receptor binding.** The antigen binding affinities of anti-HER2 mAb and trastuzumab were compared in cell culture with the high HER2 expression breast cancer cell line SKBR3 and with recombinant HER2 extracellular domain (ECD) protein. Antibody binding to the cell surface was concentration-dependent, with similar titration profiles for anti-HER2 mAb and trastuzumab (**Fig. 2**). The estimated EC50 of the binding to cancer cell surface HER2 was low nanomolar based on 4-parameter curve fitting using GraphPad Prism 5.0 software. Anti-HER2 mAb showed comparable binding affinity to trastuzumab (**Fig. 2**). The kinetics of anti-HER2 mAb for binding to recombinant HER2 ECD protein were characterized by measuring
Figure 1. Bioanalytical characterization of glycoengineered Pichia-produced anti-HER2 mAb. (A) Comparison of anti-HER2 mAb with trastuzumab in reducing and non-reducing SDS-PAGE. Lane 1: protein marker, lane 2 anti-HER2 mAb at non-reducing condition, lane 3: trastuzumab at non-reducing condition, lane 4: anti-HER2 mAb at reducing condition, lane 5: trastuzumab at reducing condition. (B) Size exclusion chromatography (SEC)-HPLC shows high similarity between the two samples. (C) A comparison of deconvoluted masses between reduced anti-HER2 mAb and trastuzumab. The antibodies were deglycosylated by PNGase F digestion; heavy chain has 2 daltons difference due to incomplete reduction.
Table 1. N-Linked glycan comparison between trastuzumab and anti-HER2 mAb

| Samples        | Man5 | G0  | G0F | G1  | G1F | G2  | G2F | Hybrid |
|---------------|------|-----|-----|-----|-----|-----|-----|--------|
| Trastuzumab   | 2.2  | 2.6 | 45  | 1.5 | 41  | 0.7 | 7.8 | ND     |
| Anti-HER2 mAb | 7    | 59  | ND  | 23  | ND  | 3   | ND  | 8      |

Man, manose; GlcNAc, N-acetyl-D-glucosamine; Gal, galactose; Fuc, fucose. Man5, Man5GlcNAc2; G0 GlcNAc2Man3GlcNAc2; G1 GlcNAc2Man3GlcNAc2Gal2; G2, GlcNAc2Man3GlcNAc2Gal2Fuc; G1F, GlcNAc2Man3GlcNAc2GalFuc; G2F, GlcNAc2Man3GlcNAc2GalFuc; Hybrid, GlcNAc2Man5GlcNAc or GlcNAc2Man5GlcNAcGal; ND, not detected.

The equilibrium dissociation constant (K_D) was calculated as the ratio between dissociation and association rate constants. Similar kinetic binding constants were found for both antibodies with KD values of 0.44 ± 0.07 nM and 0.49 ± 0.11 nM for anti-HER2 mAb and trastuzumab, respectively (Table 2).

Table 2. Kinetic constants of anti-HER2 mAb in comparison with trastuzumab (n = 6)

| Name          | k_a (E+04) | k_d (E-05) | K_D nM |
|---------------|------------|------------|--------|
| anti-HER2 mAb | 8.75 ± 1.6 | 3.79 ± 0.2 | 0.44 ± 0.07 |
| trastuzumab   | 8.46 ± 1.0 | 4.15 ± 0.8 | 0.49 ± 0.11 |

Inhibition of HER2 signaling. Inhibition of HER2 signaling by anti-HER2 mAb was measured by inhibition of phosphorylation of HER2 tyrosine kinase domain and downstream signaling kinase AKT. To compare the efficacy between anti-HER2 mAb and trastuzumab, a head-to-head comparison using BT474.m1 breast cancer cells was performed. The concentration-dependent inhibition of HER2 (3A) and AKT (3B) phosphorylation by anti-HER2 mAb and trastuzumab is shown in Figure 3. Both antibodies showed similar potency of inhibition with maximum association and dissociation rate constants k_a (on rate) and k_d (off rate) by Biacore. The equilibrium dissociation constant (K_D) was calculated as the ratio between dissociation and association rate constants. Similar kinetic binding constants were found for both antibodies with K_D values of 0.44 ± 0.07 nM and 0.49 ± 0.11 nM for anti-HER2 mAb and trastuzumab, respectively (Table 2).

The ADCC activities mediated by anti-HER2 mAb and trastuzumab were compared using three types of human effector cell populations, PBMC, NK cells and monocytes. The greatest difference between the antibodies was observed with NK cell-mediated ADCC activity (>5-fold shift in IC_50), with estimated IC_50 values at 1.09 ng/mL and 5.69 ng/mL for anti-HER2 mAb and trastuzumab, respectively (Fig. 4). The estimated IC_50 values for both anti-HER2 mAb and trastuzumab were approximately 1.0 nM, as in the case of signaling inhibition, anti-HER2 mAb showed comparable potency to CHO-produced trastuzumab.

Inhibition of cancer cell proliferation. The HER2 overexpressing BT474.m1 breast cancer cell line is known to be sensitive to trastuzumab inhibition. Inhibition of BT474.m1 cell proliferation studies with both anti-HER2 mAb and trastuzumab showed dose-dependent inhibition in the concentration range of 0.01–10 nM with maximal inhibition of 50% at >10 nM (Fig. 4). The estimated IC_50 values for both anti-HER2 mAb and trastuzumab were approximately 1.0 nM, as in the case of signaling inhibition, anti-HER2 mAb showed comparable potency to CHO-produced trastuzumab.

Fcγ receptor binding affinities and ADCC activities. Fcγ receptors are expressed on all immune cells and can engage with antibody Fc domain to mediate effector functions such as ADCC and complement dependent cytotoxicity (CDC). The affinities of antibody-receptor interactions have a direct impact on effector activities. To determine the effect of glycan differences between anti-HER2 mAb and trastuzumab on human Fcγ receptor binding, the affinities (K_D) of different Fcγ receptors were determined by surface plasmon resonance. As expected, anti-HER2 mAb with afucosylated glycans showed higher affinity for FcγRIIIA and IIIB than trastuzumab, but little difference in FcγRI and RIIA (R isoform) and RIIB. On the other hand, the affinity to FcγRIIA (H) was about 2-fold lower for anti-HER2 mAb in comparison with trastuzumab (Table 3).

The ADCC activities mediated by anti-HER2 mAb and trastuzumab were compared using three types of human effector cell populations, PBMC, NK cells and monocytes. The greatest difference between the antibodies was observed with NK cell-mediated ADCC activity (>5-fold shift in IC_50), with estimated IC_50 values at 1.09 ng/mL and 5.69 ng/mL for anti-HER2 mAb and trastuzumab, respectively (Fig. 5A). Unlike NK cells, which express FcγRIIIA but not inhibitory FcγRIIB, monocytes express both activating (FcγRIIA, IIIA) and inhibitory (FcγRIIB) receptors. The balancing effects of the inhibitory Fcγ receptors likely contributed to the more modest increase (3.2-fold) in ADCC for anti-HER2 mAb compared with trastuzumab (Fig. 5B). The estimated IC_50 values for monocyte-effector cell mediated ADCC were 16.4 ng/mL and 52.1 ng/mL for anti-HER2 mAb.
and trastuzumab, respectively. As expected, with PBMC, which contain both NK cells and monocytes in addition to lymphocytes, ADCC differences were intermediate between those observed for NK cells and monocytes (Fig. 5C), with estimated IC₅₀ values at 2.8 ng/mL for anti-HER2 mAb and 11.7 ng/mL for trastuzumab, which represents a 4-fold increase in ADCC for the anti-HER2 mAb.

Anti-tumor efficacy in a mouse xenograft model. The anti-tumor efficacies of anti-HER2 mAb and trastuzumab were compared in the BT474.m1 xenograft model in nude mice. Groups of nude mice (10 mice per group) were inoculated with BT474.m1. When tumors reached ~200 mm³ (~7 days after inoculation), mice were randomly assigned to seven treatment groups: vehicle, trastuzumab (0.3, 0.03 and 0.003 mg/kg), anti-HER2 mAb (0.3, 0.03 and 0.003 mg/kg) and mice were treated biweekly for four weeks. The doses were selected to allow comparison at a submaximal dose (which does not saturate the anti-tumor response and does not lead to cures). The time course for the average tumor growth is shown in Figure 6A. At the 0.003 mg/kg dose, both trastuzumab and anti-HER2 mAb did not show tumor inhibition, whereas both were maximally effective at the 0.3 mg/kg dose. At the intermediate 0.03 mg/kg dose, both antibodies resulted in similar tumor inhibitory efficacy and the differences were not statistically significant when using t-tests for individual time points at the p = 0.05 level. To compare the entire growth curves and not just individual days, tumor growth for each mouse was summarized by the slope of the line fitted through the time course of the logarithms of tumor volumes. The intercepts, by virtue of initial randomization, were the same. The averages and standard deviations of the slopes for each dose group are depicted in Figure 6B. Again, no statistical differences at the p = 0.05 significance level were recorded between treatments at any of the dose levels.

Pharmacokinetics. Anti-HER2 mAb was compared to trastuzumab in a PK study in wild-type C57BL6 mice following intravenous (iv) administration. The results showed that blood time concentration curve of anti-HER2 mAb was almost super-imposable on that of trastuzumab. As a result, the key PK parameters (CL, t½, AUC and Vss) were comparable between these two mAbs (Fig. 7A). A second PK study was conducted in non-human primates to evaluate the possible impact of target-mediated clearance on PK. Cynomolgus monkeys have circulating extracellular domain (ECD) of HER2 with which trastuzumab cross-react. Monkeys were dosed with anti-HER2 mAb or trastuzumab via iv administration at 5 mg/kg. PK analyses showed that the t½ was comparable between these two molecules. The serum concentrations and the systemic exposure of anti-HER2 were slightly higher than those of trastuzumab, albeit not statistically different (Fig. 7B).

Discussion

Pichia pastoris has been used for large scale protein production and is a well established fermentation process and technology platform. A wild-type Pichia produced anti-IL6 mAb ALD518 has been evaluated in Phase 2 trials. To avoid issues associated with hypermannose type of glycan, its Fc was mutated, resulting in an aglycosylated mAb. Since wild-type P. pastoris produces antibodies with hypermannosylated glycans, the resultant immunogenicity risk and fast clearance have limited the utility of P. pastoris for production of full-length therapeutic antibodies where glycan is essential for immunological functions. This limitation has been overcome with the development of glycoengineered P. pastoris with glycosylation pathways engineered to mimic those of mammalian expression hosts. This report presents the first comprehensive functional characterization of an anti-HER2 mAb produced in glycoengineered P. pastoris in comparison with CHO-produced trastuzumab. We show here that Pichia-produced anti-HER2 mAb and CHO-produced trastuzumab have comparable affinities for recombinant and cell surface HER2, as well as comparable potencies in receptor inhibition assays in vitro, including HER2 and AKT phosphorylation and tumor cell proliferation. Furthermore, anti-HER2 mAb is as efficacious as trastuzumab in tumor growth inhibition
HER2, but they do bind HER2 from non-human primates. In vivo in a mouse xenograft model. PK studies demonstrated replacement fungal glycans with mammalian-type glycans; however, reflect both FcRn and target-mediated clearance mechanisms. Since cynomolgus monkeys used in this study had significant levels of circulating HER2 extracellular domain, the PK parameters for anti-HER2 mAb was greater than 80%, with the content for anti-HER2 mAb was greater than 80%, with the remainder consisting of human Man5GlcNAc2 and hybrid-type glycoforms. Anti-HER2 mAb also contained a small number of O-linked single mannose residues (average of fewer than 3 per IgG molecule). These differences in glycosylation had no detectable impact, however, on antibody PK and HER2 antagonist activities in vitro and in vivo.

ADCC has been hypothesized as one of the mechanisms of action for trastuzumab. In vitro cell based ADCC activity shows a strong correlation with Fc gamma receptors affinity and antibody binding to FcγRIIIA in particular has shown to positively correlate with ADCC activity. Trastuzumab produced as Fc engineered or afucosylated mAb showed increased ADCC and improved tumor inhibition in a mouse xenograft model with human immuno-effector cells. A large number of studies with Fc engineered antibodies has firmly established that increased affinity for FcγRIIIA leads to increased NK cell or PBMC-mediated ADCC in vitro, and can result in better efficacy in vivo in models dependent on immune effector functions. Junttila et al. recently reported that afucosylated trastuzumab produced in engineered CHO cells with deletion of the FUT8 gene had increased its affinity for human FcγRIIIA polymorphism F158 and V158 of 29.2- and 6.5-fold, respectively, and increased in vitro ADCC. The ability to mediate ADCC improved 1.9- to 7.2-fold when F/F effectors were used and 2.1- to 7.7-fold when V/V donors were used. When purified NK cells from F/F donors were used as effector cells, an 11.3-fold enhancement in ADCC was observed for afucosylated trastuzumab. In the current study, afucosylated anti-HER2 mAb showed 6- and 8-fold increases in affinity for FcγRIIIA polymorphism F158 and V158, respectively. Compared to trastuzumab, anti-HER2 mAb had a 6-fold increase in NK cell-mediated ADCC activity and 4-fold and 3-fold increase in ADCC with PBMC and monocyte effector cells, respectively. The differences in increased ADCC observed for the afucosylated antibodies produced from engineered CHO cell line (FUT8−/−), and glycoengineered Pichia could be due to the difference in the compositions of the glycan profiles, variations in assay conditions and the effector cell donor-dependent variability. However, afucosylated antibodies from both platforms have consistently increased affinity for human FcγRIIIA, which results in enhanced ADCC.

The increased ADCC activity of Pichia-expressed anti-HER2 mAb did not translate into better in vivo efficacy in xenograft models in nude mice. Since both the expression profile and the IgG specificity of mouse Fc gamma receptors differ from those of the human receptors and mouse NK cells do not express the human homolog of FcγRIIIA, this apparent lack of ADCC contribution to xenograft tumor efficacy was not entirely unexpected. A similar result was reported for afucosylated trastuzumab from FUT8−/− CHO cells when tested in xenografts in severe combined immunodeficiency (SCID) mice. On the other hand, this same afucosylated antibody showed significantly better activity in a xenograft tumor model in transgenic mice expressing human FcγRIII [FcγRIIFcγRIIIFag2−/− Tg(hu FcγRIIIA)]. While the FcγRs in these mice only approximate the human FcγR phenotype (e.g., the mice still express mFcγRIV, the mouse homolog of hFcγRIIIA), these data at least
suggest that the increased ADCC activity of afucosylated antibodies could yield clinical benefits.

Several clinical studies have indeed suggested that ADCC may play a significant role in the clinical mechanism of action of trastuzumab. In particular, a retrospective analysis of FcγR polymorphisms (V/V vs. F/V and F/F) and patient response suggested a role for FcγRIIA, but this correlation was not confirmed in a larger study of FcγR polymorphisms and outcomes of more than 1,000 patients with early or metastatic breast cancer treated with trastuzumab (BCIRG006 trial; NCT00021255). It should be noted, however, that while the analysis of the BCIRG006 trial does not support the original hypothesis of ADCC as a significant contributor to the clinical efficacy of trastuzumab, these results do not rule out a possible clinical benefit for engineered anti-HER2 antibodies with ADCC activity significantly higher than that of trastuzumab. One attractive approach to generating such follow-on antibodies with increased ADCC activity is through the use of glycoengineered Pichia pastoris. In addition to the advantages of yeast expression, which include ease of genetic manipulation, stable expression, rapid cell growth and high yield of secreted protein, low-cost scalable fermentation processes and no risk of human pathogenic virus contamination, the Pichia platform enables enhancement of ADCC without introduction of amino acid mutations in the Fc domain. In this report, we show that anti-HER2 mAb expressed in glycoengineered P. pastoris displays the expected increase in ADCC while maintaining all the in vitro antagonist activities of trastuzumab, as well as an identical PK profile in both rodents and non-human primates.

Materials and Methods

Antibody generation. Anti-HER2 heavy and light chain genes (Herceptin® monograph, www.roche-canada.com) were codon optimized and synthesized for expression in glycoengineered P. pastoris GFI5.0 host YGLY8316 using methanol inducible AOX1 promoter. S. cerevisiae α-mating factor signal sequences were applied to both heavy and light chains. The expression cassette was integrated into P. pastoris Trp2 locus. By electroporation and then selected on a Zeocin containing media as reported previously in reference 4. The transformed clones were screened in 96-deep well plates as described in reference 40. Antibody titer was measured as described by Barnard et al. Anti-HER2 mAb fermentation, antibody purification, analytical methods and glycan analysis were conducted using the protocols described before in references 4, 5 and 41–43.

Binding kinetic constants with HER2 mAb extracellular domain (ECD) protein. The binding affinity of anti-HER2 for HER2 ECD using BIAcore T100 (Biosystems & ABI) were performed based on protocols suggested by manufacturer with optimization. The detail procedures are supplied in Supplemental material.

Anti-HER2 mAb binding to cell surface HER2 receptor. Anti-HER2 mAb and trastuzumab binding to cell surface HER2 receptor was conducted using SKBR3 human breast cancer cells (ATCC) and procedures are described in the paper by Jiang et al.

Figure 5. Antibody-dependent cell-mediated cytotoxicity activities using human NK (A), monocyte (B) and PBMC (C) effector cells. X-axis represents the antibody concentrations and y-axis is the % of target cell lysis. Solid line with solid circle represents anti-HER2 mAb treatment and dotted line with open circle represents treatment of trastuzumab. Error bars indicates standard deviations calculated from three replicates (n = 3).

Anti-HER2 mAb inhibition of cell proliferation assay. Exponentially growing BT474.m1 cells (California Pacific Medical Center) were harvested and plated onto 96-well plates (Costar 3603, Corning Inc.) at 5,000 cells/well with 100 μL of RPMI cell culture medium (Mediatech, Catalog # 10-040-CM) with 10% FBS. After 24 h culturing, cells were treated with anti-HER2 mAb in 2-fold dilution series, with final concentrations ranging from 33.3–0 nM. After 96 h incubation, 10 μL of alamarBlue (Invitrogen, DAL1100) were added to each well and incubation was continued for an additional 4 h before reading the plates. Fluorescence emission intensity was then measured at Ex/Em of 535/590 nm using a plate reader (Perkin Elmer, Victor 3).
Inhibition of AKT and HER2 phosphorylation in breast cancer cell line BT474.m1. BT474.m1 cells were seeded at 6 x 10^5 cells per mL in 96-well cell culture plate containing 100 μL RPMI media with 10% FBS. Anti-HER2 mAb and trastuzumab were added at various concentrations, starting from 100 μg/mL for pHER2 assay and 450 μg/mL for pAKT assay, in 3-fold serial dilution in 100 μl complete media per well for 2 h at 37°C. At the end treatment, the cells were lysed by adding 35 μL of cell extraction buffer from Invitrogen (cat.#FNN0011) containing fresh 1 mM PMSF, protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Roche) for 60 min with gentle rocking at 4°C. The cell lysates were centrifuged in 96-well plate at 3,000 rpm for 10 min to collect supernatant. Quantitation of phosphorylation of HER2 and AKT is described in Supplemental material.

Fcγ receptor binding affinities by BIAcore T100. Extracellular domain proteins of human FcγRIIA (131R and 131H), FcγRIIIA (158F and 158V), FcγRIIB and FcγRIIIB were expressed in Pichia using the method of producing FcγRIIA reported by Li et al. with a 6x HIS tagged at the C-terminus of each protein. Binding of all the Fc receptors was measured using BIAcore T100 with CM5 biosensor chips (GE Healthcare, USA). Detailed procedure of Fc gamma receptor binding assay can be found in Supplemental material.

ADCC activity. ADCC activities were assayed with the human ovarian adenocarcinoma cell line SKOV3 (ATCC) as target cells and human NK cells, monocyte or PBMC as effector cells (Biologics Specialty). SKOV3 target cells were grown as adherent cells in RPMI medium supplemented with 10% FBS and effector cells were used on the same day delivered. SKOV3 cells were seeded into 96-well E-plate at 15,000/well with 100 μL of media per well.

Figure 6. Tumor growth inhibition by anti-HER2 mAb in comparison with trastuzumab in the BT474m1 breast cancer xenograft tumor model (each data point is calculated as mean from group of 10 mice). (A) Mean tumor volume of anti-HER2 mAb and trastuzumab treated BT474m1 xenografted mice at dosage of 0.003 mg/kg, 0.03 and 0.3 mg/kg. (B) Slope of mean tumor volume of all the treatment at all three different dosage.
days later, when their tumors had reached ~200 mm$^3$, mice were randomized. Groups of ten mice were assigned to treatment with vehicle, trastuzumab (at 0.3, 0.03, 0.003 mg/kg) or anti-HER2 mAb (same dose levels). Treatment was twice weekly for four weeks after which the treatment stopped and the tumors were allowed to grow without further interference. Tumor growth was measured weekly. Tumor volume was calculated using the formula: $V = \frac{1}{2}LW^2$ (L = length of the tumor and W = width of tumor) and expressed as average of tumor size in each group. Average tumor size was computed for each group and increased over time was used to judge differences between treatments. Alternatively, the tumor growth for each individual mouse was summarized by the slope of the linear correlation between the logarithm of the tumor volume and time.

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**Note**

Supplemental materials can be found at: www.landesbioscience.com/journals/mabs/article/15532
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