

Hersintuzumab: A novel humanized anti-HER2 monoclonal antibody induces potent tumor growth inhibition

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Summary Humanized monoclonal antibodies (mAbs) against HER2 including trastuzumab and pertuzumab are widely used to treat HER2 overexpressing metastatic breast cancers. These two mAbs recognize distinct epitopes on HER2 and their combination induces a more potent blockade of HER2 signaling than trastuzumab alone. Recently, we have reported characterization of a new chimeric mAb (c-1T0) which binds to an epitope different from that recognized by trastuzumab and significantly inhibits proliferation of HER2 overexpressing tumor cells. Here, we describe humanization of this mAb by grafting all six complementarity determining regions (CDRs) onto human variable germline genes. Humanized VH and VL sequences were synthesized and ligated to human $\gamma 1$ and κ constant region genes using splice overlap extension (SOE) PCR. Subsequently, the humanized antibody designated hersintuzumab was expressed and characterized by ELISA, Western blot and flow cytometry. The purified humanized mAb binds to recombinant HER2 and HER2-overexpressing tumor cells with an affinity comparable with the chimeric and parental mouse mAbs. It recognizes an epitope distinct from those recognized by trastuzumab and pertuzumab. Binding of hersintuzumab to HER2 overexpressing tumor cells induces G1 cell cycle arrest, inhibition of ERK and AKT signaling pathways and growth inhibition.

Moreover, hersintuzumab could induce antibody-dependent cell cytotoxicity (ADCC) on BT-474 cells. This new humanized mAb is a potentially valuable tool for single or combination breast cancer therapy.

Keywords Antibody humanization · Monoclonal antibody · Breast cancer · Immunotherapy · HER2

Introduction

Human epidermal growth factor receptor-2 (HER2) is a tumor-associated antigen (TAA) and a member of the ErbB family of receptor tyrosine kinases (RTKs) which encodes a 185 kDa transmembrane glycoprotein including an extracellular ligand binding region, a single transmembrane region and a cytoplasmic tyrosine kinase domain. HER2 has been implicated in the development of several malignancies in human [1]. Specifically, 15% of invasive breast cancers, 54–100% of colorectal cancers, 25% of ovarian cancers, 17–82% of pancreatic cancers and 34% of prostate cancers overexpress HER2 and this property is associated with poor prognosis [2]. Formation of homodimers and heterodimers of HER2 in a ligand-independent fashion leads to stimulation of intrinsic tyrosine kinase activity, cell growth and proliferation of HER2 overexpressing tumor cells. Therefore, HER2 has emerged as an important therapeutic target in oncology.

Initial work for the development of antibody against extracellular domain (ECD) of HER2 leads to production of the murine monoclonal antibody (mAb) 4D5, which had antiproliferative effects on HER2-overexpressing breast tumor cell lines [3]. Humanization of this antibody resulted in the development of trastuzumab (Herceptin, Genentech Inc., San Francisco, CA) [3] which was approved by the United States Food and Drug Administration (FDA) in 1998, for therapeutic

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use in patients with HER2-overexpressing breast cancer. Despite its promising results, de novo and acquired resistance remain major obstacles in the clinic. Many patients eventually develop disease progression within 1 year of initiating trastuzumab therapy, which could be due to the inefficiency of trastuzumab to inhibit HER2 binding to other members of the HER family [4]. Pertuzumab is another humanized anti-HER2 mAb which binds to an epitope different from trastuzumab [5]. Recently, combination of pertuzumab and trastuzumab has improved survival in breast cancer patients [6]. Based on these results, FDA has recently approved pertuzumab in combination with trastuzumab for patients with HER2-positive metastatic breast cancer [7]. According to the anti-cancer results acquired from pertuzumab alone and in combination with trastuzumab, generation of new mAbs with distinct epitope specificities might improve efficacy of HER2-targeted therapy in breast cancer.

In previous studies we produced several mouse mAbs against HER2 ECD that recognize different epitopes than trastuzumab [8]. Also, we demonstrated that two of these mAbs (1 T0 and 2A8) in combination with trastuzumab more significantly inhibit proliferation of HER2 overexpressing tumor cells than each mAb alone [9]. However, due to induction of the human anti-murine antibody (HAMA) response [10], which is generated by up to 50% of patients following administration of murine hybridoma-derived antibodies [11], the use of murine mAbs is limited. The HAMA response is significantly reduced or totally lifted by chimerization and humanization of these antibodies. Moreover, chimerized and humanized mAbs display increased the potential for antibody dependent cell cytotoxicity (ADCC) and complement dependent cell cytotoxicity (CDC), reduced immunogenicity and prolonged plasma half-life as compared to mouse mAbs.

We have recently reported chimerization of an inhibitory anti-HER2 mAb (c-1 T0) [4]. In the present study, we report humanization of the m-1 T0 mAb by grafting of the murine CDRs into the corresponding regions of a human framework germ line scaffold while preserving the donor residues that contribute to antigen binding. The humanization method in this study successfully generated a humanized mAb (Hersintuzumab), which retained the parental mouse antigen binding specificity, affinity and functionality.

Materials and methods

Cell lines

CHO-K1 cells and MDA-MB-231 (National Cell Bank of Iran, Pasture Institute of Iran, Tehran, Iran) were grown in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco) and 10% fetal bovine serum (Gibco) at 37 °C in a

humidified atmosphere of 5% CO₂. HER2-overexpressing human breast cancer cell line BT-474 (National Cell Bank of Iran, Pasture Institute of Iran, Tehran, Iran) was cultured using the serum culture medium supplemented with 10 µg/mL insulin (Exir Co., Boroojerd, Iran).

Molecular modeling of the variable regions of mouse 1 T0

The homology modeling of mouse 1 T0 variable regions (Fv) was performed using PIGS (<http://www.biocomputing.it/pigs>), a web-based automatic antibody modeling program [12]. The VH and VL protein sequences of 1 T0 were compared with all known antibody structures. Subsequently, the three-dimensional (3D) structure of 1 T0 Fv was constructed by homology modeling based on the structures of the VH (PDB No. 1IGT) of a mouse anti-canine lymphoma mAb and the VL structure of a mouse anti-ampicillin single-chain Fv fragment (PDB No. 1H8O). The final 3D structure of 1 T0 was loaded and visualized with Visual Molecular Dynamics (VMD) [13], a molecular visualization program for identification of the residues located within a 5 Å distance from the CDRs contributing to VH-VL interaction and antigen binding [14].

Humanization of the mouse 1 T0 monoclonal antibody

A humanized version of 1 T0 (Hersintuzumab) was generated by grafting all six complementarity determining regions (CDRs) onto human variable germline genes as described elsewhere [15–17]. Briefly, to select the appropriate human VH and VL frameworks 1–3 sequences, the amino acid sequences of VH and VL murine 1 T0 were separately aligned to the entire human Ig germline V gene segments in ImMunoGeneTics database (IMGT, <http://www.imgt.org/>) and the human heavy and light chain germline V gene segments were chosen based on their highest framework similarities with those of murine 1 T0 VH and VL. Framework 4 of human VH and VL was selected from human heavy and light chain J gene segments, respectively, according to the highest similarities between human J gene segments and murine 1 T0 VH and VL CDR3. Finally, CDRs of murine 1 T0 VH and VL were grafted onto the frameworks of selected germline V and J gene segments of human heavy and light chains antibody, respectively. Codon optimized DNA encoding the two humanized variable domains (VH hersintuzumab, VL hersintuzumab) was synthesized by Eurofins MWG Operon (Ebensburg, Germany). Finally, the hersintuzumab VH and VL were grafted onto human gamma 1 heavy chain CH and kappa light chain CL, respectively, to assemble the whole humanized Ab gene, resulting in humanized 1 T0 (Hersintuzumab).

Amplification of h-VH, h-V κ , C γ 1 and C κ and construction of the humanized antibody expression vector using splice overlap extension (SOE) PCR

Appropriate restriction site, leader and Kozak sequences and 15 bp of CH and CL for SOE PCR embedded in the humanized VH (h-VH) and humanized VL (h-VL) genes, were synthesized (Eurofins MWG Operon, Ebersberg, Germany) and used as the template for amplification of h-VH and h-VL. The h-VH gene was amplified using the primers VHT0-hum-SalI-S and VHT0-hum-AS and the h-VL gene was amplified using the primers V κ -kpn-T0-hum-S and V κ -T0-hum-AS (Table 1). PCR reactions were performed in 50 μ l volume, containing 10 \times reaction buffer, 2 μ l of gene synthesis vector, 2 μ M of sense and anti-sense primers, 4 mM MgSo4 concentration, 2 unit Pfu DNA polymerase (Fermentas, Thermo Fisher Scientific Inc., USA). After 5 min denaturation at 95 $^{\circ}$ C, the PCR reaction was followed by 30 cycles of 1 min at 92 $^{\circ}$ C, 1 min at 58 $^{\circ}$ C, 1 min at 72 $^{\circ}$ C and a final 72 $^{\circ}$ C for 5 min.

CH of human IgG1 and C κ of human kappa chain were amplified using C κ -S, XhoIC κ -AS, CH-S and BamHICH-AS primers (Table 1) and c-1 T0 vector [4] was used as a template. Splice overlap extension (SOE) PCR allows the fusion of two sequences of DNA with 15 bp complementarity region without the use of restriction enzymes [18]. PCR products of h-VH, h-VL and CH, C κ genes were amplified, extracted from the gel and were used as a template for first round PCR (PCR1). They were subsequently hybridized and extended to produce full-length humanized h-VH-CH and h-VL-CL sequences by external primers (VHT0-hum-SalI-S, BamHICH-AS for VH-CH and V κ -kpn-T0-hum-S, C κ -S for VL-CL) in the second round of PCR (PCR2). The PCR product of h-VH-CH was digested and inserted into pBudCE4.1 vector (Invitrogen, Grand Island, NY, USA) at SalI/BamHI restriction sites to generate pBud-h-VH-CH, which contains the heavy chain sequence of the humanized antibody. After

sequencing and confirmation of this construct, PCR product of h-VL-CL was subsequently digested and inserted into pBud-h-VH-CH at KpnI/XhoI sites and after sequence confirmation, pBud-h-VH-CH-h-VL-CL (pBud-hersintuzumab) was generated.

In SOE PCR, first round PCR reactions were performed in 20 μ l volume, containing 10 \times reaction buffer, 1 μ l of cDNA extracted from h-VH and CH or h-VL and CL, 2 mM MgSo4 concentration and 1 unit Pfu DNA polymerase (Fermentas). After 3 min denaturation at 95 $^{\circ}$ C, the PCR reaction was followed by 5 cycles of 1 min at 94 $^{\circ}$ C, 1 min at 58 $^{\circ}$ C, 1.5 min at 72 $^{\circ}$ C. The temperature was held on 94 $^{\circ}$ C for 3 min and after addition of external primers in 5 μ l volume containing 10 \times reaction buffer, PCR2 reaction was performed by 30 cycles of 1 min at 92 $^{\circ}$ C, 1 min at 58 $^{\circ}$ C, 1.5 min at 72 $^{\circ}$ C and a final cycle at 72 $^{\circ}$ C for 5 min.

Transfection of humanized construct in CHO cells and screening of antibody production by ELISA

pBud-hersintuzumab construct was prepared using Plasmid Maxiprep (Qiagen, Stockholm, Sweden). CHO-K1 cells were grown to 80% confluency in 12-well culture plate and transfected with 3 μ g DNA using 4 μ l Lipofectamine 2000 (Life Technologies, Rockville, MD) transfection reagent according to the manufacturer's instruction. After 24 h incubation, supernatant was collected and transient expression of hersintuzumab was assessed by ELISA. For selection of stable transfectants, transfected cells were cultured in culture medium supplemented with 1 mg/ml of Zeocin (Gibco, Grand Island, NY, USA) for a minimum of 2 weeks. Humanized antibody was then detected using an indirect enzyme-linked immunosorbent assay (ELISA) method which has been described elsewhere [4].

Table 1 Sequences of PCR primers employed in this study

Primer	Amplified genes	Sequence	Amplicon size
C κ -S XhoIC κ -AS	C κ C κ (SOE PCR)	ACTGTGGCTGCACCATCTGTCTTCATCTTCCC CTCGAGCTAACACTCTCCCTGTTGA AGCTCTTTGTGACGGGCGA	318 bp
CH-S BamHICH-AS	C γ 1 C γ 1 (SOE PCR)	GCCTCCACCAAGGGCCCATCGGTC GGATCCTCATTTACCCGGAGACAGGG AGAGGCTCTT	990 bp
V κ -kpn-T0-hum-S J κ -T0-hum-AS	V κ T0-hum (SOE PCR) V κ T0 (SOE PCR)	CTCTTTCGGAACCCGCACCAAG ACAGATGGTGCAGCCACAGTTC	458 bp
VHT0-hum-SalI-S JH-T0-hum-AS	VHT0-hum (SOE PCR) VHT0-hum (SOE PCR)	GCTCCAAGTGTGTTGAGACTCTGGAAGAG GAAGACCGATGGGCCCTTGG	486 bp

Structural characterization of humanized antibody by ELISA and SDS-PAGE

Hersintuzumab producing stable cells were adapted to serum free medium (EX-CELL™ Sp2/0, Sigma, St Louis, MO, USA) and IgG was purified using a 1 ml HiTrap Protein G HP column (Amersham Biosciences, New Jersey, USA). Briefly, the cell culture supernatant was passed through the HiTrap column and subsequently washed thoroughly with PBS. Bound recombinant humanized antibody was eluted by 10 ml of elution buffer (0.1 M glycine/HCl, pH 2.5) and immediately dialysed against PBS. ELISA method was used for detection of human IgG and IgC κ in culture supernatant. Briefly, 5 μ g/mL mouse monoclonal antibody against human IgG in PBS was coated in a 96-well ELISA plate. SPG purified hersintuzumab as well as trastuzumab as positive control were added at different concentrations and incubated at 37 °C for 1.5 h. After washing, horseradish peroxidase (HRP)-conjugated rabbit anti-human Ig and sheep anti-human IgC κ were added separately for evaluation of human IgG and IgC κ and then the plate was incubated for 1 h at 37 °C. After further washings, the reaction was revealed with TMB substrate. Sulfuric acid was added to stop the reaction and ODs were measured as mentioned above.

The structure of hersintuzumab antibody was assessed by SDS-PAGE as previously described [4]. Briefly, 1 μ g of SPG purified c-1 T0 and hersintuzumab antibodies were separated on 10% SDS-PAGE under reducing and non-reducing conditions and visualized by silver staining. The parental mouse 1 T0 antibody (m-1 T0) was used as a control.

Western blot analysis of humanized antibody

Comparative HER2 binding of m-1 T0, c-1 T0 and hersintuzumab mAbs was assessed by Western blot technique [4]. Fifty nanogram of rHER2 under reducing and nonreducing conditions was separated on 10% SDS-PAGE and transferred to PVDF membrane (Roche Diagnostics, Mannheim, Germany). After blocking of membrane with blocking buffer (PBS-Tween-20 containing 5% non-fat skim milk) at 4 °C overnight, m-1 T0, c-1 T0 and hersintuzumab antibodies as well as trastuzumab as a positive control were added at 10 μ g/mL in blocking buffer and incubated at room temperature for 1.5 h while shaking. Washing steps were repeated and HRP-conjugated sheep anti-mouse immunoglobulin (prepared in our lab) for m-1 T0 and HRP-conjugated sheep anti-human Ig (prepared in our lab) for c-1 T0, hersintuzumab and trastuzumab were added and incubated at room temperature for 1.5 h on a shaker. After washing, PVDF membrane was treated with ECL (Amersham Biosciences, New Jersey, USA) and the bands were visualized on Kodak X-ray film (Eastman Kodak, Rochester, NY, USA).

Affinity constant determination by ELISA

An ELISA-based method [19] was used to compare the binding affinity of m-1 T0, c-1 T0 and hersintuzumab. Briefly, serial concentrations (2–0.031 μ g/mL) of recombinant extracellular part of HER2 were coated in microtiter ELISA plate. After blocking with PBS-T plus 3% non-fat skim milk, serial concentrations of m-1 T0, c-1 T0 and hersintuzumab (10–0.07 μ g/mL) in blocking buffer were added into coated wells and incubated at 37 °C for 1.5 h. Washing was repeated and wells incubated with either HRP-conjugated sheep anti-mouse Ig or sheep anti-human Ig (prepared in our lab) for 1.5 h at 37 °C. After the final wash step, TMB substrate solution was added followed by stopping solution and ODs were measured as described above. Sigmoidal curves of ODs versus the logarithm of antibody concentrations were constructed. The antibody concentration giving 50% of the maximum absorbance value ([Ab]_{0.5}) at a particular antigen coating concentration was chosen for the affinity measurement using the formula $K_{\text{aff}} = 1/2(2 [\text{Ab}]_0 - [\text{Ab}]_t)$. [Ab]₀ and [Ab]_t represent the antibody concentrations resulting in 50% of the maximum absorbance value at two consecutive concentrations of coated antigen where [Ag] = 2[Ag]₀. The mean of such calculations for three non-overlapping antigen concentrations was taken as the final K_{aff} value [4].

Analysis of cell surface binding of humanized antibody by flow cytometry

10⁶ BT-474 cells were trypsinized and washed two times with washing buffer (PBS, 0.1% NaN₃) and incubated with 100 μ L of 10 μ g/mL of m-1 T0, c-1 T0 or hersintuzumab antibodies as primary antibodies at 4 °C for 1 h. Mouse IgG1 mAb (2F9G5) and human IgG of irrelevant specificities (produced in our lab) were included at the same concentration as negative controls. After washing, cells were stained with FITC-conjugated sheep anti-mouse Ig or sheep anti-human Ig (prepared in our lab) at 4 °C for 1 h. Finally, the cells were scanned by a flow cytometer (Partec, Nuremberg, Germany). Flomax flow cytometry analysis software (Partec) was used to analyze the data.

Epitope mapping, cross-inhibition and cross-reactivity of hersintuzumab

A sandwich ELISA was performed for epitope mapping of m-1 T0 and hersintuzumab mAbs. Briefly, recombinant full extracellular domain, single and paired subdomains of HER2-ECD, with 90 bp overlap, including DI, DII, DIII, DIV, DI + II and DIII + IV were amplified from HER2-pCMV-XL4 construct (OriGene Technologies, Rockville, MD, USA) and subcloned in mammalian expression pSecTag2A vector and then

transiently expressed in CHO-K1 cell line and their presence in supernatants was confirmed by ELISA (data not published). Five micrograms of purified m-1 T0, hersintuzumab, trastuzumab and pertuzumab were coated and the plate incubated for 1.5 h at 37 °C and after blocking for 1 h at 37 °C, the supernatant of recombinant proteins were added and the plate incubated at 37 °C for another 1 h. After washing step, since all recombinant proteins had a 6 × histidine tag in their C-terminal, HRP-anti His-Tag antibody (SinaBiotech, Tehran, Iran) was used as a detector layer. TMB substrate was added into the wells and after stopping the reaction by HCl, OD was measured as described above.

Cross-reactivity of m-1 T0 and hersintuzumab mAbs were also assessed with other members of ErbB family (EGFR, HER3 and HER4) as well as Cynomolgus monkey HER2 by sandwich ELISA. Recombinant EGFR, HER3 and HER4 (Speed BioSystems, Rockville, MD, USA) and Cynomolgus HER2 (Sino Biological Inc., Beijing, China) proteins were used for this purpose.

Localization of the epitopes recognized by trastuzumab, pertuzumab and hersintuzumab were also assessed by an ELISA. Briefly, ELISA plates were coated with 1 µg/mL of recombinant HER2-ECD and then blocked as previously described. Serial concentrations (50, 12.5, 3.1, 0.8, 0.2, 0.05, 0.012 and 0.006 µg/mL) of hersintuzumab and m-1 T0 were added separately and incubated at 37 °C for 1.5 h. After three times washing, appropriate dilution of HRP-conjugated trastuzumab or HRP-conjugated pertuzumab (conjugated in our lab) was added to wells and incubated for 1 h at 37 °C. Washing steps were repeated and ODs were calculated after adding TMB substrate solution followed by stopping solution. Serial concentrations of unconjugated trastuzumab or pertuzumab were also used as positive control.

Assessment of inhibition of tumor growth and proliferation by XTT and radioactive thymidine incorporation assays

5×10^4 BT-474 cells were seeded in 96-well flat-bottom tissue culture plates in serum-containing RPMI-1640 medium (Gibco, Grand Island, NY, USA). The cells were treated with different concentrations of m-1 T0, c-1 T0, hersintuzumab and trastuzumab (50, 10 and 2.5 µg/ml) for 72 h at 37 °C in a humidified atmosphere of 5% CO₂. After incubation, the RPMI medium was replaced with serum free medium containing XTT solution (Roche, Indianapolis, IN) for 4 h at 37 °C as recommended by the manufacturer. After incubation with XTT, microtiter plates were read by an ELISA reader (Organon Teknika, Turnhout, Belgium) at 450 nm with the reference wavelength of 650 nm. Controls wells included cells without antibody or with irrelevant antibody. All experiments were performed in triplicate. The following formula was used to estimate the tumor growth inhibition rate induced by anti-HER2 antibodies:

Tumor growth inhibition (%) =

$$\left[\frac{(\text{OD without antibody} - \text{OD with antibody})}{\text{OD without antibody}} \right] \times 100$$

For assessment of tumor proliferation inhibition, antibodies were added to wells at different concentrations of m-1 T0, hersintuzumab and trastuzumab (12, 6 and 0.7 µg/ml) for 72 h at 37 °C in a humidified atmosphere of 5% CO₂. After incubation, 3H-thymidine (PerkinElmer, MA, USA) was added at 0.5 µCi per well for 18 h. Cultures were then harvested and transferred to scintillation fluid for measurement of 3H-thymidine incorporation by a β-counter (Wallac 1410 Liquid Scintillation Counter, Pharmacia, Sweden). The following formula was used to estimate the proliferation inhibition rate:

$$\text{Proliferation inhibition (\%)} = \left(\frac{[\text{Counts per minute [CPM] without antibody} - \text{CPM with antibody}]}{\text{CPM without antibody}} \right) \times 100$$

50 and 12 µg/ml for single and half concentration of each mAb for combination treatment were respectively used for XTT and LTT experiments. The cells only, trastuzumab and pertuzumab were used as background and positive controls, respectively, and all experiments were performed three times independently in triplicate.

Antibody-dependent cell cytotoxicity (ADCC) assay

ADCC was measured by lactate dehydrogenase-releasing assay kit (CytoTox96, Promega, Madison, WI) according to the manufacturer's instructions. The BT-474 breast cancer cell

line was chosen as the target cells. Briefly, 2×10^4 target cells were incubated with either anti-HER2 antibody (m-1 T0, c-1 T0, hersintuzumab or trastuzumab) at various concentrations or medium alone for 20 min in phenol red-free RPMI-1640 culture medium on ice. Peripheral blood mononuclear cells (PBMC) were isolated from healthy adult volunteers by density gradient centrifugation using Histopaque 1077 (Sigma Chemical Co, St. Louis, MO, USA). PBMCs and cancer cells were co-incubated at 50:1 effector:target ratio for 6 h in 200 µl phenol red-free RPMI containing 5% FBS in a 96-well U-bottomed plate. All experiments were performed in triplicate at 37 °C. Percentage of cytotoxicity was calculated according

to the amount of LDH released into the culture supernatant. Maximum release of target cells was determined by adding the

lysis buffer provided in the kit. Percentage of specific lysis was calculated according to the following formula:

$$\% \text{lysis} = (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release}) \times 100.$$

The spontaneous release represents the level of LDH detected in culture supernatant of untreated cells.

Cell cycle analysis

Flow cytometry was employed to analyze the cell cycle of antibody treated cells. BT474 cells were treated with 20 $\mu\text{g}/\text{ml}$ of hersintuzumab, c-1 T0, m-1 T0 and trastuzumab for 48 or 72 h. Human IgG and mouse IgG (2F9G5) served as control antibodies for chimeric or humanized and mouse monoclonal antibodies, respectively. Rapamycin is a potent inducer of G1 arrest in BT474 cells [20] and was used as a positive control in this experiment at 100 nM/ml concentration. After antibodies treatment, cells were fixed in 70% ethanol and stored overnight at 4 °C. Cells were then resuspended in staining solution containing propidium iodide (20 $\mu\text{g}/\text{mL}$), Triton X-100 (0.1% v/v) and RNase A (50 $\mu\text{g}/\text{mL}$) and incubated at 37 °C for 45 min. DNA content was determined by flow cytometry (Partec, Nuremberg, Germany).

The effect of hersintuzumab on downstream signaling pathways

We evaluated phosphorylation of ERK and AKT as two important downstream signaling pathways of HER2 which play important roles in development and progression of human tumors [21]. In brief, 3×10^6 BT474 cells were seeded in culture flask and treated with 50 $\mu\text{g}/\text{ml}$ of hersintuzumab, m-1 T0, trastuzumab and combination of trastuzumab and m-1 T0 or hersintuzumab. After 24 h incubation at 37 °C in a humidified atmosphere of 5% CO₂, cells were washed with ice-cold PBS, trypsinized and lysed using the M-PER mammalian protein extraction reagent (Thermo Scientific). Halt™ Protease and Phosphatase Inhibitor (Thermo Scientific) was added to cell lysates according to the manufacturer's instruction. BCA protein assay reagent (Thermo Scientific) was used to measure concentration of lysates. The lysates were loaded on 10% SDS-PAGE and transferred to the PVDF nitrocellulose membrane and then blocked with 5% nonfat dry milk in TBS without Tween20 for 1 h at room temperature. Blots were incubated with P-AKT, P-ERK and β -actin mAbs (Cell Signaling Technology, Danvers, MA, USA) as primary antibodies for overnight at 4 °C and then with HRP-conjugated anti-rabbit antibody (Cell signaling Technology) as secondary

antibody for 1 h at room temperature. The target proteins were detected with the advanced ECL kit. After striping [22], the blots were stained with anti-total AKT and ERK antibodies (Cell signaling Technology) and HRP-anti-rabbit antibody as described. Finally, protein bands were scanned and band densities were analyzed with ImageJ software. The experiments were repeated three times independently and the results are presented as mean \pm standard deviation.

Analysis of the humanness of hersintuzumab by Z-score

The degree of humanness of hersintuzumab variable domains sequences is predicted by a web-based tool reported by Abhinandan and Martin [23]. We compared the Z-scores of VH and VL sequences of hersintuzumab with parental murine 1 T0 mAb by this tool (<http://www.bioinf.org.uk/abs/shab/>).

Statistics

The results were analyzed with One-Way ANOVA test using SPSS software (version 20, IBM SPSS statistics data editor). Differences between groups were significant at p values less than 0.05 ($p < 0.05$). The results are presented as mean \pm standard deviation.

Results

Molecular modeling of variable regions of mouse 1 T0

The molecular model of 1 T0 Fv was determined based on the most homologous mouse VH and VL sequences within the database of known antibody structures. The VH of the mouse antibody (PDB No. 1IGT) [24] with 81% identity and the VL of the mouse scFv (PDB No. 1H8O) [25] with 84% identity were selected for final 3D structure modeling. Finally, the 3D structure of 1 T0 Fv was visualized using VMD (Fig. 1). The residues within 5 Å distance from all six CDRs that are of potential importance to VH-VL interaction and antigen-binding were replaced with the parental rodent amino acids. As shown in Fig. 1, the four residues in VL (L42, L43, L60 and L63) and five residues in VH (H44, H49, H60, H73 and H77), which were near or in direct contact with the CDRs, were retained in the humanized version of 1 T0.

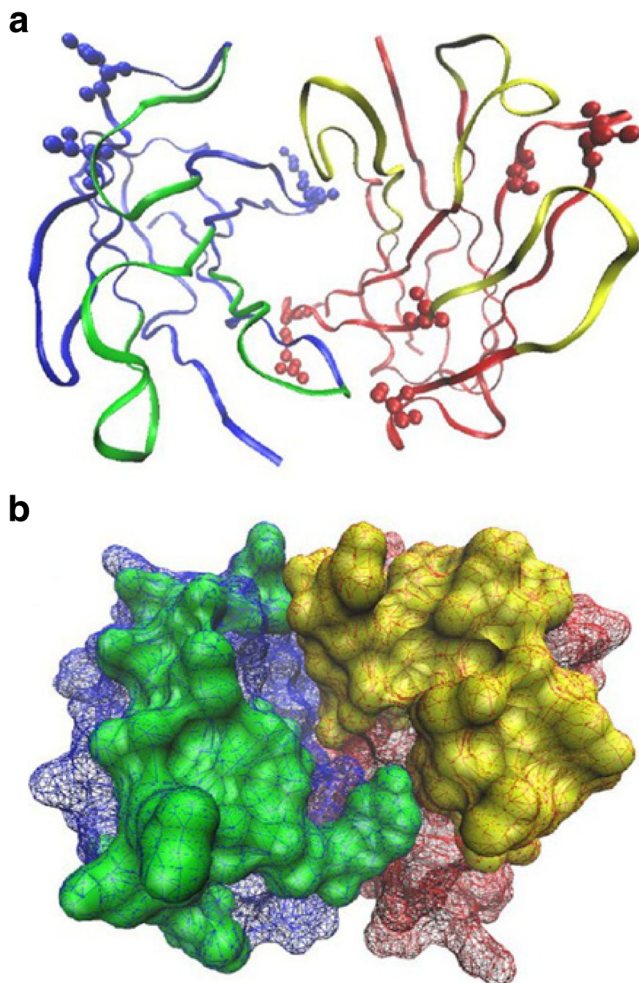


Fig. 1 Molecular modeling of the 1 T0 variable regions. Ribbon (a) and surface (b) representations of the variable domains. In both panels, the light chain is shown in blue with corresponding CDRs highlighted in green, while the heavy chain is shown in red with its CDRs highlighted in yellow. Nine FR residues, L42, L43, L60, L63, H44, H49, H60, H73 and H77, which are within 5 Å distance from the CDRs contributing to VH-VL interaction and antigen binding, are specified in the ribbon representation. The structure is depicted from the antigen-binding point of view

Humanization of mouse 1 T0 monoclonal antibody

In this study, we used CDR grafting approach for humanization of the anti-HER2 mouse mAb 1 T0. The amino acid sequences of the VH and VL of 1 T0 were aligned with human Ig germline V and J genes. Human heavy chain V gene segment IGHV3–69-1*01 (IMGT accession number: AJ879484), J gene segment IGHJ6 (IMGT accession number: J00256), human light chain V gene segment IGKV1–17*02 (IMGT accession number: D88255) and J gene segment IGKJ4 (IMGT accession number: AF103571) were selected as acceptor frameworks for the CDRs of murine 1 T0 VH and VL, respectively. The overall homology of m-1 T0 VH, VL, JH and JK with IGHV3–69-1*01, IGKV1–

17*02, IGHJ6 and IGKJ4 were 79, 80, 70 and 86%, respectively. The VH and VL were subsequently ligated to the human gamma 1 heavy chain and human kappa light chain, respectively, to form a full humanized hersintuzumab construct.

Amplification of the VH- C γ 1 and VL- C κ genes

The VH and VL genes were amplified using specific primers designed for the upstream and downstream regions of the synthetic DNA containing sequences of the humanized VH and VL genes. The primers do not contain any sequence of restriction site, Kozak sequence or ribosome binding sites. The PCR products of VH and VL are 486 bp and 458 bp, respectively, while the ORF of the VH region of the mAb hersintuzumab is 405 bp in length, encoding a 135-aa polypeptide and the ORF of VL is 381 bp in length, encoding a 127-aa polypeptide, including the leader peptides. Leader peptides of both VH and VL m-1 T0 are localized to their N-terminal region, encoding 19- and 20-aa peptides, respectively. The C κ and C γ 1 were amplified with specific primers from c-1 T0 vector [4] encoding 107- and 330-aa polypeptides, respectively. Finally, the VL-C κ and VH-C γ 1 segments were linked to each other by SOE PCR as described in the Materials and Methods.

The hersintuzumab construct was transfected in CHO-K1 cells using Lipofectamin 2000 transfection reagent. After selection in Zeocin and four rounds of subcloning, a stable transfected cell line which produces high levels of humanized antibody was selected. (data not shown).

Structural characterization of the humanized antibody

The humanized hersintuzumab antibody was purified from serum-free culture supernatant of transfected CHO cells by affinity chromatography using SPG column. The ELISA results showed that the purified antibody contains human IgG1 and Ig κ proteins. Further analysis was performed by SDS-PAGE under non-reducing and reducing conditions. Silver staining of SDS-PAGE gel (Fig. 2a) shows monomeric (~150 kDa) form of the humanized antibody under non-reducing condition (lane 1). The monomeric light (~25 kDa) and heavy chains (~50 kDa) were detected under reducing conditions (lane 2). The parental mouse 1 T0 and c-1 T0 mAb gave a similar pattern under non-reducing and reducing conditions.

Western blot analysis revealed that hersintuzumab and the parental mouse 1 T0 and chimeric c-1 T0 mAbs react with the non-reduced recombinant extracellular HER2 protein (Fig. 2b). Lack of reactivity with the reduced HER2 protein indicates recognition of a conformational

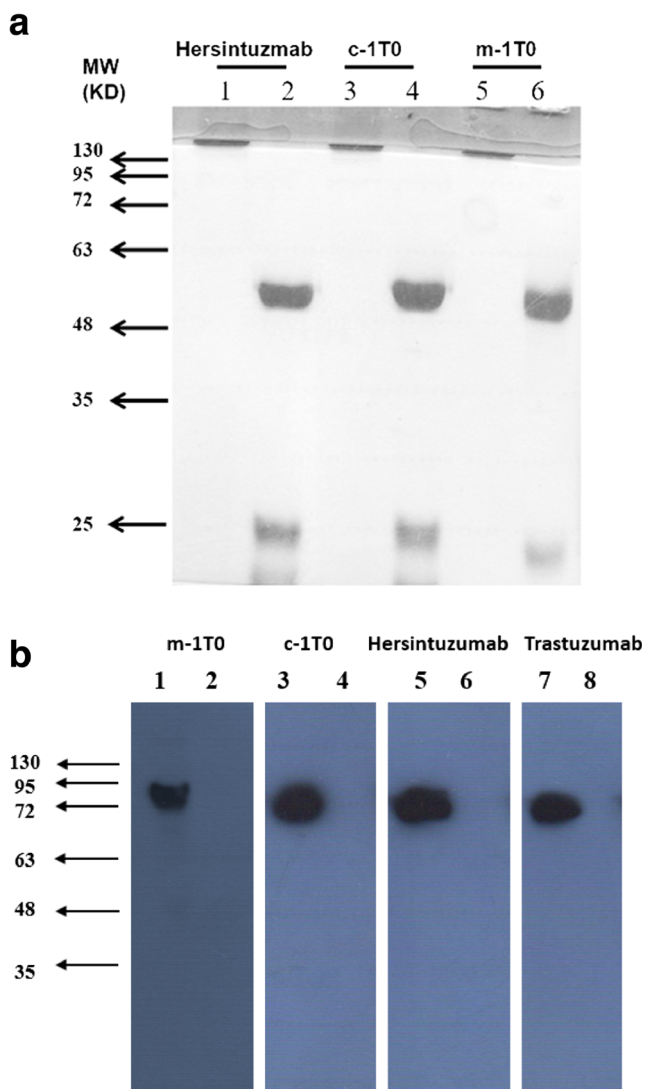


Fig. 2 SDS-PAGE electrophoresis pattern and western-blot analysis of hersintuzumab. **a** SPG purified hersintuzumab, c-1 T0 and mouse 1 T0 IgG preparations were separated on 10% gel at non-reducing (1, 3 and 5) and reducing conditions (2, 4 and 6). MW: molecular weight ladder. **b** Non-reduced (Lane 1, 3, 5 and 7) and reduced (Lane 2, 4, 6 and 8) forms of rHER2 extracellular protein was separated on 10% SDS-PAGE gel and transferred onto nitrocellulose membrane. The membrane was blotted with hersintuzumab (Lane 1 and 2), c-1 T0 (Lanes 3 and 4), m-1 T0 (Lanes 5 and 6) and trastuzumab (Lanes 7 and 8) and then visualized by ECL, as described in the Materials and Methods

epitope by our mAb. A similar pattern of reactivity was observed for trastuzumab, which was used as a control.

Affinity constant determination

The binding affinity of the humanized antibody was determined by an ELISA method as described in the Materials and Methods. Based on the binding curves obtained for the humanized, chimeric and mouse parental mAbs (Fig. 3a, b and c), the mean K_{aff} of m-1 T0,

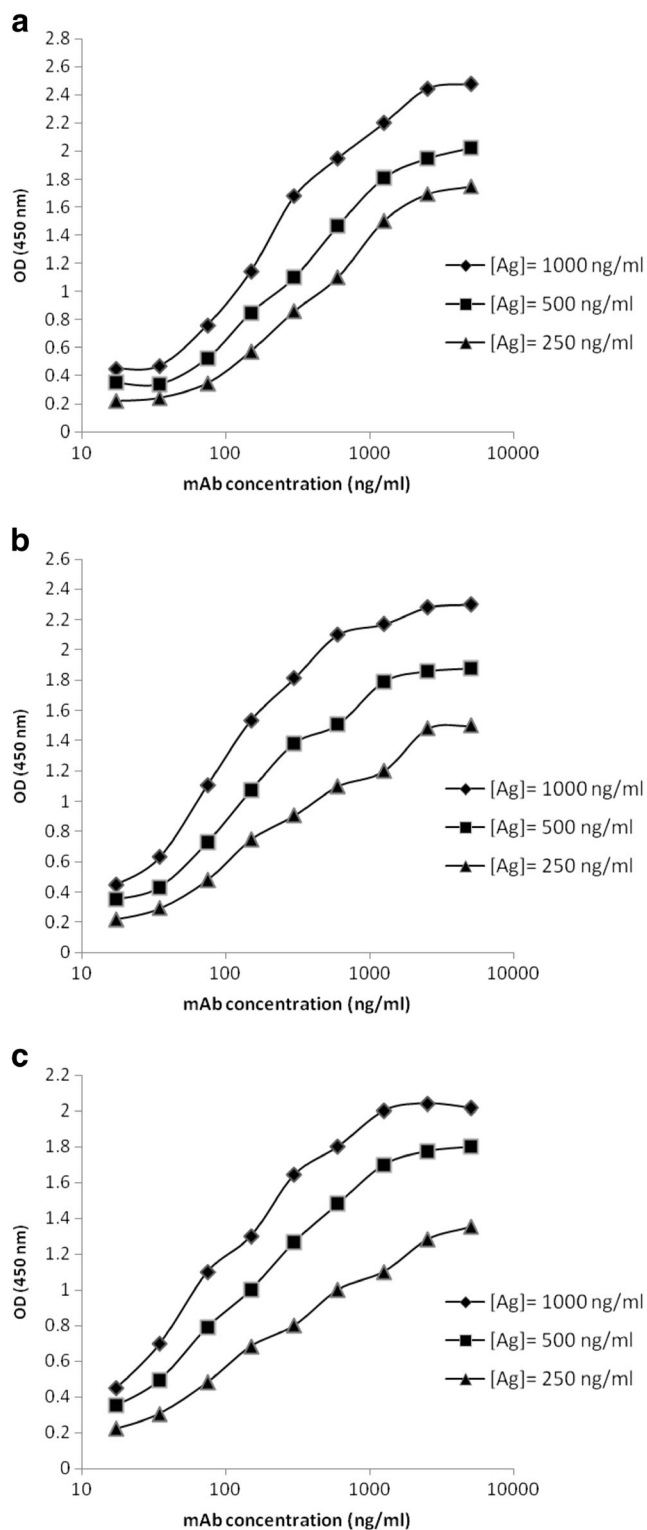


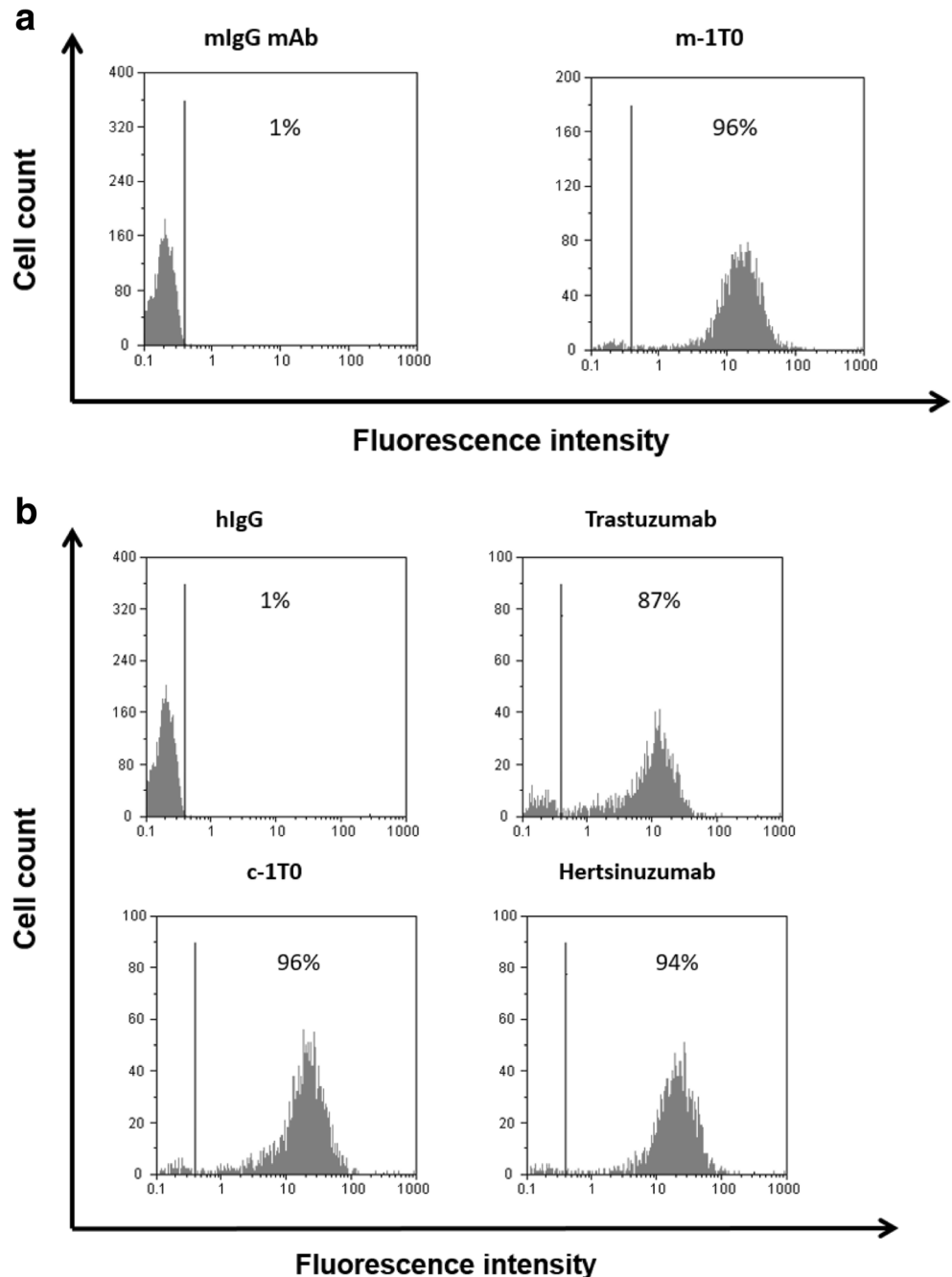
Fig. 3 Affinity constant determination by ELISA. Experimental dose-response curves for mouse 1 T0 (a), c-1 T0 (b) and hersintuzumab (c) monoclonal antibodies at three different concentrations of recombinant extracellular region of HER2

c-1 T0 and hersintuzumab were $0.6 \times 10^9 M^{-1}$, $0.9 \times 10^9 M^{-1}$ and $0.6 \times 10^9 M^{-1}$, respectively.

Assessment of cell binding activity by flow cytometry

In order to determine the binding reactivity of hersintuzumab to native HER2, we performed flow cytometric analysis using hersintuzumab, c-1 T0 and m-1 T0 as first layer and sheep-anti human Ig-FITC and sheep-anti mouse Ig-FITC as second layer, respectively. In parallel to trastuzumab as positive control, hersintuzumab showed positive reactivity and detected HER2 on the surface of BT-474 cells similar to c-1 T0 and the parental m-1 T0 antibody (Fig. 4).

Fig. 4 Detection of binding activity of hersintuzumab by flow cytometry. BT-474 cells were harvested and stained with mouse 1 T0 (a), c-1 T0 and hersintuzumab (b). Irrelevant mouse mAb (mIgG), irrelevant human IgG (hIgG) and trastuzumab were used with the same concentration as negative and positive controls, respectively. The figures represent percent of positive cells



Epitope mapping of hersintuzumab

A sandwich ELISA was designed to determine the reactivity of the m-1 T0 and hersintuzumab mAbs with HER2 extracellular subdomains including I, II, III, IV, I + II and III + IV as well as full HER2-ECD. Trastuzumab and pertuzumab were used as positive controls. The ELISA results showed that hersintuzumab similar to the mouse 1 T0 detected subdomain DI + II of HER2 but not DI or DII alone, which indicates that the conformational epitope could be formed when the two adjacent subdomains are expressed together. Similarly,

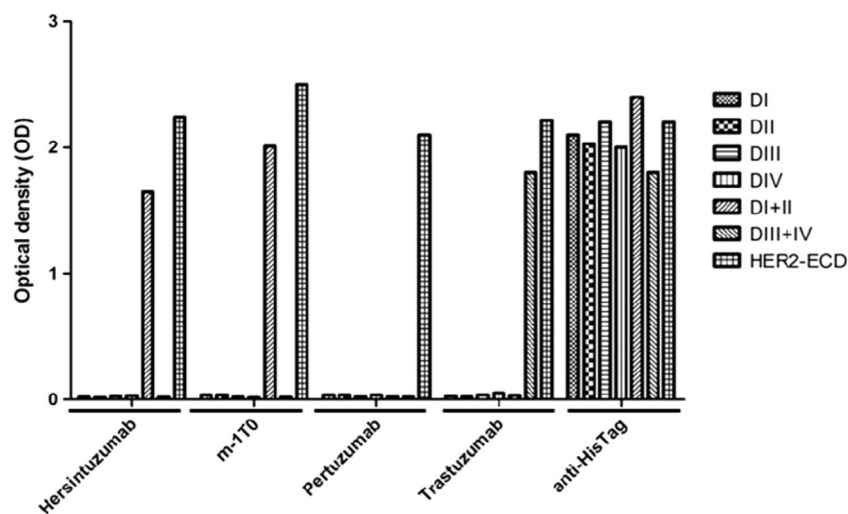
trastuzumab did not recognize isolated subdomain DIV while it reacted with the paired DIII + IV subdomain of HER2. On the other hand, pertuzumab did not recognize any isolated or paired recombinant subdomains of HER2 (Fig. 5). The results of cross reactivity ELISA showed that hersintuzumab as well as m-1 T0, trastuzumab and pertuzumab react similarly with recombinant human HER2 and Cynomolgus monkey HER2. However, none of the mAbs recognized other members of the human HER family, including HER1, HER3 and HER4 (data not presented).

The results of inhibition ELISA suggested that while, as low as 0.5 $\mu\text{g/ml}$ of unconjugated trastuzumab and pertuzumab induced approximately 50% inhibition of binding of the HRP-conjugated trastuzumab and pertuzumab to HER2, respectively, no inhibition was observed for hersintuzumab or m-1 T0 even at the highest concentration (50 $\mu\text{g/ml}$) employed in the experiment (data not presented).

Tumor cell growth inhibition by hersintuzumab

Colorimetric (XTT) and radioactive thymidine incorporation assays were performed to assess the effect of hersintuzumab on growth of BT-474 cell line. The growth and proliferation inhibition rate of triplicate wells were determined and percent of inhibition was calculated according to the formula described in the Materials and Methods. Accordingly, hersintuzumab induced a dose dependent growth and proliferation inhibition, similar to c-1 T0 and the parental m-1 T0 mAb (Fig. 6a and b). Moreover, combination of hersintuzumab and trastuzumab can inhibit growth and proliferation of tumor cells more efficiently than combination of commercial mAbs, trastuzumab and pertuzumab (Fig. 6c and d).

Fig. 5 Epitope mapping and cross-reactivity of hersintuzumab. Reactivity of hersintuzumab with isolated and paired subdomains as well as full extracellular domain of HER2 (HER2-ECD) was assessed by ELISA. Trastuzumab, pertuzumab and anti-HisTag antibodies were used as controls



Induction of antibody-dependent cell cytotoxicity (ADCC)

To determine the capacity of c-1 T0 and hersintuzumab to trigger ADCC, BT-474 cells were incubated for 6 h with decreasing concentrations of c-1 T0 and hersintuzumab (50, 5, 0.5, 0.05 and 0.005 $\mu\text{g/ml}$). Trastuzumab and m-1 T0 were used as positive and negative controls, respectively. The results demonstrate that in the presence of normal PBMC, hersintuzumab similar to trastuzumab induces ADCC activity against HER2-positive BT-474 target cells, dose dependently. No ADCC activity, however, was detected in presence of mouse 1 T0 antibody (Fig. 7) or MDA-MB-231 cell line as HER2-negative target cells (data not shown).

Cell cycle arrest by hersintuzumab

BT474 cells were treated with 20 $\mu\text{g/ml}$ of hersintuzumab. DNA content was measured in comparison with untreated cells and cells treated with irrelevant mouse IgG1 and human IgG after 48 and 72 h. G1 accumulation occurred in BT-474 cells treated with trastuzumab or Rapamycin after 48 or 72 h (Fig. 8). A similar profile was observed in cells treated with hersintuzumab, c-1 T0 and m-1 T0 mAbs. G1 accumulation was more prominent after 72 h compared to 48 h antibody treatment.

The effect of hersintuzumab on ERK and AKT downstream signaling pathways

To determine the signaling pathways involved in hersintuzumab inhibitory activity, phosphorylation of ERK and AKT proteins was analyzed by Western blotting. While trastuzumab induced a weak inhibition on ERK and AKT

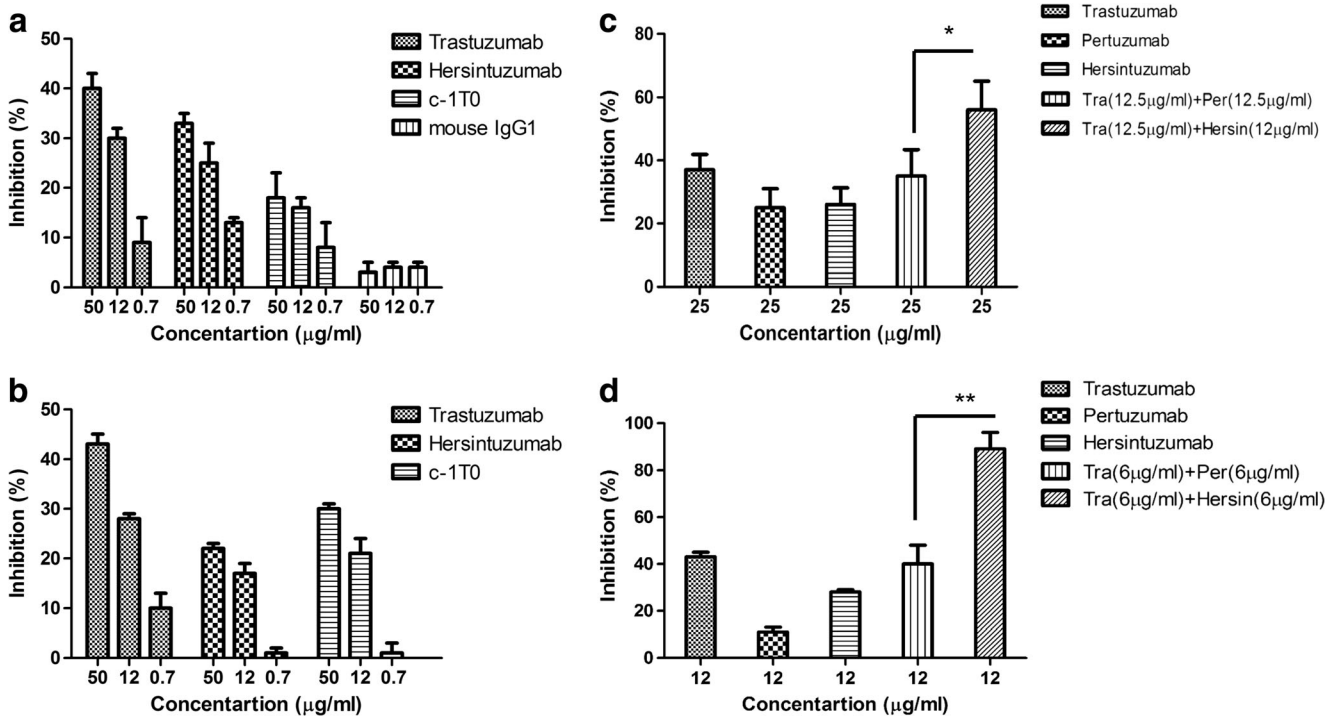


Fig. 6 Assessment of tumor growth inhibition by XTT and ^3H -thymidine incorporation assays. Serial concentrations of hersintuzumab were added to BT-474 cells. After 72 h incubation, cells were treated either with XTT reagent or ^3H -thymidine. Optical density and radioactive thymidine incorporation were measured by multiscan ELISA reader and β -counter, respectively. Percent of cell growth inhibition was measured as described in the Materials and Methods.

The results of XTT (**a** and **c**) and thymidine incorporation (**b** and **d**) are presented as percent inhibition. Serial concentrations of irrelevant mouse IgG1 (2F9G5), m-1 T0, c-1 T0 and trastuzumab were employed as controls. Error bars represent the mean \pm SD of three independent experiments performed in triplicate. * $p = 0.035$ and ** $p = 0.001$. Hersin: Hersintuzumab; Tras: Trastuzumab; Per: Pertuzumab

phosphorylation, hersintuzumab inhibited phosphorylation of both proteins, particularly ERK, more potently (Fig. 9a and b). Interestingly, the combination of hersintuzumab and trastuzumab almost completely inhibited phosphorylation of both ERK and AKT proteins (* $p < 0.01$ and ** $p < 0.001$, respectively).

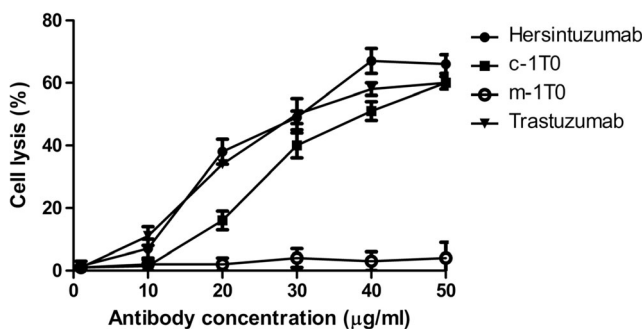


Fig. 7 Antibody-dependent cell cytotoxicity (ADCC) induced by hersintuzumab. ADCC was determined by measuring the level of lactate dehydrogenase (LDH) released from the BT-474 cells incubated with normal PBMC at 1:50 target to effector ratio after 6 h incubation. Percent of specific lysis was calculated as described in the Materials and Methods. Error bars represent the mean \pm SD of an experiment in duplicate

Z-score analysis

Comparison between Z-score of VH and VL sequences of 1 T0 and hersintuzumab showed that they increased from -0.401 to 1.027 and -1.901 to -0.346 which clearly indicates that the humanness was increased after humanization.

Discussion

HER2 is a well known oncoprotein overexpressed in breast cancer and many other malignancies. It has been employed as a therapeutic target in several clinical studies using the humanized HER2-specific mAb, trastuzumab [26]. Trastuzumab binds to the extracellular subdomain IV of HER2 and induces antitumor activity in several ways, including inhibition of signal transduction and induction of antibody-dependent cell cytotoxicity (ADCC) [27]. Despite encouraging clinical results, the tumor cells in many patients are primarily resistant to trastuzumab [28]. One strategy to overcome trastuzumab resistance is through the simultaneous blockade of HER2 by multiple mAbs recognizing different epitopes of HER2. Several studies have shown that some combinations of mAbs against HER2 work better than individual antibodies

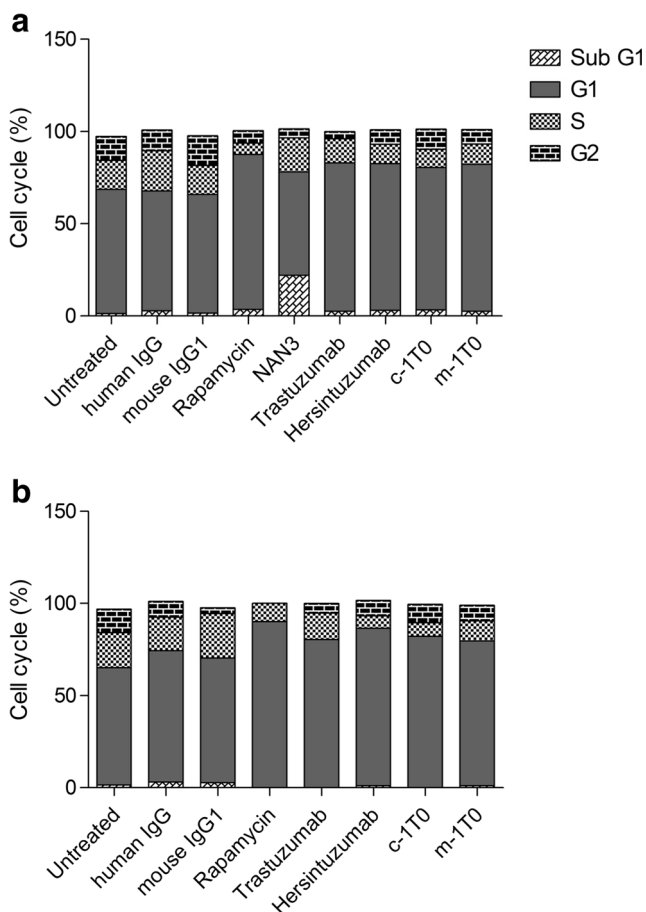


Fig. 8 Cell cycle arrest induced by hersintuzumab. BT-474 cells were incubated with 20 $\mu\text{g}/\text{ml}$ of hersintuzumab, c-1 T0, m-1 T0 and trastuzumab for 48 and 72 h. Cells were then fixed, stained and analyzed by Partec flow cytometer. The bar diagrams represent distribution of the cells at different phases of the cell cycle 48 h (a) and 72 h (b) after antibody treatment

[29–31]. Pertuzumab, another humanized mAb, binds to an epitope on domain II of the extracellular region of HER2, different from the binding site of trastuzumab [5]. Combination of pertuzumab with trastuzumab have demonstrated promising anti-tumor efficacy in treatment of breast cancer and was recently approved by FDA [7].

We have recently produced a panel of anti-HER2 mouse mAbs with non-overlapping epitope binding on HER2. Two of these mAbs (1 T0 and 2A8) binds to an epitope different from those recognized by trastuzumab and pertuzumab. Moreover, they specifically inhibit proliferation of tumor cells overexpressing HER2 either alone or in combination with trastuzumab [8, 9].

For successful clinical application of murine mAbs, immunogenicity of these mAbs should be reduced by chimerization and humanization technologies [32]. An early effort to reduce immunogenicity of murine mAbs is production of chimeric mAbs, which consists of murine variable regions and human constant regions [4]. However, the clinical experiences have

shown that murine variable regions in chimeric mAbs are sufficient to produce strong human anti-murine antibody (HAMA) responses [33]. To further reduce the immunogenicity, humanized mAbs have been generated with different approaches [34, 35].

In an attempt to reduce immunogenicity of murine mAbs for therapeutic purposes, we previously chimerized the anti-HER2 mAb (1 T0) [4] and subsequently humanized it by CDR grafting method in this study. In CDR grafting technology, the antigen-binding specificity of a murine antibody is transferred to a human antibody by grafting the CDR loops [36]. However, replacement of the six CDRs of murine antibodies onto appropriate frameworks of human antibodies often lead to reduced or abolished affinity or specificity for the target antigen [37–39]. The successful CDR grafting approach depends on identification and retaining the murine framework residues that may contribute to antigen binding either through direct antigen contact or indirectly through packing with CDR residues [40]. Wedemayer and coworkers [41] have shown that the affinity of an anti-hapten antibody could be increased through somatic mutations located 10Å away from the hapten-binding site. Moreover, Foote and Winter have suggested that antibody residues in the β -sheet framework underlying the CDRs play a critical role in adjustment of the loop structures of the CDRs that commonly called the “Vernier residues” [42]. They found that mutations in some residues that are in direct contact with the CDRs, could have a significant effect on the conformation and position of CDR loops and therefore the antibody affinity [43]. For identification of these residues, the X-ray crystallographic data of free or antigen-complexed antibodies is required. Since the crystal structure of the new mouse antibody is not available, computer-aided molecular modeling of the antibody was used to recognize such residues. In different works, various high-affinity humanized antibodies were successfully developed by molecular modeling method while retaining FRs residues [44–47].

In the present study, we used an antibody modeling program (PIGS, Prediction of ImmunoGlobulin Structure) to construct the 3D structure of 1 T0 Fv. This modeling system enabled us to define important residues located within a 5 Å distance of the VH-VL CDRs in the antigen-binding site [14, 48]. Subsequently, m1 T0 mAb genes were humanized based on similarity of frameworks to human immunoglobulin germ line and finally, VH and VL genes of hersintuzumab were synthesized and ligated to human IgG and IgC κ by SOE PCR [18]. Application of PIGS web-based program for Fv antibody modeling has been reported in several studies [12, 48, 49]. The first attempt to produce humanized mAbs against HER2 was made by Carter and coworkers [50]. They humanized 4D5, the original murine hybridoma clone of trastuzumab, by CDRs grafting in to consensus sequences derived from the VL kappa subgroup I and VH subgroup

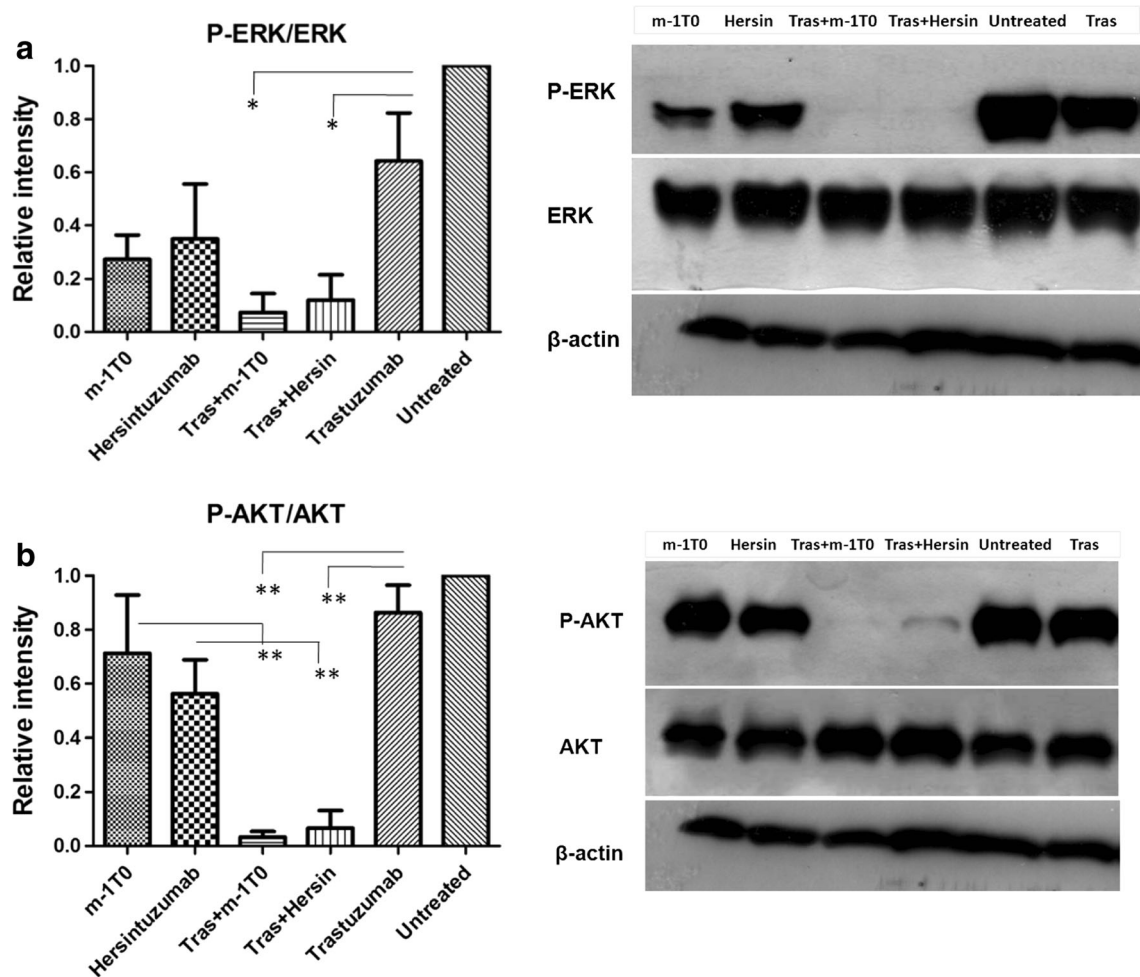


Fig. 9 Hersintuzumab inhibits ERK and AKT phosphorylation. BT-474 cells were incubated either with 50 $\mu\text{g/ml}$ of each mAb or combination of 25 $\mu\text{g/ml}$ of two mAbs. The cell lysates were then run on 10% gel and then transferred to PVDF membrane. The blots were probed with primary and secondary antibodies and band densities were analysed and subsequently relative density of p-ERK/ERK (**a**) and p-AKT/AKT (**b**)

was calculated. Untreated cells served as negative control. All experiments were performed independently three times and representative Western blot images are shown. Error bars represent the mean \pm SD of three independent experiments. Hersin: Hersintuzumab; Tras: Trastuzumab. * $p < 0.01$, ** $p < 0.001$

III. For identification of important amino acid residues, molecular model of mouse 4D5 (mumAb4D5) was constructed by using seven Fab crystal structures from the Brookhaven Protein Data Bank. Finally, 8 variants of humanized 4D5 were constructed by replacing selected human residues in humAb4D5 with their mumAb4D5 counterparts. In 2006, another humanized mAb against HER2, pertuzumab, was also produced by Adams and colleagues [51] with the same protocol that was described for 4D5.

We assessed expression of hersintuzumab by ELISA using recombinant HER2 as an immobilized antigen. Similar to our work, expression of the two humanized mAbs, trastuzumab and pertuzumab, was assessed by immobilized recombinant HER2 by ELISA method [9, 50]. The HER2 binding activity of hersintuzumab was also determined by immunoblotting and flowcytometry techniques. Similar to its parental murine and chimeric counterpart, hersintuzumab recognizes a

conformational epitope on extracellular domain of HER2 (Fig. 2b). The flow cytometry results show that hersintuzumab binds to native HER2 expressed on the surface of tumor cells as efficiently as the mouse and chimeric counterparts and trastuzumab (Fig. 4). As mentioned above, several humanized forms of 4D5 with different affinities were generated [50, 51]. The most humanized, but least potent humAb4D5 variant with low affinity to HER2 was constructed by simply grafting mumAb4D5 CDRs into consensus human frameworks. Another variant (humAb4D5–8), the most potent humanized variant, contains five FR residues from mouse 4D5 and shows a 3-fold higher affinity than parental mouse 4D5mAb. In another study [51], several variants of humanized mouse 2C4 were generated. Similar to Carter's work, transferring mouse CDRs to consensus human frameworks results in complete loss of binding to HER2-ECD and altering three residues from mouse 2C4 mAb restored binding affinity to HER2-ECD

which was comparable to the chimeric mAb. These results from Carter and Adam studies showed that transferring of CDRs alone might not be enough to retain affinity and there is potentially important residue that would play main role in maintaining the conformation of CDRs. In our study, we generated humanized monoclonal antibody by CDRs grafting and retaining all potentially important residues of mouse 1 T0, obtained from molecular modeling, in frameworks of humanized mAb which resulted in comparable affinity to that of the parental mouse mAb.

To further define the fine specificity of hersintuzumab, we designed and constructed different subdomains of HER2-ECD including DI, DII, DIII, DIV, DI + II and DIII + IV. The constructs were then transiently expressed in CHO-K1 cells. The ELISA results suggested that hersintuzumab similar to m-1 T0 recognizes DI + II, but not DI or DII alone, which indicates that the epitope is located within DI or DII subdomain of HER2. Based on trastuzumab results, it seems that the conformational epitope is created when the two subdomains are expressed together. Similar to our work, Hu and coworker [52] characterized new anti-HER2 mAb (A21) by producing several eukaryotic recombinant single and paired domains of HER2. The results showed that the A21 mAb binds to DI + II, but not to isolated DI or DII subdomains [52]. They also reported that trastuzumab recognized paired subdomains DIII + IV of HER2, but they did not assess the reactivity of trastuzumab with the isolated subdomain DIV. On the other hand, Ko and coworker [53] reported that trastuzumab binds to the isolated subdomain DIV as well as paired DIII + DIV subdomain. However, they did not explain how the subdomains were produced. DIV might be produced as an Fc fusion protein in 293F system, which could maintain its natural three dimensional structure.

Our results indicate that hersintuzumab similar to its mouse parental mAb recognizes an epitope different from those identified by trastuzumab and pertuzumab. Hersintuzumab, similar to trastuzumab and pertuzumab, did not cross-react with other members of ErbB family, but strongly bound to Cynomolgus monkey HER2.

The *in vitro* biological activity of our humanized antibody was assessed by XTT and radioactive thymidine incorporation methods. Hersintuzumab inhibits the proliferation of BT-474 cells in a dose dependent manner similar to trastuzumab, c-1 T0 and m-1 T0. In addition, the inhibitory effect of hersintuzumab and trastuzumab combination was significantly ($*p = 0.035$ and $**p = 0.001$) higher than the combination of trastuzumab and pertuzumab.

Analysis of the cell cycle demonstrates that it induces arrest at the G1 phase, similar to trastuzumab [54–56]. These inhibitory effects seem to be partly delivered through AKT and ERK signaling pathways [53, 57, 58]. Phosphorylation of both proteins was reduced in tumor cells by hersintuzumab, more potently than trastuzumab. Interestingly, combination of

these two mAbs almost completely inhibited both AKT and ERK phosphorylation, which explains the drastic proliferation and growth inhibitory effects observed for the combination of these two mAbs compared to each antibody alone (Fig. 9).

In addition to these direct anti-tumor effects, hersintuzumab can also induce ADCC similar to trastuzumab which strengthens its anti-tumor potential through activation of the immune system.

Altogether, the diverse tumor inhibitory effects induced by hersintuzumab, particularly in combination with trastuzumab, suggest that it could have potential therapeutic value for treatment of breast cancer and other HER2-overexpressing malignancies, either alone or in combination with other anti-HER2 mAbs, such as trastuzumab and/or chemotherapeutic agents.

References

- Hynes NE, MacDonald G (2009) ErbB receptors and signaling pathways in cancer. *Curr Opin Cell Biol* 21(2):177–184. <https://doi.org/10.1016/j.ceb.2008.12.010>
- Ladjemi MZ, Jacot W, Chardes T, Pelegrin A, Navarro-Teulon I (2010) Anti-HER2 vaccines: new prospects for breast cancer therapy. *Cancer Immunol Immunother* 59(9):1295–1312. <https://doi.org/10.1007/s00262-010-0869-2>
- Kruser TJ, Wheeler DL (2010) Mechanisms of resistance to HER family targeting antibodies. *Exp Cell Res* 316(7):1083–1100. <https://doi.org/10.1016/j.yexcr.2010.01.009>
- Amiri MM, Jeddi-Tehrani M, Kazemi T, Bahadori M, Maddah M, Hojjat-Farsangi M, Khoshnoodi J, Rabbani H, Shokri F (2013) Construction and characterization of a new chimeric antibody against HER2. *Immunotherapy* 5(7):703–715. <https://doi.org/10.2217/imt.13.67>
- Ceran C, Cokol M, Cingoz S, Tasan I, Ozturk M, Yagci T (2012) Novel anti-HER2 monoclonal antibodies: synergy and antagonism with tumor necrosis factor-alpha. *BMC Cancer* 12:450. <https://doi.org/10.1186/1471-2407-12-450>
- Baselga J, Cortes J, Kim SB, Im SA, Hegg R, Im YH, Roman L, Pedrini JL, Pienkowski T, Knott A, Clark E, Benyunes MC, Ross G, Swain SM (2012) Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. *N Engl J Med* 366(2):109–119. <https://doi.org/10.1056/NEJMoa1113216>
- Traynor K (2012) FDA approves pertuzumab for breast cancer. *Am J Health Syst Pharm* 69(14):1178. <https://doi.org/10.2146/news120049>
- Kazemi T, Tahmasebi F, Bayat AA, Mohajer N, Khoshnoodi J, Jeddi-Tehrani M, Rabbani H, Shokri F (2011) Characterization of novel murine monoclonal antibodies directed against the extracellular domain of human HER2 tyrosine kinase receptor. *Hybridoma (Larchmt)* 30(4):347–353. <https://doi.org/10.1089/hyb.2011.0023>
- Tahmasebi F, Kazemi T, Amiri MM, Khoshnoodi J, Bayat AA, Jeddi-Tehrani M, Rabbani H, Shokri F (2014) *In vitro* assessment of the effects of anti-HER2 monoclonal antibodies on proliferation of HER2-overexpressing breast cancer cells. *Immunotherapy* 6(1):1–7
- Schroff RW, Foon KA, Beatty SM, Oldham RK, Morgan AC Jr (1985) Human anti-murine immunoglobulin responses in patients receiving monoclonal antibody therapy. *Cancer Res* 45(2):879–885

11. Khazaeli MB, Conry RM, LoBuglio AF (1994) Human immune response to monoclonal antibodies. *J Immunother Emphasis Tumor Immunol* 15(1):42–52
12. Marcatili P, Rosi A, Tramontano A (2008) PIGS: automatic prediction of antibody structures. *Bioinformatics* 24(17):1953–1954. <https://doi.org/10.1093/bioinformatics/btn341>
13. Humphrey W, Dalke A, Schulten K (1996) VMD: visual molecular dynamics. *J Mol Graph* 14(1):33–38
14. Hou S, Li B, Wang L, Qian W, Zhang D, Hong X, Wang H, Guo Y (2008) Humanization of an anti-CD34 monoclonal antibody by complementarity-determining region grafting based on computer-assisted molecular modelling. *J Biochem* 144(1):115–120. <https://doi.org/10.1093/jb/mvn052>
15. Almagro JC, Fransson J (2008) Humanization of antibodies. *Front Biosci* 13:1619–1633
16. Hu WG, Chau D, Wu J, Jager S, Nagata LP (2007) Humanization and mammalian expression of a murine monoclonal antibody against Venezuelan equine encephalitis virus. *Vaccine* 25(16):3210–3214. <https://doi.org/10.1016/j.vaccine.2007.01.034>
17. Queen C, Schneider WP, Selick HE, Payne PW, Landolfi NF, Duncan JF, Avdalovic NM, Levitt M, Junghans RP, Waldmann TA (1989) A humanized antibody that binds to the interleukin 2 receptor. *Proc Natl Acad Sci* 86(24):10029–10033
18. Jones ML, Barnard RT (2005) Chimerization of multiple antibody classes using splice overlap extension PCR. *BioTechniques* 38(2):181–182
19. Saboor-Yaraghi AA, Ghods R, Gharagozlou S, Roohi A, Khoshnoodi J, Towfighi F, Jeddi-Tehrani M, Shokri F (2004) Identification of cross-reactive and restricted epitopes localized on human chorionic gonadotropin beta-subunit by monoclonal antibodies. *Hybrid Hybridomics* 23(2):101–107. <https://doi.org/10.1089/153685904774129702>
20. García-Morales P, Hernando E, Carrasco-García E, Menéndez-Gutiérrez MP, Saceda M, Martínez-Lacaci I (2006) Cyclin D3 is down-regulated by rapamycin in HER-2-overexpressing breast cancer cells. *Mol Cancer Ther* 5(9):2172–2181
21. Caromile LA, Dortche K, Rahman MM, Grant CL, Stoddard C, Ferrer FA, Shapiro LH (2017) PSMA redirects cell survival signaling from the MAPK to the PI3K-AKT pathways to promote the progression of prostate cancer. *Sci Signal* 10(470). <https://doi.org/10.1126/scisignal.aag3326>
22. Yeung YG, Stanley ER (2009) A solution for stripping antibodies from polyvinylidene fluoride immunoblots for multiple reprobing. *Anal Biochem* 389(1):89–91. <https://doi.org/10.1016/j.ab.2009.03.017>
23. Abhinandan KR, Martin AC (2007) Analyzing the "degree of humanness" of antibody sequences. *J Mol Biol* 369(3):852–862. <https://doi.org/10.1016/j.jmb.2007.02.100>
24. Harris LJ, Larson SB, Hasel KW, McPherson A (1997) Refined structure of an intact IgG2a monoclonal antibody. *Biochemistry* 36(7):1581–1597. <https://doi.org/10.1021/bi962514+>
25. Burmester J, Spinelli S, Pugliese L, Krebber A, Honegger A, Jung S, Schimmele B, Cambillau C, Pluckthun A (2001) Selection, characterization and x-ray structure of anti-ampicillin single-chain Fv fragments from phage-displayed murine antibody libraries. *J Mol Biol* 309(3):671–685. <https://doi.org/10.1006/jmbi.2001.4663>
26. Fiszman GL, Jasnis MA (2011) Molecular Mechanisms of Trastuzumab Resistance in HER2 Overexpressing Breast Cancer. *Int J Breast Cancer* 2011:352182. <https://doi.org/10.4061/2011/352182>
27. Cortes J, Fumoleau P, Bianchi GV, Petrella TM, Gelmon K, Pivot X, Verma S, Albanell J, Conte P, Lluch A, Salvagni S, Servent V, Gianni L, Scaltriti M, Ross GA, Dixon J, Szado T, Baselga J (2012) Pertuzumab monotherapy after trastuzumab-based treatment and subsequent reintroduction of trastuzumab: activity and tolerability in patients with advanced human epidermal growth factor receptor 2-positive breast cancer. *J Clin Oncol* 30(14):1594–1600. <https://doi.org/10.1200/JCO.2011.37.4207>
28. Pohlmann PR, Mayer IA, Mernaugh R (2009) Resistance to Trastuzumab in Breast Cancer. *Clin Cancer Res* 15(24):7479–7491. <https://doi.org/10.1158/1078-0432.CCR-09-0636>
29. Harwerth IM, Wels W, Schlegel J, Muller M, Hynes NE (1993) Monoclonal antibodies directed to the erbB-2 receptor inhibit in vivo tumour cell growth. *Br J Cancer* 68(6):1140–1145
30. Drebin JA, Link VC, Greene MI (1988) Monoclonal antibodies reactive with distinct domains of the neu oncogene-encoded p185 molecule exert synergistic anti-tumor effects in vivo. *Oncogene* 2(3):273–277
31. Kasprzyk PG, Song SU, Di Fiore PP, King CR (1992) Therapy of an animal model of human gastric cancer using a combination of anti-erbB-2 monoclonal antibodies. *Cancer Res* 52(10):2771–2776
32. Chames P, Van Regenmortel M, Weiss E, Baty D (2009) Therapeutic antibodies: successes, limitations and hopes for the future. *Br J Pharmacol* 157(2):220–233. <https://doi.org/10.1111/j.1476-5381.2009.00190.x>
33. De Groot AS, Martin W (2009) Reducing risk, improving outcomes: bioengineering less immunogenic protein therapeutics. *Clin Immunol* 131(2):189–201. <https://doi.org/10.1016/j.clim.2009.01.009>
34. Ahmadzadeh V, Farajnia S, Feizi MA, Nejad RA (2014) Antibody humanization methods for development of therapeutic applications. *Monoclon Antib Immunodiagn Immunother* 33(2):67–73. <https://doi.org/10.1089/mab.2013.0080>
35. Dennis MS (2010) CDR repair: A novel approach to antibody humanization. In: *Current trends in monoclonal antibody development and manufacturing*. Springer, New York, pp 9–28
36. Gonzales NR, Padlan EA, De Pascalis R, Schuck P, Schlom J, Kashmiri SV (2004) SDR grafting of a murine antibody using multiple human germline templates to minimize its immunogenicity. *Mol Immunol* 41(9):863–872. <https://doi.org/10.1016/j.molimm.2004.03.041>
37. Verhoeven M, Milstein C, Winter G (1988) Reshaping human antibodies: grafting an antilysozyme activity. *Science* 239(4847):1534–1536
38. Yoon SO, Lee TS, Kim SJ, Jang MH, Kang YJ, Park JH, Kim KS, Lee HS, Ryu CJ, Gonzales NR, Kashmiri SV, Lim SM, Choi CW, Hong HJ (2006) Construction, affinity maturation, and biological characterization of an anti-tumor-associated glycoprotein-72 humanized antibody. *J Biol Chem* 281(11):6985–6992. <https://doi.org/10.1074/jbc.M511165200>
39. Jones PT, Dear PH, Foote J, Neuberger MS, Winter G (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* 321(6069):522–525
40. Tiwari A, Khanna N, Acharya SK, Sinha S (2009) Humanization of high affinity anti-HBs antibody by using human consensus sequence and modification of selected minimal positional template and packing residues. *Vaccine* 27(17):2356–2366. <https://doi.org/10.1016/j.vaccine.2009.02.019>
41. Wedemayer GJ, Patten PA, Wang LH, Schultz PG, Stevens RC (1997) Structural insights into the evolution of an antibody combining site. *Science* 276(5319):1665–1669
42. Foote J, Winter G (1992) Antibody framework residues affecting the conformation of the hypervariable loops. *J Mol Biol* 224(2):487–499
43. An Z (2011) *Therapeutic monoclonal antibodies: from bench to clinic*. Wiley, New York
44. Kipriyanov SM, Le Gall F (2004) Generation and production of engineered antibodies. *Mol Biotechnol* 26(1):39–60. <https://doi.org/10.1385/MB:26:1:39>
45. Graves SS, Goshorn SC, Stone DM, Axworthy DB, Reno JM, Bottino B, Searle S, Henry A, Pedersen J, Rees AR (1999)

- Molecular modeling and preclinical evaluation of the humanized NR-LU-13 antibody. *Clin Cancer Res* 5(4):899–908
46. Li B, Wang H, Zhang D, Qian W, Hou S, Shi S, Zhao L, Kou G, Cao Z, Dai J (2007) Construction and characterization of a high-affinity humanized SM5-1 monoclonal antibody. *Biochem Biophys Res Commun* 357(4):951–956
 47. S-o L, Goldenberg DM, Dion AS, Pellegrini MC, Shevitz J, Shih LB, Hansen HJ (1995) Construction and characterization of a humanized, internalizing, B-cell (CD22)-specific, leukemia/lymphoma antibody, LL2. *Mol Immunol* 32(17):1413–1427
 48. Hu WG, Yin J, Chau D, Negrych LM, Cherwonogrodzky JW (2012) Humanization and characterization of an anti-ricin neutralization monoclonal antibody. *PLoS One* 7(9):e45595. <https://doi.org/10.1371/journal.pone.0045595>
 49. Kuroda D, Shirai H, Jacobson MP, Nakamura H (2012) Computer-aided antibody design. *Protein Eng Des Sel* 25(10):507–521. <https://doi.org/10.1093/protein/gzs024>
 50. Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, Rowland AM, Kotts C, Carver ME, Shepard HM (1992) Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc Natl Acad Sci U S A* 89(10):4285–4289
 51. Adams CW, Allison DE, Flagella K, Presta L, Clarke J, Dybdal N, McKeever K, Sliwkowski MX (2006) Humanization of a recombinant monoclonal antibody to produce a therapeutic HER dimerization inhibitor, pertuzumab. *Cancer Immunol Immunother* 55(6):717–727
 52. Hu S, Zhu Z, Li L, Chang L, Li W, Cheng L, Teng M, Liu J (2008) Epitope mapping and structural analysis of an anti-ErbB2 antibody A21: Molecular basis for tumor inhibitory mechanism. *Proteins* 70(3):938–949. <https://doi.org/10.1002/prot.21551>
 53. Ko BK, Lee SY, Lee YH, Hwang IS, Persson H, Rockberg J, Borrebaeck C, Park D, Kim KT, Uhlen M, Lee JS (2015) Combination of novel HER2-targeting antibody 1E11 with trastuzumab shows synergistic antitumor activity in HER2-positive gastric cancer. *Mol Oncol* 9(2):398–408. <https://doi.org/10.1016/j.molonc.2014.09.007>
 54. Sherr CJ, Roberts JM (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* 13(12):1501–1512
 55. Le XF, Claret FX, Lammayot A, Tian L, Deshpande D, LaPushin R, Tari AM, Bast RC Jr (2003) The role of cyclin-dependent kinase inhibitor p27Kip1 in anti-HER2 antibody-induced G1 cell cycle arrest and tumor growth inhibition. *J Biol Chem* 278(26):23441–23450. <https://doi.org/10.1074/jbc.M300848200>
 56. Valabrega G, Montemurro F, Aglietta M (2007) Trastuzumab: mechanism of action, resistance and future perspectives in HER2-overexpressing breast cancer. *Ann Oncol* 18(6):977–984. <https://doi.org/10.1093/annonc/mdl475>
 57. Li R, Hu S, Chang Y, Zhang Z, Zha Z, Huang H, Shen G, Liu J, Song L, Wei W (2016) Development and Characterization of a Humanized Anti-HER2 Antibody HuA21 with Potent Anti-Tumor Properties in Breast Cancer Cells. *Int J Mol Sci* 17(4):563. <https://doi.org/10.3390/ijms17040563>
 58. Nahta R, Hung MC, Esteva FJ (2004) The HER-2-targeting antibodies trastuzumab and pertuzumab synergistically inhibit the survival of breast cancer cells. *Cancer Res* 64(7):2343–2346