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Development and characterization of a fully functional small anti-HER2 antibody

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The penetrating of monoclonal antibodies (mAbs) into solid tumor may be hampered by their large size. The antibody mimetics, composed of two complementarity-determining regions (CDRs) through a cognate framework region (FR), have been demonstrated to have the capacity to penetrate tumors superior to its parental intact IgG. In this study, we used CDR and FR sequences from the humanized anti-HER2 monoclonal antibody trastuzumab to design four antibody mimetics. Then these antibody mimetics were fused to human IgG Fc to generate mimetics-Fc small antibodies. One of the four mimetics-Fc antibodies binds well to HER2-overexpressing SK-BR3 cells and effectively inhibits the binding of trastuzumab. This mimetics-Fc, denoted as HMTI-Fc, was shown to be effective in mediating antibody-dependent cellular cytotoxicity and exhibit an antiproliferative effect in SK-BR3 cells. To our knowledge, the HMTI-Fc antibody shown here is the smallest fully functional antibody and may have a potential for treatment of cancer. [BMB reports 2009; 42(10): 636-641]

INTRODUCTION

Although over 85% of human cancers are solid tumors, only 25% of the 8 monoclonal antibodies (mAbs) approved for cancer therapy are directed at solid tumor surface antigens (1). This shortfall may be due to barriers to achieving adequate exposure in solid tumors. Solid tumors are characterized by features such as heterogeneous vessel distribution, increasing density of extracellular matrix and tighter collagen organization, which are severe barriers for mAbs diffusion in solid tumors (2). The limited ability of native antibodies to penetrate solid cancers has stimulated the search for smaller alternatives, as a major determinant of speed of mAbs diffusion through tumors is molecular size (3). The rate of diffusion is inversely proportional to the molecular radius, or approximately to the cube root of molecular weight. For example, single-chain antibody (scFv) diffuses approximately 6 times faster than IgG, due to their smaller size and other factors (4).

Recent design variations of engineered antibodies have included reduction in size to Fab', scFv or dissection into minimal binding fragments such as V_H domains (5, 6). These antibody fragments have significantly diminished the molecular weight of parent monoclonal antibody. Dramatically, Qiu and colleagues have further whittled bulky IgG antibodies to small antibody mimetics of ~3 kDa, about 1/50 of their normal size (7). These antibody mimetics, which were composed of two complementarity-determining regions (CDRs), V_HCDR1 and V_LCDR3, through a cognate framework region (V_HFR2), retain the antigen recognition of their parent molecules, but have a superior capacity to penetrate tumors. These antibody mimetics are the smallest known antigen-binding fragments of antibodies so far (8).

Antibody has six CDRs residues all of which are more or less involved in antigen recognition. Whereas antibody mimetics composed of CDRs represent a particularly interesting target, the mimetics design remains a big problem (9, 10). Qiu and colleagues proposed four guidelines for mimetic designing (7). First, mimetic should contain at least two antigen-binding sites: one from the V_H and the other from the V_L domain. Second, the CDR3 loop is an essential component of the mimetic. Third, the CDR3 loop should be complemented by either the CDR1 or CDR2 loop of the other variable domain. Fourth, the C-terminus of the selected CDR1 or CDR2 loop and the N-terminus of the selected CDR3 loop should be joined with a framework region selected from either the V_H or the V_L to approximate the linkage of CDR in the parent molecule. Referring to the above guidelines, we used CDR and framework region sequences from trastuzumab, the humanized anti-HER2 monoclonal antibody, to design four small antibody mimetics comprising two interacting V_H- and V_L-derived CDRs. HER2 (erbB2/neu) gene encodes an epidermal growth factor receptor (EGFR)-related ty-

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rosine kinase that is overexpressed in 20-25% of invasive breast cancers (11, 12). HER2 has become an important therapeutic target in breast cancer for it is associated with an aggressive tumor phenotype and reduced survival rate (13, 14). Then these small antibody mimetics were fused to human IgG Fc fragment to generate mimetics-Fc antibodies. The results showed that one of the four minetics-Fc antibodies, denoted as HMTI-Fc, exhibited a significant antiproliferative effect and had the capacity to induce antibody-dependent cellular cytotoxicity (ADCC) against HER2-overxpressing tumor cells. As a small fully functional antibody, HMTI-Fc is expected to have improved penetration properties in solid tumor with respect to full-size IgG.

RESULTS

Construction and characterization of mimetics-Fc antibodies

The mimetics-Fc antibodies were generated by fusing the small antibody mimetics with a human IgG Fc domain. The purity and the molecular weight of the purified mimetics-Fc antibodies were determined by SDS-PAGE (Supplementary Fig. 1A). The mimetics-Fc antibody showed a molecular weight of 60 kDa under non-reducing conditions. Under reducing conditions, it migrated with the expected molecular size of about 30 kDa, indicating that the fusion protein was expressed as a disulphide linked dimer. Western blot analysis also demonstrated that the purified mimetics-Fc antibody had a molecular mass of about 60 kDa (Supplementary Fig. 1B). Compared with the intact IgG molecule (~150 kDa), the mimetics-Fc antibodies had a significantly reduced size.

In vitro binding activity and specificity of mimetics-Fc antibodies

The binding of mimetics-Fc antibodies to HER2-overexpressing SK-BR3 cells was measured by flow cytometry. As shown in Fig. 1, HHMTI-Fc bound well to SK-BR3 cells, though its binding activity was weaker than that of the parent intact IgG, trastuzumab. HMTII-Fc also retained some binding activity. However, HMTIII-Fc and HMTIV-Fc totally lost the binding capacity to SK-BR3 cells. Competitive binding assays further indicated that HMTI-Fc were able to effectively compete with trastuzumab for binding to SK-BR3 cells (Fig. 2). As shown in Supplementary Table 1, the avidity of HMTI-Fc was much weaker compared with that of the parent antibody ($P < 0.01$) and reduced approximately 10-fold. The data demonstrated that this small antibody could retain the binding specificity of the parental antibody though their molecular mass was one-third of the normal IgG molecule.

Biological effects of mimetics-Fc antibodies on HER2-overexpressing breast tumor cells

The effect of HMTI-Fc on tumor cell growth was assessed by measuring the survival of SK-BR3 cells treated with increasing concentrations of HMTI-Fc. As shown in Fig. 3A, HMTI-Fc inhibited the growth of SK-BR3 cells in a dose-dependent fashion, though its antiproliferative activity was lower than that observed for trastuzumab ($P < 0.01$, Supplementary Table 2). Both of the two antibodies had no effect on the growth of HER2-negative MCF-7 cells (Fig. 3B).

To determine the capacity of HMTI-Fc to trigger ADCC, SK-BR3 and MCF-7 cells were incubated for 4 h with increasing amounts of effector PBMC in the absence or presence of 3 μg/ml HMTI-Fc. As shown in Fig. 4, HMTI-Fc effectively induced ADCC activity against HER2-positive SK-BR3 target cells in the presence of PBMC. The extent of lysis reached 20% of treated cells at a ratio of 100 : 1 (effector to target cells), whereas trastuzumab, used as a positive control, induced about 60% lysis at a ratio of 100 : 1. At the ratio of 50 : 1 and 100 : 1, HMTI-Fc induced significantly higher cell lysis than HMTII-Fc ($P < 0.05$) and negative control ($P < 0.05$), whereas

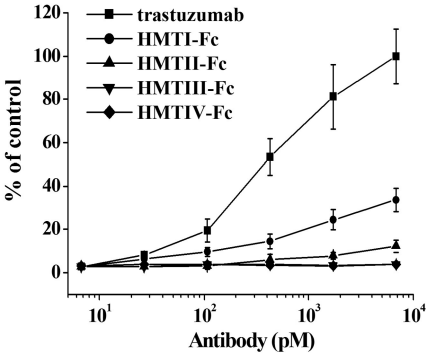

Fig. 1. Antigen binding assays for mimetics-Fc antibodies. SK-BR3 cells were incubated with a series concentration of the antibodies for 45 min at 4°C. Cells were washed and incubated with FITC labeled goat anti-human IgG for 45 min at 4°C. Cells were then washed and analyzed by FCM. The control is the mean fluorescence intensity of trastuzumab at the highest concentration (6830 pM). All data expressed as % of control and as the mean ± SD (n = 3).

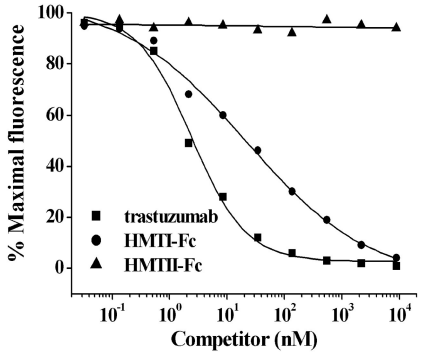

Fig. 2. Binding of FITC-trastuzumab to human SK-BR3 cells in the presence of increasing concentrations of the antibodies. SK-BR3 cells were incubated with 1 μg/ml FITC-trastuzumab and increasing concentrations of competing antibodies for 45 min at 4°C. The cells were then washed and analyzed by FCM. Maximal fluorescence means the mean fluorescence intensity obtained in the absence of competitor antibodies. All data were expressed as the mean of triplicate samples.

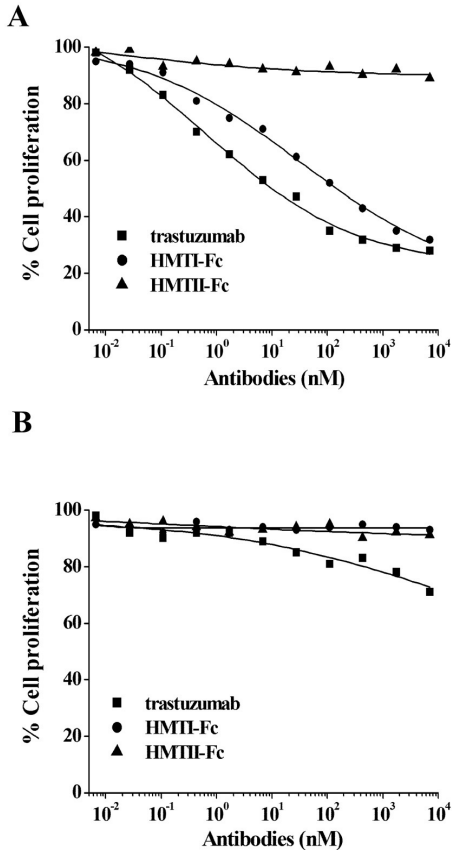

Fig. 3. Cell growth inhibition assays. SK-BR3 (A) and MCF-7 cells (B) at a density of 1.5×10^4 per well were seeded in 96-well plates overnight. A series concentration of antibodies was added to the cells and incubated for 24 h. Then cell viability was determined by Cell Titer 96 non-radioactive cell proliferation assay kit. All data were expressed as the mean of triplicate samples.

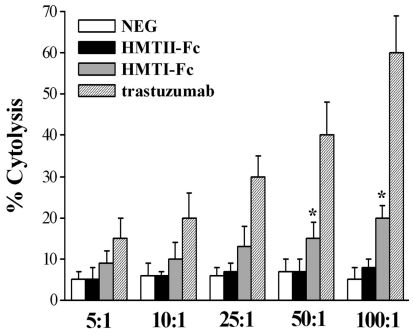

Fig. 4. ADCC tests. ADCC activities of antibodies were measured by lactate dehydrogenase (LDH)-releasing assay using the CytoTox 96 non-Radioactive Cytotoxicity Assay kit. Percentage of specific lysis was calculated according to the following formula: % lysis = (experimental release - spontaneous release)/(maximum release - spontaneous release) × 100. *P < 0.05. All data were expressed as the mean ± SD (n = 3).

cell lysis was not induced by HMTII-Fc and considered as nearly background. No effect was detectable in parallel assays carried out with HER2-negative MCF-7 cells (data not shown). These results demonstrate the specificity of the HMTI-Fc-dependent cell-mediated cytolytic activity, clearly based on both binding abilities of the mimetics-Fc antibody: (I) to the cognate receptor with its antigen binding sites; (II) to natural killer cells with its Fc effector domain.

DISCUSSION

Monoclonal antibodies have become the protein therapeutics of choice for targeting tumor (1). Currently, eight mAbs have been approved for cancer therapy. However, only trastuzumab, cetuximab and bevacizumab are approved to treat solid tumors and actually bevacizumab is directed at a soluble ligand (vascular endothelial growth factor), not at a surface protein within solid tumors. Full-length antibodies, especially the highest-affinity binders, do not generally penetrate far into solid tumors (15, 16). Such a dilemma reflects that there is an urgent demand in improving the penetration of mAbs in solid tumors (3).

Reducing the size of mAbs represents a promising way to improve the penetration of mAbs (7, 8). Recent design variations of engineered antibodies have included reduction in size to single-chain antibody, dissection into minimal binding fragments such as V_H domains, and rebuilding of scFvs into multivalent high avidity oligomeric scFvs (5). These recombinant antibody fragments, such as scFvs, whose activities depend on the correct folding of the proteins, are not very convenient in expression and purification (15). Antibodies have six CDRs (three in each light and heavy chain), which determine their binding affinity and specificity. On each chain, the CDRs are interspersed with four framework regions (FRs) that maintain the CDRs in their proper orientations. Small antibody mimetics composed of various CDRs combination with or without FRs exhibit weak binding affinity but retain antigen-specificity (7, 10). It has been proposed that two CDRs alone (one from the heavy chain and one from the light chain) might retain antigen specificity if separated by an FR that allows them to assume a conformation similar to that of the parental antibody after antigen binding (8).

In the present study, we designed four small antibody mimetics of anti-HER2 humanized antibody trastuzumab and fused them to the human IgG Fc, yielding fully functional antibodies (minetics-Fc). One of the four mimetics-Fc antibodies, HMTI-Fc, was shown to bind well to HER2-positive cells, though its affinity was reduced compared with the full size parental antibody. The three dimensional structure of HMT-I was built by homology modeling and shown in ribbon representation (Supplementary Fig. 2). V_LFR2, which links V_LCDRl and V_HCDR3 in the native Fab, plays a determinant role to connect the V_LCDRl and V_HCDR3 and keep them in a "quasi-physiological" binding-site surface (Supplementary Fig. 2), which is in consistent with the results reported by Qiu *et al* (7). Previous stud-

ies found that the lowest-affinity scFv had the most uniform distribution throughout the tumor, whereas the highest affinity scFv was found mainly in the perivascular region of the tumor (16). To avoid such a "binding-site barrier", we have retained weakened affinity instead of enhanced affinity of parental antibody against targets.

The Fc region of the antibody directly participates in recruiting immune cells in ADCC (8). We fused Fc region to the small mimetics antibody HMT-I to produce HMTI-Fc, aiming to triggering its ADCC function. Our results showed that HMTI-Fc effectively mediated ADCC against HER2-positive breast cancer cells. The *in vitro* antitumor activity of HMTI-Fc was also demonstrated by the growth inhibition assay.

In summary, the mimetics-Fc antibody HMTI-Fc, which has moderate binding affinity and antitumor activity, is the smallest fully functional antibody so far. The data shown here suggest that this small antibody may have the potential as a novel antitumor agent in solid tumor.

MATERIALS AND METHODS

Materials

Two human breast cancer cell lines, SK-BR3 and MCF-7, and the Chinese hamster ovary (CHO)-K1 cell line were obtained from the American Type Culture Collection (ATCC). The anti-HER2 humanized antibody (trastuzumab) was purchased from Roche Ltd. Trastuzumab was labeled with FITC to produce FITC-trastuzumab. FITC-goat anti-human IgG and horseradish peroxidase (HRP)-conjugated goat anti-human IgG were purchased from Zymed (San Francisco, CA).

Rational design of the mimetics-Fc antibody

Referring to the above guidelines proposed by Qiu *et al*. (7), we used CDR and framework region sequences from trastuzumab to design four small antibody mimetics comprising two interacting V_H- and V_L-derived CDRs, denoted as HMT-I, HMT-II, HMT-III and HMT-IV. The heavy and light chain variable region genes of trastuzumab (17) were synthesized by Shanghai Sangon Biological Engineering Technology Company (Shanghai, China). The following primers were used to generate the small antibody mimetics using trastuzumab variable gene as template: for HMT-I (LCDR1-LFR2-HCDR3), the primers were 5′-AAGCTT CACCATGGGATTCAGCAGGATCTTTCTCTTCCTCCTGTCAG TAACTACAGGTGTCCACTCCCGTGCCAGTCAGGATGT-3′ and 5′-GCTAGCGTAGTCCATAGCATAGAAGCCGTCCCCTC CCCAGTAAATCAGTAGTTTCGGAG-3′; for HMT-II (HCDR1-HFR2-LCDR3), the primers were 5′-AAGCTTCACCATGGGAT TCAGCAGGATCTTTCTCTTCCTCCTGTCAGTAACTACAGGT GTCCACTCCGACACCTATATACACTG-3′ and 5′-GCTAGCCG TGGGAGGAGTAGTATAATGTTGCTGTGCAACCCATTCCAG GCCCTTA-3′; for HMT-III (HCDR2-HFR3-LCDR3), the primers were 5′-AAGCTTCACCATGGGATTCAGCAGGATCTTTCTCTT CCTCCTGTCAGTAACTACAGGTGTCCACTCCAAGGATTTAT CCTACGAA-3′ and 5′-GCTAGCGTGGGAGGAGTAGTATAATG TTGCTGACTAGAACAATAATAGACG-3′; for HMT-IV (LCDR2-LFR3-HCDR3), the primers were 5′-AAGCTTCACCATGGGAT TCAGCAGGATCTTTCTCTTCCTCCTGTCAGTAACTACAGGT GTCCACTCCCTCGGCATCCTTCCTCTA-3′ and 5′-GCTAGCT AGTCCATAGCATAGAAGCCGTCCCCTCCCCATCAGTAATA AGTTGCGAAG-3′. The restriction endonuclease recognition sites (*Hind*III and *Nhe*I) were underlined. The resultant antibody mimetics genes were genetically in frame fused to the human IgG Fc gene. Then the mimetics-Fc fusion genes were cloned into the pcDNA3.1 (+) vector (Invitrogen, Carlsbad, CA) to yield the expression vectors.

Expression and purification of mimetics-Fc antibodies

The expression vectors for mimetics-Fc antibodies were transfected into Chinese hamster ovary (CHO)-K1 cells according the manufacture's protocols. In brief, cells grown in RPMI containing 10% FCS at 80-90% confluency were transfected with 0.8 μg of expression vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Stable transfectants producing the highest amount of recombinant antibodies were selected in the presence of G418 (Sigma, St. Louis, MO) and grown in serum-free medium. Recombinant antibodies were purified by Protein A affinity chromatography as described previously (18).

SDS-PAGE and Western blot

The purified mimetics-Fc antibodies were analyzed by SDS-PAGE and quantified by MicroBCA protein assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions as described previously (19). For Western blot analysis, purified mimetics-Fc antibodies were electrophoresed on a 10% SDS-PAGE under non-reducing conditions and then electrophoretically transferred onto polyvinylidine difluoride (PVDF) membranes (Amersham Biosciences). After blockade of its free protein-binding sites, the PVDF membranes were treated with HRP-conjugated goat anti-human IgG. Finally, the bands were detected by enhanced chemiluminescence reagents (Amersham Biosciences).

Flow cytometry

To examine the binding activity of mimetics-Fc antibodies, 1×10^5 SK-BR3 cells were incubated with increasing concentrations of purified mimetics-Fc antibodies for 45 min at 4°C. After washed twice with PBS, the cells were incubated with FITC-goat anti-human IgG (Zymed). To examine the specificity of mimetics-Fc antibodies, competitive binding assay was performed as follows. 1×10^5 SK-BR3 cells were incubated with 1 μg/ml FITC-trastuzumab and increasing concentrations of purified competing antibodies for 45 min at 4°C. Thereafter, the cells were washed and analyzed by FACScan flow cytometer (Becton-Dickinson, San Jose, CA). The IC_{50} values of competitors were calculated using a four parameter algorithm.

Cell growth inhibition assays

Breast cancer cells (SK-BR3 and MCF-7 cells) at a density of 1.5×10^4 per well were seeded in 96-well plates in a 5% CO_2 in-

cubator at 37°C. After an overnight incubation, different concentrations of mimetics-Fc antibodies were added and incubated for another 24 h. Then cell viability was determined by Cell Titer 96 non-radioactive cell proliferation assay kit according to the manufacturer's protocol (Promega, Madison, WI) as described previously (20). Briefly, 20 μl MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt)/phenazine methosulfate solution was added into each well. After incubation for 2 h at 37°C, the absorbance was measured at 490 nm using BIO-TEK ELx800 Universal Microplate Reader (BioTek Instruments Inc., Winooski, VT). The IC_{50} values of competitors were calculated using a four parameter algorithm.

ADCC test

ADCC activities of mimetics-Fc antibodies were measured by lactate dehydrogenase (LDH)-releasing assay using the CytoTox 96 non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI) according to the manufacturer's instructions as described previously (21). Briefly, the cells were incubated with the small antibody mimetics for 1 h in phenol red-free DMEM culture medium in a 5% CO_2 incubator at 37°C, followed by the addition of human peripheral blood mononuclear cells (PBMC) as effector cells (effector to target, 5 : 1; 10 : 1; 25 : 1; 50 : 1; 100 : 1 for ADCC assay). Controls included target cells incubated in the absence of effector. After an additional incubation for 4 h at 37°C, the cell lysis was determined by measuring the amount of LDH released into the culture supernatant. Maximum LDH release was determined by lysis in 0.2% TritonX-100. Percentage of specific lysis was calculated according to the following formula: % lysis = (experimental release - spontaneous release)/(maximum release - spontaneous release) × 100.

Molecular modeling

The three-dimensional structure of HMT-I were built by homology modeling based on the crystal structure of trastuzumab using Insight II/Homology Software Package (Accelrys Inc., San Diego, CA). In order to minimize steric clashes and ensure correct bond lengths and angles after modeling, the side chains of the model structure were subjected to 5, 000 steps of energy minimization using steepest descent method and 10, 000 steps of energy minimization using conjugate gradient method, while the α carbon atoms of the main chain were held fixed in position (the convergence criterion is 0.5 and 0.01 KJ/mol respectively).

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REFERENCES

1. Adams, G. P. and Weiner, L. M. (2005) Monoclonal antibody therapy of cancer. *Nat. Biotechnol.* **23**, 1147-1157.
2. Jang, S. H., Wientjes, M. G., Lu, D. and Au, J. L. (2003) Drug delivery and transport to solid tumors. *Pharm. Res.* **20**, 1337-1350.
3. Beckman, R. A., Weiner, L. M. and Davis, H. M. (2007) Antibody constructs in cancer therapy: protein engineering strategies to improve exposure in solid tumors. *Cancer* **109**, 170-179.
4. Graff, C. P. and Wittrup, K. D. (2003) Theoretical analysis of antibody targeting of tumor spheroids: importance of dosage for penetration, and affinity for retention. *Cancer Res.* **63**, 1288-1296.
5. Kortt, A. A., Dolezal, O., Power, B. E. and Hudson, P. J. (2001) Dimeric and trimeric antibodies: high avidity scFvs for cancer targeting. *Biomol. Eng.* **18**, 95-108.
6. Holt, L. J., Herring, C., Jespers, L. S., Woolven, B. P. and Tomlinson, I. M. (2003) Domain antibodies: proteins for therapy. *Trends Biotechnol.* **21**, 484-490.
7. Qiu, X. Q., Wang, H., Cai, B., Wang, L. L. and Yue, S. T. (2007) Small antibody mimetics comprising two complementarity-determining regions and a framework region for tumor targeting. *Nat. Biotechnol.* **25**, 921-929.
8. Ladner, R. C. (2007) Antibodies cut down to size. *Nat. Biotechnol.* **25**, 875-877.
9. Casset, F., Roux, F., Mouchet, P., Bes, C., Chardes, T., Granier, C., Mani, J. C., Pugnière, M., Laune, D., Pau, B., Kaczorek, M., Lahana, R. and Rees, A. (2003) A peptide mimetic of an anti-CD4 monoclonal antibody by rational design. *Biochem. Biophys. Res. Commun.* **307**, 198-205.
10. Qin, W., Feng, J., Li, Y., Lin, Z. and Shen, B. (2006) Fusion protein of CDR mimetic peptide with Fc inhibit TNF-a induced cytotoxicity. *Mol. Immunol.* **43**, 660-666.
11. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A. and McGuire, W. L. (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**, 177-182.
12. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E. (1989) Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **244**, 707-712.
13. Press, M. F., Bernstein, L., Thomas, P. A., Meisner, L. F., Zhou, J. Y., Ma, Y. (1997) HER-2/neu gene amplification characterized by fluorescence in situ hybridization: poor prognosis in node-negative breast carcinomas. *J. Clin. Oncol.* **15**, 2894-2904.
14. Press, M. F., Slamon, D. J., Flom, K. J., Park, J., Zhou, J. Y. and Bernstein, L. (2002) Evaluation of HER-2/neu gene amplification and overexpression: comparison of frequently used assay methods in a molecularly characterized cohort of breast cancer specimens. *J. Clin. Oncol.* **20**, 3095-3105.
15. Jurado, P., de Lorenzo, V. and Fernández, L. A. (2006) Thioredoxin fusions increase folding of single chain Fv antibodies in the cytoplasm of Escherichia coli: evidence that chaperone activity is the prime effect of thioredoxin. *J. Mol. Biol.* **357**, 49-61.
16. Adams, G. P., Schier, R., McCall, A. M., Simmons, H. H., Horak, E. M., Alpaugh, R. K., Marks, J. D. and Weiner, L. M. (2001) High affinity restricts the localization and tumor penetration of single-chain Fv antibody molecules. *Cancer Res.* **61**, 4750-4755.

17. Carter, P., Presta, L., Gorman, C. M., Ridgway, J. B., Henner, D., Wong, W. L., Rowland, A. M., Kotts, C., Carver, M. E. and Shepard, H. M. (1992) Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4285-4289.
18. Li, B., Wang, H., Da, i. J., Ji, J., Qian, W., Zhang, D., Hou, S. and Guo, Y. (2005) Construction and characterization of a humanized anti-human CD3 monoclonal antibody 12F6 with effective immunoregulation functions. *Immunology* **116**, 487-498.
19. Gao, J., Kou, G., Chen, H., Wang, H., Li, B., Lu, Y., Zhang, D., Wang, S., Hou, S., Qian, W., Dai, J., Zhao, J., Zhong, Y. and Guo, Y. (2008) Treatment of hepatocellular carcinoma in mice with PE38KDEL type I mutant-loaded poly(lactic-co-glycolic acid) nanoparticles conjugated with humanized SM5-1 F(ab') fragments. *Mol. Cancer Ther.* **7**, 3399-3407.
20. Kou, G., Gao, J., Wang, H., Chen, H., Li, B., Zhang, D., Wang, S., Hou, S., Qian, W., Dai, J., Zhong, Y. and Guo, Y. (2007) Preparation and characterization of paclitaxel-loaded PLGA nanoparticles coated with cationic SM5-1 single-chain antibody. *J. Biochem. Mol. Biol.* **40**, 731-739.
21. Li, B., Shi, S., Qian, W., Zhao, L., Zhang, D., Hou, S., Zheng, L., Dai, J., Zhao, J., Wang, H. and Guo, Y. (2008) Development of novel tetravalent anti-CD20 antibodies with potent antitumor activity. *Cancer Res.* **68**, 2400-2408.