Superior antitumor activity of a novel bispecific antibody co-targeting human epidermal growth factor receptor 2 and type I insulin-like growth factor receptor

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**Running title:** Bispecific antibody co-targeting HER2 and IGF-IR

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Abstract

The humanized anti-HER2 monoclonal antibody (mAb) Herceptin (trastuzumab) effectively inhibits HER2-positive breast tumors. However, many patients responding to treatment often develop resistance. Crosstalk between IGF-IR and HER2 and elevated IGF-IR signaling have been implicated in tumor cell resistance to Herceptin therapy. Previously, we reported that the anti-IGF-IR mAb m590 inhibits proliferation and migration of breast cancer MCF-7 cells \textit{in vitro}. Here, we generated a “knobs-into-holes” bispecific antibody (Bi-Ab) against HER2 and IGF-IR by engineering Herceptin and m590. We compared the effects of Bi-Ab treatment \textit{in vitro} and in SKOV-3 HER2- and IGF-IR-overexpressing cancer xenograft mouse model with those of m590 and Herceptin treatment alone or in combination (Comb). Bi-Ab effectively inhibited proliferation of HER2- and IGF-IR-overexpressing ovarian cancer SKOV-3 cells \textit{in vitro} by ablating receptor phosphorylation and downstream PI3K/Akt and MAPK signaling. Bi-Ab more effectively inhibited cancer growth in SKOV-3 HER2- and IGF-IR-overexpressing cancer xenograft mouse model than m590 and Herceptin alone or in combination. Mice bearing SKOV-3 HER2- and IGF-IR-overexpressing xenografts showed extensive and sustainable tumor regression when treated with Bi-Ab. Our results suggest that Bi-Ab has superior antitumor activity compared with monospecific antibodies, and co-targeting HER2 and IGF-IR may be clinically beneficial in minimizing the acquired resistance to Herceptin therapy.
**Introduction**

Human Epidermal Growth Factor Receptor 2 (HER2), encoded by the *ErbB2* gene, is a member of the epidermal growth factor receptor (EGFR/ErbB) family (1). HER2 is structurally similar to other EGFR family members, including HER1 (EGFR, ErbB1), HER3 (ErbB3), and HER4 (ErbB4), and also acts as a receptor tyrosine kinase. Homodimerization of HER1 and HER4 upon ligand binding activates intrinsic, intracellular protein-tyrosine kinase activity, resulting in receptor autophosphorylation and downstream signaling, including signaling pathways such as, the phosphatidylinositol 3-kinase (PI3K), the c-Jun NH(2)-terminal kinase (JNK), and the mitogen-activated protein kinase (MAPK), which promote DNA synthesis, cell proliferation and inhibition of cell apoptosis. HER3 does not have a tyrosine kinase domain, so it transfers signals upon ligand binding through heterodimerization with other EGFR family members that have kinase activity. Unlike HER1, HER3 and HER4, HER2 is unable to bind ligands and form homodimers. However, HER2 possesses tyrosine kinase activity, and appears to be the major signaling partner for EGFR family members through the formation of heteromeric complexes (2). Heterodimerization between two EGFR family members requires ligand binding (3, 4), but the crystal structure of a truncated HER2 ectodomain suggests that HER2 is constitutively in the activated conformation and readily interacts with HER3 mostly and other EGFR family members (5). Overexpression of HER2 promotes ligand-independent formation of a HER2/HER3 receptor complex, a major oncogenic driver in HER2-overexpressing breast tumor cells (6). Cleavage of HER2 by the extracellular protease, ADAM10, produces the HER2 ectodomain and a truncated, constitutively active HER2 receptor (p95HER2) shown to drive carcinogenesis (7). HER2 overexpression is associated with strong activation of
the PI3K pathway, which stimulates cell proliferation by activating the protein kinase Akt and down-regulating the cyclin-dependent kinase (CDK) inhibitor, p27 (8). HER2 can also activate the MAPK pathway via interaction with SHC and GRB2 adaptor proteins (9). Overexpression of HER2 was found in breast and ovarian cancers, and associated with cancer metastasis (10-12), poor clinical outcome and decreased survival rate (13-15).

Type I Insulin-like Growth Factor Receptor (IGF-IR) is a tyrosine kinase receptor composed of 2 α subunits and 2 β subunits. Upon binding to either of the two ligands, Insulin-like growth factor I (IGF-I) or IGF-II, the extracellular domain of the α chains induces tyrosine autophosphorylation of the β chains in the cytoplasm. This activates the kinase activity of IGF-IR, and triggers downstream signaling via the PI3K/Akt and Ras/MAPK pathways, resulting in increased cell survival and cell proliferation (16, 17). Elevated IGF-IR is found in many tumor malignancies, including breast, prostate and lung cancers (18, 19). Additionally, overexpression of IGF-IR has been associated with disease progression and cancer metastasis (20, 21).

HER2 is a widely used diagnostic marker and validated target for therapy. The humanized anti-HER2 mAb Herceptin (trastuzumab) has been effective in treating HER2-overexpressing breast cancers (22, 23). Binding of Herceptin to HER2 causes internalization and degradation of the receptor in SKBR3 and MDA453 cells (24). Herceptin binds to domain IV of the extracellular segment of HER2, leading to disruption of HER2/HER3 dimerization and ablation of downstream PI3K/Akt signalling (6). Herceptin can also inhibit cleavage of HER2 ectodomain in breast cancer cells, thus block the generation of a constitutively active truncated receptor (p95HER2) (7, 25, 26). In addition, Fc-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) may partially contribute to the antitumor activity of Herceptin in vivo (27).
Only 25-30% of breast cancer patients overexpress HER2, and patients treated with Herceptin can develop resistance as the disease progresses. Various mechanisms may account for this resistance, which likely involve the PI3K/Akt pathway, including elevated HER2-associated receptors and other receptors (28, 29), cross activation between HER2 and other receptors (30-32), blockage of Herceptin by membrane-associated glycoproteins such as mucin-4, removal of the Herceptin epitope by cleavage or loss of HER2 expression, and increased HER2 expression. Accumulating evidence shows that crosstalk between HER2 and IGF-IR, including receptor heterodimerization and transactivation, and elevated IGF-IR signalling are associated with Herceptin resistance (31, 33-35). Overexpression of IGF-IR in HER2-overexpressing breast cancer cell lines results in Herceptin resistance in vitro (36). Inhibition of IGF-1R activity enhances the response to Herceptin in HER-2-positive breast cancer cells (37). A phase II clinical trial of HER2-positive breast cancer patients revealed that overexpression of IGF-IR in the primary tumor was associated with resistance to Herceptin (38). Therefore, co-targeting IGF-IR and HER2 may be clinically beneficial in minimizing the acquired resistance to Herceptin therapy.

We previously described a human/mouse chimeric mAb m590 that specifically bound with high affinity to IGF-IR and blocked the binding of IGF-I and IGF-II to IGF-IR. This inhibited ligand-induced phosphorylation of IGF-IR in breast cancer MCF-7 cells (39). In this study, we generated a bispecific antibody (Bi-Ab) by engineering the m590 and Herceptin, and tested Bi-Ab in vitro and in SKOV-3 HER2- and IGF-IR-overexpressing xenograft mouse model. We found that co-targeting HER2 and IGF-IR with Bi-Ab was more effective than targeting HER2 or IGF-IR alone with the monospecific antibodies or cotargeting HER2 and IGF-IR with a
combination (Comb) of the two monospecific antibodies in ablating tumor cell proliferation *in vitro* and *in vivo*.

**Materials and methods**

**Cell lines, antibodies and chemicals**

Breast cancer MCF-7 cells were cultured in DMEM medium (Invitrogen) supplemented with 10% heat-inactivated FBS and 1% penicillin / streptomycin (P/S). Ovarian cancer SKOV-3 cells were cultured in McCoy’s 5A medium (Hyclone) supplemented with 10% heat-inactivated FBS and 1% P/S. Both MCF-7 and SKOV-3 cell lines were obtained from National Cancer Institute, National Institutes of Health. No authentication was done by the authors. The following primary mAbs were purchased from Cell Signal Technology: rabbit anti-Phospho-AKT (Thr308) (C31E5E), rabbit anti-Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (D13.14.4E) XP, rabbit anti-Akt (pan) (11E7), rabbit anti-P44/42 MAPK (ERK1/2) (137F5), and rabbit anti-GAPDH mAb (14C10). The secondary antibodies were purchased from Jackson ImmunoResearch. Xenolight D-luciferin was purchased from Caliper Life Sciences.

**Expression and purification of recombinant IGF-IR and HER2 ectodomain**

The gene encoding the extracellular domain (ectodomain) of IGF-IR was amplified from the pBlueScript-IGF-IR construct P08069 (39), and subcloned into the pSecTag2C vector at the EcoR I and Not I sites. The gene encoding HER2 ectodomain was amplified from SKOV-3 cell line by reverse transcriptase PCR, and subsequently cloned into the pSecTag2A vector at the Xho I and Sfi I sites. Both constructs were confirmed by DNA sequencing. Recombinant
ectodomains of IGF-IR and HER2 were produced by transient transfection of 293T cells. Expression in transfectants was enhanced by the transduction of vaccinia virus vTF7-3 encoding bacteriophage T7 RNA polymerase. 72 h post transfection, culture supernatants were collected and His-tagged ectodomains purified by immobilized metal-affinity chromatography.

Construction of “knobs-into-holes” CH3 variants

The humanized Herceptin heavy chain variable region (VH) and light chain (LC) [DrugBank: Trastuzumab (DB00072) (BIOD00098, BTD00098)] genes were synthesized and cloned into the mammalian expression plasmid pDR12 containing human IgG1 heavy chain genomic DNA constant regions. Two mutations were introduced in the CH3 domains of pDR12-Herceptin (T366Y) and pDR12-m590 (Y407T) using a site-directed mutagenesis kit (Stratagene). The primers for the mutagenesis were: T366Y-F: 5'-CCAGGTCAGCCTGTACTGCCTGGTCAAAG-3', and T366Y-R: 5'-CTTTGACCAGGCAGTACAGGCTGACCTGG -3'; and Y407T-F: 5'-CTCCTTCTTCCTCACCAGCAAGCTCACC CG-3', and Y407T-R: 5'-CGGTGAGCTTGCTGGTGAGGAAGAAGGAG -3'. The mutations were confirmed by DNA sequencing. The resultant plasmids were designated as pDR12-Herceptin-366 and pDR12-m590-407, respectively.

Expression and purification of Herceptin and m590, and Bi-Ab

Herceptin and m590 were expressed by transient transfection of 293F cells (Invitrogen) with recombinant plasmids pDR12-Herceptin and pDR12-m590, respectively. Bi-Ab was expressed by transiently co-transfecting 293F cells with pDR12-Herceptin-366 and pDR12-m590-407
plasmid DNA. Recombinant antibodies were purified from the culture supernatants by Protein A (GE Healthcare) affinity purification.

**Indirect ELISA**

Recombinant ectodomains of IGF-IR or HER2 (2 μg/ml in both cases) were coated on 96-well high-binding ELISA plates at 4°C overnight. The plates were washed and blocked with 3% BSA in PBS at 37°C for 2 h. Two-fold serially diluted mAb Herceptin or m590 were added to the wells and the bound mAbs were detected by HRP conjugated anti-human Fc as secondary antibody and TMB substrate. Optical density at 450 nm (OD450nm) was measured after color development at RT for 30 min. In the case of Bi-Ab, recombinant IGF-IR ectodomains were coated on the plates. Following addition of 2-fold serially diluted antibodies and incubation at RT for 2 h, plates were washed and biotinylated HER2 ectodomain (2 μg/ml) was added to each well. Bound HER2 ectodomains were detected by HRP conjugated streptavidin and TMB substrate.

**Western blotting**

MCF-7 or SKOV-3 cells in complete medium were seeded in 6-well plates. When cells reached 70-80% confluence, they were incubated in serum-free medium overnight. Cells were treated with antibodies for 30 min, followed by addition of 1.5 nM IGF-I and further incubation for 30 min. Cells were then lysed and 10 μl of cell lysates from each sample was resolved by 12% SDS-PAGE. Once the proteins were transferred, PVDF membranes were blocked with 5% skim milk in PBS for 30 min, incubated with primary antibodies, and then secondary antibodies.
The membranes were extensively washed after each incubation step. The Western blot signal was detected by Western Bright ECL-HRP substrate (Advansta).

Flow cytometry

MCF-7 or SKOV-3 cells were detached using enzyme-free cell-disassociation buffer (Invitrogen), washed twice with PBS, and incubated at 4°C for 2 h with antibodies in fluorescence-activated cell sorting (FACS) buffer (1% FBS in PBS). Cell surface bound antibodies were detected using PE conjugated to anti-human Fc by incubation at 4°C for 1 h followed by washing twice with FACS buffer and fixation with 2% paraformaldehyde in FACS buffer. The stained cells were analyzed with a BD flow cytometer and FlowJo software.

ADCC assay

The flow cytometry-based ADCC assay has been described previously (40). Here, we used SKOV-3 cells as target cells and healthy human volunteers peripheral blood mononuclear cells (PBMCs) as effector cells at an E/T ratio of 25/1. Briefly, SKOV-3 cells were stained with PKH-67, then mixed with antibodies and PBMCs. Following 2 h of incubation, 7-AAD was added to the mixture. Following several washes, the samples were analyzed by FACS AriaIII flow cytometer using BD FACS Diva software. Percent cell death was determined by software analysis of four identifiable cell populations, live effector cells (no dye), dead effector cells (7-AAD positive), live target cells (PKH-67 positive) and dead target cells (PKH-67 and 7-AAD double positive). Percent ADCC was calculated as [(% experimental lysis - % spontaneous lysis) / (% maximum lysis - % spontaneous lysis)]x100, in which “% spontaneous lysis” referred to percent dead target cells mixed with effectors in the absence of antibodies, and “% maximum
lysis” referred to percent dead target cells mixed with effectors in the presence of 1% Triton X-100. “% experimental lysis” referred to percent dead target cells mixed with effectors in the presence of antibodies. The assay was performed in duplicate and repeated once. One representative set of data was shown in this report.

**Cell proliferation assay**

Antibodies were serially diluted in culture medium containing 2% FBS and mixed with equal volume of SKOV-3 cells containing 3 nM IGF-I. Cell/Ab mixtures were then plated onto 96-well cell culture plates with a final concentration of 2,000 cells per well and 1.5 nM IGF-I. The plates were incubated at 37°C with 5% CO₂ for 72 h, and cell proliferation level was detected by Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit (Promega).

**Generation of the luciferase-expressing SKOV-3 stable cell line**

293T cells were transiently co-transfected with recombinant plasmid encoding HIV-1 Gag and Polymerase (Gag-pol), Luc-expressing plasmid, and VSV backbone plasmid at a ratio of 2/2/1 (Gag-pol / Luc / VSV). 36-48 h post transfection(), culture supernatant containing Luc-lentivirus was collected, and equally mixed with fresh culture medium followed by addition of polybrene to a final concentration of 8 μg/ml. Nine milliliters of the mixture were added to SKOV-3 cells seeded in 100-mm dishes and incubated at 37°C for 6 h in 5% CO₂. Three milliliters of culture medium containing 8 μg/ml polybrene were then added to the dish. Following overnight incubation, infection medium was removed and cells cultured in fresh McCoy’s 5A medium containing 1 μg/ml puromycin. After 3-5 passages, limiting dilution was performed and single
cell clones were screened by luciferase assay. The single cell clone expressing the highest level of luciferase was expanded and titrated by imaging in a 96-well plate.

**Establishment of a breast tumor xenograft mouse model and the mouse study**

This study was approved by HKU Committee on Using Live Animals in Teaching and Research (CULATR # 2514-11). Nude BALB/c female mice, 4-6-weeks-old, were obtained from the Animal Centre of the University of Hong Kong. To establish a breast cancer xenograft mouse model, a pilot experiment was carried out by subcutaneously injecting different numbers of SKOV3-Luc cells into nude mice and imaging the mice at different time points. The optimal cell numbers that yielded sustainable and increasing luminescence intensity in the regions of interest (ROI) were determined.

The mouse study was carried out as follow: on day 0, SKOV-3-Luc cells were resuspended in plain McCoy’s 5A medium and 3 million of the cells were injected subcutaneously into each nude mouse. On day 1, KETAMINE/XYCAZINE/PBS at a ratio of 1/1.2/7.8 were mixed and 40 μl (2.5 μl/g body weight) of the anesthetic mixture was injected subcutaneously into each mouse. D-luciferin (100 μl) at a concentration of 30 mg/ml in DPBS (5 μl/g body weight) was then injected intra-peritoneally (i.p.) into each mouse (each mouse received 300 mg luciferin/kg body weight). About 15 min post injection, mice were imaged for luminescence intensity in the regions of interest (ROI) using Xenogen IVIS 100 *in vivo* imaging system. Mice were then randomized, so that each group of mice had approximately the same average luminescence intensity. Each group had 7 mice and a total of 4 groups were formed corresponding to Herceptin, m590, Bi-Ab, and Comb treatment conditions. Each antibody (100 μg) or antibody combination (100 μg total) were injected by i.p. into each mouse on days 1, 4, 6 and 8. Mouse imaging was
repeated on days 4, 6, 8, 11, 15, 25, and 35. A control group had 5 mice and were not treated with any antibodies post injection of tumor cells, but imaged at the same time with antibody treated groups.

Results

Bi-Ab co-targets IGF-IR and HER2

We used the “knobs-into-holes” approach to generate an anti-IGF-IR/anti-HER2 hybrid IgG (41-43). A “knob” mutant was created by replacing a Threonine with Tyrosine (T366Y) in the CH3 domain of Herceptin. A “hole” mutant was made by replacing a Tyrosine with Threonine (Y407T) in the CH3 domain of m590. Co-transfection of 293F cells with the “knob” and “hole” plasmids resulted in the production of stable heterodimers that exhibited bispecificity for both HER2 and IGF-IR (Fig. 1). The bispecific antibody (Bi-Ab) bound to recombinant IGF-IR and HER2 ectodomains (Fig. 1C) and to overexpressed, membrane-associated IGF-IR and HER2 on SKOV-3 cells (Fig. 1B and 1D). Compared to breast cancer MCF-7 cells used in our previous study, ovarian cancer SKOV-3 cells express a high level of HER2 and a modest level of IGF-IR (Fig. 1A). Similar to the two parental antibodies, m590 and Herceptin, Bi-Ab bound to SKOV-3 in a dose-dependent manner (Fig. 1D). Moreover, flow cytometry analysis revealed that Herceptin and Bi-Ab have a similar binding profile which shows two peaks in the histogram (Fig. 1B), suggesting that cell surface HER2 proteins may have multiple conformational or organizational states.
Bi-Ab inhibits receptor phosphorylation and down-regulates downstream PI3K/Akt and MAPK signaling

We previously reported that m590 blocked ligand-induced IGF-IR phosphorylation in breast cancer MCF-7 cells (39), and inhibited MCF-7 cell proliferation and migration (44). We extended these findings in this study by analyzing the downstream signaling of IGF-IR. We found that m590 inhibited ligand-induced phosphorylation of Akt and ERK in MCF-7 cells (Fig. 2A), and synergized with Herceptin in inhibiting ligand-induced Akt phosphorylation in MCF-7 cells (Fig. 2B). Next, we then compared the effects of Bi-Ab, m590 and Herceptin on phosphorylation of IGF-IR in SKOV-3 cells (Fig. 2C) and downstream signaling in MCF-7 (Fig. 2D) and SKOV-3 cells (Fig. 2E) in the presence of ligand (IGF-I) or absence of ligand (Fig. 2F). Both m590 and Bi-Ab inhibited phosphorylation of IGF-IR in SKOV-3 cells compared with non-specific human IgG control. Treatment with Herceptin or Comb did not result in decreased levels of phosphorylated IGF-IR in SKOV-3 cells (Fig. 2C). In MCF-7 cells, m590 and Bi-Ab, as well as Comb inhibited ligand-induced phosphorylation of Akt, but only Bi-Ab inhibited ligand-induced phosphorylation of ERK (Fig. 2D). Treatment with Herceptin slightly enhanced ligand-induced phosphorylation of Akt in MCF-7 cells (Fig. 2D, lane 2). None of the antibodies showed inhibitory effects on ligand-induced phosphorylation of Akt and ERK in SKOV3 cells at the antibody concentration (100 μg/ml) tested (Fig. 2E). When we compared the effects of Bi-Ab, m590 and Herceptin on phosphorylation of ERK in MCF-7 and SKOV-3 cells (Fig. 2F) in the absence of ligand, we found that in MCF-7 cells, both m590 and Bi-Ab inhibited ERK phosphorylation, and Comb treatment slightly reduced ERK phosphorylation (data not shown), but in SKOV-3 cells, Bi-Ab inhibited ERK phosphorylation (Fig. 2F), and this inhibition was dose-dependent (Fig. 2G). Herceptin did not have inhibitory effect on ERK phosphorylation in
both cell lines (data not shown and Fig. 2F). These results indicate that co-expression of both high levels of HER2 and IGF-IR in cancer cells raise the bars for antibodies to interfere with the receptor phosphorylation and downstream signaling, especially when the ligand (IGF-I) is present. Herceptin was ineffective in inhibiting phosphorylation of Akt and ERK in both MCF-7 and SKOV-3 cells in the presence or absence of IGF-I. Bi-Ab, m590 and Comb were equally effective in inhibiting phosphorylation of Akt and ERK in MCF-7 cells in the absence of ligand (data not shown) and in inhibiting ligand-induced Akt phosphorylation in MCF-7 cells (Fig. 2D), but Bi-Ab was more effective than m590 and Comb in inhibiting ligand-induced ERK phosphorylation in MCF-7 cells (Fig. 2D), and more effective than Comb in inhibiting ERK phosphorylation in SKOV-3 cells in the absence of ligand (Fig. 2F).

**Bi-Ab more effectively inhibits cancer cell proliferation than Herceptin and m590 in vitro, and preserves ADCC activity**

The attenuation of the PI3K/Akt and MAPK pathways by Bi-Ab prompted us to analyze the effects of Bi-Ab on cancer cell proliferation. Bi-Ab treatment effectively inhibited SKOV-3 cell proliferation in vitro (Fig. 3A). Notably, although Comb treatment showed enhanced inhibition of SKOV-3 proliferation compared with Herceptin treatment alone, both treatment conditions were significantly less potent than Bi-Ab, especially at high antibody concentrations (over 25 μg/ml) (Fig. 3A). M590 inhibited SKOV-3 cell proliferation, but its effect decreased sharply as m590 concentration decreased (Fig. 3A).

To investigate whether “knob” and “hole” mutations affected Fc-mediated effector function, we tested Bi-Ab for ADCC activity in a flow cytometry based assay using SKOV-3 as target cells and healthy human PBMCs as effector cells. The assay revealed that Bi-Ab had ADCC
activity comparable to or slightly higher than that of m590, Herceptin, and the combination of m590 and Herceptin (Comb) (Fig. 3B). Notably, percent ADCC with Bi-Ab was significantly higher than that with Comb at 1 μg/ml (P-value < 0.05). These results suggest Bi-Ab remains effective in killing HER2- and / or IGF-IR-overexpressing tumor cells through ADCC in vivo.

Bi-Ab inhibits tumor growth in SKOV-3 HER2- and IGF-IR-overexpressing xenograft mouse model

To establish a HER2- and IGF-IR-overexpressing cancer xenograft mouse model for testing the effect of Bi-Ab in vivo, we generated a SKOV-3-Luc stable cell line expressing luciferase. We tested Bi-Ab, m590, Herceptin, and Comb in this mouse model following the protocol shown in Fig. 4A. There were a total of 4 experimental groups, and each group had 7 nude mice. A control group that had 5 nude mice was included in the study. Three million of SKOV-3-Luc cells were injected subcutaneously to each nude mouse, and antibodies (100 μg per mouse) were injected by i.p. on days 1, 4, 6 and 8 post inoculations. Mouse body weight and luminescence intensity in the regions of interest (ROI) were measured on days 1, 4, 6 and 8 prior to antibody injections, and repeated on days 11, 15, 25 and 35 post inoculations. The average body weight of Bi-Ab treated mice did not decrease throughout the study, while the average mouse body weight of the other three experimental groups decreased on day 4. Nevertheless, there was no significant difference in average body weight among the 4 groups at comparable time points (data not shown).

The average luminescence intensities varied across all the experimental groups (Fig. 4B). Notably, the Bi-Ab treated group experienced a dramatic inhibition of tumor growth which lasted for a much longer time compared with the other three groups. The Bi-Ab group showed
significantly lower average luminescence intensities than the m590 group (P-value < 0.001) from day 6 to day 35, the end of the mouse study, while the Comb group only showed significantly lower average luminescence intensities than the m590 group on days 6, 8, and 11, and the Herceptin group only showed significant lower average luminescence intensities than the m590 group on days 8 and 11 (Fig. 5A). The average luminescence intensity of the m590 group also decreased on day 11, and further decreased on day 15, but returned to high levels on days 25 and 35. These decreases may not be caused by m590 treatment because the control group also showed slightly decreases in average luminescence intensity on day 11 and 15 (Fig. 4B). The Comb group showed relapse (increased average luminescence intensity) starting on day 25, while the Herceptin group relapsed earlier on day 15. The average luminescence intensity of the Bi-Ab on day 35 slightly increased, but it was still significantly lower than that of the m590 group (Fig. 5A).

We then investigated individual mouse in each group and counted the number of mouse that had 2-fold higher luminescence intensity than the baseline level (no inoculation) (Fig. 5B). The Bi-Ab group showed early decrease in luminescence intensity. 5 out 7 mice in the Bi-Ab group had luminescence intensities below 2-fold of the baseline level on day 4, while only 3 out 7 mice in the Comb and Herceptin groups had the same low levels of luminescence intensity on day 4 (Fig. 5B). The rest 2 mice in the Bi-Ab showed luminescence intensities below 2-fold of the baseline level on days 8 and 15, and the luminescence intensities of all mice in the Bi-Ab group remained low till the end of the mouse study except that one mouse relapsed on day 35 (Fig. 5B). 5 out of 7 mice in the Comb group showed luminescence intensities below 2-fold of the baseline level on days 6 and 8, but one of these 5 mice in the Comb group relapsed on day 35. 6 out of 7 mice in the Herceptin group showed luminescence intensities below 2-fold of the baseline level
on day 8, but these mice relapsed one by one as the study progressed. There was only one mouse left in the Herceptin group that had the luminescence intensity below 2-fold of the baseline level on day 35 (Fig. 5B). None of mice in the m590 ever exhibited luminescence intensity below 2-fold of the baseline level throughout the study. These results indicate that dual targeting HER2 and IGF-IR is more effective than single targeting in inhibiting HER2- and IGF-IR-overexpressing tumor growth and postponing tumor relapse in vivo, and Bi-Ab more effectively inhibits tumor growth and prevents tumor relapse than simple combination of two monospecific antibodies.

**Discussion**

Breast cancer is one of the most common cancers in women. It accounts for 22.9% of all cancer cases in women worldwide and was responsible for 13.7% of cancer deaths in 2008. EGFR and IGF-IR activation contributes to the initiation and progression of breast cancer. Herceptin (trastuzumab) has been in clinical use for treatment of HER2-overexpressing breast cancers, but patients often develop resistance to the therapy. Thus, there is a need for novel therapies. In this study, we generated and tested Bi-Ab, a “knobs-into-holes” bispecific antibody that co-targets HER2 and IGF-IR, in *in vitro* assays and in the tumor xenograft mouse model overexpressing both HER2 and IGF-IR. Our results indicate that co-targeting HER2 and IGF-IR may be of clinical relevance in treating HER2- and / or IGF-IR-overexpressing human cancers.

This study was promoted by our observation that m590 synergized with Herceptin in inhibiting Akt phosphorylation in MCF-7 cells (Fig 2B). But when we come to SKOV-3 cell line that expresses much higher level of HER2 than MCF-7, the inhibition of Akt and ERK
phosphorylation by antibodies becomes complicated. This may attribute to two reasons. First, anti-HER2 mAb Herceptin down-regulates the phosphorylation of Ras, Raf, MEK and MAPK (ERK) (6). ERK kinase can phosphorylate dual specific phosphatases Cdc25c. Down-regulated ERK phosphorylation reduces the activity of Cdc25c, resulting in increased phosphorylation of receptor tyrosine kinase (RTK), such as IGF-IR. Therefore, although the MAPK pathway is blocked by Herceptin, following the activation of IGF-IR, the PI3K/Akt pathway is triggered, leading to a compensatory effect, which rescues tumor cells from apoptosis. This feedback loop was first reported by Prahallad et al. in 2012 (45). We observed the same phenomenon in this study (Fig. 2D-2F). The increased Akt phosphorylation caused by the feedback loop makes anti-IGF-IR mAb m590 less effective in inhibiting Akt phosphorylation, especially in the presence of IGF-I (Fig 2E and 2F). Nevertheless, m590 and Bi-Ab inhibit ligand-induced phosphorylation of IGF-IR in SKOV-3 cells (Fig. 2F), and Bi-Ab inhibits ERK phosphorylation in a dose-dependent manner in SKOV-3 cells in the absence of IGF-I (Fig. 2G). Second, high levels of HER2 and IGF-IR in SKOV-3 cells may promote the formation of HER2/IGF-IR heterodimer. Indeed, Browne et al. reported the existence of HER2/IGF-IR complexes in Herceptin-resistant breast cancer SKBR3 cells and found that co-targeting both receptors improved the efficacy of Herceptin in vitro (37). HER2/IGF-IR heterodimer may be less sensitive to monospecific antibodies, Herceptin and m590. To inhibit the signaling of HER2/IGF-IR heterodimer, Bi-Ab is expected to be more effective than Comb because there may be steric hindrance when two IgG molecules need to bind simultaneously to the same heterodimer. This may be one of the reasons why Bi-Ab is more effective than Comb in inhibiting cancer cell proliferation in vitro and tumor growth in the tumor xenograft mouse model.
Different methodologies have been described to generate bispecific antibodies by design (42, 46). We took advantage of the “knobs-into-holes” strategy for generating Bi-Ab (42). Co-transfection of 293F cells with the “knob” and “hole” mutant plasmids yielded Bi-Ab. Protein A affinity-purified Bi-Ab showed a single peak in gel filtration (data not shown). More importantly, “knobs-into-holes” mutations located in the CH3 domain did not affect antibody effector functions which are mediated mainly by the CH2 domain of antibodies as evidenced by the high ADCC activity of Bi-Ab. ADCC is an important mechanism of action for therapeutic antibodies in vivo (47-49). The result from our ADCC assay demonstrates that Bi-Ab has comparable or slightly enhanced ADCC activity compared with monospecific antibodies, m590 and Herceptin, and the Comb. Notably, this is the first report showing that the “knobs-into-holes” strategy did not affect ADCC activity of the resultant bispecific antibody. Nude mice have relatively normal NK cells and human IgG1 can bind to mouse Fc gamma receptor III (FcrRIII, CD16) on NK cells. Thus, administration of human IgG1s or human/mouse chimeric antibodies into mice can induce ADCC and Ab-dependent cellular phagocytosis in NK cells, polymorphonuclear leukocytes, and macrophages (50). Both Herceptin and m590 contain human IgG1 Fc, therefore, the inhibitory effect observed in this mouse study may result from Fab-mediated direct effects of the antibodies and Fc-mediated ADCC and phagocytosis.

In the current mouse study, tumor growth was more effectively inhibited by Bi-Ab than Herceptin and Comb. Out of 7 mice in each experimental group, 6 mice in the Bi-Ab group were still having low luminescence intensities (below 2-fold of the baseline level) in the end of the study (day 35 post inoculations of SKOV-3-Luc cells), while this number was 4 in the Comb group and 1 in the Herceptin group. Although m590 alone failed to bring the luminescence intensity to such low level, co-administration of m590 with Herceptin (Comb) significantly
enhanced the inhibitory effect of Herceptin. Co-targeting IGF-IR- and HER2-overexpressing
tumor xenografts with Bi-Ab worked even better than the simple mixture of two monospecific
antibodies (Comb), which may be ascribed to the more effective inhibition of ERK
phosphorylation and ligand-induced IGF-IR phosphorylation by Bi-Ab in SKOV-3 cells (Fig. 2C
and 2D), and enhanced ADCC activity of Bi-Ab compared to Comb. These results indicate that
Bi-Ab has superior antitumor activity compared to the monospecific antibodies alone or in
combination, and co-targeting HER2 and IGF-IR with Bi-Ab may be clinically beneficial in
minimizing the acquired resistance to the current Herceptin therapy.

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Author contributions

MYZ conceived and designed the experiments; CC, YZ, YZ, JL and MYZ performed the
experiments; CC, YZ and MYZ analyzed the data; SWT discussed the study and provided
helpful suggestions; MYZ wrote the paper.

References


Figure legends

Figure 1. Flow cytometry of SKOV-3 and MCF-7 cells stained with m590 or Herceptin and characterization of Bi-Ab for binding to recombinant HER2 and IGF-IR ectodomains and membrane-associated HER2 and IGF-IR in comparison with m590 and Herceptin. (A) Flow cytometry of breast cancer MCF-7 and ovarian cancer SKOV-3 cells stained with m590 and Herceptin at 10 μg/ml. (B) Simultaneous binding of Bi-Ab to recombinant IGF-IR and HER2 ectodomains by indirect ELISA. Recombinant IGF-IR ectodomain was coated in microplates. Three-fold serially diluted Bi-Ab, Herceptin and m590 were added to the wells followed by
addition of 100 ng per well of biotinylated recombinant HER2 ectodomain. Bound HER2 ectodomain was detected by HRP conjugated to streptavidin and TMB. (C) Histogram overlay of SKOV-3 cells stained with m590, Herceptin and Bi-Ab at 2 and 10 μg/ml. (D) Binding kinetics of Bi-Ab, m590 and Herceptin to membrane-associated IGF-IR and HER2 on SKOV-3 cells. Mean Fluorescence Intensity (MFI) of SKOV-3 cells stained with the antibodies at different concentrations was measured.

**Figure 2.** Inhibition of cancer cell signaling by Bi-Ab in comparison with m590 and Herceptin alone, or in combination (Comb). (A-B) Inhibition of IGF-I (1.5 nM) induced phosphorylation of Akt and ERK by m590 (40 nM) alone (A) or in combination with Herceptin (6.7 nM) (B) in MCF-7 cells. (C) Inhibition of IGF-I (1.5 nM) induced phosphorylation of IGF-IR in SKOV-3 cells. (D-E) Inhibition of IGF-I (1.5 nM) induced phosphorylation of Akt and ERK in MCF-7 (D) and SKOV-3 (E) cells. (F) Inhibition of phosphorylation of Akt and ERK by the antibodies in SKOV-3 cells in the absence of IGF-I. All antibodies were tested at 100 μg/ml and incubated with cells for 24 h (C-F). (G) Dose-dependent inhibition of ERK phosphorylation, but not Akt phosphorylation by Bi-Ab in SKOV-3 cells in the absence of IGF-I. Bi-Ab: 200, 40, 8, 1.6, 0.32 and 0 μg/ml.

**Figure 3.** Characterization of Bi-Ab for inhibitory activity in SKOV-3 cell proliferation and ADCC activity in comparison with m590 and Herceptin alone, or in combination (Comb). (A) Inhibition of SKOV-3 cell proliferation in MTT assay. Percent inhibition at each antibody concentration was used in ANOVA (one-way Analysis of Variance) statistical analysis to test if there was significant difference between any two antibodies at the same concentration. Two paired antibodies with significant difference (P-value < 0.001) in percent inhibition are indicated. (B) Percent ADCC of the antibodies at 1 and 5 μg/ml. The same ANOVA statistical analysis was
done to test if there was significant difference in percent ADCC between any two antibodies at the same concentration. Percent ADCC between Bi-Ab and Comb at 1 μg/ml showed significant difference (P-value < 0.05), which is indicated. Each antibody dilution was tested in triplicate and the assays were repeated once.

**Figure 4.** Diagram of the mouse study and tumor growth kinetics in each group of mice treated with or without antibodies (control) (A) Diagram of the mouse study. 3 million of SKOV-3-Luc cells were injected subcutaneously into each nude mouse on day 0. 100 μg of Bi-Ab, or m590, or Herceptin, or Comb were injected by i.p. on day 1, 4, 6 and 8 post inoculations. Mouse imaging was performed on day 1, 4, 6 and 8 prior to antibody injections, and on day 11, 15, 25 and 35 post inoculations. Seven mice were included in each antibody treated group, but only five mice were in the control group. (B) Average luminescence intensity in each group of mice at different time point. Logarithmic values of the average luminescence intensities and standard variations were shown.

**Figure 5.** Inhibition of cancer growth by Bi-Ab in SKOV-3 HER2- and IGF-IR-overexpressing xenograft mouse model in comparison with m590 and Herceptin alone, or in combination (Comb). (A) Average luminescence intensity in each group of mice at different time point. Logarithmic values of the average luminescence intensity were used in ANOVA statistical analysis to test if there was significant difference between any two groups at the same time point. Two paired groups with significant difference (P-value < 0.001) are indicated. (B) Number of mice in each group with luminescence intensity 2-fold higher than the baseline level.
Figure 1

A: SKOV-3

- 2nd Ab only
- m590
- Herceptin

B: MCF-7

- 2nd Ab only
- Herceptin
- m590

C: SKOV-3

- 2nd Ab only
- Ab: 2µg/ml
- m590
- Bi-Ab
- Herceptin

D: SKOV-3

- 2nd Ab only
- Ab: 10µg/ml
- m590
- Bi-Ab
- Herceptin

Antibody concentration (nM) vs. OD450/nm

Antibody concentration (µg/ml) vs. MFI
Figure 2

A (MCF-7)

P-Akt
Akt
p-ERK
ERK
IGF-I − + + +
m590 − − + +

B (MCF-7)

P-Akt
Akt
IGF-I + + + +
Herceptin − + − +
m590 − − + +

C (SKOV-3)

m590
Herceptin
Bi-Ab
Comb
IgG control

P-IGF-IR
GAPDH
IGF-I + + + + +

D (MCF-7)

no ligand
Herceptin
m590
Bi-Ab
Comb
IgG control

P-Akt
Akt
p-ERK
ERK
GAPDH
IGF-I − + + + +

E (SKOV-3)

no ligand
Herceptin
m590
Bi-Ab
Comb
IgG control

P-Akt
Akt
p-ERK
ERK
GAPDH
IGF-I − + + + +

F (SKOV-3)

IgG control
Herceptin
m590
Bi-Ab
Comb

P-Akt
Akt
p-ERK
ERK
GAPDH
IGF-I − − − − − −

G (SKOV-3)

200 40 8 1.6 0.32 0 μg/ml

P-Akt
Akt
p-ERK
ERK
GAPDH
IGF-I − − − − − −
Figure 3

(A) Percent inhibition (%) of m590, Herceptin, Bi-Ab, and Comb against antibody concentrations of 200, 100, 50, 25, 12.5, and 6.25 μg/ml.

(B) Percent ADCC (%) of Bi-Ab, Comb, Herceptin, and m590 against antibody concentrations of 1 μg/ml and 5 μg/ml.
Figure 4

A
Inject tumor cells

Infect tumor cells

Imaging

Day 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 25 35

B

Luminescence intensity (p/s/cm²/sr)

Time post inoculation (day)

m590

Bi-Ab

Herceptin

Comb

control
Figure 5

A

![Graph showing luminescence intensity over time post inoculation for different treatments.]

B

![Graph showing the number of mice with positive signal over time post inoculation for different treatments.]
Molecular Cancer Therapeutics

Superior antitumor activity of a novel bispecific antibody co-targeting human epidermal growth factor receptor 2 and type I insulin-like growth factor receptor

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