Four-in-One Antibodies Have Superior Cancer Inhibitory Activity against EGFR, HER2, HER3, and VEGF through Disruption of HER/MET Crosstalk

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Abstract

The anti-HER receptor antibodies cetuximab, trastuzumab, and pertuzumab are used widely in clinic to treat metastatic cancer. However, activation of the extensive crosstalk among the HER receptors as well as other RTKs, particularly HER-MET crosstalk, has emerged as a likely source of drug resistance. In this study, we developed two new types of tetra-specific antibodies that recognize EGFR, HER2, HER3, and VEGF. These tetra-specific antibodies, termed FL518 (four-in-one antibody) and CRTB6 (tetra-specific, tetravalent antibody), not only inhibited signaling mediated by these receptors in vitro and in vivo but unexpectedly also disrupted HER-MET crosstalk. When compared with two-in-one antibodies and a series of bispecific antibodies in multiple tumor models, FL518 and CRTB6 were more broadly efficacious. We further showed that tetra-specific antibodies were far more effective than bispecific antibodies in inhibiting the growth of anti-HER-resistant cancer cells, which exhibited elevated levels of MET activation both in vitro and in vivo. Overall, our results establish a new principle to achieve combined HER inhibition and limit drug resistance using a single antibody. Cancer Res; 75(1); 1–12. ©2014 AACR.

Introduction

Deregulation of the human epidermal growth factor receptor (HER) family plays a significant role in tumorigenesis and progression (1). The basic and clinical research into two of the family members, epidermal growth factor receptor (EGFR/HER1) and HER2, has led the way in transforming therapeutic biomolecules from bench to bedside, including the anti-EGFR antibodies cetuximab and panitumumab, the anti-HER2 antibodies trastuzumab and pertuzumab (2, 3), and the EGFR tyrosine kinase inhibitors erlotinib and gefitinib. Another family member, HER3, has recently emerged as a novel drug target because its upregulation is an adverse prognostic factor in many tumor types and is associated with worse survival (4–6). Although HER inhibitors have been widely evaluated in clinic, these promising anticancer drugs are effective only for a limited time, due to the resistance developed by tumor cells (7). Signaling shift has been proved for when such intensely proliferative cancer cells faced a strong signaling inhibitor. For instance, HER3 has also been implicated in the development of resistance to anti-EGFR or anti-HER2 therapy (8–10). A shift from HER1 homodimer to HER1/HER3 heterodimer signaling in response to sustained treatment with EGFR inhibitors leads to reactivation of the PI3K/AKT pathway and bypasses the therapeutic action of these compounds. Upregulation of HER3 seems to be driven by negative feedback from AKT (11). Amplification of the tyrosine kinase receptor MET induces HER3 phosphorylation and PI3K activation, representing another mechanism by which HER3 confers resistance to EGFR-targeting therapies (11, 12).

Data increasingly indicate the significant status of combined inhibition strategies for directing key signaling networks of the HER family, including cetuximab plus trastuzumab (13), cetuximab plus bevacizumab (14), and trastuzumab plus pertuzumab (2). Recently, generation of bispecific antibodies that have the capacity to bind two different antigens simultaneously has become a new trend of engineering antibodies (15), and a number of recombinant strategies have been developed to synthesize bispecific antibodies (8, 16, 17). Indeed, in 2009, catumaxomab (Removab, Neovii Biotech) was approved as the first bispecific antibody drug for the treatment of malignant ascites (18). For anti-HER-targeted therapy, previous studies have shown that bispecific antibodies engineered from anti-EGFR or -HER2 may give promising results (19, 20). Moreover, two-in-one antibodies, which created a surface that specifically binds two target antigens with high affinity using a single antibody binding site (21, 22),
have aroused abundant interest in the field. Moreover, the in vivo properties, such as pharmacokinetics, stability, and effector function, of the IgG-based bispecific antibodies are expected to be similar to those of standard monoclonal antibodies, which are crucial for clinical use. Thanks to the design of two-in-one antibodies, we were able to create IgG-based tetra-specific antibodies using current antibody engineering methodologies.

Materials and Methods

Cell lines, antibodies, and animals

All cancer cell lines were obtained from the American Type Culture Collection (ATCC), except HCC827 and gastric cancer cell line MNK45, which were purchased from the German Collection of Microorganisms and Cell Cultures. All the cell lines were authenticated twice by morphologic and isoenzyme analyses during the study period. Cell lines were routinely checked for contamination by mycoplasma using Hoechst staining and consistently found to be negative. Cells were cultured in DMEM containing 5% FCS in 5% CO2 at 37.8°C in a humidified incubator. Cetuximab was purchased from Merck. Trastuzumab and bevacizumab were purchased from Roche Ltd.

Six-week-old female BALB/c nude mice and eight- to 12-week-old C.B-17 SCID mice were obtained from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China). All animals were treated in accordance with guidelines of the Committee on Animals of the Chinese Academy of Sciences.

Designed antibodies

Four-in-one CrossMab FL518 were generated from MEHD7945A (23) and bh1-44 (24) as previously described (25). Four-in-one dual-variable domain antibodies (DVD-Ig) methodology (26) was utilized to generate DVD Ig CRTB6, together with the "knobs into holes" (KH) methodology and the CrossMab methodology. Bispecific crossmabs CT9, CB12, RT6, and RB4 were generated from cetuximab/trastuzumab, cetuximab/bevacizumab, RG7116 (anti-HER3)/trastuzumab, and RG7116/bevacizumab.

Construction, expression, and protein purification

Residues 1–619 of the extracellular domain of EGFR (EGFR-ECD), residues 1–513 of the HER3-ECD, residues 1–646 of HER2-ECD, and residues 8–109 of human VEGF were prepared as described previously (27, 28) except that we employed the pcDNA3.1(+) expressing vector (Invitrogen) and the FreeStyle 293 expression system (Invitrogen). Four-in-one antibodies and bispecific crossmabs were cloned, expressed, and purified following reported procedures (29).

Immunoblotting

Cells were incubated with the indicated antibodies in serum-free medium for 1 hour at 36.5°C, then treated with EGF (0.5 nmol/L), HRG (0.5 nmol/L), VEGF (0.5 nmol), or not for 15 minutes. After washing, the cells were lysed and the lysates were subjected to SDS-PAGE and immunoblotted with antibodies against EGFR, phospho-EGFR-Tyr1068, phospho-HER2-Tyr1221/1222, phospho-HER3-Tyr1289, phospho-MET-Tyr1234/5, AKT, phospho-AKT-Ser473, phospho-STAT3-Ser705, p44/42 MAPK, phospho-p44/42 ERK1/2 Thr202/Tyr204, EGFR, HER2, and MET (all from Cell Signaling Technology), and HER3 (Santa Cruz). All assays were performed independently three times.

Coimmunoprecipitation assays

Coimmunoprecipitation was used to evaluate the HER/MET heterodimerization as previously described (28, 30). Cells were lysed in 1 mL RPMI lysis buffer [1% v/v Triton X-100, 1% w/v CHAPS, 10 mmol/L HEPEs (pH 7.2)], in RPMI medium containing 0.2 mmol/L PMSF, 10 μg/mL leupeptin, 10 U/mL aprotinin, and 1 mmol/L Na2VO4. Heterodimers were immunoprecipitated from 500 μL of lysate using indicated antibodies covalently coupled to agarose (Pierce ultralink) for 1 hour at 36.5°C. Recombinant human EGF and HRG (R&D Systems) were added at a final concentration of 0.5 nmol/L, respectively. Complexes were washed twice in lysis buffer and resuspended in SDS sample buffer and boiled. Samples were separated on a 4% to 12% polyacrylamide gel (Novex) and electro-blotted onto nitrocellulose membranes. Blots were blocked in 10% BSA/TBST and probed with indicated antibodies. Western blot and protein immunoprecipitation analyses were performed after the CST protocol.

Cell proliferation assay

H1666 cells (4,000 cells/well) and H1993 cells (3,000 cells/well) were plated in 96-well plates. The next day, cells were treated with indicated concentrations of antibody in 1% serum-containing medium. Depending on the cell line, ligand was added simultaneously. After 3 to 4 days, AlamarBlue (Inviotgen) was added to the wells and fluorescence was read using a 96-well fluorometer with excitation at 530 nm and emission of 590 nm. The results are shown as relative fluorescence units (RFU). For MTS assay, cells were treated with 10 μg/mL recombinant antibodies. Recombinant human EGF, HRG, and VEGF (R&D Systems) were added to a final concentration of 0.5 nmol/L, respectively. After an additional 4- to 7-day incubation, cell proliferation was determined by Cell Titer 96 AQeous One Solution Cell Proliferation Assay Kit (Promega). All assays were performed independently three times.

In vivo therapy study

C.B-17 SCID mice were inoculated with 5 × 106 BxPC3 cells suspended in HBSS. For MDA-MB-231, BT474, MCF-7, HCC1954, or ReHCC1954 in vivo xenograft studies, 3 × 106 BT474 or MCF-7 cells, 4 × 106 HCC1954, or ReHCC1954 cells were inoculated into the mammary fat pads of female BALB/c nude mice implanted with 0.11 mg 60-day release 17β-estradiol pellets (Innovative Research of America; ref. 31). For HCC827 or ReHCC827 xenograft studies, 5 × 106 HCC827 or ReHCC827 cells were inoculated into BALB/c nude mice. Mice were randomly split into groups of 10 mice each as tumor volumes reached an average of about 150 mm3. Multiple dose studies comprised 4 weeks of treatment, with the first dose (day of randomization) being a 2× loading dose. Tumors were measured with calipers at least once a week for the duration of the study. Tumors were measured with digital calipers, and tumor volumes were calculated by the formula: volume = length × (width)2/2.

Statistical analysis

Statistical analysis was performed by the Student unpaired t test to identify significant differences unless otherwise indicated, with P value of less than 0.05 considered significant difference. Additional methods can be found in Supplementary Materials and Methods.
Design and characterization of a four-in-one antibody in an IgG-like format

We first sought to generate a four-in-one antibody using two two-in-one antibodies, including MEHD7945A (21) directed against EGFR and HER3 and bH1-44 that binds to HER2 and VEGF. Models were generated from PDB entry 3PII, 3POY, 1YY9, 1M6B, 3BE1, and 3BDY. B, schematic representation of the four-in-one antibody FL518. C, schematic representation of tetra-specific, tetravalent DVD-Ig CRTB6.

Results

Design and characterization of a four-in-one antibody in an IgG-like format

We first sought to generate a four-in-one antibody using two two-in-one antibodies, including MEHD7945A (21) directed against EGFR and HER3 and bH1-44 (22), which is described as binding VEGF and HER2 (Fig. 1A). Two methodologies were used in the generation of the four-in-one antibody FL518. C. schematic representation of tetra-specific, tetravalent DVD-Ig CRTB6. D, FL518 binding to immobilized EGFR-ECD, HER3-ECD, HER2-ECD, or VEGF in the presence of the indicated soluble competitor. 1x = 0.01 μg/mL; 10x = 0.1 μg/mL; 100x = 1 μg/mL; 1,000x = 10 μg/mL. Results are expressed as FL518 concentration versus optical density (OD). E, four-in-one antibodies binding to immobilized indicated protein in the presence of the indicated biotin-competitor antibodies. Detection was carried out with alkaline phosphatase-conjugated avidin. Results are expressed as antibody concentration versus optical density. F–I, immunoblots examining the ability of 10 μg/mL of control IgG, MEHD7954A, cetuximab, RG7116, trastuzumab, bevacizumab, FL518, or CRTB6 to inhibit the EGF-stimulated phosphorylation of EGFR and ERK1/2 in Caco-2 cells (F), the HRG-stimulated phosphorylation of HER3, AKT, and ERK1/2 in MCF-7 cells (G), the ligand-independent phosphorylation of HER2 and AKT in BT474 cells (H), and the ligand-independent phosphorylation of HER2 and AKT in BT474 cells (I).
Figure 2.
Four-in-one antibodies have superior antitumor activity in vitro and in vivo. A, schematic representation of bispecific CrossMabs. B and C, H1666 cells (B) or H1993 cells (C) were treated with increasing concentrations of the indicated antibodies in the presence of EGF (0.5 nmol/L), HRG (0.5 nmol/L), and VEGF (0.5 nmol/L). The concentrations shown on the x-axis reflect the concentration of a single antibody or the combination of antibodies. (Continued on the following page.)
chain interactions was achieved using the CrossMab methodology (Fig. 1B; ref. 34).

Because one Fab of FL518 can bind either EGFR or HER3 and the other Fab can bind either HER2 or VEGF, a certain concentration of FL518 antibodies can directly bind to EGFR, HER3, HER2, and VEGF on the surface of the cell membrane during the same period. However, this kind of four-in-one antibody has its own limits in that one antibody molecule can only bind two of the targets. Thus, we employ the DVD-lg methodology (35), the KiH methodology, and the CrossMab methodology to make a tetra-specific, tetravalent antibody termed CRTB6 (Fig. 1C). For this antibody design, one IgG-like antibody molecular now can bind to four targets. We use the V region of cetuximab and RG7116 (36) in the Fab arms for EGFR and HER3, for their ability to block the EGF- and HRG-dependent homo- or hetero-dimerization and signaling and the V region of trastuzumab, which has shown its unique effect on ligand-independent EGFR/HER2 or HER3/HER2 signaling (37). In addition, anti-VEGF antibody bevacizumab was added to make this tetra-specific, tetravalent antibody more comparable with FL518. Because the dimerization and communication among HER family members are the key events of EGFR, HER2, and HER3, there is reason to suspect that this tetra-specific, tetravalent antibody may be more efficacious than FL518.

FL518 binding to immobilized HER3-ECD or immobilized HER2-ECD was observed to reduce in a dose-dependent manner with increasing amounts of EGFR-ECD or VEGF, respectively. Conversely, FL518 was competed away from immobilized EGFR-ECD or immobilized VEGF by soluble HER3-ECD or HER2-ECD protein, respectively. As expected, given their relative binding constants, higher concentrations of soluble EGFR-ECD or VEGF were needed to compete with the binding of FL518 to immobilized HER3-ECD or HER2-ECD. These data were consistent with the parental antibodies MEHD7945A and beH1-44 (Fig. 1D). In contrast, no such data can be detected for DVD-lg CRTB6 (Supplementary Fig. S1). Competitive binding assays employing antibodies were also performed and showed that the epitopes of these four-in-one antibodies overlap with the epitopes of MEHD7945A and beH1-44 on EGFR, HER3, HER2, and VEGF (Fig. 1E).

The affinities of the four-in-one antibodies and the parental antibodies for EGFR, HER3, HER2, and VEGF were determined by surface plasmon resonance (SPR). As suggested by molecular modeling, the affinities of the four-in-one antibodies to their respective ligands did not differ significantly from those of the parental antibodies and simultaneous binding to both ligands could be shown by SPR assay (Supplementary Table S1). In addition, as summarized in Supplementary Table S2, the main PK parameters of FL518 and CRTB6 in mice were very similar to those of the parental antibodies and simultaneous binding to both ligands could be shown by SPR assay (Supplementary Table S1). In addition, as summarized in Supplementary Table S2, the main PK parameters of FL518 and CRTB6 in mice were very similar to those of the parental antibodies and simultaneous binding to both ligands could be shown by SPR assay (Supplementary Table S1).

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(Continued.) Cell proliferation relative to an untreated control was measured after 7 or 6 days using AlamarBlue staining. D, MTS assay examining the effects of 10 μg/mL of the indicated antibodies on breast or pancreatic cell proliferation in the absence or presence of EGF, HRG, and VEGF (0.5 nmol/L each). Results are shown as the percentage of control cell proliferation. Error bars, SD; *P < 0.05; **P < 0.01, compared with control (CTRL) group. All assays were performed independently three times. E, BxP3, MDA-MB-231, and MCF-7 tumor-bearing mice were treated weekly using control IgG (10 mg/kg), CT9 (10 mg/kg), CB12 (10 mg/kg), RT6 (10 mg/kg), RB4 (10 mg/kg), MEHD7945A (10 mg/kg), beH1-44 (10 mg/kg), MEHD7945A plus beH1-44 (5 mg each), FL518 (10 mg/kg), or CRTB6 (10 mg/kg). The first dose was given as a 2× loading dose. Arrows, treatments; data are presented as the mean tumor volume ± SEM. Percent tumor growth inhibition was evaluated at day 26.
in the cell proliferation assay. However, the greatest inhibition of cell growth was obtained with the four-in-one antibodies (Fig. 2B).

Intriguingly, four-in-one antibodies potentely inhibited cell proliferation at lower antibody concentrations than the combination of MEHD7945A and bH1-44, and the tetravalent DVIdg CRTB6 was more effective than FL518. The greater potency may be due to the four-in-one antibodies’ unique ability to simultaneously engage more receptors on the surface of cells. The inhibition mechanism underlying the tetravalent antibody is hard to predict because it depends not only on the affinity for the antigens but also on the local antigen concentration. Similar inhibition results can be observed for H1993 cells, which also exhibited a high level of expression of EGFR, HER2, and HER3 (Fig. 2C; ref. 25). The combination of MEHD7945A and bH1-44 caused significant inhibition of cell proliferation; however, four-in-one antibodies had a better inhibition profile.

Next, we performed similar proliferation assays for breast cancer MDA-MB-231 (high EGFR expression; ref. 36), BT474 (high HER2 expression), and MCF-7 (low HER2 expression) cells as well as for BxPC3 cells (21), a pancreatic cancer cell line driven by HER3 activation (Fig. 2D). In MDA-MB-231 cells, bispeciﬁc antibodies did not exhibit signiﬁcant inhibition, but the combination of MEHD7945A and bH1-44 had a considerable toxic effect. However, four-in-one antibodies, particularly the DVIdg CRTB6, showed a unique inhibitory effect on these cells. In BT474 cells, MCF-7 cells and BxPC3 cells, anti-HER3 antibodies RT6, RB4, and MEHD7945A had better performance than anti-EGFR/HER2 antibodies or anti-VEGF antibodies, whereas FL518 inhibited proliferation more potently than bispeciﬁc antibodies and the combination of MEHD7945A and bH1-44. CRTB6 exerted the greatest inhibitory effect in all the cell lines. Taken together, these data suggested that four-in-one antibodies exhibit increased antiproliferative activity compared with bispeciﬁc antibodies in vitro.

To test four-in-one antibody activity in vivo, BxPC3 cells were injected subcutaneously into nude mice, and established tumors were treated once a week with four-in-one antibodies or bispeciﬁc antibodies. For BxPC3 cells, RT6 inhibited tumor growth by 55% compared with the vehicle control (P < 0.001; Fig. 2E, left), and RB4 suppressed tumor growth by 66% (P < 0.05), whereas CT9, CB12, and RB4 had only marginal effects. bH1-44 had a small effect and MEHD7945A signiﬁcantly inhibited tumor growth by 72%, whereas the four-in-one antibody FL518 inhibited tumor growth by nearly 92% compared with the vehicle control (P < 0.0001), which was even more effective than the combination of parental antibodies (P < 0.001, MEHD7945A plus bH1-44, TGI 77%). Notably, tumor growth was inhibited nearly 100% with CRTB6 treatment compared with the vehicle control (P < 0.0001), and the established tumors were ultimately eradicated in tumor-bearing mice.

To further investigate and compare the potency of four-in-one antibodies with other antibodies in additional xenograft models, breast cancer MDA-MB-231 and MCF-7 cells were injected subcutaneously into female BALB/c nude mice. In our assay, the tumor growth of MDA-MB-231 xenografts (Fig. 2E, middle) was inhibited by MEHD7945A (TGI 57%), bH1-44 (TGI 28%), CT9 (TGI 12%), CB12 (TGI 5%), RT6 (TGI 43%), and RB4 (TGI 34%), whereas the combined blockade in cell signaling caused by FL518 and CRTB6 was most effective in inhibiting tumor growth (TGI 83% and TGI 97%, respectively). The combination of bispeciﬁc antibodies did not signiﬁcantly enhance inhibition (MEHD7945A plus bH1-44, TGI 66%). Moreover, the tumor growth of MCF-7 xenografts (Fig. 2E, right) was inhibited by bispeciﬁc antibodies MEHD7945A (TGI 39%), bH1-44 (TGI 38%), CT9 (TGI 19%), CB12 (TGI 33%), RT6 (TGI 29%), and RB4 (TGI 22%). Although the combined blockade in cell signaling by a combination of bispeciﬁc antibodies resulted in a gain in the inhibitory effect [MEHD7945A plus BH1-44 (TGI 50%)], FL518 was more effective in inhibiting tumor growth (TGI 73%, P < 0.001), and the tetravalent antibody CRTB6 had the best inhibition rate (TGI 100%, P < 0.00001) of tumor xenografts in nude mice.

Only four-in-one antibodies have the ability to disrupt HER/MET crosstalk

The above results showed that simultaneously targeting several proliferation and survival signals will provide better clinical outcomes; however, two observations drew our attention. First, more signiﬁcant inhibition of AKT and ERK1/2 signaling was attained using a single agent of four-in-one antibodies than with any other antibody applied in the same concentration. Second, in the in vitro and in vivo cancer cell proliferation assays, four-in-one antibodies, especially DVIdg CRTB6, had a far greater inhibition rate than other antibodies, even the combination of parental antibodies. These data show that simultaneously targeting EGFR, HER2, HER3, and VEGF may involve a mechanism of inhibition other than only the phosphorylation of EGFR, HER2, HER3, and VEGFR2. Interestingly, in MKN45 cells (39) and HCC827 (40) cells stimulated with EGF and HRG, although phosphorylation of EGFR, HER2, HER3 could be inhibited by MEHD7945A or bH1-44, downstream ERK1/2 or STAT3 signaling was not interrupted (Fig. 3A), even at an extremely high dose (Fig. 3B). Moreover, phosphorylation of receptors was even signiﬁcantly inhibited by the combination of these two types of antibodies. Downstream ERK1/2 was only inhibited with an IC50 value of 18.6 μg/mL, and STAT3 signaling was not interrupted in MKN45 cells (Fig. 3C). Particularly, phosphorylation of MET, the oncogene that is often coexpressed in tumors with EGF family receptors, was only inhibited when a signiﬁcant high dose of a combination of MEHD7945A and bH1-44 was administered (IC50 13.5 μg/mL). However, the inhibition of MET was attained by the four-in-one antibodies FL518 and CRTB6 with an IC50 value of 0.37 μg/mL and 0.14 μg/mL, respectively. Downstream phosphorylation of STAT3 occurred with an IC50 value of 0.17 and phosphorylation of ERK1/2 occurred with an IC50 value of 0.28 μg/mL (Fig. 3D and E). Accordingly, only four-in-one antibodies have the unique ability to disrupt the MET/EGFR or MET/HER3 heterodimers in MKN45 cells (Fig. 3F). We concluded that blocking MET/HER crosstalk is a novel mechanism underlying the superior inhibition ability of four-in-one antibodies.

A MET/HER signaling shift is crucial for the resistance to HER-directed therapeutic antibodies

Because anti-HER antibodies cetuximab, trastuzumab, and pertuzumab are three widely used clinical antibody drugs, we first generated cetuximab-resistant HCC827 cells (ReHCC827) using previously described methods (40). Next, we modeled the development of acquired resistance treating the trastuzumab-insensitive breast cancer cell line HCC1954 cells in vivo by exposing xenografts to increasing concentrations of trastuzumab and
pertuzumab to generate dual-resistance cells ReHCC1954. Afterward, we validated the resistant phenotype in vivo and in vitro (Fig. 4A and B). Importantly, although the cancer cell line HCC1954 can be inhibited by trastuzumab plus pertuzumab (Fig. 4B), cancer cells developed resistance to this current-evaluated treatment strategy in clinic in our assay. Next, we tested phosphotyrosine signaling in parental and resistant cell lines and found that the amounts of MET, phosphorylation of MET, phosphorylation of HER3, phosphorylation of ERK1/2, and phosphorylation of STAT3 were significantly enhanced in the resistant cells compared with the parental cells (Fig. 4C). Consistent with this, the resistant cells showed a dramatic increase in EGFR/MET and HER3/MET heterodimers (Fig. 4C). In addition, the resistant cells expressed higher levels of EGF-like ligands (EGF, HRG, BTC, and HGF) than the parental cells (Fig. 4D). These data indicate that overexpression of MET and EGF-like ligands may be associated with resistant phenotypes.

Next, we examined the effect of siRNA knockdown of MET on the resistant cell lines. Transfection of MET siRNA dramatically downregulated the amount of MET in both cell lines (Fig. 4E). Treatment with MET siRNA effectively inhibited EGF-induced MET/EGFR signaling and cell growth (Fig. 4F) in ReHCC827 cells. Our data also showed that treatment with MET siRNA resulted in effective inhibition of HRG-mediated HER3 signaling and cell growth (Fig. 4G) in ReHCC1954 cells. In addition, MET siRNA treatment in both cell lines considerably resensitized resistant cells to cetuximab or dual trastuzumab and pertuzumab treatment (Fig. 4F and G). Together, these data further suggest that enhanced MET/EGFR and MET/HER3 crosstalk and signaling may be acquired antibody resistance mechanisms.

Four-in-one antibodies, but not bispecific antibodies, overcome resistance to HER-directed therapeutic antibodies in vitro and in vivo

Our experiments have established that for cetuximab administration–induced acquired resistance ReHCC827 cells, EGFR signaling has shifted to EGF/MET signaling, whereas for ReHCC1954 cells, HER2/HER3 signaling has shifted to HER3/MET signaling. Because four-in-one antibodies have been shown to block MET signaling, inhibiting MET/EGFR or MET/HER3 crosstalk, next we tested whether they could overcome acquired resistance to the HER direct therapy antibodies. Similar to the previous result, only four-in-one antibodies potently inhibited EGF-induced MET/EGFR heterodimerization/signaling and HRG-dependent MET/HER3 heterodimerization/signaling as well as downstream phosphorylation of ERK1/2, STAT3, and AKT in the resistant cells (Fig. 5A). Accordingly, the extent of in vitro growth inhibition by four-in-one antibodies was similar to the parental and resistant cells, whereas bispecific antibodies did not show significant inhibition in the resistant cells (Fig. 5B). Four-in-one antibody treatment significantly inhibited the tumor growth in both established parental and resistant cell tumors in all tumor-bearing mice (Fig. 5C), whereas bispecific antibody treatment failed to lead to significant tumor inhibition. We also showed that the combination of bispecific antibodies was significantly less effective than four-in-one antibodies in inhibiting the in vitro growth.
Figure 4.
MET/HER signaling shift is crucial for the resistance to HER-directed therapeutic antibodies. A and B, left, MTS assay evaluating cell proliferation of the indicated parental cancer cell lines and resistant sublines upon treatment with 10 μg/mL of control IgG, 10 μg/mL of cetuximab, or trastuzumab plus pertuzumab (5 μg/mL each). Error bars, SD. Right, tumor volume of HCC827, ReHCC827, HCC1954, or ReHCC1954 tumor xenografts after treatment with 10 mg/kg of control IgG or cetuximab or trastuzumab plus pertuzumab. Data are shown as the mean ± SEM. C, immunoblots comparing major cell signaling changes between the indicated parental breast cancer cell lines and their corresponding resistant sublines. D, real-time quantitative PCR analysis of expression of EGF-like ligands. Data are shown as the mean ± SD. E, ReHCC827 and ReHCC1954 cells were transfected with 100 pmol control or MET siRNA or were not transfected. (Continued on the following page.)
proliferation of resistant cells (Fig. 5B and C). Consistent with this, the in vivo antitumor activity of the antibodies in combination was much lower than that of four-in-one antibodies in the resistant xenograft mouse model (Fig. 5B and C).

Discussion

Herein, we provide evidence that a four-in-one antibody, FL518, generated from two-in-one antibodies, MEHD7945A and bH1-44, is capable of binding one EGFR and one HER2, one EGFR and one VEGF, one HER3 and one VEGF, or one HER3 and one HER2 simultaneously. Moreover, we generated another four-in-one DVD-Ig-like antibody, CRTB6, which is capable of binding one EGFR, one HER2, one HER3, and one VEGF simultaneously. To our knowledge, this is the first study to describe this type of antibody. Interestingly, four-in-one antibodies were more active than all the bispecific antibodies in all in vitro and in vivo models. Unexpectedly, our cell signaling assays revealed that four-in-one antibodies not only exhibited a blocking effect on EGFR, HER2, and one VEGF, one HER3 and HER2, or one HER3 and one VEGF simultaneously. Moreover, we generated another four-in-one DVD-Ig-like antibody, CRTB6, which is capable of binding one EGFR, one HER2, one HER3, and one VEGF simultaneously. To our knowledge, this is the first study to describe this type of antibody. Interestingly, four-in-one antibodies were more active than all the bispecific antibodies in all in vitro and in vivo models. Unexpectedly, our cell signaling assays revealed that four-in-one antibodies not only exhibited a blocking effect on EGFR, HER2,
HER3, and VEGF signaling but also had the capacity to block HER/ MET crosstalk. Combination use of the anti-EGFR/HER3 antibody MEHD7945A plus the anti-HER2/VEGF antibody bH1-44 has a similar effect but required an extremely high dose. Although in the DVD-lg design of four-in-one antibodies, cetuximab, trastuzumab, bevacizumab, and a new anti-HER3 antibody RG7116 were used to be comparable with FL518. However, as the efficacy of adding bevacizumab in anti-HER-targeted therapy has been controversial and the cardiac safety may also arise as a disadvantage, using other options for this arm may gain more potent therapeutic biomacromolecules, especially those antibodies that recently have been suggested to enhance the immune response of antibody drugs, such as anti-CTLA4 antibody ipilimumab or anti-CD3 antibody.

Sensitivity to a specific anticancer agent evolves during administration (8, 39). A variety of compensatory mechanisms may underlie acquired resistance to EGFR- or HER2-targeted agents, including switching dimerization partners (7), upregulation of ligands for HER3 or EGFR (24, 26, 41), or overexpression of particular receptors, especially HER3, which are identified as an increased response to current anti-HER/Erbb4 agents needing early and direct blockade (42). In a previous study, a bispecific antibody generated from trastuzumab and pertuzumab also supported this hypothesis, overcoming acquired resistance to trastuzumab by effectively blocking HER2/HER3 heterodimerization (19). Moreover, receptor crosstalk of MET plays a critical role in the development of resistance to EGFR family inhibitors. Amplification of MET occurs in patients with NSCLC who develop resistance to the RTK inhibitors such as gefitinib or erlotinib (11, 43, 44). In NSCLC cells selected for resistance to gefitinib or erlotinib in vitro, MET is amplified and activated and stimulates MET/EGFR or HER3 phosphorylation and signaling to AKT (11, 25). In Met-amplified gastric cancer cell lines, EGFR and HER3 are basally phosphorylated, which is abrogated in the presence of Met inhibitors (23). Stimulation with both HGF and EGF promotes downstream activation of several signaling pathways, including AKT, ERK1/2, and STAT3 (45). Met is also expressed at elevated levels in HER2-amplified human breast cancers, and cooperative signaling between MET and HER2 might be a mechanism by which MET promotes cancer progression (46). HER3/MET crosstalk and signaling were observed to play a crucial role in the resistance to direct HER2 blocking therapy, especially for the cancer cell lines resistant to the dual therapy of trastuzumab plus pertuzumab, which is currently under evaluation in clinics and has promoted good outcomes (2). Because MET was proved to be a select partner of HER family members, it is not surprising that use of anti-HER agents would result in the activation of HER/MET dimerization and signaling. In our present report, only simultaneous targeting of EGFR, HER2, and HER3 proved efficient to block the crosstalk between HER/MET, and superiority of four-in-one antibodies was also observed. Although the combination of two-in-one antibodies to block EGFR, HER2, and HER3 can also inhibit such crosstalk, four-in-one antibodies have the unique advantage of low dose.

Direct oncogenic-signal stress through competing with natural ligands of HERs and inducing blockade of the HER signal pathway, and antibody-dependent cellular cytotoxicity (ADCC) effect mediated through the Fc portion of the antibody, are the two mechanisms that underlie the antitumor effect of anti-HER antibodies. However, in our in vivo data, xenografts were using immunodeficiency mice for human cancer cells, we cannot directly evaluate ADCC effect between four-in-one antibodies and bispecific antibodies. An evaluation of whether targeted antibody treatment of cancer could reduce tumor burden in syngeneic wild-type mice (47) is ongoing. Moreover, it is also interesting that whether other RTK crosstalks may involve in the HER receptors that can be disrupted by novel antibodies, especially recent evidence had shown that the RTK AXL may be a key mediator of cetuximab resistance (48), providing a rationale for the cetuximab usage that would raise the crosstalk between EGFR and other RTKs. However, a detailed mechanism needs further investigation. In addition, it is well known that binding of cetuximab to EGFR also resulted in induction of apoptosis, whereas HER2/HER3 signaling inhibits cell death through the PI3K–AKT–mTOR pathway. AKT includes three distinct enzymes, each of which is a member of the protein kinase family that is specific for serine/ threonine and that inhibits apoptosis. Nevertheless, trastuzumab alone did not efficiently induce cancer cell apoptosis, partly due to its weak effect on the HRG-dependent HER2/HER3 activation. However, combined therapy to block HER2/HER3 signaling, such as trastuzumab plus pertuzumab, is currently under investigation in clinical use. Thus, four-in-one antibodies, particularly the tetraspecific, tetravalent antibody, which combined anti-HER antibodies in a single antibody, would amplify the apoptosis effect. All in all, our data greatly support the hypothesis that simultaneously targeting more than one proliferation and survival signal or targeting a resistance mechanism will provide better clinical outcomes. The unique potential of FL518 and CRTb6 to target EGFR, HER2, HER3, and VEGF to interrupt MET/HER crosstalk and delay direct anti-HER drug resistance warrants their consideration as promising anticancer therapies in the clinic.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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