A Bispecific HER2 Targeting FynomAb with Superior Antitumor Activity and Novel Mode of Action

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Abstract

Upregulation of HER2 is a hallmark of 20% to 30% of invasive breast cancers, rendering this receptor an attractive target for cancer therapy. Although HER2 targeting agents have provided substantial clinical benefit as cancer therapeutics, there is a need for the development of new agents aiming at circumventing anti-HER2 resistance. Based on the approved antibody pertuzumab we have created a panel of bispecific FynomAbs which target two epitopes on HER2. FynomAbs are fusion proteins of an antibody and a Fyn SH3-derived binding protein. One bispecific FynomAb, COVA208, was characterized in detail and showed a remarkable ability to induce rapid HER2-internalization and apoptosis in vitro. Moreover, it elicited a strong inhibition of downstream HER2 signaling by reducing HER2, HER3 and EGFR levels in vitro and in vivo. Importantly, COVA208 demonstrated superior activity in four different xenograft models as compared to the approved antibodies trastuzumab and pertuzumab. The bispecific FynomAb COVA208 has the potential to enhance the clinical efficacy and expand the scope of HER2-directed therapies, and delineates a paradigm for designing a new class of antibody-based therapeutics for other receptor targets.
Introduction

Since the introduction of trastuzumab (Herceptin®) for the treatment of HER2 positive metastatic breast cancer in 1998, HER2 targeted therapies have transformed the clinical practice of HER2 positive tumor treatment. With the recent approval of pertuzumab (Perjeta®) (1), an antibody which binds to domain II of HER2 and blocks its dimerization (2, 3), and the antibody drug conjugate ado-trastuzumab emtansine (Kadcyla®) (4), multiple therapeutic options for HER2 positive breast cancer patients have become available. Still, the vast majority of patients with metastatic breast cancer relapse after treatment with the currently available HER2 targeted therapies, and there remains a significant medical need for better treatment regimens (5).

The observation that patients relapsing on trastuzumab treatment benefit from the addition of lapatinib, an EGFR/HER2 kinase inhibitor, was a first indication that HER2 blockade is neither complete nor durable with single agent treatments (6). Furthermore, several reported examples of synergistic activity of anti-HER2 antibody combinations in preclinical animal models support the concept of targeting HER2 with more than one therapeutic agent at the same time (7-10). In particular, the combination of trastuzumab and pertuzumab, the latter of which displayed no significant clinical activity as a single agent (11), showed impressive therapeutic activity in trastuzumab-resistant mouse tumor models (12) and prolonged overall survival in metastatic breast cancer patients (1). These observations prompted us to explore whether bispecific HER2 targeting Fynomer-antibody fusion proteins (FynomAbs) would allow for an even more effective treatment of HER2 positive tumors.

Fynomers are small 7 kDa globular proteins derived from the SH3 domain of the human Fyn kinase (Fyn SH3) that can be engineered to bind with antibody-like affinity and specificity to virtually any target of choice through random mutation of two loops (RT- and src-loop) on the surface of the Fyn SH3 domain (13-15). The Fyn SH3 domain is a particularly attractive scaffold for the generation of binding proteins since the derived Fynomers (i) achieve very
high expression levels, (ii) are monomeric and do not aggregate when stored in solution, (iii) are very stable (Tm ~70 °C), (iv) lack cysteine residues, and (v) are of human origin featuring a framework amino acid sequence completely conserved from mouse to man and are considered to have a low immunogenic potential (14). Importantly, Fynomers are ideal building blocks to generate multispecific Fynomer-antibody fusions (FynomAbs) which are obtained by fusion of Fynomers to any of the four antibody light- or heavy chain termini. The resulting FynomAbs can be produced with comparable yield as antibodies, and they have favorable biophysical and pharmacokinetic properties (the Fynomer technology is described in Supplementary Materials and references 13-15).

Here, we have fused the HER2-specific Fynomer C12 which targets an epitope in domain I of HER2, to all termini of pertuzumab to generate bispecific HER2 targeting FynomAbs (Fig. 1A). These constructs showed superior in vitro and in vivo activity as compared to pertuzumab or trastuzumab, while the simultaneous targeting of both epitopes on HER2 with a mixture of the monospecific agents did not result in superior activity. Interestingly, we found that the activity of the HER2 targeting FynomAbs was depending on the FynomAb architecture, i.e. the relative spatial arrangement of the binding sites of antibody and the Fynomer C12. Further investigations with the optimized FynomAb, COVA208, established that this molecule acts through a novel mode of action, i.e. it is capable of inducing apoptosis and rapid HER2 internalization, and leads to a broad and efficacious blockade of HER2 downstream signaling accompanied by a reduced level of HER3 and EGFR. The therapeutic potential of COVA208 has been demonstrated in four different HER2 mouse models, where COVA208 exhibited promising anti-tumor activity.
Materials & Methods

Cell lines and reagents

OE19 cell line was purchased from HPA cultures, all other cell lines from ATCC. KPL-4 cell line was a kind gift from Prof. Kurebayashi (Kawasaki Medical School, Kurashiki, Japan) (16). Cell lines were authenticated by frequent cell morphology analysis and FACS analysis of several surface markers. Chemicals were from Sigma except otherwise indicated, Trastuzumab (Herceptin®, Roche) was purchased from the pharmacy. Bispecific FynomAbs were transiently expressed in CHO cells and purified on protein A affinity chromatography columns.

In vitro cell assays: Viability, Apoptosis, Antibody-dependent cellular cytotoxicity (ADCC)

For viability assays, 5000 to 7000 cells/well were seeded in 96 well plates, and treated for 5 days. % viability relative to PBS control was determined by XTT (Roche).

Caspase assays were performed using a caspase-3/7 kit (Promega). 100 nM anti-HER2 agents were added to 45’000 NCI-N87 cells in 96-well plates, and incubated for 24, 48 or 144 h. Staurosporine (1 µM, Applichem) served as a positive control.

ADCC was measured by calcein-AM release on BT-474 cells, using human PBMCs as effector cells at an E/T ratio of 25:1 as described in Tai et al. (17).

Internalization assays

NCI-N87 cells were surface labeled on ice for 1 h with 100 nM anti-HER2 agent, followed by 5 h incubation at 37 °C. 1 µM geldanamycin served as a positive control. Cells were fixed, permeabilized, and antibody distribution analyzed using an anti-human IgG-Alexa488 conjugate (Invitrogen), followed by laser scanning microscopic analysis. Images were processed and merged using Image J (Fiji).
The amount of anti-HER2 agents which localized into distinct dots was quantified in 3D using the software Imaris 7.4.0 (Bitplane). The percentage of anti-HER2 agents present in dots was expressed using the formula:

\[
\% \text{ anti-HER2 agents in dots} = \left( \frac{\text{volume of dots}}{\text{volume of total anti-HER2 staining}} \right) \times 100
\]

**Immunoblot analysis of cell lysates**

5x10^5 MCF-7 cells were starved in medium without serum for 3 h, then treated with 40 µg/ml anti-HER2 agents for 1 h in 6-well plates. After 45 min, 2 nM human recombinant heregulin-1β (HRG-1β; R&D systems) was added for 15 min. 1x10^6 NCI-N87 were treated with 40 µg/ml anti-HER2 agents for 72 h in 6-well plates. Total cell lysate was analyzed by immunoblotting. Primary antibodies were purchased from Cell Signaling Technology, except anti-Vinculin, anti-HER3 (both Millipore) and anti-EGFRY1172 antibodies (Abcam).

**In vivo tumor xenograft and pharmacokinetic studies**

Mouse experiments conducted at Covagen AG were performed in accordance with Swiss guidelines and were approved by the Veterinarian Office of Zürich, Switzerland. For pharmacokinetic analysis, 200 µg anti-HER2 agent was injected i.v. into C57BL/6 mice. Serum concentrations after 10 min, 6, 24, 48, 96, 120, 144 and 168 hours were determined by ELISA on immobilized HER2 (18).

KPL-4 and NCI-N87 tumors were enoculated as described previously (7, 12). GXA-3039 (gastric carcinoma) and CXF-1991 (colon carcinoma) patient-derived tumor models were conducted by Oncotest in Freiburg, Germany. At 50 - 250 mm³ tumor volume, 3 week treatment was initiated (12). For statistical analysis, relative tumor volumes (RTV) were compared on the day of optimal tumor-to-control ratio (T/C) observed for the COVA208 therapy group. Statistical significance was tested using the non-parametric Mann-Whitney
test for pairwise comparisons (GraphPad Prism 6, version 6.0a), and p-values adjusted for multiple comparisons using Holm-Bonferroni (19).

For pharmacodynamic analysis, 5 animals from each GXA-3039 treatment group were re-dosed with 30 mg/kg anti-HER2 compound 48 h before termination. Tumors were harvested and analyzed by immunoblot (20). Statistical significance was tested with the non-parametric Kruskal-Wallis test using Dunn’s correction for multiple comparison (GraphPad Prism 6, version 6.0a).
Results

**Bispecific anti-HER2 targeting FynomAbs suppress tumor cell growth in vitro with high potency.**

The HER2-specific Fynomer C12 is a 63 amino acid polypeptide derived from the SH3 domain of the human Fyn kinase (Fyn SH3). Using recombinant HER2 (extracellular domain, ECD) as target antigen, Fynomer C12 was isolated by phage display from a repertoire of mutated Fyn SH3-derived domains (14, 15) in which selected residues located within or in close proximity of either the RT-loop or the src-loop were randomized to one of the twenty natural amino acids ([Supplementary Fig. S1A](#)). Fynomer C12 was further characterized and showed specific binding to HER2 (recombinant and cell-surface expressed HER2), high stability (T_M = 74 °C) and was found to be produced at high levels (>100 mg/l) in *E.coli* shake flask cultures under non-optimized conditions ([Supplementary Fig. S1B-D](#)). Using alanine scanning mutagenesis, the binding site of Fynomer C12 on HER2 was mapped to five amino acid residues on the surface of domain I, representing an epitope distinct from pertuzumab within the dimerization domain II (3) or from trastuzumab within domain IV (21) ([Supplementary Fig. S1E](#)). As expected from the binding site mapping studies, Fynomer C12 is neither blocked by pertuzumab nor trastuzumab in competitive cell binding assays, thus confirming that Fynomer C12 binds to a different epitope ([Supplementary Fig. S1F](#)).

We constructed HER2 targeting bispecific FynomAbs by genetically fusing Fynomer C12 to each of the four termini of pertuzumab, thereby generating FynomAbs with different architectures due to the different relative orientation of the two binding specificities ([Fig. 1A](#)). All four C12-pertuzumab FynomAbs termed COVA201, COVA202, COVA207 and COVA208 could be purified as described in Materials & Methods at comparable yields as the parental antibody pertuzumab (in the range of 100 mg/l from CHO cell supernatant). The FynomAbs were capable of engaging both the pertuzumab as well as the C12 epitope on HER2 overexpressing cells with similar efficacy ([Supplementary Fig. S2A](#)). In addition, all
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four FynomAbs showed improved binding to HER2 overexpressing cells with EC_{50} values improved by a factor 4.2 – 7.9 as compared to pertuzumab (Supplementary Fig. S2B). This was in agreement with binding dissociation constants (K_D) determined by BIACore, which were 7- to 60- fold lower than for pertuzumab (Supplementary Fig. S2C).

The bispecific FynomAbs were tested for cell growth inhibition of HER2 overexpressing NCI-N87 gastric carcinoma cells in vitro (Fig. 1B). Even though targeting the C12 epitope on HER2 with a bivalent Fc-C12 fusion protein did not have any effect and did not synergize with pertuzumab when used in combination, the bispecific FynomAbs potently inhibited tumor cell growth with a maximal reduction of relative cell viability of 39 % – 82 % as compared to 23 % observed for pertuzumab (Fig. 1B). The greatest effect was seen with COVA208 in which the Fynomer C12 was fused to the N-terminus of the pertuzumab light chain. In order to establish whether the superior in vitro activity of COVA208 seen on NCI-N87 cells translates into superior anti-tumor activity in vivo, we compared the anti-tumor activity of COVA208 and pertuzumab in vivo in a NCI-N87 tumor xenograft therapy study. COVA208 led to rapid tumor regression which was not achieved by pertuzumab (p < 0.0001; Fig 2A). Importantly, COVA208 and pertuzumab had identical PK profiles and similar serum half life (244 h for COVA208 vs 187 h for pertuzumab, Fig 2B). Also, antibody-dependent cellular cytotoxicity was identical for COVA208 and pertuzumab (Fig 2C). In addition, COVA208 maintained the favorable biophysical properties of pertuzumab such as long-term stability and monomeric aggregation state (data not shown) and potently suppressed growth of additional cell lines, including OE19 oesophageal cancer, Calu-3 lung cancer, MDA-MB-175 VII breast cancer and BT-474 breast cancer cells, the latter being highly sensitive to trastuzumab (Supplementary Fig. S3).
COVA208 is unique in its ability to induce apoptosis and rapid internalization of HER2 on the NCI-N87 cell line

We then focused on COVA208 to elucidate its mode of action and its effect on tumor cell biology and HER2 signaling. Apoptosis was investigated in NCI-N87 cells by Caspase 3/7 activity assay and by Tunel staining. Upon exposure to COVA208 in vitro, NCI-N87 cells markedly upregulated Caspase-3/7 activity in a dose-dependent fashion (Fig. 3A, and Supplementary Fig. S4A). The activity was clearly above background after 2 days and further increased after 6 days of incubation, which was not observed with trastuzumab or pertuzumab. Around 65% of NCI-N87 cells contained fragmented DNA and were Tunel positive within 3 days after addition of COVA208 (Supplementary Fig. S4B). Addition of soluble HER2 ECD interfered with induction of apoptosis (data not shown), supporting that it is a specific effect of the COVA208–HER2 interaction. Thus, COVA208 potently induces apoptosis in NCI-N87 cells.

To investigate whether COVA208 is able to induce rapid internalization, we surface labeled NCI-N87 cells with COVA208, pertuzumab or trastuzumab, and tracked the distribution of the anti-HER2 agents with a fluorescently labeled detection antibody using confocal laser scanning microscopy (Fig. 3B,C). After 5 hours, pertuzumab and trastuzumab remained confined to the cell membrane and showed minimal cell uptake (9.5% or 10%, respectively). In contrast, 52% of COVA208 relocalized to the intracellular area, appearing in a punctate pattern typical of internalized material and resembling the pattern of the positive control pertuzumab plus geldanamycin, a Hsp90 inhibitor which leads to rapid internalization and degradation of HER2 (22). These observations confirm that COVA208 efficiently induces apoptosis and internalizes rapidly into cells, suggesting a novel mode of action.

COVA208 inhibits ligand-induced as well as ligand-independent HER2 downstream signaling

Trastuzumab inhibits ligand-independent activation occurring in HER2 overexpressing tumor cells (23). In contrast, pertuzumab but not trastuzumab is capable of blocking ligand-induced
activation (2). We analyzed the effect of COVA208 on HER2 downstream signaling by focusing on key nodes of the HER3-PI3K-AKT and MAPK pathways. MCF-7 cells in the presence of the HER3 ligand heregulin-1β (HRG-1β) was used as model for ligand-induced activation, and NCI-N87 cells were used as model for ligand-independent activation.

COVA208 retained the activity of the parental antibody pertuzumab and efficiently prevented HRG-1β-induced phosphorylation of HER3, AKT and ERK in MCF-7 cells, whereas trastuzumab did not show any inhibitory activity, as expected (Fig. 4A). In NCI-N87 cells, COVA208 reduced the level of basal phosphorylated HER3 (pHER3) and pAKT similar to trastuzumab, while pertuzumab did not have any effect (Fig. 4B). Strikingly, COVA208 treated cells displayed a pronounced reduction of total HER3 levels. Thus, in addition to retaining pertuzumab’s ability to inhibit ligand-induced signaling, COVA208 was found to inhibit ligand-independent signaling similar to trastuzumab. However, the mechanism leading to this effect involves downregulation of total HER3 levels and thus is different from trastuzumab.

**COVA208 elicits a more complete HER2 signaling inhibition as compared to trastuzumab and pertuzumab**

In order to identify additional HER2 signaling mediators that are specifically modulated by COVA208, lysates of treated NCI-N87 cells were analyzed by reverse phase protein array (RPPA) for differences in 167 phosphoprotein or total protein levels (24). COVA208 clustered separately from trastuzumab and pertuzumab on a two-dimensional heat map, indicating that the effect of COVA208 on NCI-N87 cells is distinct from the effect achieved with conventional anti-HER2 antibodies (Supplementary Fig. S5). Out of the 167 proteins analyzed, 22 proteins (13.1%) were found to be modulated, (log2 change > 0.5 compared to PBS) by COVA208, trastuzumab or pertuzumab, of which 15 proteins (68%) were specifically modulated by COVA208 but neither by trastuzumab nor pertuzumab (Supplementary Table 1). RPPA data suggested that COVA208 induced downmodulation of HER2, phosphorylated EGFR, FOXM1, CDC2 (CDK1), cyclin D1 and phosphorylated RB1.
In addition, it prevented upregulation of STAT3, JNK (MAPK18) and P38 (MAPK14) as observed with trastuzumab and pertuzumab. Furthermore, phosphorylated SRC levels were slightly reduced by COVA208.

NCI-N87 lysates were analyzed by immunoblot to confirm these findings. Firstly, COVA208 specifically reduced HER2 and pHER2 protein, the latter being at least in part a consequence of reduced HER2 levels (Fig. 4C). Furthermore, COVA208 strongly reduced EGFR and pEGFR levels which remained unaffected by trastuzumab or pertuzumab. Finally, COVA208 reduced pSRC, FOXM1 and CDC2, and prevented upregulation of pSTAT3, which are all oncogenic proteins or are described to be involved in tumor progression and resistance (25-28). In summary, COVA208 inhibits HER2 downstream signaling in a more complete and more efficacious manner as compared to the anti-HER2 antibodies trastuzumab and pertuzumab.

COVA208 inhibits tumor growth in different HER2 overexpressing xenograft models and downregulates HER2 and HER3 in vivo.

In vivo efficacy of COVA208 was tested in additional HER2 overexpressing xenograft models of breast, gastric and colorectal cancers. The breast carcinoma KPL-4 model carries an activating PI3KCA mutation associated with trastuzumab resistance which constitutively activates the PI3K pathway (12, 23, 29-31). The gastric GXA-3039 model and the colorectal CXF-1991 model are patient-derived human tumor xenografts which are passaged subcutaneously in nude mice. As a consequence, these xenografts retain most of the characteristics of the parental patient tumors such as gene expression profiles, histology and sensitivity to anti-cancer drugs.

In the KPL-4 model, trastuzumab and pertuzumab had only moderate activity on the growth of established xenograft tumors (Fig. 5A), while COVA208 strongly inhibited tumor growth (p-value = 0.0009) and showed significantly better anti-tumor activity than pertuzumab or trastuzumab (p-value = 0.0279 and 0.0015, respectively). COVA208 was also highly active in the GXA-3039 model (p-value = 0.0136) and was clearly superior to pertuzumab (p-value =
0.0279) and trastuzumab (p-value = 0.0015; Fig 5B). Finally, COVA208 showed improved tumor growth inhibition compared to pertuzumab and trastuzumab in the CXF-1991 model and resulted in a significant tumor growth inhibition (p-value = 0.0045, Fig. 5C). Thus, we observed significant in vivo activity of COVA208 in three patient-derived tumor models in which trastuzumab or pertuzumab showed no or little activity.

To establish whether HER2, HER3 or EGFR downregulation observed in vitro may contribute to the in vivo activity of COVA208, five GXA-3039 tumors from each treatment group were re-treated 48 hours before the treatment groups were intended to be terminated. Tumors were collected and lysates were analyzed by immunoblot (Fig. 6). HER2 and HER3 protein levels were reduced in COVA208 treated tumors, whereas pertuzumab and trastuzumab had no or only a weak effect. This reduction correlated with lower pHER2, pHER3 and pAKT levels. EGFR levels and consequently pEGFR levels were also reduced by COVA208, but the difference was only weak. Hence, COVA208 leads to a reduction in total and phosphorylated HER family receptors, and resulted in decreased pAKT levels in vivo.
Discussion

The widespread use of HER2 targeted therapies has improved the treatment outcome of breast and gastric carcinoma patients in the clinic. Still, the development of resistance to these therapies limits their clinical efficacy and urges the development of improved HER2 targeted therapies. By fusing the small 7 kDa anti-HER2 Fynomer C12 to the clinically validated antibody pertuzumab, we generated a bispecific HER2 targeting FynomAb with a novel mode of action and superior activity as compared to the parental antibody pertuzumab and also to trastuzumab, the antibody which forms the backbone of today’s clinical management of HER2 positive malignancies. COVA208 was highly active in the KPL-4 xenograft model, a tumor model with an activating PI3KCA mutation which is associated with trastuzumab resistance in the clinic, as well as in the gastric GXA 3039 model, where trastuzumab showed no anti-tumor effect (30, 31).

The \textit{in vivo} activity of COVA208 may be explained by its unique effect on HER2 trafficking and HER2 downstream signaling. COVA208 induced rapid HER2 internalization \textit{in vitro} and reduced HER2 levels \textit{in vitro} and \textit{in vivo}. In addition, COVA208 treatment was accompanied by a reduction in pHER2 levels \textit{in vitro} and \textit{in vivo}, suggesting that COVA208, unlike pertuzumab or trastuzumab, reduces phosphorylated HER2 levels and consequently the activation of oncogenic downstream signaling. This is exemplified by the reduction in pAKT signal \textit{in vitro} and \textit{in vivo}, and of pSRC, FOXM1, CDC2 and STAT3 \textit{in vitro}. This finding is of importance since it is known from other receptors that endocytosis does not necessarily block the capability of receptors to participate in active signaling complexes (32).

In addition, we have found that COVA208 reduced HER3 and pHER3 surface levels \textit{in vitro} and \textit{in vivo}. In light of the importance of HER3 to propagate HER2 mediated signaling (33, 34) and of the mounting evidence that HER3 is involved in resistance to HER2 directed therapies, possibly through the interruption of a negative AKT-HER3 feedback loop (35), this observation offers the potential that COVA208 may prevent HER3-mediated resistance.
Similarly, COVA208 treatment resulted in reduced EGFR levels \textit{in vitro}, which may prevent resistance through a transition from HER2 to EGFR-dependence of HER2 overexpressing tumors (36, 37). The effect of the broad and efficient inhibition of HER2 downstream signaling which transmits pro-survival signals via the HER3-PI3K-AKT axis (38) is further illustrated by the observation that in contrast to pertuzumab or trastuzumab, COVA208 is a potent inducer of apoptosis and strongly inhibited tumor cell growth \textit{in vitro}.

Under physiological conditions, several studies found that HER2 internalizes rather slowly, and monoclonal anti-HER2 agents have little or no effect on internalization (39, 40), even though Lub-de Hooge et al showed that targeting HER2 with trastuzumab (carrying a residualizing \textsuperscript{111}Indium label) may internalize efficiently (41). This contradiction could be explained by the rapid degradation and leakage of catabolites when using non-residualizing labels. Importantly, synergistic activity and accelerated internalization induced by anti-HER2 antibody combinations have previously been established in several studies (7-10). This observation was attributed to extensive receptor cross-linking producing large receptor clusters on the cell surface that are targeted for degradation. Due to the observed rapid internalization of COVA208 we speculate that the formation of HER2 clusters is an important underlying mechanism by which COVA208 exerts its activity. However, the Fynomer C12 and the pertuzumab paratope could in principle bind to the same receptor, thereby constraining the receptor in an inactive conformation. We cannot exclude that both binding mechanisms take place and contribute to the activity of COVA208.

More recently, two groups reported on the \textit{in vitro} and \textit{in vivo} activity of bispecific HER2-targeting agents (37, 42). To our knowledge, COVA208 is the first bispecific HER2 targeting agent which showed promising activity in patient-derived trastuzumab-resistant tumor models, highlighting the therapeutic potential of COVA208 in metastatic breast cancer. Whereas most of the previous studies showed antibody-mediated downmodulation of HER2 \textit{in vitro}, we present a comprehensive analysis of the impact of COVA208-induced HER2
downmodulation on downstream signaling and apoptosis and compare it with the clinically validated antibodies trastuzumab and pertuzumab.

Based on our findings, we speculate that multi-epitope targeting of other disease-associated cell surface receptors with reagents such as designed bispecific FynomAbs is capable of modulating molecular processes such as receptor trafficking, downstream signaling or induction of apoptosis in a novel, unique manner and may result in superior anti-cancer therapeutics.

The study presented here also has important implications for the discovery of bi- or multispecific antibodies. Even though the four C12-pertuzumab FynomAbs were essentially indistinguishable in binding to cell surface HER2, we found that the in vitro activity of the bispecific FynomAbs was different and influenced by the FynomAb architecture, thus depending on the position at which the Fynomer was fused to the antibody. This is likely due to the different FynomAb architectures which lead to different geometries and/or stoichiometries of FynomAb-HER2 complexes. The importance of the molecular architecture has also been shown with monospecific trastuzumab derivatives bearing non-natural Fab arm arrangement (43) as well as for other EGFR-specific arms (44-46). We also found that the activity of bispecific reagents cannot be predicted from single agent activities. Together, these observations suggest the investigation of bispecific molecules with different architectures to discover optimal lead candidates.

In summary, we have shown that the fusion of a small Fynomer to a clinically validated antibody can dramatically improve the therapeutic activity of this antibody. Such FynomAbs represent a novel approach to convert existing antibodies into bispecific therapeutic agents with superior efficacy. The anti-HER2 FynomAb COVA208 is a highly active HER2 targeting agent with a novel, differentiated mode of action, which results in a strong anti-tumor effect in vitro and in vivo in models resistant to conventional anti-HER2 antibodies. COVA208 treatment leads to a qualitatively distinct molecular response from those of
trastuzumab or pertuzumab: COVA208 i) elicits rapid internalization of HER2, ii) induces apoptosis and iii) more effectively inhibits HER2 signaling than pertuzumab or trastuzumab.

We thus believe that COVA208 is a promising HER2 targeting agent for further development in metastatic breast cancer.
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**Figure Legends**

**Figure 1:** C12-pertuzumab FynomAbs show architecture-dependent activity and are superior to pertuzumab in vitro. (A) Schematic structure of the four different C12-pertuzumab FynomAb architectures COVA201, COVA202, COVA207 and COVA208 which are obtained by fusing Fynomer C12 (blue spheres) to the C- or N-termini of the antibody heavy and light chain, respectively. (B) *In vitro* XTT viability assay on NCI-N87 gastric tumor cells. Dose response curves are plotted as mean % viability (n = 3) ± SD relative to PBS treated cells on the y-axis, and concentration on the x-axis.

**Figure 2:** COVA208 is superior to pertuzumab in vivo. (A) Tumor growth inhibition was assessed in mice bearing established NCI-N87 tumor xenografts. COVA208 (red triangles) and pertuzumab (dark-blue circles) were injected intraperitoneally (i.p.) at 30 mg/kg loading dose followed by three weekly 15 mg/kg maintenance doses. Average relative tumor volumes (n = 8) ± SEM are presented. Statistical analysis was performed with Mann-Whitney U test (**** p-value < 0.0001). (B) Serum concentrations of COVA208 and pertuzumab at different time points after a single i.v. injection into C57Bl/6 mice. Mean serum concentrations are plotted versus time, error bars represent standard deviations (n=5). The six last time points were used to calculate the terminal half-life of COVA208 (244 h) and pertuzumab (187 h). (C) *In vitro* ADCC assay on BT-474 cells with COVA208, pertuzumab, trastuzumab and an isotype control. The mean % cytotoxicity (n=3) was plotted against the antibody concentration. Error bars represent standard deviation.

**Figure 3:** COVA208 but not pertuzumab or trastuzumab induces apoptosis and rapid internalization of HER2 in NCI-N87 cells in vitro. (A) Induction of apoptosis determined
by Caspase 3/7 activity. 100 nM COVA208, pertuzumab and trastuzumab were incubated for one, two, or six days. Staurosporine was added for two days as positive control. Caspase 3/7 activity was determined using a fluorescent enzymatic assay, and the mean activity (n=3) ± SD was plotted. (B) NCI-N87 gastric tumor cells were surface-labeled with pertuzumab, trastuzumab or COVA208 at 4 °C, followed by incubation at 37 °C for 0 h or 5 h to allow for internalization. As a positive control, 1 µM geldanamycin was added to pertuzumab-labeled cells. After cell fixation and permeabilization, anti-HER2 agents were detected with a fluorescently labeled antibody directed against human IgG1, cell nuclei were stained with Hoechst dye and images acquired by confocal laser scanning microscopy. Images are shown in two different magnifications, the larger of which shows a five-fold digital zoom of the region boxed in the smaller magnification. Scale bars represent 10 μm. (C) Quantification of the dot-like structures after 5 h incubation with anti-HER2 agents at 37 °C. Optical sections were collected and four 3D regions per condition were analyzed. The amount of anti-HER2 agent that localized into distinct dots was quantified with the software Imaris 7.4.0 (Bitplane), using the surface tool of Imaris to detected spheroid dots. Error bars represent SD.

Figure 4: COVA208 inhibits ligand-induced as well as ligand-independent activation of HER2 downstream signaling. (A) MCF-7 cells were treated with 40 µg/mL of the indicated HER2-targeting agents for 1 h. After 45 min incubation, HRG-1ß was added for 15 min to induce ligand-dependent HER2 signaling. Total cell lysates were immunoblotted for the specified phosphoprotein or total protein levels. (B, C) NCI-N87 tumor cells were treated with 40 µg/mL of the indicated HER2-targeting agents for 72 hrs. Total cell lysates were analyzed by immunoblot for the specified phosphoprotein or total protein levels. Vinculin served as loading control in (A) – (C).

Figure 5: COVA208 promotes superior anti-tumor activity in vivo. The activity of COVA208 was compared to pertuzumab and trastuzumab in mouse models of breast (KPL-
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4), gastric (GXA-3039) and colon (CXF-1991) carcinoma. Mice bearing KPL-4 (A), GXA-3039 (B) and CXF-1991 tumors (C) were treated intraperitoneally (i.p.) with 30 mg/kg loading and three subsequent 15 mg/kg maintenance doses of COVA208 (red triangles), pertuzumab (dark-blue circles) and trastuzumab (light-blue diamonds) administered once per week. Average relative tumor volumes (KPL-4: n = 8; GXA-3039 and CXF-1991: n =10 except for pertuzumab in GXA-3039 and trastuzumab in CXF-1991 study (n=9)) ± SEM are presented. For statistical analysis, all treatment groups were compared to COVA208 using Mann-Whitney U test followed by Holm-Bonferroni correction of p-values (*** p-value ≤ 0.001; ** p-value ≤ 0.01; * p-value ≤ 0.05).

Figure 6: COVA208 reduces HER2 and HER3 levels in vivo. (A) Immunoblots assessing the impact of COVA208 on HER family members and on downstream pAKT in human patient-derived GXA-3039 gastric tumor xenografts in vivo. Total tumor lysates were immunoblotted for phosphoprotein and for total protein levels as specified. Vinculin served as loading control. (B) Quantification of the immunoblot bands shown in (A). Every dot represents the signal intensity relative to the vinculin signal for one tumor. Mean (n=5) and SEM are indicated by whiskers. (* p-value = 0.0232 for both comparisons).
Figure 1

A

COVA201  COVA202  COVA207  COVA208

B

![Graph showing relative viability vs. concentration for different treatments.](image)

- PBS
- Fc-C12
- Fc-C12 + pertuzumab
- Pertuzumab
- Trastuzumab
- COVA201
- COVA202
- COVA207
- COVA208

Relative viability (XTT) vs. Concentration (nM)
Figure 3
Figure 4
Figure 5

(A) KPL-4 (breast carcinoma) with relative tumor volume over days, showing the effect of the indicated doses. (B) GXA-3039 (gastric carcinoma) with relative tumor volume over days. (C) CXF-1991 (colorectal carcinoma) with relative tumor volume over days.

Legend:
- PBS
- trastuzumab
- pertuzumab
- COVA208

Dose (mg/kg): 30, 15, 15, 15.
Molecular Cancer Therapeutics

A Bispecific HER2 Targeting FynomAb with Superior Antitumor Activity and Novel Mode of Action

Simon Brack, Isabella Attinger-Toller, Babette Schade, et al.

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