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Engineered Multivalency Enhances Affibody-Based HER3 Inhibition and Downregulation in Cancer Cells

John S. Schardt[†], Jinan M. Oubaid[†], Sonya C. Williams[†], James L. Howard[†], Chloe M. Aloimonos[†], Michelle L. Bookstaver[†], Tek N. Lamichhane[†], Sonja Sokic[†], Mariya S. Liyasova[‡], Maura O'Neill[§], Thorkell Andresson[§], Arif Hussain^{||,⊥}, Stanley Lipkowitz[‡], and Steven M. Jay^{*,†,⊥,#}

[†]Fischell Department of Bioengineering, University of Maryland, College Park, Maryland 20742, United States

[‡]Women's Malignancies Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, United States

[§]Protein Characterization Laboratory, Frederick National Laboratory for Cancer Research, Frederick, Maryland 21702, United States

^{||}Baltimore VA Medical Center, Baltimore, Maryland 21201, United States

[⊥]Marlene and Stewart Greenebaum Comprehensive Cancer Center, University of Maryland School of Medicine, Baltimore, Maryland 21201, United States

[#]Program in Molecular and Cellular Biology, University of Maryland, College Park, Maryland 20742, United States

Abstract

The receptor tyrosine kinase HER3 has emerged as a therapeutic target in ovarian, prostate, breast, lung, and other cancers due to its ability to potently activate the PI3K/Akt pathway, especially via dimerization with HER2, as well as for its role in mediating drug resistance. Enhanced efficacy of HER3-targeted therapeutics would therefore benefit a wide range of patients. This study evaluated the potential of multivalent presentation, through protein engineering, to enhance the effectiveness of HER3-targeted affibodies as alternatives to monoclonal antibody therapeutics. Assessment of multivalent affibodies on a variety of cancer cell lines revealed their broad ability to improve inhibition of Neuregulin (NRG)-induced HER3 and Akt phosphorylation compared to monovalent analogues. Engineered multivalency also promoted enhanced cancer cell growth inhibition by affibodies as single agents and as part of combination therapy approaches. Mechanistic

*Corresponding Author: smjay@umd.edu.

ORCID: Steven M. Jay: 0000-0002-3827-5988

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Supporting Information

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DNA and protein sequences for affibody constructs, purified affibody mass spectra, surface plasmon resonance data, and immunoblot quantification (PDF)

investigations revealed that engineered multivalency enhanced affibody-mediated HER3 downregulation in multiple cancer cell types. Overall, these results highlight the promise of engineered multivalency as a general strategy for enhanced efficacy of HER3-targeted therapeutics against a variety of cancers.

Graphical Abstract

Keywords

HER3; multivalent; affibody; cancer; antibody

INTRODUCTION

HER3, a member of the ErbB receptor tyrosine kinase (RTK) family, which also includes epidermal growth factor receptor (EGFR), HER2, and HER4,¹ has emerged as a therapeutic target in ovarian, prostate, breast, lung, and other cancer subtypes.² HER3 is unique in the ErbB family in that it has a weak tyrosine kinase domain,^{1,3} necessitating its partnership with other ErbB members for downstream signal transduction. Despite this, HER3 is a key node in the ErbB network, characteristically dimerizing with HER2 or other partner receptors (e.g., c-MET, EGFR) to mediate cancer progression and/or metastasis.⁴ Structurally, HER3 contains six phosphotyrosine sites in its C-terminal tail,^{5–7} allowing for potent mitogenic signaling through the PI3K/Akt pathway. Further, HER3 activation can enable acquired resistance to cancer therapeutics, including ErbB-targeted drugs.^{8–13}

Numerous monoclonal antibody (mAb) approaches directed at HER3 are in development,¹⁴ and it is probable that some of these will continue to emerge as effective therapeutics, either alone or in combination with other drugs. However, these approaches are still likely to be ineffective in a significant proportion of patients; for example, FDA-approved HER2-targeted therapies have been reported to show efficacy in ~24–64% of patients with HER2-overexpressing tumors.¹⁵ Thus, development of alternative therapeutic approaches against HER3 as part of antitumor targeting strategies, particularly where HER3 acts as a facilitator and modulator of oncogenic signaling cascades, would fill a critical clinical need.

Toward meeting this need, one promising concept for improved design of pharmaceuticals is engineered multivalency, which can be achieved through chemical biology and protein engineering approaches. Multivalency refers to the phenomenon by which a single molecule can be involved in multiple simultaneous molecular recognition events, which is

hypothesized to enhance therapeutic efficacy by means of improved avidity, residence time, selectivity, and differential receptor trafficking.^{16–20} The therapeutic potential of multivalent ligands against HER3 specifically is supported by the possibility of inducing HER3 sequestration by engaging HER3 into non-signaling homotypic interactions. This phenomenon may be promoted by the tendency of HER3 to exist predominantly in homotypic clusters on the cell surface prior to ligand stimulation.²¹ Our previous work demonstrated the therapeutic potential of multivalent HER3 engagement by using an engineered bivalent Neuregulin-1 β (NRG) molecule, which induced decreased proliferation and chemokinesis and increased apoptosis in multiple cancer cell lines.^{22,23} However, employing NRG as a HER3-binding domain is problematic from a translational perspective, as it could activate HER3 to induce pro-neoplastic signaling.^{24–26} The therapeutic potential of bivalent NRG is further limited by potential off-target effects on HER4 as well as challenges in protein production due to aggregation caused by sequence repetition and improper formation of multiple disulfide bonds.

Thus, in this work, we sought to extend the concept of multivalent HER3 engagement by employing HER3-binding domains with greater translational potential and lower risk of pro-neoplastic side effects. Utilizing the Z₀₅₄₁₃ affibody, a 58 amino acid blocking peptide with subnanomolar affinity to HER3 developed by Lofblom and colleagues,²⁷ as the HER3 binding domain, we developed novel multivalent HER3 ligands and evaluated their functional efficacy in multiple cancer cell lines in comparison to monovalent molecules. We further investigated the relevance of the specific molecular conformation employed as well as the mechanisms contributing to the efficacy of the multivalent ligands. Our results indicate that multivalency enhances HER3 signaling inhibition and induces rapid downregulation of the receptor by affibody molecules, further establishing multivalent engagement of HER3 as a promising therapeutic strategy.

EXPERIMENTAL SECTION

Protein Production and Purification

Coding DNA sequences for affibody constructs were inserted into the pET45b vector (Merck Millipore), and Q5 directed mutagenesis (New England Biolabs) was used to generate bivalent affibodies with varying domain size. pET45b-affibody constructs were transformed into *E. coli* strain B121 (DE3) (New England Biolabs). For protein expression, 500 mL of LB broth with 100 μ g/mL ampicillin was inoculated from a 3 mL overnight starter culture, incubated at 37 °C, shaking at 220 rpm, grown to an OD₆₀₀ of 0.6–0.8, induced with 0.4 μ M IPTG, and grown for 4 h at 30 °C. The cell pellet was isolated by centrifugation at 6000 rpm for 15 min and frozen at –20 °C. Soluble protein lysates were generated using B-PER complete protein extraction reagent (Thermo Fisher Scientific) following manufacturer's protocol and purified by immobilized metal affinity chromatography using TALON metal affinity resin (Clontech), and refined/buffer exchanged in PBS using ENrich SEC 650 size-exclusion (Bio-Rad) high-performance liquid chromatography NGC Quest 10 Plus system (Bio-Rad).

Protein Molecular Weight and Sequence Identity Determination by Mass Spectrometry

For molecular weight determination, monovalent, bivalent, and trivalent affibodies were mixed and then separated with a ProSwift RP-4H monolithic column. Trivalent affibody spectrum was further analyzed with poroshell C3 microbore column. Mass spectra were acquired with a Thermo Scientific Orbitrap Fusion Lumos mass spectrometer at $R = 120,000$ (m/z 200). Protein molecular weight was calculated using XTract. For sequence identity determination, monovalent, bivalent, and trivalent affibodies were diluted with 0.1 M triethylaminebicarbonate (TEAB) and digested with trypsin/LysC Mix (Promega V5073) at 35 °C overnight. Digest was acidified with glacial acetic acid and analyzed by NanoLC-MS/MS using a trap column (Thermo Scientific Acclaim PepMap 100, 5 μ m, 100 Å, 300 μ m $\times$ 5 mm) and an analytical column (Thermo Scientific Acclaim PepMap 100, 3 μ m, 100 Å, 75 μ m $\times$ 250 mm). Data were processed using Proteome Discoverer (v 2.1) and Sequest HT.

HER3 Binding Affinity Determination by Surface Plasmon Resonance

Interactions between the affibodies and HER3 were analyzed by surface plasmon resonance using a Biacore T200 system (GE Healthcare). HER3 (Human, His-tagged; Sino Biological) at 50 μ g/mL in acetate 4.5 was covalently immobilized on a CM5 chip via amine coupling to a final response of 1600 RU in 25 mM Hepes pH 7.3 at 25 °C. Affibody samples were prepared in degassed, filtered HBS-P+ buffer (GE Healthcare). Single cycle kinetic experiments were carried out using five injections (30 μ L/min) of increasing concentration of affibodies (0.1–10 nM) passed over the sensor chip for 120 s association followed by 420 s dissociation. Following buffer and reference subtraction, kinetic constants and binding affinities were determined utilizing the Biacore T200 evaluation software (GE Healthcare).

Cell Lines and Reagents

The OvCAR8 cell line was obtained from Dr. Christina Annunziata (National Cancer Institute). Du145, BT474, and H1975 cell lines were purchased from ATCC. OvCAR8, Du145, and H1975 were maintained in RPMI 1640 (ATCC), and BT474 cells were maintained in Hybri-Care Medium (ATCC).

Cell Signaling Studies

Cells were serum starved for 4 h, treated with the indicated concentrations of affibody or the small molecule pan-HER kinase inhibitor *N*-(4-((3-chloro-4-fluorophenyl)amino)pyrido[3,4-*d*]pyrimidin-6-yl)2-butyramide (Millipore 324840) (Total Inhibitor) for 30 min, stimulated with 10 nM human heregulin-1 β (NRG) (Peprotech) for 10 min, lysed, and probed by immunoblot for pHER3, pHER2, pAkt, and pERK1/2. For immunoblotting, membranes were probed with primary antibodies (21D3 pHER3, tyr877 pHER2, D9E pAkt, pERK1/2 #9101, 13E5 β -actin, and D65A4 β -tubulin) all from Cell Signaling, and secondary antibody IRDye 800 CW (Licor), and scanned using an Odyssey CLx image system (Licor).

Cell Proliferation Assays

OvCAR8, Du145, and H1975 cells were seeded at 9000 cells per well, and BT474 cells were seeded at 80 000 cells per well in media supplemented with 2% FBS, 1% Pen-Strep, and 200 pM NRG in a 24-well plate (VWR 10062-900), treated with the indicated

concentrations of affibody, PI3K inhibitor NVP-BKM120, combination, or no treatment control (media alone), incubated in 5% CO₂ at 37°C for 5 days, and analyzed using Alamar Blue (Bio-Rad) following the manufacturer's protocol.

HER3 Downregulation Assays

To examine HER3 levels, cells were treated with the indicated concentrations of affibody, pan-HER kinase inhibitor (Millipore 324840), or no treatment (media alone) control, lysed at the indicated time points, and probed by immunoblot for HER3 as previously described using primary antibodies (D22C5 HER3 and 13E5 β -actin). To investigate the mechanism of HER3 downregulation, a cycloheximide pulse-chase experiment was performed where cells were treated with 10 nM affibodies, 178 μ M cycloheximide (Cell Signaling 2112), or the combination, lysed at the indicated time points, and probed by immunoblot. For HER3 degradation studies, cells were pretreated with 200 μ M leupeptin (Sigma) or 5 μ M MG-132 (Sigma) for 3 h, treated with 10 nM affibodies, 200 μ M leupeptin (Sigma), 5 μ M MG-132 (Sigma), or combinations of affibody plus inhibitor, lysed at the indicated time points, and probed by immunoblot.

RESULTS

Engineered Multivalency Enhances Affibody-Induced pHER3 Inhibition

The general hypothesis motivating these studies is that engineered multivalency can enhance HER3-targeted therapeutic efficacy by means of enhanced avidity and HER3 sequestration into nonproductive homotypic interactions, given that heterodimerization is requisite for HER3-mediated signal transduction. Thus, employing the Z₀₅₄₁₃ affibody²⁷ as the HER3 binding domain and a flexible protease resistant peptide spacer²² as a linker domain, bivalent and trivalent HER3 affibodies were designed (Figure 1A). Multivalent affibodies were expressed as recombinant proteins in B121 (DE3) *E. coli* and purified by immobilized metal ion chromatography and size-exclusion HPLC (Figure 1B). All HER3 affibodies consistently displayed molecular weights higher than their predicted values by SDS-PAGE, which could be a result of electrophoretic interference caused by their helix-loop-helix motifs.²⁷ However, mass spectrometry analysis indicated that all affibodies showed molecular weight and amino acid composition consistent with predicted values (Figures S1-S3). Binding affinities for HER3 were characterized for all affibodies by surface plasmon resonance. Monovalent and bivalent affibodies had similar affinities to HER3 (monovalent K_D = 316 pM, bivalent K_D = 327 pM, mean values), whereas trivalent affibodies displayed substantially lower affinity (trivalent K_D = 2340 pM) (Figure S4). These data suggest that any impact of multivalency on the cellular effects of HER3 affibodies is not due to enhanced HER3 binding.

The Z₀₅₄₁₃ affibody is reported to disrupt NRG-mediated HER3 signaling.²⁷ To assess the effect of multivalency on this phenomenon, cells were treated with the indicated equivalent concentrations of affibody domains (100 nM monovalent affibody molecule has an equivalent affibody domain concentration to 50 nM bivalent and 33 nM trivalent affibody molecules), stimulated with NRG, and then probed for phosphorylated HER3 (pHER3) by immunoblot. The results indicate that multivalency enhanced affibody-mediated pHER3

signal inhibition in a variety of cancer cell lines, OvCAR8 (ovarian), Du145 (prostate), BT474 (breast), and H1975 (lung), which were selected based on the reported role of HER3 signaling in proliferative and/or metastatic phenotypes as well as in promoting drug resistance.^{9,26,28,29} The most significant differential effects were seen between multivalent versus monovalent treatment groups (Figures 1C and S5); differences between the multivalent affibody groups (bivalent versus trivalent) were less pronounced (Figures 1C and S5). Additionally, assessment of downstream signaling revealed that, in all cell lines tested, increased valency resulted in enhanced pAkt inhibition, with the most differential effects again seen between multivalent compared to monovalent treatment groups (Figures 1C and S5).

One potential outcome of HER3 engagement by multivalent ligands is deprivation of productive dimerization with HER2, as observed for bivalent NRG in our previous work.²² Thus, immunoblots for phosphorylated HER2 (pHER2) were performed, with the results indicating that pHER2 levels were not consistently affected by affibody treatments in the BT474 cell line relative to control (10 nM NRG) levels (Figures 2 and S6A). Downstream pERK1/2 in this cell line was similarly not significantly affected by affibody treatments (Figures 2 and S6B). Notably, pHER2 was not detected by immunoblot in the OvCAR8, Du145, and H1975 cell lines after NRG stimulation (these cell lines are reported to have substantially less HER2 expression compared to the BT474 cell line; Broad-Novartis Cancer Cell Line Encyclopedia). Previous reports indicate that monovalent HER3 affibodies inhibit NRG-induced pHER2 in MCF-7 and SKBR-3 cells;^{30,31} the MCF-7 data match our own observations. Taken together, pHER2 inhibition by HER3 affibodies may be cell line specific and does not appear to be a requirement for HER3 signaling inhibition.

Linker Domain Size Has Limited Impact on Bivalent Affibody Efficacy

Linker domain size is reported to be an important parameter in multivalent ligand design.¹⁷ The flexible, glycine rich, protease resistant spacer domain employed for the bivalent and trivalent affibodies described in Figure 1 contains three repeat units and has a predicted length of approximately 20 nm (nm), long enough to traverse the theoretical 10 nm distance required to sequester a cross-facing HER3 homodimer.²² To systematically investigate the role of linker domain size on efficacy, a panel of bivalent affibodies was engineered by varying the number of repeat units in the spacer peptide from 1 to 4 (each unit having a predicted length of ~7 nm) (Figure 3A,B). Inhibitory efficacy against HER3 and Akt phosphorylation across multiple cell types with bivalent affibodies of various linker domain sizes was observed to be similar, with no consistent trends relating spacer domain length to inhibitory efficacy (Figures 3C and S7). Interestingly, the increased pHER3 inhibition associated with multivalency was still observed for the smallest linker size tested, suggesting that even lower MW multivalent HER3 affibodies have enhanced therapeutic potential. Nevertheless, the original ~20 nm linker was used for all subsequent studies.

Multivalency Increases HER3 Affibody-Mediated Inhibition of Cancer Cell Growth Alone and in Combination Therapies

We next examined the effect of multivalent ligands on *in vitro* cell viability in comparison to monovalent affibody treatment. Interestingly, despite the observation of HER3 signaling

inhibition by multivalent affibodies in all cell lines tested, cell viability was differentially impacted. In OvCAR8 cells, both bivalent and trivalent HER3 affibodies (10 nM and 50 nM) significantly reduced viability compared to monovalent affibodies (Figure 4A). In Du145 cells, multivalent and monovalent ligands significantly reduced cell viability compared to untreated controls (Figure 4B). In H1975 cells, multivalent affibody treatment had more limited antiproliferative effects compared to monovalent treatment and untreated controls, as only the trivalent affibodies caused statistically significant growth inhibition (Figure 4C). In BT474 cells, neither multivalent nor monovalent treatment reduced proliferation below control levels (Figure 4D).

Given the role of HER3 in mediating drug resistance, it was hypothesized that HER3-targeted multivalent affibodies might further improve therapeutic efficacy in combination inhibitory strategies. The use of small molecules to target the PI3K/Akt pathway is of clinical interest for the treatment of a variety of cancers, including ovarian, breast, and prostate cancers.^{29,32} However, these small molecule drugs have limited efficacy due to compensatory signaling and alternative activation of other RTKs, including HER3.^{10,32,33} Thus, the “vertical” combination of HER3-targeted multivalent affibody treatment with the PI3K inhibitor BKM120 (buparlisib) against OvCAR8 cells was explored. Enhanced antiproliferative activity was observed when affibody treatments (50 nM) were combined with BKM120 (1 μ M) against OvCAR8 cells, with multivalent affibodies having greater inhibitory effects compared to monovalent affibodies (Figure 4E).

Multivalency Induces Increased Affibody-Mediated HER3 Downregulation

It has been reported that multivalent binding events can induce differential receptor trafficking.¹⁷ Thus, the impact of multivalent affibodies on HER3 expression was investigated as a potential mechanism of their increased HER3 signaling inhibition compared to monovalent analogues. The results of these studies indicate that, in comparison to monovalent affibodies, both bivalent and trivalent affibodies more potently downregulate HER3 protein levels in all cell lines tested. Specifically, substantial HER3 downregulation was observed 3 h after application of 10 nM equivalent doses of trivalent and bivalent affibodies compared to monovalent affibody-treated cells and untreated controls, and this effect persisted over 72 h (Figures 5 and S8).

To verify whether the observed reduction in HER3 levels was post-translational in nature, cycloheximide, an inhibitor of protein synthesis, was used in a pulse-chase assay. The half-life of HER3 was not significantly affected by cycloheximide treatment over 8 h, and multivalent affibodies induced HER3 downregulation after 2, 4, and 8 h treatments. The down-regulation of HER3 by multivalent ligands at a much faster rate than the HER3 half-life in OvCAR8 cells suggests that multivalent ligands cause reduced HER3 levels at least in part by post-translational downregulation (Figures 6A and S9A). To investigate the possibility of enhanced HER3 degradation, OvCAR8 cells were treated with multivalent affibodies in the presence or absence of the lysosomal inhibitor leupeptin or the proteasomal inhibitor MG-132, and HER3 protein levels were examined by immunoblot. Surprisingly, HER3 downregulation by multivalent affibody treatment persisted in the presence of

leupeptin or MG-132, suggesting that HER3 receptor down-regulation may occur via an alternative mechanism such as receptor shedding or cleavage (Figures 6B–C and S9B–C).

DISCUSSION

HER3, a potent activator of the PI3K/Akt pathway,⁵ and a mediator of resistance to ErbB and non-ErbB targeted therapies,^{8–13} is an important target for cancer therapy.¹⁴ New and improved HER3-targeted therapeutics would provide additional treatment options for patients with cancers refractory to current therapies. In this study, we demonstrated that engineered multivalency could enhance the therapeutic potential of HER3-targeted affibodies. Further, we identified multivalent ligand-induced HER3 downregulation as a potential mechanism of action that could be exploited by other HER3-targeted therapeutics.

Cellular effects of various HER3 affibodies have previously been characterized, and the ability of monovalent affibodies to inhibit NRG-induced HER3 and Akt phosphorylation and cell growth is well established.^{31,34} Bivalent and bispecific affibodies have also been described, with a bivalent HER3 affibody incorporating an albumin-binding domain shown to efficiently inhibit phosphorylation of both HER3 and HER2.³⁰ The studies presented here significantly extend these findings to show that therapeutically relevant advantages of HER3-targeted affibodies endowed by multivalency are robust across multiple cancer cell lines, potentially independent of linker domain identity and potentially the result of enhanced HER3 downregulation. These studies also extend our own previous work demonstrating the efficacy of bivalent HER3 ligands,^{22,23} showing that multivalent engagement may be a generally useful strategy for HER3-targeted therapeutics that does not require employment of NRG. Given the potential for a NRG domain to initiate undesired activation of HER3 as the result of unexpected proteolytic cleavage or unanticipated binding conformations, multivalent affibodies represent a lower risk approach to HER3-targeted therapy via engineered multivalent ligands.

In terms of efficacy, both NRG- and affibody-based multivalent ligands promote pHER3 inhibition and reduce cancer cell viability; however, their mechanisms of action appear to be different. Multivalent affibodies had limited impact on HER2 phosphorylation in the BT474 cell line (Figure 2), whereas bivalent NRG efficacy is more closely associated with pHER2 inhibition in several cell lines,²² possibly via sequestering HER3 into homotypic interactions and preventing HER2/HER3 heterodimer formation. Alternatively, bivalent NRG, with the ability to bind both HER3 and HER4, could be inhibiting pHER2 as a result of blocking HER4-mediated signaling. In addition, we observed that bivalent affibodies with predicted linker domain lengths of less than 10 nm, theoretically too small to induce a HER3 dimer on a one molecule per dimer basis, exhibited similar pHER3 inhibition as constructs with longer predicted linker lengths (Figure 3). Taken together with the pHER2 data, pHER3 inhibition by multivalent affibodies may be independent of the ability of HER3 to interact with HER2.

Another significant result of this study is the demonstration that HER3-targeted multivalent affibodies cause rapid and prolonged HER3 downregulation, whereas monovalent affibodies have little impact on HER3 levels (Figure 5). HER3 mediated-resistance to HER2 and EGFR

targeted-therapies is reported to occur by means of increased cell surface HER3 and a shift in HER3 phosphorylation equilibrium.¹³ Therefore, strategies to internalize and reduce membrane-expressed HER3 levels could limit this resistance mechanism. HER3 can exist both on the cell surface and within intracellular compartments,³⁶ and surface HER3 expression was not explicitly quantified here. However, the substantial downregulation of total HER3 in combination with the phenotypic effects observed suggest multivalent affibodies may be effective in combatting HER3-mediated resistance. Further, the finding that HER3-targeted multivalent affibody efficacy is due at least in part to enhanced post-translational receptor downregulation (Figure 6) and is not dependent on pHER2 inhibition, linker domain length, or increased binding affinity to the target molecule, providing evidence for potential broad utility of multivalent affibodies for targets beyond HER3.

The therapeutic potential of any cancer drug could be further enhanced by selective deployment against the most vulnerable cancer types. Our results revealed disparate efficacies on the cell lines used in this study, despite the fact that these cells were chosen based on biological features that were expected to correlate with HER3-targeted affibody efficacy. The BT474 cancer cell line is reported to have moderate to high levels of HER2 and HER3,^{28,29} which together form the most potent mitogenic unit of the HER family.³⁵ The H1975 cell line expresses HER2 and HER3 and was previously shown to be susceptible to HER3 biasing by bivalent NRG treatment.²² Additionally, high basal NRG expression has been demonstrated as a biomarker for HER3 oncogenic addiction and susceptibility to HER3-targeted therapies;²⁸ both the OvCAR8 and Du145 cell lines are reported to have high basal NRG expression,²⁸ and the OvCAR8 cell line has been documented to depend on a HER3/NGR autocrine loop.²⁶ In our studies, multivalent affibodies induced significant reductions in cell viability in the OvCAR8, H1975, and Du145 cell lines, while BT474 cells were not significantly affected (Figure 4). In BT474 cells, this lack of efficacy occurred despite enhanced HER3 downregulation (Figure 5). Thus, our data support previous work indicating that HER3 targeted therapies are most efficacious as single agents against cancers that exhibit NRG autocrine signaling.²⁸ However, the ability of multivalent affibodies to induce HER3 downregulation indicates therapeutic potential for combination therapy approaches, for example, with HER2-targeted drugs, as well as potential utility in drug conjugate approaches.

Of course, any discussion of multivalent ligands should include monoclonal antibodies (mAbs), which are bivalent in nature. The capability of inducing HER3 downregulation is variable in HER3 mAbs described in the literature; however, a tetravalent HER3 antibody has been shown to induce greater HER3 internalization compared to its bivalent counterpart.³⁷ This same study reported diminished efficacy of several mAbs against HER3 in the presence of NRG, in contrast to the data presented here and highlighting a potential limitation of targeting HER3 individually with mAbs. Nevertheless, it is notable that several mAbs targeting HER3 have reached later stage clinical trials.³⁸ Interestingly, a prior report has shown that combinations of two distinct HER3 antibodies did not induce an additive effect,³⁹ as has been observed for EGFR⁴⁰ and other receptors. The same report indicated that acceleration of HER3 degradation is a potentially critical determinant of tumor growth inhibition by mAbs, reinforcing the significance of the multivalent affibody-induced HER3 downregulation observed in the present work. Complete understanding of the mechanistic

differences between HER3-targeted multivalent affibodies (or other multivalent HER3 ligands) and antibodies requires further study; however, such future studies could also incorporate evaluation of combinations of these molecules for potential therapeutic synergy.

Affibodies, like all molecules, have strengths and weaknesses with regard to their therapeutic potential. The structure of affibodies makes them highly stable and soluble,⁴¹ which is beneficial from a biomanufacturing perspective. The smaller size of affibodies relative to mAbs may increase their tumor penetration,^{42,43} although the circulating half-life of affibodies is much shorter than of the typical mAb. However, affibodies are easily amenable to formatting for improved pharmacokinetics, such as via fusion to an albumin-binding domain,^{30,44} and such constructs are compatible with multivalent presentation. Yet, the enhanced HER3 downregulation mediated by engineered multivalency reported here is a mechanism that can potentially be broadly exploited independently of specific HER3 binding domains, linker domain sizes, and other molecular idiosyncrasies. Overall, these results position multivalent HER3-binding affibodies for further development and exemplify a general and versatile strategy to improve the efficacy of HER3-targeted cancer therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Multivalency enhances affibody-mediated pHER3 and pAkt inhibition. (A) Schematic representing monovalent, bivalent, and trivalent affibody constructs. (B) Purified protein products are displayed on a Coomassie stained gel loaded at $7.3 \mu\text{g}/\text{lane}$. Monovalent, bivalent, and trivalent ligands have respective theoretical molecular weights of 7.6, 19.1, and 30.5 kDa. (C) OvCAR8, Du145, H1975, and BT474 cells were treated with the indicated concentrations of trivalent, bivalent, or monovalent ligand, 100 nM pan HER kinase inhibitor (total inhibitor), or media alone (no treatment) for 30 min, then stimulated with 10 nM NRG for 10 min, lysed, and probed for pHER3, pAkt, and β -actin by immunoblot. All multivalent ligand concentrations refer to the concentration of individual affibody domain. Results shown are representative of three independent experiments. All blots were cropped.

Figure 2.

HER3 affibodies do not influence pHER2 levels regardless of valency in BT474 cells.

BT474 cells were treated with the indicated concentrations of trivalent, bivalent, or monovalent affibodies, 100 nM pan HER kinase inhibitor (total inhibitor), or media alone (no treatment) for 30 min, then stimulated with 10 nM NRG for 10 min, lysed, and probed by immunoblot for pHER2 or pERK1/2. β -Tubulin levels were assessed for normalization purposes. Results shown are representative of two independent experiments. All blots were cropped.

Figure 3.

Linker domain size has limited impact on bivalent affibody activity. (A) Schematic representing bivalent affibodies of varying linker domain size: 1 link (1 repeat unit), 2 link, 3 link, and 4 link. (B) Purified protein products of bivalent affibodies with the indicated linker domain size are displayed on a Coomassie stained gel loaded at 7.3 $\mu\text{g}/\text{lane}$. One link, 2 link, 3 link, and 4 link bivalent affibodies have respective theoretical molecular weights of 16.0, 17.5, 19.1, and 20.6 kDa. (C) OvCAR8, Du145, H1975, and BT474 cells were serum starved for 4 h, treated with the indicated concentrations of trivalent, bivalent, or monovalent affibody, pan HER kinase inhibitor (total inhibitor), or media alone for 30 min, then stimulated with 10 nM NRG for 10 min, lysed, and probed by immunoblot. Results are representative of two independent experiments. All blots were cropped.

Figure 4.

Multivalency increases functional efficacy of HER3 affibodies as single agents and as part of combination therapy. (A) OvCAR8, (B) Du145, (C) H1975, and (D) BT474 cells were exposed to 200 pM NRG and treated with the indicated concentrations of trivalent, bivalent, or monovalent affibodies, incubated for 5 days and analyzed using the Alamar Blue assay following the manufacturer's protocol. (E) OvCAR8 cells were treated with 50 nM of the indicated affibodies in combination with 1 μ M BKM120 (BKM) or with 1 μ M BKM alone and incubated and analyzed as above. Results shown are mean $\pm$ SD and are representative of three independent experiments; $n = 10$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ in reference

to the equivalent dosage monovalent affibody group, and $^{###}P < 0.001$ in reference to the BKM only group by one way ANOVA with Bonferonni posthoc test.

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Figure 5. Multivalency induces affibody-mediated HER3 downregulation. (A) OvCAR8, (B) Du145, (C) H1975, and (D) BT474 cells were treated with 10 nM trivalent, bivalent, or monovalent affibody, pan HER kinase inhibitor (total inhibitor), or media alone (no treatment), lysed at the indicated time points, and probed by immunoblot for HER3. β -actin levels were assessed for normalization. Results shown are representative of three independent experiments. All blots were cropped.

Figure 6.

Multivalent affibody-mediated HER3 downregulation is at least in part post-translational. (A) OvCAR8 cells were treated with 10 nM trivalent, bivalent, or monovalent affibody, or in combination with 178 μ M cycloheximide or 178 μ M cycloheximide alone, lysed at the indicated time points, and probed by immunoblot for HER3 and β -actin. (B) OvCAR8 cells were pretreated with 200 μ M leupeptin for 3 h or no treatment, treated with 10 nM trivalent, bivalent, or monovalent affibody, or in combination with 200 μ M leupeptin or 200 μ M leupeptin alone, lysed at the indicated time points, and probed by immunoblot for HER3 and β -actin. (C) OvCAR8 cells were pretreated with 5 μ M MG-132 for 3 h or no treatment, treated with 10 nM trivalent, bivalent, or monovalent affibody alone, or in combination with

5 μ M MG-132 or 5 μ M MG-132 alone, lysed at the indicated time points, and probed by immunoblot for HER3 and β -actin. Results shown are representative of two independent experiments. All blots were cropped.

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