BISPECIFIC EGFR/IGFIR BINDING MOLECULES

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on November 23, 2009, is named COTH52WO.txt, and is 552,529 bytes in size.

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Nos. 61/200,164, filed November 24, 2008; 61/200,282, filed November 26, 2008; 61/212,966, filed April 17, 2009; 61/178,279, filed May 14, 2009; and 61/227,330, filed July 21, 2009, which applications are hereby incorporated by reference in their entireties.

FIELD OF THE INVENTION

The present invention relates to EGFR binding domains and bispecific molecules comprising an EGFR binding domain and a distinct IGFIR binding domain for use in diagnostic, research and therapeutic applications. The invention further relates to cells comprising such proteins, polynucleotide encoding such proteins or fragments thereof, and vectors comprising the polynucleotides encoding the innovative proteins. Exemplary EGFR binding domains and bispecific molecules include antibody-like protein dimers based on the tenth fibronectin type III domain.

INTRODUCTION

Activation of receptor tyrosine kinase signaling is central to cancer development (see e.g., Grimberg A. Cancer Biol Ther. 2003 2(6):630-5 and Mendelsohn J. J Clin Oncol. 2003 21(14):2787-99). Receptor tyrosine kinases have a conserved domain structure including an extracellular domain, a transmembrane domain and an intracellular tyrosine kinase domain. The extracellular domain can bind to a ligand, such as to a polypeptide growth factor or to a cell membrane-

associated molecule. Typically, either ligand binding or ligand binding induced dimerization of receptor tyrosine kinases activates the intracellular catalytic tyrosine kinase domain of the receptor and subsequent signal transduction.

Examples of receptor tyrosine kinases include, but are not limited to ERBB receptors (e.g., EGFR, ERBB2, ERBB3, ERBB4), erythropoietin-producing hepatocellular (EPH) receptors, fibroblast growth factor (FGF) receptors (e.g., FGFR1, FGFR2, FGFR3, FGFR4, FGFR5), platelet-derived growth factor (PDGF) receptors (e.g., PDGFR-A, PDGFR-B), vascular endothelial growth factor (VEGF) receptors (e.g., VEGFR1/FLT1, VEGFR2/FLK1, VEGF3), tyrosine kinase with immunoglobulin-like and EGF-like domains (TIE) receptors , insulin-like growth factor (IGF) receptors (e.g., INS-R, IGFIR, IR-R), Discoidin Domain (DD) receptors , receptor for c-Met (MET), recepteur d'origine nantais (RON); also known as macrophage stimulating 1 receptor, Flt3 fins-related tyrosine kinase 3 (Flt3), colony stimulating factor 1(CSF1) receptor, adhesion related kinase receptor (e.g., Axl), receptor for c-kit (KIT) and insulin receptor related (IRR) receptors.

Inhibition of receptor tyrosine kinases has emerged as an effective treatment strategy for certain human malignancies (for a review see Roussidis AE, In Vivo. 2002 16(6):459-69).

While targeted monotherapy may initially be effective in treating cancer, therapeutic resistance often follows, possibly as a result of upregulation of other signaling cascades (see e.g., Nahta R et al., Breast Cancer Res. 2006 8(6):215 and Horn L et al., Clin Lung Cancer. 2007 8:S68-73). Accordingly, there exists a need for developing improved cancer therapeutics.

SUMMARY OF THE INVENTION

In one aspect, the application provides EGFR binding tenth fibronectin type III domains (^{10}Fn3) having novel sequences. EGFR binding ^{10}Fn3 having a consensus sequence are also provided. Such EGFR binding ^{10}Fn3 may be monomeric or may be included as part of a fusion protein.

In another aspect, the application provides bispecific molecules that bind EGFR and IGFIR, referred to herein as "E/I binders". E/I binders encompassed by the invention include bispecific antibodies and dimers of ligand binding scaffold

proteins (e.g., tendamistat, affibody, fibronectin type III domain, anticalin, tetranectin, and ankyrin). When constructed as a single polypeptide chain, the E/I binders may be constructed in any orientation, e.g., from N-terminus to C-terminus either in the E-I arrangement or the I-E arrangement.

In one aspect, antibody-like protein dimers are provided comprising an EGFR binding ^{10}Fn3 covalently or non-covalently linked to an IGFIR binding ^{10}Fn3. The ^{10}Fn3 bind their target (EGFR or IGFIR) with a K_D of less than 500 nM. Each of the individual ^{10}Fn3 independently has an amino acid sequence at least 70, 80, 85, 90, 95, 98, or 100% identical to SEQ ID NO: 32, wherein n is an integer from 1-20, o is an integer from 1-20, and p is an integer from 1-40. In some embodiments, n is an integer from 8-12, o is an integer from 4-8, and p is an integer from 4-28. In some embodiments, n is 10, o is 6, and p is 12.

In some embodiments, the antibody-like protein dimers comprise IGFIR binding ^{10}Fn3 covalently linked to EGFR binding ^{10}Fn3 via a polypeptide linker or a polyethylene glycol moiety. In some embodiments, the antibody-like protein dimer comprises an amino acid sequence at least 80, 90, 95, or 100% identical to any one of SEQ ID NOs: 20-31, 53-58, 87-92, 98-105, 118-133, 149-154, 164-169, 179-184, 192-197, 205-210 and 211-216.

In some embodiments, the E/I binder comprises an amino acid sequence having any one of SEQ ID NOs: 20-31, 53-58, 87-92, 98-105, 118-133, 149-154, 164-169, 179-184, 192-197, 205-210 and 211-216, wherein (i) the EGFR binding ^{10}Fn3 and/or the IGF-IR binding ^{10}Fn3 comprises a ^{10}Fn3 scaffold having from has anywhere from 0 to 20, from 0 to 15, from 0 to 10, from 0 to 8, from 0 to 6, from 0 to 5, from 0 to 4, from 0 to 3, from 0 to 2, or from 0 to 1 substitutions, conservative substitutions, deletions or additions relative to the corresponding scaffold amino acids of SEQ ID NO: 1, and/or (ii) the EGFR binding ^{10}Fn3 has anywhere from 0 to 15, from 0 to 10, from 0 to 8, from 0 to 6, from 0 to 5, from 0 to 4, from 0 to 3, from 0 to 2, or from 0 to 1 substitutions, conservative substitutions, deletions or additions relative to the corresponding loop sequences of any one of SEQ ID NOs: 5-8, 52, 66-68, 106-108, 112-114, 140-142, 155-157, 170-172, 182, 185-187, 198-200, or 219-327 and/or the IGF-IR binding ^{10}Fn3 has anywhere from 0 to 15, from 0 to 10, from 0 to 8, from 0 to 6, from 0 to 5, from 0 to 4, from 0 to 3, from 0 to 2, or from 0

to 1 substitutions, conservative substitutions, deletions or additions relative to the corresponding loop sequences of SEQ ID NO: 3.

In one aspect, pharmaceutically acceptable compositions are provided comprising an antibody-like protein dimer as described herein and a pharmaceutically acceptable carrier, wherein the composition is essentially pyrogen free.

In a further aspect, methods for treating hyperproliferative disorders, such as cancer, in a subject are provided comprising administering to a subject in need thereof a therapeutically effective amount of a pharmaceutically acceptable composition comprising an antibody-like protein dimer as described herein.

In another aspect, the application provides a nucleic acid encoding an antibody-like protein dimer as described herein. Also provided is a vector comprising a nucleic acid encoding an antibody-like dimer as described herein. Suitable vectors include, for example, expression vectors. Also provided are host cells comprising a nucleic, vector, or expression vector, comprising a nucleic acid encoding an antibody-like protein dimer as described herein. Suitable host cells include prokaryotic and eukaryotic host cells. Exemplary prokaryotic cells are bacterial cells, such as E. coli. Exemplary eukaryotic cells are mammalian cells, such as CHO cells. Also provided are methods for producing an antibody-like protein dimer as described herein, comprising culturing a host cell comprising a nucleic, vector, or expression vector, comprising a nucleic acid encoding the antibody-like protein dimer and recovering the expressed antibody-like protein dimer from the culture.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. SDS-PAGE Analysis of I1-GS10-E2. Samples from the lysis of HMS174(DE3) bacterial cell pellet from which I1-GS10-E2 was expressed and purified by a HisTrap chromatography column were run on a 4-12% NuPAGE minigel and stained by Sypro-Orange and visualized by STORM imager. Mark 12 molecular weight standards (Lane 1); Lysate-soluble (Lane 2); Lysate- insoluble (Lane 3); HisTrap load (Lane 4); HisTrap non-bound (Lane 5); Pooled HisTrap

Eluate (Lane 6); Dialyzed into 50mM NaOAc, 150mM NaCl, pH 4.5 (Lane 7); Dialyzed into PBS (Lane 8); Dialyzed into Tris, 150mM NaCl, pH 8.5 (Lane 9).

Figure 2. A. SEC Analysis of midscale purified I1-GS10-E2. 22 μg of HisTrap purified I1-GS10-E2 dialyzed into PBS, pH 7.4 was loaded onto a Superdex 200 10/30 SEC Column (GE Healthcare) with a mobile phase of 100 mM $NaPO_4$, 100 mM $NaSO_4$, 150 mM NaCl, pH 6.8 and measured using A280. I1-GS10-E2 eluted predominantly as a single monomeric species at a molecular weight range of approximately 24.6 kDa vs. globular Gel Filtration standards (BioRad). B. SEC analysis of E2-GS10-I1.

Figure 3. A. Differential Scanning Calorimetry (DSC) of midscale purified I1-GS10-E2 in PBS was performed to determine the T_m. A 1 mg/mL solution of I1-GS10-E2 was scanned from 5 °C to 95 °C at a rate of 1 degree per minute under 3 atm pressure. The data was analyzed versus a control run of the PBS buffer. B. DSC of E2-GS10-I1.

Figure 4. Inhibition of IGFR activity in H292 cells. Cells were stimulated with 100 ng/mL of IGF-1 and 100ng/mL of EGF and treated with either ● I1, □ E1, or Δ E1-GS10- I1 HTPP preparations. Phosphorylation of IGFIR on tyrosine 1131 was determined by ELISA.

Figure 5. Inhibition of EGFR activity in H292 cells. Cells were stimulated with 100ng/mL of IGF-1 and 100ng/mL of EGF and treated with either ● I1, □ E1, or Δ E1-GS10-I1 HTPP preparations. Phosphorylation of EGFR on tyrosine 1068 was determined by ELISA.

Figure 6. Inhibition of AKT phosphorylation in H292 cells. Cells were stimulated with 100ng/mL of IGF-1 and 100ng/mL of EGF and treated with either ● I1, □ E1, or Δ E1-GS10-I1 HTPP preparations. Phosphorylation of AKT on serine 473 was determined by ELISA.

Figure 7. Inhibition of RH41 cell proliferation. Cells were treated with either ● I1, □ E1, or Δ E1-GS10-I1 HTPP preparations and percent inhibition of proliferation was determined.

Figure 8. Inhibition of H292 cell proliferation. Cells were treated with either ● I1, □ E1, or Δ E1-GS10-I1 HTPP preparations and percent inhibition of proliferation was determined.

Figure 9. Summarizes IC50 values in cell based functional assays for isolated EGFR mononectins, E/I ^{10}Fn3-based binders with serine at the C-terminal position without PEG added and E/I ^{10}Fn3-based binders with cysteine at the C-terminal position conjugated to a 40 kDa branched PEG. Representative data is shown.

Figure 10. Immunoblot analysis of PEGylated E/I ^{10}Fn3-based binder with E2 in the N-terminal and C-terminal positions. Despite both constructs demonstrating comparable activity in the H292 cell assay for inhibiting EGFR, the E/I ^{10}Fn3-based binder with E2 at the C-terminal position did not degrade EGFR while the E/I ^{10}Fn3-based binder with E2 at the N-terminal position did. Both constructs show very weak to no IGFR degradation in this cell line. β-actin was included to demonstrate equal loading across all lanes. The phosphorylation state of EGFR, ERK and Shc was also examined.

Figure 11. Inhibition of EGF-stimulated EGFR phosphorylation in H292 cells. Both constructs demonstrated comparable activity in the H292 cell assay for inhibiting EGFR. E2-GS10-I1 (with PEG) (○), I1-GS10-E2 (with PEG) (□), panitumumab (----).

Figure 12. Results of tumor xenograft studies. Figure 12A: Preclinical anti-tumor activity in the H292 human tumor xenograft model. Mean tumor sizes calculated from groups of 8 mice is shown in mg for control animals (■), E3-GS10-I1 (w/ PEG) dosed at 100mg/kg (○), E2-GS10-I1 (with PEG) dosed at 100 mg/kg (□), panitumumab dosed at 1mg/mouse (△) or 0.1mg/mouse (▽). The letter a on the x-axis indicates doses of E/I binders administered and the p indicates doses of panitumumab administered. Figure 12B: Average weight change is shown for each group over the course of the study. Symbols are as described in Figure 12A legend.

Figure 13. Pharmacodynamic effects in the H292 NSCLC tumor xenograft model. Levels of the indicated analytes were determined in tumor lysates as described in Example 12. (A) phosph-EGFR, (B) phospho-ErbB2, (C) phospho-IGFR, and (D) total EGFR. Checkered bars = panitumumab, empty bars = E2-GS10-I1 (with PEG), hatched bats = E3-GS10-I1 (with PEG).

Figure 14. Western blot analysis of MCF7r cells compared to MCF7 parental cells.

Figure 15. MCF7 (Panel A) and MCF7r (Panel B) human tumor xenograft studies in nude mice. Mean tumor size is shown for both studies calculated from 8 mice per group.

Figure 16. GEO human tumor xenograft studies in nude mice.

Figure 17. H292 human tumor xenograft studies in nude mice.

Figure 18. Colony formation assay with H292 NSCLC cells. A. Representative data is shown from a single plate. B. IC50 from one E/I ^{10}Fn3-based binder is shown with error bars calculated from triplicate measurements.

Figure 19. Epitope mapping assay. Location of epitope binding for various EGFR binding antibodies are shown in panel A. A description of the antibodies is provided in Example 18, Table 11. The left column of table 11 provides a number for each anti-EGFR antibody which correlates with the numbered antibodies shown in panel A. Panel B shows an exemplany epitope mapping assay as described in Example 18.

Figure 20. DSC analysis of the E/I ^{10}Fn3-based binder, I1-GS10-E5 pegylated, measured with a scan range of 15-95 °C at 1mg/ml protein concentration in PBS, resulted in a Tm measurement of 55.2 °C

Figure 21. Evaluation of E/I ^{10}Fn3-based binders for inhibition of AKT phosphorylation in H292 cells as measured by ELISA. I1-GS10-E5-pegylated (○) was more potent than I1-pegylated alone (■) or E5-pegylated alone (▲) for blocking IGF1-stimulated AKT phosphorylation.

Figure 22. Evaluation of E/I ^{10}Fn3-based binders for inhibition of cell proliferation in H292 cells. I1-GS10-E5-pegylated (□) was more potent than I1-pegylated alone (▲) and E5-pegylated alone (●) had only weak effects for inhibiting the growth of H292 cells. Assays were carried out in triplicate. Representative data is shown.

Figure 23. Evaluation of E/I ^{10}Fn3-based binders for inhibition of cell proliferation in RH41 cells. I1-GS10-E5-pegylated (□) was slightly more potent than I1-pegylated alone (▲) and E5-pegylated alone (●) or panitumumab (dashed line) had almost no effect for inhibiting the growth of RH41 cells. Assays were carried out in triplicate. Representative data is shown.

Figure 24. Inhibition of ligand stimulated signaling by ^{10}Fn3-based binders (pegylated). Effect of E/I ^{10}Fn3-based binder (I1-GS10-E5 pegylated) on receptor activation and cell signaling in DiFi (Panel A), H292 (Panel B) or BxPC3 (Panel C) cells. Cells were serum starved and treated for 2 hours with 1 μM ^{10}Fn3-based binders before stimulation with either EGF, IGF1 or a combination of EGF + IGF1. GAPDH was probed to illustrate equal loading in all lanes.

Figure 25. Inhibition of ligand stimulated signaling in H292 cells by ^{10}Fn3-based binders (unpegylated). Effect of E/I ^{10}Fn3-based binder (E2-GS10-I1) on receptor activation and cell signaling in H292 cells. Cells were serum starved and treated for 2 hours with 1 μM ^{10}Fn3-based binders before stimulation with either EGF, IGF1 or a combination of EGF + IGF1. GAPDH was probed to illustrate equal loading in all lanes

Figure 26. Competition binding studies with E/I ^{10}Fn3-based binders. A. The EGFR ^{10}Fn3-based binder does not compete for binding of EGFR antibodies to EGFR. Initial injection of the EGFR ^{10}Fn3-based binder shows binding to EGFR on the surface of the chip. A second injection of EGFR ^{10}Fn3-based binder mixed with an equal amount of cetuximab, panitumumab, or nimotuzumab shows no competition for binding of antibodies to EGFR by the EGFR ^{10}Fn3-based binder. B. The E/I ^{10}Fn3-based binder can bind EGFR and IGF-IR simultaneously. Initial injection of the E/I ^{10}Fn3-based binder shows binding to EGFR immobilized on the chip surface. A second injection of the E/I ^{10}Fn3-based binder soluble IGF-IR shows binding of sIGF-IR to other end of the immobilized E/I ^{10}Fn3-based binder.

Figure 27. TGFα plasma levels 4 hours after last dose of xenograft studies. Plasma samples taken at the end of treatment from the BxPC3 (Panel A), GEO (Panel B) and H441 (Panel C) xenograft studies described in Table 24 were analyzed for circulating levels of TGFα.

Figure 28. TGFα and IGF1 plasma levels in non tumor bearing nude mice after dosing with I1-GS10-E5 pegylated. Non-tumor bearing mice were given a single dose of I1-GS10-E5 pegylated ^{10}Fn3-based binder and analyzed for circulating levels of TGFα (Panel A) and IGF1 (Panel B).

Figure 29. H292 xenograft study using E/I 10Fn3-based binders as compared to panitumumab. H292 xenografts were either untreated (■) or dosed three times a

week with ^{10}Fn3-based binders formulated in PBS with the individual constructs as described in the figure or dosed every three days i.p. with panitumumab at 1 mg/mouse (○) or 0.1 mg/mouse (□). Actual doses of ^{10}Fn3-based binders and panitumumab (▲) are indicated on the x-axis with the panitumumab doses closest to the x-axis below the triangles indicating doses of ^{10}Fn3-based binders. Figure 29A shows measurements out to day 43. Figure 29B shows measurements out to day 27.

Figure 30. Pharmaokinectic parameters profile of E2-GS10-Il pegylated in mice.

Figure 31. Comparison of half-life at 100 mg/kg and 10 mg/kg IP, and 10 mg/kg and 64 mg/kg SC in various E/I ^{10}Fn3-based binders.

Figure 32. Antitumor efficacy of E2-GS10-I1 pegylated in the RH41 model.

Figure 33. Measurement of pharmacodynamic endpoints in tumors. At the end of treatment, tumors were removed 4 hours following the final dose from DiFi xenograft model (panel A) and H292 xenograft model (panel B) and examined for levels of phospho-EGFR, phospho-IGFR, total EGFR and total IGFR. Equal amounts of total protein lysate was loaded into each lane of the gels and blots were also probed with GAPDH to demonstrate equal loading across all lanes.

Figure 34. Sequence of anti-EGFR binder 679F09 (SEQ ID NO: 490). Loop residues which were varied are underlined.

Figure 35. BC loop Sequence Analysis I. Frequency of amino acids at each position in the BC loop from EGFR binding sequences. Image created using WebLogo (Crooks GE, Hon G, Chandonia JM, Brenner SE. WebLogo: A sequence logo generator. *Genome Research*, 14:1188-1190, 2004).

Figure 36. DE loop Sequence Analysis 1. Frequency of amino acids at each position in the DE loop from EGFR binding sequences (263 unique DE loop sequences analyzed).

Figure 37. FG loop (10-aa length) Sequence Analysis I. Frequency of amino acids at each position in the FG loop from EGFR binding sequences with 10-amino acid long FG loops (228 unique 10-amino acid long FG loops analyzed).

Figure 38. FG loop (15-aa length) Sequence Analysis I. Frequency of amino acids at each position in the FG loop from EGFR binding sequences with 15-amino acid long FG loops (349 unique 15-amino acid long FG loops analyzed).

Figure 39. BC loop Sequence Analysis II. Frequency of amino acids at each position in the BC loop from all "potent" sequences (85 unique BC loop sequences analyzed).

Figure 40. DE loop Sequence Analysis II. Frequency of amino acids at each position in the DE loop from all "potent" sequences (60 unique DE loop sequences analyzed).

Figure 41. FG loop (10-aa length) Sequence Analysis II. Frequency of amino acids at each position in the FG loop from all "potent" sequences with 10-amino acid long FG loops (6 unique 10-amino acid long FG loops analyzed).

Figure 42. FG loop (15-aa length) Sequence Analysis II. Frequency of amino acids at each position in the FG loop from all "potent" sequences with 15-amino acid long FG loops (65 unique 15-amino acid long FG loops analyzed).

Figure 43. Table summarizing various characteristics of E/I ^{10}Fn3-based binders as described in Example 22.

Figure 44. Table summarizing various pharmacokinetic parameters of E/I ^{10}Fn3-based binders as described in Example 30.

Figure 45. Amino acid sequences of E monomers as described in Example 32. The BC, DE and FG loops in each sequence are underlined.

Figure 46. Alignment of wild-type core sequence (amino acids 9-94 of SEQ ID NO: 1) with I1 core (SEQ ID NO:65), E1 core (SEQ ID NO:66), E2 core (SEQ ID NO:67), E3 core (SEQ ID NO:68), E4 core (SEQ ID NO:108), E5 core (SEQ ID NO:114), E85 core (SEQ ID NO:141), E90 core (SEQ ID NO:156), E96 core (SEQ ID NO:171), E105 core (SEQ ID NO:186), and E112 core (SEQ ID NO:199). The BC, DE and FG loops in the wild-type sequences are shown in bold and underlined. The amino acid residues actually changed in comparison to wild-type for the I and E cores are shown i bold and underlined.

Figure 47. Nucleic acid sequences of E and I monomers. Unless otherwise specified, the nucleotide sequences encode a monomer having an N+10 N-terminal extension, a Ser tail, and a his tag.

Figure 48. Nucleic acid sequence of E/I ^{10}Fn3-based binders. All nucleotide sequences encode an E/I ^{10}Fn3-based binder having an N+10 N-terminal extension on the first monomer in the construct and a Cys tail and his tag on the second

monomer in the construct. GS10 is SEQ ID NO: 11; GSGCGS8 is SEQ ID NO: 218; and GSGC is SEQ ID NO: 489.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, the following terms and phrases shall have the meanings set forth below. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art.

The singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise.

The terms "comprise" and "comprising" are used in the inclusive, open sense, meaning that additional elements may be included.

The term "including" is used to mean "including but not limited to". "Including" and "including but not limited to" are used interchangeably.

The term "antibody-like protein" refers to a non-immunoglobulin protein having an "immunoglobulin-like fold", i.e., comprises about 80-150 amino acid residues that are structurally organized into a set of beta or beta-like strands, forming beta sheets, where the beta or beta-like strands are connected by intervening loop portions. The beta sheets form the stable core of the antibody-like protein, while creating two "faces" composed of the loops that connect the beta or beta-like strands. As described herein, these loops can be varied to create customized ligand binding sites, and such variations can be generated without disrupting the overall stability of the protein. An example of such an antibody-like protein is a "fibronectin-based scaffold protein", by which is meant a polypeptide based on a fibronectin type III domain (Fn3). In one aspect, an antibody-like protein is based on a tenth fibronectin type III domain (^{10}Fn3).

By a "polypeptide" is meant any sequence of two or more amino acids, regardless of length, post-translation modification, or function. "Polypeptide," "peptide," and "protein" are used interchangeably herein.

"Percent (%) amino acid sequence identity" herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in a selected sequence, after aligning the sequences and

introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are obtained as described below by using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc. has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087, and is publicly available through Genentech, Inc., South San Francisco, Calif. The ALIGN-2 program should be compiled for use on a UNIX operating system, preferably digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

For purposes herein, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows: 100 times the fraction X/Y where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A.

The term "therapeutically effective amount" refers to an amount of a drug effective to treat a disease or disorder in a mammal. In the case of cancer, the therapeutically effective amount of the drug may reduce the number of cancer cells; reduce the tumor size; inhibit (i.e., slow to some extent and preferably stop) cancer

cell infiltration into peripheral organs; inhibit (i.e., slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the disorder. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. For cancer therapy, efficacy in vivo can, for example, be measured by assessing the time to disease progression (TTP) and/or determining the response rates (RR).

The half-life of an amino acid sequence or compound can generally be defined as the time taken for the serum concentration of the polypeptide to be reduced by 50% in vivo due to, e.g., degradation of the sequence or compound and/or clearance or sequestration of the sequence or compound by natural mechanisms. The half-life can be determined in any manner known in the art, such as by pharmacokinetic analysis. See e.g., M Gibaldi & D Perron "Pharmacokinetics", published by Marcel Dekker, 2nd Rev. edition (1982).

The term "E/I binder" refers to a bispecific molecule that comprises an EGFR binding domain and a distinct IGFIR binding domain. The two domains may be covalently or non-covalently linked. An exemplary E/I binder is an antibody-like dimer comprising an EGFR binding ^{10}Fn3 and an IGFIR binding ^{10}Fn3, i.e., an E/I ^{10}Fn3 based binder.

Overview

The epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor (IFGR) play key roles in the tumorigenesis of several types of human cancer. Inhibition of either receptor effectively reduces tumor growth in preclinical models as well as clinically. Blocking the EGFR pathway induces switching to the IGFR pathway to drive growth with in vitro tumor models. Therefore, blocking both receptors simultaneously may achieve superior efficacy to blocking either pathway alone by overcoming pathway switching. In exemplary embodiments, the activity of an E/I binder is synergistic in comparison to the monomeric components of the E/I binder.

The specification describes, *inter alia*, bispecific molecules that bind EGFR and IGFIR, referred to herein as "E/I binders". Applicants have discovered that such bispecific molecules inhibit proliferation of a cancer model cell line with greater

potency than the corresponding. monospecific binders (see e.g., Example 9 and Figure 8).

E/I binders will be useful in numerous therapeutic applications, especially in the treatment of cancer. In addition to therapeutic applications, E/I binders may be used in any circumstance where it is desirable to detect EGFR and/or IGFIR.

E/I binders have an EGFR binding domain and a distinct IGFIR binding domain. Typical binding domains include antibodies; therefore, bispecific antibodies may be generated to function as E/I binders. Bispecific antibodies comprising complementary pairs of V_H and V_L regions are known in the art. These bispecific antibodies comprise two pairs of V_H and V_L, each $V_{H/L}$ pair binding to a single antigen. (see e.g., Hu et al., Cancer Res. 1996 56:3055-306; Neri et al., J. Mol. Biol. 1995 246:367-373; Atwell et al., Mol. Immunol. 1996 33:1301-1312; and Carter et al., Protein Sci. 1997 6:781-788). An exemplary bispecific antibody is a diabody, i.e., a small antibody fragment with two antigen-binding sites, which fragments comprise a heavy-chain variable domain connected to a light-chain variable domain in the same polypeptide chain (Hollinger et al., Proc. Natl. Acad. Sci. USA 1993 90: 6444-6448).

E/I binders also encompass dimers of ligand binding scaffold proteins. Scaffold proteins are well described in the literature and include, e.g., tendamistat, affibody, fibroncectin type III domain, anticalin, tetranectin, and ankyrin. Additional scaffold proteins that may be used to generate E/I binders are reviewed in Binz et al., Nature Biotech 23:1257-1268 (2005). Scaffold proteins are based on a rigid core structure or 'framework' that is important in determining and stabilizing the three-dimensional structure. In between the fixed or conserved residues of the scaffold lie variable regions such as loops, surfaces or cavities that can be randomized to alter ligand binding. A large diversity of amino acids is provided in the variable regions between the fixed scaffold residues to provide specific binding to a target molecule.

An exemplary ligand binding scaffold protein is based on a fibronectin type III domain (Fn3). Fibronectin is a large protein which plays essential roles in the formation of extracellular matrix and cell-cell interactions; it consists of many repeats of three types (types I, II, and III) of small domains.

Fn3 is small, monomeric, soluble, and stable. It lacks disulfide bonds and, therefore, is stable under reducing conditions. The overall structure of Fn3 resembles the immunoglobulin fold. Fn3 domains comprise, in order from N-terminus to C-terminus, a beta or beta-like strand, A; a loop, AB; a beta or beta-like strand, B; a loop, BC; a beta or beta-like strand, C; a loop, CD; a beta or beta-like strand, D; a loop, DE; a beta or beta-like strand, E; a loop, EF; a beta or beta-like strand, F; a loop, FG; and a beta or beta-like strand, G. The seven antiparallel β-strands are arranged as two beta sheets that form a stable core, while creating two "faces" composed of the loops that connect the beta or beta-like strands. Loops AB, CD, and EF are located at one face and loops BC, DE, and FG are located on the opposing face. Any or all of loops AB, BC, CD, DE, EF and FG may participate in ligand binding. There are at least 15 different modules of Fn3, and while the sequence homology between the molecules is low, they all share a high similarity in tertiary structure.

Adnectins™ (Adnexus, a Bristol-Myers Squibb R&D Company) are ligand binding scaffold proteins based on the tenth fibronectin type III domain, i.e., the tenth module of Fn3, (^{10}Fn3). The amino acid sequence of a naturally occurring human ^{10}Fn3 is set forth in SEQ ID NO: 1.

VSDVPRDLEVVAATPTSLLI**SWDAPAVTVR**YYRITYGETGGNSPVQEFTV**P**
GSKST ATISGLKPGVDYTITVYAV**TGRGDSPASSKP**ISINYRT (SEQ ID NO:1) (BC, FG, and DE loops are emphasized)

In SEQ ID NO:1, the AB loop corresponds to residues 15-16, the BC loop corresponds to residues 21-30, the CD loop corresponds to residues 39-45, the DE loop corresponds to residues 51-56, the EF loop corresponds to residues 60-66, and the FG loop corresponds to residues 76-87. (Xu et al., Chemistry & Biology 2002 9:933-942). The BC, DE and FG loops align along one face of the molecule and the AB, CD and EF loops align along the opposite face of the molecule. In SEQ ID NO: 1, beta strand A corresponds to residues 9-14, beta strand B corresponds to residues 17-20, beta strand C corresponds to residues 31-38, beta strand D corresponds to residues 46-50, beta strand E corresponds to residues 57-59, beta strand F corresponds to residues 67-75, and beta strand G corresponds to residues 88-94. The strands are connected to each other through the corresponding loop, e.g.,

strands A and B are connected via loop AB in the formation strand A, loop AB, strand B, etc. Residues involved in forming the hydrophobic core (the "core amino acid residues") include the amino acids corresponding to the following amino acids of SEQ ID NO: 1: L8, V10, A13, L18, I20, W22, Y32, I34, Y36, F48, V50, A57, I59, L62, Y68, I70, V72, A74, I88, I90 and Y92, wherein the core amino acid residues are represented by the single letter amino acid code followed by the position at which they are located within SEQ ID NO: 1. See e.g., Dickinson et al., J. Mol. Biol. 236: 1079-1092 (1994).

As described above, amino acid residues corresponding to residues 21-30, 51-56, and 76-87 of SEQ ID NO: 1 define the BC, DE and FG loops, respectively. However, it should be understood that not every residue within the loop region needs to be modified in order to achieve a ^{10}Fn3 binder having strong affinity for a desired target, such as IGF-IR or EGFR. For example, in many of the examples described herein, only residues corresponding to amino acids 23-30, 52-55 and 77-86 of SEQ ID NO: 1 were modified to produce high affinity ^{10}Fn3 binders (see Figure 46. Accordingly, in certain embodiments, the BC loop may be defined by amino acids corresponding to residues 23-30 of SEQ ID NO: 1, the DE loop may be defined by amino acids corresponding to residues 52-55 of SEQ ID NO: 1, and the FG loop may be defined by amino acids corresponding to residues 77-86 of SEQ ID NO: 1.

^{10}Fn3 are structurally and functionally analogous to antibodies, specifically the variable region of an antibody. While ^{10}Fn3 domains may be described as "antibody mimics" or "antibody-like proteins", they do offer a number of advantages over conventional antibodies. In particular, they exhibit better folding and thermostability properties as compared to antibodies, and they lack disulphide bonds, which are known to impede or prevent proper folding under certain conditions. Exemplary E/I ^{10}Fn3 based binders are predominantly monomeric with Tm's averaging ~50 °C.

The BC, DE, and FG loops of ^{10}Fn3 are analogous to the complementary determining regions (CDRs) from immunoglobulins. Alteration of the amino acid sequence in these loop regions changes the binding specificity of ^{10}Fn3. The protein sequences outside of the CDR-like loops are analogous to the framework regions

from immunoglobulins and play a role in the structural conformation of the ^{10}Fn3. Alterations in the framework-like regions of ^{10}Fn3 are permissible to the extent that the structural conformation is not so altered as to disrupt ligand binding. Methods for generating ^{10}Fn3 ligand specific binders have been described in PCT Publication Nos. WO 00/034787, WO 01/64942, and WO 02/032925, disclosing high affinity TNFα binders, PCT Publication No. WO 2008/097497, disclosing high affinity VEGFR2 binders, and PCT Publication No. WO 2008/066752, disclosing high affinity IGFIR binders. Additional references discussing ^{10}Fn3 binders and methods of selecting binders include PCT Publication Nos. WO 98/056915, WO 02/081497, and WO 2008/031098 and U.S. Publication No. 2003186385.

Antibody-like proteins based on the ^{10}Fn3 scaffold can be defined generally by the sequence:
VSDVPRDLEVVAATPTSLLI$(X)_n$YYRITYGETGGNSPVQEFTV$(X)_o$ATISGLKP GVDYTITVYAV$(X)_p$ISINYRT (SEQ ID NO: 32), wherein n is an integer from 1-20, o is an integer from 1-20, and p is an integer from 1-40. The BC, DE, and FG loops are represented by $(X)_n$, $(X)_o$, and $(X)_p$, respectively.

^{10}Fn3 generally begin with the amino acid residue corresponding to number 1 of SEQ ID NO: 1. However, domains with amino acid deletions are also encompassed by the invention. In some embodiments, amino acid residues corresponding to the first eight amino acids of SEQ ID NO: 1 are deleted. Additional sequences may also be added to the N- or C-terminus. For example, an additional MG sequence may be placed at the N-terminus of ^{10}Fn3. The M will usually be cleaved off, leaving a G at the N-terminus. In some embodiments, sequences may be placed at the C-terminus of the ^{10}Fn3 domain, e.g., EIDKPSQ (SEQ ID NO: 9), EIDKPCQ (SEQ ID NO: 10), EGSGS (SEQ ID NO: 96) or EGSGC (SEQ ID NO: 97).

The non-ligand binding sequences of ^{10}Fn3, i.e., the "^{10}Fn3 scaffold", may be altered provided that the ^{10}Fn3 retains ligand binding function and/or structural stability. In some embodiments, one or more of Asp 7, Glu 9, and Asp 23 are replaced by another amino acid, such as, for example, a non-negatively charged amino acid residue (e.g., Asn, Lys, etc.). These mutations have been reported to have the effect of promoting greater stability of the mutant ^{10}Fn3 at neutral pH as

compared to the wild-type form (See, PCT Publication No. WO 02/04523). A variety of additional alterations in the ^{10}Fn3 scaffold that are either beneficial or neutral have been disclosed. See, for example, Batori et al., Protein Eng. 2002 15(12):1015-20; Koide et al., Biochemistry 2001 40(34):10326-33.

The ^{10}Fn3 scaffold may be modified by one or more conservative substitutions. As many as 5%, 10%, 20% or even 30% or more of the amino acids in the ^{10}Fn3 scaffold may be altered by a conservative substitution without substantially altering the affinity of the ^{10}Fn3 for a ligand. For example, the scaffold modification preferably reduces the binding affinity of the ^{10}Fn3 binder for a ligand by less than 100-fold, 50-fold, 25-fold, 10-fold, 5-fold, or 2-fold. It may be that such changes will alter the immunogenicity of the ^{10}Fn3 in vivo, and where the immunogenicity is decreased, such changes will be desirable. As used herein, "conservative substitutions" are residues that are physically or functionally similar to the corresponding reference residues. That is, a conservative substitution and its reference residue have similar size, shape, electric charge, chemical properties including the ability to form covalent or hydrogen bonds, or the like. Preferred conservative substitutions are those fulfilling the criteria defined for an accepted point mutation in Dayhoff et al., Atlas of Protein Sequence and Structure 5:345-352 (1978 & Supp.). Examples of conservative substitutions are substitutions within the following groups: (a) valine, glycine; (b) glycine, alanine; (c) valine, isoleucine, leucine; (d) aspartic acid, glutamic acid; (e) asparagine, glutamine; (f) serine, threonine; (g) lysine, arginine, methionine; and (h) phenylalanine, tyrosine.

E Binders

In one aspect, the disclosure provides antibody-like proteins comprising an EGFR binding ^{10}Fn3 domain. In certain embodiments, an EGFR binding ^{10}Fn3 may be provided as part of a fusion protein or multimer. For example, an EGFR binding ^{10}Fn3 may be covalently or non-covalently linked to at least a second ^{10}Fn3 binding domain. The second ^{10}Fn3 binding domain may bind to EGFR or to a different target. In an exemplary embodiment, an EGFR binding ^{10}Fn3 may be covalently or non-covalently linked to an IGF-IR binding ^{10}Fn3.

In exemplary embodiments, the EGFR binding ^{10}Fn3 proteins described herein bind to EGFR with a K_D of less than 500 nM, 100 nM, 50 nM, 10 nM, 1 nM, 500 pM, 100 pM. 100 pM, 50 pM or 10 pM.

In exemplary embodiments, the BC loop of the EGFR binding ^{10}Fn3 proteins correspond to amino acids 23-30 of SEQ ID NO: 1, the DE loop of the EGFR binding ^{10}Fn3 proteins correspond to amino acids 52-55 of SEQ ID NO: 1, and the FG loop of the EGFR binding ^{10}Fn3 proteins correspond to amino acids 77-86 of SEQ ID NO: 1.

In one embodiment, the invention provides an EGFR binding ^{10}Fn3 comprising a BC loop having a YQ at the positions corresponding to amino acids 29 and 30 of SEQ ID NO: 1.

In another embodiment, the invention provides an EGFR binding ^{10}Fn3 comprising a BC loop having a YQ at the positions corresponding to amino acids 29 and 30 of SEQ ID NO: 1 and an FG loop that is fifteen amino acids in length, e.g., an FG loop that is extended in length by five amino acids due to an insertion of five amino acids between residues corresponding to amino acids 77-86 of SEQ ID NO: 1.

In another embodiment, the invention provides an EGFR binding ^{10}Fn3 comprising a BC loop having a YQ at the positions corresponding to amino acids 29 and 30 of SEQ ID NO: 1 and a DE loop having a V, I, L, M or A residue at the position corresponding to amino acid 54 of SEQ ID NO: 1.

In another embodiment, the invention provides an EGFR binding ^{10}Fn3 comprising a BC loop having a YQ at the positions corresponding to amino acids 29 and 30 of SEQ ID NO: 1, a DE loop having a V, I, L, M or A residue at the position corresponding to amino acid 54 of SEQ ID NO: 1, and an FG loop that is fifteen amino acids in length, e.g., an FG loop that is extended in length by five amino acids due to an insertion of five amino acids between residues corresponding to amino acids 77-86 of SEQ ID NO: 1.

In another embodiment, the invention provides an EGFR binding ^{10}Fn3 comprising a BC loop having a YQ at the positions corresponding to amino acids 29 and 30 of SEQ ID NO: 1 and an FG loop comprising a D or N at the position corresponding to amino acid 77 of SEQ ID NO: 1.

In another embodiment, the invention provides an EGFR binding ^{10}Fn3 comprising a BC loop having a YQ at the positions corresponding to amino acids 29 and 30 of SEQ ID NO: 1 and an FG loop (i) that is fifteen amino acids in length, e.g., an FG loop that is extended in length by five amino acids due to an insertion of five amino acids between residues corresponding to amino acids 77-86 of SEQ ID NO: 1 and (ii) comprises a D or N at the position corresponding to amino acid 77 of SEQ ID NO: 1.

In another embodiment, the invention provides an EGFR binding ^{10}Fn3 comprising a DE loop comprising a V, I, L, M or A residue at the position corresponding to amino acid 54 of SEQ ID NO: 1 and an FG loop comprising a D or N at the position corresponding to amino acid 77 of SEQ ID NO: 1.

In another embodiment, the invention provides an EGFR binding ^{10}Fn3 comprising a DE loop comprising a V, I, L, M or A residue at the position corresponding to amino acid 54 of SEQ ID NO: 1 and an FG loop (i) that is fifteen amino acids in length, e.g., an FG loop that is extended in length by five amino acids due to an insertion of five amino acids between residues corresponding to amino acids 77-86 of SEQ ID NO: 1 and (ii) comprises a D or N at the position corresponding to amino acid 77 of SEQ ID NO: 1.

In another embodiment, the invention provides an EGFR binding ^{10}Fn3 comprising a BC loop having a YQ at the positions corresponding to amino acids 29 and 30 of SEQ ID NO: 1, a DE loop comprising a V, I, L, M or A residue at the position corresponding to amino acid 54 of SEQ ID NO: 1, and an FG loop comprising a D or N at the position corresponding to amino acid 77 of SEQ ID NO: 1.

In another embodiment, the invention provides an EGFR binding ^{10}Fn3 comprising a BC loop having a YQ at the positions corresponding to amino acids 29 and 30 of SEQ ID NO: 1, a DE loop comprising a V, I, L, M or A residue at the position corresponding to amino acid 54 of SEQ ID NO: 1, and an FG loop (i) that is fifteen amino acids in length, e.g., an FG loop that is extended in length by five amino acids due to an insertion of five amino acids between residues corresponding to amino acids 77-86 of SEQ ID NO: 1 and (ii) comprises a D or N at the position corresponding to amino acid 77 of SEQ ID NO: 1.

In another embodiment, the invention provides an EGFR binding ^{10}Fn3 comprising a BC loop comprising the amino acid sequence XXXXXXYQ, a DE loop comprising the amino acid sequence XX(V/I/L/M/A)X, and an FG loop comprising the amino acid sequence (D/N)X_n, wherein X is any amino acid and n is 9-14 amino acids. In an exemplary embodiment, n is 14 amino acids.

In another embodiment, the invention provides an EGFR binding ^{10}Fn3 comprising a BC loop corresponding to amino acids 23-30 of SEQ ID NO: 1 comprising the amino acid sequence XXXXXXYQ, a DE loop corresponding to amino acids 52-55 of SEQ ID NO: 1 comprising the amino acid sequence XX(V/I/L/M/A)X, and an FG loop corresponding to amino acids 77-86 of SEQ ID NO: 1comprising the amino acid sequence (D/N)X_n, wherein X is any amino acid and n is 9-14 amino acids. In an exemplary embodiment, n is 14 amino acids.

In another embodiment, the invention provides an EGFR binding ^{10}Fn3 comprising a BC loop comprising the amino acid sequence XXXXXXYQ, a DE loop comprising the amino acid sequence XX(V/I/L/M/A)X, and an FG loop comprising an amino acid sequence selected from:

i.

(D/N)(Y/M)(Y/A/M)(Y/H/F)(K/Q/V)(E/P/R)(Y/T/K)X(E/Y/Q)(Y/G/H); and

ii.

D(Y/F/W)(Y/F/K)(N/D/P)(P/H/L)(A/T/V)(T/D/S)(H/Y/G)(E/P/V)(Y/H)(T/K/I)(Y/F)(H/N/Q)(T/Q/E)(T/S/I);

wherein X is any amino acid.

In another embodiment, the invention provides an EGFR binding ^{10}Fn3 comprising a BC loop comprising the amino acid sequence XXXXXXYQ, a DE loop comprising the amino acid sequence (G/Y/H)(D/M/G)(V/L/I)X, and an FG loop comprising an amino acid sequence (D/N)(Y/M)(Y/A/M)(Y/H/F)(K/Q/V)(E/P/R)(Y/T/K)X(E/Y/Q)(Y/G/H), wherein X is any amino acid.

In another embodiment, the invention provides an EGFR binding ^{10}Fn3 comprising a BC loop comprising the amino acid sequence XXXXXXYQ, a DE loop comprising the amino acid sequence (G/Y/H)(D/M/G)(V/L/I)X, and an FG loop comprising an amino acid sequence

D(Y/F/W)(Y/F/K)(N/D/P)(P/H/L)(A/T/V)(T/D/S)(H/Y/G)(E/P/V)(Y/H)(T/K/I)(Y/F)(H/N/Q)(T/Q/E)(T/S/I), wherein X is any amino acid.

In another embodiment, the invention provides an EGFR binding ^{10}Fn3 comprising a BC loop comprising the amino acid sequence XXXXXXYQ, a DE loop comprising the amino acid sequence XX(V/I/L/M/A)X, and an FG loop comprising an amino acid sequence selected from:

i. DY(A/Y)GKPYXEY (SEQ ID NO: 473);

ii. DY(A/Y)Y(K/R/Q/T)PYXEY (SEQ ID NO: 474);

iii. (D/N)Y(A/Y)(Y/F)(K/R/Q/T)EYXE(Y/H) (SEQ ID NO: 475);

iv. DYY(H/Y)X(R/K)X(E/T)YX (SEQ ID NO: 476);

v. DYY(H/Y)(K/H/Q)(R/K)T(E/T)Y(G/P) (SEQ ID NO: 477);

vi. (D/N)MMHV(E/D)YXEY (SEQ ID NO: 478);

vii. DYMHXXYXEY (SEQ ID NO: 479); and

viii. D(M/Y)YHX(K/R)X(V/I/L/M)YG (SEQ ID NO: 480);

wherein X is any amino acid.

In another embodiment, the invention provides an EGFR binding ^{10}Fn3 comprising a BC loop comprising the amino acid sequence XXXXXXYQ, a DE loop comprising the amino acid sequence XX(V/I/L/M/A)X, and an FG loop comprising an amino acid sequence selected from:

i. D(Y/F)(Y/F)NPXTHEYXYXXX (SEQ ID NO: 481);

ii. D(Y/F)(Y/F)D(P/L)X(T/S)HXYXYXXX (SEQ ID NO: 482); and

iii. D(Y/F)(K/R)PHXDGPH(T/I)YXE(S/Y) (SEQ ID NO: 483);

wherein X is any amino acid.

In another embodiment, the invention provides an EGFR binding ^{10}Fn3 comprising a BC loop comprising the amino acid sequence XXXXXXYQ, a DE loop comprising the amino acid sequence XX(V/I/L/M/A)X, and an FG loop comprising the amino acid sequence (D/N)(M/Y)(M/A/W)(H/F/Y)(V/K)EY(A/Q/R/S/T)E(Y/H/D), wherein X is any amino acid.

In another embodiment, the invention provides an EGFR binding ^{10}Fn3 comprising a BC loop comprising the amino acid sequence XXXXXXYQ, a DE loop comprising the amino acid sequence XX(V/I/L/M/A)X, and an FG loop

comprising the amino acid sequence D(Y/F/W)(Y/F/K)(N/P/D)(P/H/L)X(T/D/S)(H/G/Y)(E/P/Y)(Y/H)XYXXX, wherein X is any amino acid.

In various embodiments, the DE loop of the EGFR binding ^{10}Fn3 may comprise the sequence (G/Y/H)(D/M/G)(V/L/I)X.

In another embodiment, the invention provides an EGFR binding ^{10}Fn3 comprising an FG loop comprising an amino acid sequence selected from:

i. D(Y/F)(Y/F)NPXTHEYXYXXX (SEQ ID NO: 481);

ii. D(Y/F)(Y/F)D(P/L)X(T/S)HXYXYXXX (SEQ ID NO: 482); and

iii. D(Y/F)(K/R)PHXDGPH(T/I)YXE(S/Y) (SEQ ID NO: 483);

wherein X is any amino acid.

In certain embodiments, the EGFR binding ^{10}Fn3 comprises any of the consensus sequences provided above, with the proviso that the EGFR binding ^{10}Fn3 does not comprise one or more of the following sequences:

i.

VSDVPRDLEVVAATPTSLLISWQVPRPMYQRYYRITYGETGGNSPVQ EFTVPGGVRTATISGLKPGVDYTITVYAVTDYMHSEYRQYPISINYRT (SEQ ID NO: 484), and

ii.

VSDVPRDLEVVAATPTSLLISWQVPRPMYQYYRITYGETGGNSPVQE FTVPGGVRTATISGLKPGVDYTITVYAVTDYMHSEYRQYPISINYRT (SEQ ID NO: 485), and

iii.

VSDVPRDLEVVAATPTSLLISWQVPRPMYQRYYRITYGETGGNSPVQ EFTVPGGVRTATISGLKPGVDYTITVYAVTDYMHSEYRQYPISINYRTEIDKP CQ (SEQ ID NO: 486).

In certain embodiments, an EGFR binding ^{10}Fn3 comprising one of the consensus sequences provided above has at least 40%, 50%, 60%, 70%, 75%, or 80% identity to SEQ ID NO: 1. In certain embodiments, the overall structure of an EGFR binding ^{10}Fn3 comprising one of the consensus sequences provided above resembles the immunoglobulin fold. In certain embodiment, an EGFR binding ^{10}Fn3 comprising one of the consensus sequences provided above further comprises

the core amino acid residues of the scaffold. In certain embodiments, an EGFR binding ^{10}Fn3 comprising one of the consensus sequences provided above has at least 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to any one of SEQ ID NOs: 5-8, 52, 66-68, 106-108, 112-114, 140-142, 155-157, 170-172, 182, 185-187, 198-200, or 219-327. In certain embodiments, an EGFR binding ^{10}Fn3 comprising one of the consensus sequences provided above has at least 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to the amino acid sequence of amino acid residues corresponding to E9 of SEQ ID NO: 1 through T94 of SEQ ID NO: 1 of any one of SEQ ID NOs: 5-8, 52, 66-68, 106-108, 112-114, 140-142, 155-157, 170-172, 182, 185-187, 198-200, or 219-327. In certain embodiments, the EGFR binding ^{10}Fn3 comprising one of the consensus sequences provided above comprises a ^{10}Fn3 scaffold having from has anywhere from 0 to 20, from 0 to 15, from 0 to 10, from 0 to 8, from 0 to 6, from 0 to 5, from 0 to 4, from 0 to 3, from 0 to 2, or from 0 to 1 substitutions, conservative substitutions, deletions or additions relative to the scaffold amino acids residues of SEQ ID NO: 1.

In certain embodiments, the invention provides an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 23-30, a DE loop having the amino acid sequence set forth in amino acids 52-55, and an FG loop having the amino acid sequence set forth in amino acids 77-86 of any one of SEQ ID NOs: 219-327. In certain embodiments, the invention provides an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 21-30, a DE loop having the amino acid sequence set forth in amino acids 51-56, and an FG loop having the amino acid sequence set forth in amino acids 76-87 of any one of SEQ ID NOs: 219-327. In certain embodiments, the invention provides an EGFR binding ^{10}Fn3 comprising an amino acid sequence at least 60%, 75%, 80%, 85%, 90%, 95%, or 98% identical to any one of SEQ ID NOs: 219-327.

In one embodiment, an antibody-like protein is provided comprising a tenth fibronectin type III domain (^{10}Fn3) that binds EGFR with a K_D of less than 500 nM and comprises a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 5, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 5, and an FG loop having the amino acid sequence set forth in amino acids 76-92 of SEQ ID NO: 5. In some embodiments,

the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence $X_gDSGRGSYQX_h$ (SEQ ID NO: 40), a DE loop having the amino acid sequence X_iGPVHX_j (SEQ ID NO: 42), and an FG loop having the amino acid sequence $X_kDHKPHADGPHTYHEX_l$ (SEQ ID NO: 44); wherein X is any amino acid and g, h, i, j, k, and l are integers independently selected from 0 to 5. In some embodiments, the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence SWDSGRGSYQ (SEQ ID NO: 39), a DE loop having the amino acid sequence PGPVHT (SEQ ID NO: 41), and an FG loop having the amino acid sequence TDHKPHADGPHTYHESP (SEQ ID NO: 43). In some embodiments, the EGFR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, or 100% identical to SEQ ID NOs: 5 or 6.

In one embodiment, an antibody-like protein is provided comprising a tenth fibronectin type III domain (^{10}Fn3) that binds EGFR with a K_D of less than 500 nM and comprises a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 7, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 7, and an FG loop having the amino acid sequence set forth in amino acids 76-87 of SEQ ID NO: 7. In some embodiments, the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence $X_mVAGAEDYQX_n$ (SEQ ID NO: 34), a DE loop having the amino acid sequence X_oHDLVX_p (SEQ ID NO: 36), and an FG loop having the amino acid sequence $X_qDMMHVEYTEHX_r$ (SEQ ID NO: 38); wherein X is any amino acid and m, n, o, p, q, and r are integers independently selected from 0 to 5. In some embodiments, the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence SWVAGAEDYQ (SEQ ID NO: 33), a DE loop having the amino acid sequence PHDLVT (SEQ ID NO: 35), and an FG loop having the amino acid sequence TDMMHVEYTEHP (SEQ ID NO: 37). In some embodiments, the EGFR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, or 100% identical to SEQ ID NO: 7 or 8.

In one embodiment, an antibody-like protein is provided comprising a tenth fibronectin type III domain (^{10}Fn3) that binds EGFR with a K_D of less than 500 nM and comprises a BC loop having the amino acid sequence set forth in amino acids 23-30 of SEQ ID NO: 82, a DE loop having the amino acid sequence set forth in

amino acids 51-55 of SEQ ID NO: 82, and an FG loop having the amino acid sequence set forth in amino acids 76-86 of SEQ ID NO: 82. In some embodiments, the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence $X_sLPGKLRYQX_t$ (SEQ ID NO: 60), a DE loop having the amino acid sequence X_uHDLRX_w (SEQ ID NO: 62), and an FG loop having the amino acid sequence $X_yNMMHVEYSEYX_z$ (SEQ ID NO: 64); wherein X is any amino acid and s, t, u, w, y and z are integers independently selected from 0 to 5. In some embodiments, the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence LPGKLRYQ (resdiues 3-13 of SEQ ID NO: 59), a DE loop having the amino acid sequence PHDLR (residues 1-5 of SEQ ID NO: 61), and an FG loop having the amino acid sequence TNMMHVEYSEY (residues 1-11 of SEQ ID NO: 63). In some embodiments, the EGFR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, or 100% identical to SEQ ID NO: 52 or 82.

In one embodiment, an antibody-like protein is provided comprising a tenth fibronectin type III domain (^{10}Fn3) that binds EGFR with a K_D of less than 500 nM and comprises a BC loop having the amino acid sequence set forth in amino acids 23-30 of SEQ ID NO: 106, a DE loop having the amino acid sequence set forth in amino acids 51-55 of SEQ ID NO: 106, and an FG loop having the amino acid sequence set forth in amino acids 76-86 of SEQ ID NO: 106. In some embodiments, the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence $X_gHERDGSRQX_h$ (SEQ ID NO: 134), a DE loop having the amino acid sequence X_iGGVRX_j (SEQ ID NO: 135), and an FG loop having the amino acid sequence $X_kDYFNPTTHEYIYQTTX_l$ (SEQ ID NO: 136); wherein X is any amino acid and g, h, i, j, k and l are integers independently selected from 0 to 5. In some embodiments, the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence SWHERDGSRQ (SEQ ID NO: 109), a DE loop having the amino acid sequence PGGVRT (SEQ ID NO: 110), and an FG loop having the amino acid sequence TDYFNPTTHEYIYQTTP (SEQ ID NO: 111). In some embodiments, the EGFR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, or 100% identical to any one of SEQ ID NOs: 106-108.

In one embodiment, an antibody-like protein is provided comprising a tenth fibronectin type III domain (^{10}Fn3) that binds EGFR with a K_D of less than 500 nM

and comprises a BC loop having the amino acid sequence set forth in amino acids 23-30 of SEQ ID NO: 112, a DE loop having the amino acid sequence set forth in amino acids 51-55 of SEQ ID NO: 112, and an FG loop having the amino acid sequence set forth in amino acids 76-86 of SEQ ID NO: 112. In some embodiments, the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence $X_gWAPVDRYQX_h$ (SEQ ID NO: 137), a DE loop having the amino acid sequence X_iRDVYX_j (SEQ ID NO: 138), and an FG loop having the amino acid sequence $X_kDYKPHADGPHTYHESX_l$ (SEQ ID NO: 139); wherein X is any amino acid and g, h, i, j, k and l are integers independently selected from 0 to 5. In some embodiments, the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence SWWAPVDRYQ (SEQ ID NO: 115), a DE loop having the amino acid sequence PRDVYT (SEQ ID NO: 116), and an FG loop having the amino acid sequence TDYKPHADGPHTYHESP (SEQ ID NO: 117). In some embodiments, the EGFR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, or 100% identical to any one of SEQ ID NOs: 112-114.

In one embodiment, an antibody-like protein is provided comprising a tenth fibronectin type III domain (^{10}Fn3) that binds EGFR with a K_D of less than 500 nM and comprises a BC loop having the amino acid sequence set forth in amino acids 13-22 of SEQ ID NO: 141, a DE loop having the amino acid sequence set forth in amino acids 43-48 of SEQ ID NO: 141, and an FG loop having the amino acid sequence set forth in amino acids 68-84 of SEQ ID NO: 141. In some embodiments, the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence $X_gTQGSTHYQX_h$ (SEQ ID NO: 146), a DE loop having the amino acid sequence X_iGMVYX_j (SEQ ID NO: 147), and an FG loop having the amino acid sequence $X_kDYFDRSTHEYKYRTTX_l$ (SEQ ID NO: 148); wherein X is any amino acid and g, h, i, j, k and l are integers independently selected from 0 to 5. In some embodiments, the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence SWTQGSTHYQ (SEQ ID NO: 143), a DE loop having the amino acid sequence PGMVYT (SEQ ID NO: 144), and an FG loop having the amino acid sequence TDYFDRSTHEYKYRTTP (SEQ ID NO: 145). In some embodiments, the EGFR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, or 100% identical to any one of SEQ ID NOs: 140-142.

In one embodiment, an antibody-like protein is provided comprising a tenth fibronectin type III domain (^{10}Fn3) that binds EGFR with a K_D of less than 500 nM and comprises a BC loop having the amino acid sequence set forth in amino acids 13-22 of SEQ ID NO: 156, a DE loop having the amino acid sequence set forth in amino acids 43-48 of SEQ ID NO: 156, and an FG loop having the amino acid sequence set forth in amino acids 68-84 of SEQ ID NO: 156. In some embodiments, the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence X_gYWEGLPYQX_h (SEQ ID NO: 161), a DE loop having the amino acid sequence X_iRDVNX_j (SEQ ID NO: 162), and an FG loop having the amino acid sequence X_kDWYNPDTHEYIYHTIX_l (SEQ ID NO: 163); wherein X is any amino acid and g, h, i, j, k and l are integers independently selected from 0 to 5. In some embodiments, the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence SWYWEGLPYQ (SEQ ID NO: 158), a DE loop having the amino acid sequence PRDVNT (SEQ ID NO: 159), and an FG loop having the amino acid sequence TDWYNPDTHEYIYHTIP (SEQ ID NO: 160). In some embodiments, the EGFR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, or 100% identical to any one of SEQ ID NOs: 155-157.

In one embodiment, an antibody-like protein is provided comprising a tenth fibronectin type III domain (^{10}Fn3) that binds EGFR with a K_D of less than 500 nM and comprises a BC loop having the amino acid sequence set forth in amino acids 13-22 of SEQ ID NO: 171, a DE loop having the amino acid sequence set forth in amino acids 43-48 of SEQ ID NO: 171, and an FG loop having the amino acid sequence set forth in amino acids 68-84 of SEQ ID NO: 171. In some embodiments, the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence X_gASNRGTYQX_h (SEQ ID NO: 176), a DE loop having the amino acid sequence X_iGGVSX$_j$ (SEQ ID NO: 177), and an FG loop having the amino acid sequence X_kDAFNPTTHEYNYFTTX$_l$ (SEQ ID NO: 178); wherein X is any amino acid and g, h, i, j, k and l are integers independently selected from 0 to 5. In some embodiments, the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence SWASNRGTYQ (SEQ ID NO: 173), a DE loop having the amino acid sequence PGGVST (SEQ ID NO: 174), and an FG loop having the amino acid sequence TDAFNPTTHEYNYFTTP (SEQ ID NO: 175). In some embodiments, the

EGFR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, or 100% identical to any one of SEQ ID NOs: 170-172.

In one embodiment, an antibody-like protein is provided comprising a tenth fibronectin type III domain (^{10}Fn3) that binds EGFR with a K_D of less than 500 nM and comprises a BC loop having the amino acid sequence set forth in amino acids 13-22 of SEQ ID NO: 186, a DE loop having the amino acid sequence set forth in amino acids 43-48 of SEQ ID NO: 186, and an FG loop having the amino acid sequence set forth in amino acids 68-84 of SEQ ID NO: 186. In some embodiments, the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence $X_gDAPTSRYQX_h$ (SEQ ID NO: 190), a DE loop having the amino acid sequence X_iGGLSX_j (SEQ ID NO: 191), and an FG loop having the amino acid sequence $X_kDYKPHADGPHTYHESX_l$ (SEQ ID NO: 139); wherein X is any amino acid and g, h, i, j, k and l are integers independently selected from 0 to 5. In some embodiments, the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence SWDAPTSRYQ (SEQ ID NO: 188), a DE loop having the amino acid sequence PGGLST (SEQ ID NO: 189), and an FG loop having the amino acid sequence TDYKPHADGPHTYHESP (SEQ ID NO: 117). In some embodiments, the EGFR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, or 100% identical to any one of SEQ ID NOs: 185-187.

In one embodiment, an antibody-like protein is provided comprising a tenth fibronectin type III domain (^{10}Fn3) that binds EGFR with a K_D of less than 500 nM and comprises a BC loop having the amino acid sequence set forth in amino acids 13-22 of SEQ ID NO: 199, a DE loop having the amino acid sequence set forth in amino acids 43-48 of SEQ ID NO: 199, and an FG loop having the amino acid sequence set forth in amino acids 68-84 of SEQ ID NO: 199. In some embodiments, the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence $X_gDAGAVTYQX_h$ (SEQ ID NO: 203), a DE loop having the amino acid sequence X_iGGVRX_j (SEQ ID NO: 135), and an FG loop having the amino acid sequence $X_kDYKPHADGPHTYHEYX_l$ (SEQ ID NO: 204); wherein X is any amino acid and g, h, i, j, k and l are integers independently selected from 0 to 5. In some embodiments, the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence SWDAGAVTYQ (SEQ ID NO: 201), a DE loop having the amino acid

sequence PGGVRT (SEQ ID NO: 110), and an FG loop having the amino acid sequence TDYKPHADGPHTYHEYP (SEQ ID NO: 202). In some embodiments, the EGFR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, or 100% identical to any one of SEQ ID NOs: 198-200.

In certain embodiments, an EGFR binding ^{10}Fn3 domain is covalently or non-covalently linked to an EGF-IR binding ^{10}Fn3 domain. In exemplary embodiments, the IGF-IR binding ^{10}Fn3 may comrpise a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 3, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 3, and an FG loop having the amino acid sequence set forth in amino acids 76-83 of SEQ ID NO: 3. In some embodiments, the IGF-IR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence $X_aSARLKVAX_b$ (SEQ ID NO: 46), a DE loop having the amino acid sequence X_cKNVYX_d (SEQ ID NO: 48), and an FG loop having the amino acid sequence $X_eRFRDYQX_f$ (SEQ ID NO: 50), wherein X is any amino acid and a, b, c, d, e, f, g, h, i, j, k, and l are integers independently selected from 0 to 5, or wherein a is 2 and b-f are 1, or wherein a-f are zero. In some embodiments, the IGF-IR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, 98, 99, or 100% identical to SEQ ID NO: 3. In certain embodiments, the IGF-IR binding ^{10}Fn3 comprises a ^{10}Fn3 scaffold having from has anywhere from 0 to 20, from 0 to 15, from 0 to 10, from 0 to 8, from 0 to 6, from 0 to 5, from 0 to 4, from 0 to 3, from 0 to 2, or from 0 to 1 substitutions, conservative substitutions, deletions or additions relative to the scaffold amino acid residues of SEQ ID NO: 1. In certain embodiments, the IGF-IR binding ^{10}Fn3 has anywhere from 0 to 15, from 0 to 10, from 0 to 8, from 0 to 6, from 0 to 5, from 0 to 4, from 0 to 3, from 0 to 2, or from 0 to 1 substitutions, conservative substitutions, deletions or additions relative to the corresponding loop sequences of SEQ ID NO: 3.

^{10}Fn3 E/I Binders

One aspect of the disclosure provides E/I binders constructed from antibody-like protein multimers. In some embodiments, an antibody-like protein multimer comprises at least one EGFR binding ^{10}Fn3 covalently or non-covalently linked to at least one IGFIR binding ^{10}Fn3. In certain embodiments, the E/I binders described

herein may be constructed as a single polypeptide chain wherein the E and I subunits may be in either orientation, e.g., from N-terminus to C-terminus, in the E-I orientation or in the I-E orientation.

The disclosure relates, in part, to the surprising discovery that multiple ^{10}Fn3 joined via a polypeptide linker correctly fold independently of each other, retain high affinity binding, and that each of the domains retains its functional properties (see e.g., Examples 5-10). Additionally, these E/I ^{10}Fn3 based binders demonstrate desirable biophysical properties such as low aggregation and high melting temperature (T_m) (see e.g., Example 4). The Examples characterize a variety of E/I ^{10}Fn3 based binders. An exemplary IGFIR binding ^{10}Fn3 is set forth in SEQ ID NO: 4. Exemplary EGFR binding ^{10}Fn3 are set forth in SEQ ID NOs: 6, 8, 52, 107, 113, 140, 155, 170, 185 and 198.

In some embodiments, an E/I binder comprises an EGFR binding ^{10}Fn3 and an IGFIR binding ^{10}Fn3, independently having an amino acid sequence at least 40, 50, 60, 70, or 80% identical to the human ^{10}Fn3 domain, shown in SEQ ID NO: 1. Much of the variability will generally occur in one or more of the loops.

In some embodiments, an E/I binder comprises an EGFR binding ^{10}Fn3 and an IGFIR binding ^{10}Fn3, independently having an amino acid sequence at least 70, 80, 85, 90, 95, 98, or 100% identical to SEQ ID NO: 32, wherein n is an integer from 1-20, o is an integer from 1-20, and p is an integer from 1-40. In some embodiments, n is an integer from 8-12, o is an integer from 4-8, and p is an integer from 4-28. In some embodiments, n is 10, o is 6, and p is 12.

In some embodiments, the disclosure provides multimers of ^{10}Fn3 having at least one loop selected from loop BC, DE, and FG with an altered amino acid sequence relative to the sequence of the corresponding loop of the human ^{10}Fn3. By "altered" is meant one or more amino acid sequence alterations relative to a template sequence (corresponding human fibronectin domain) and includes amino acid additions, deletions, and substitutions. Altering an amino acid sequence may be accomplished through intentional, blind, or spontaneous sequence variation, generally of a nucleic acid coding sequence, and may occur by any technique, for example, PCR, error-prone PCR, or chemical DNA synthesis. In some

embodiments, an amino acid sequence is altered by substituting with or adding naturally occurring amino acids.

In some embodiments, one or more loops selected from BC, DE, and FG may be extended or shortened in length relative to the corresponding human fibronectin loop. In particular, the FG loop of the human ^{10}Fn3 is 12 residues long, whereas the corresponding loop in antibody heavy chains ranges from 4-28 residues. To optimize antigen binding, therefore, the length of the FG loop of ^{10}Fn3 may be altered in length as well as in sequence to obtain the greatest possible flexibility and affinity in antigen binding.

In some embodiments of the ^{10}Fn3 molecules, the altered BC loop has up to 10 amino acid substitutions, up to 9 amino acid deletions, up to 10 amino acid insertions, or a combination of substitutions and deletions or insertions. In some embodiments, the altered DE loop has up to 6 amino acid substitutions, up to 5 amino acid deletions, up to 14 amino acid insertions or a combination of substitutions and deletions or insertions. In some embodiments, the FG loop has up to 12 amino acid substitutions, up to 11 amino acid deletions, up to 28 amino acid insertions or a combination of substitutions and deletions or insertions.

Naturally occurring ^{10}Fn3 comprises an "arginine-glycine-aspartic acid" (RGD) integrin-binding motif in the FG loop. Preferred multimers of ^{10}Fn3 lack an RGD integrin-binding motif.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising a) an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 5, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 5, and an FG loop having the amino acid sequence set forth in amino acids 76-92 of SEQ ID NO: 5; covalently or non-covalently linked to b) an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 3, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 3, and an FG loop having the amino acid sequence set forth in amino acids 76-83 of SEQ ID NO: 3. In some embodiments, the EGFR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, 98, 99, or 100% identical to SEQ ID NO: 5. In some embodiments, the IGFIR binding ^{10}Fn3 has an amino acid sequence at

least 80, 90, 95, 98, 99, or 100% identical to SEQ ID NO: 3. In some embodiments, the E/I binder comprises an amino acid sequence at least 80, 85, 90, 95, 98, 99, or 100% identical to SEQ ID NOs: 20, 21, 23, 24, 90, 92, 101 or 103.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising a) an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 7, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 7, and an FG loop having the amino acid sequence set forth in amino acids 76-87 of SEQ ID NO: 7; covalently or non-covalently linked to b) an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 3, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 3, and an FG loop having the amino acid sequence set forth in amino acids 76-83 of SEQ ID NO: 3. In some embodiments, the EGFR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, 98, or 100% identical to SEQ ID NO: 7. In some embodiments, the IGFIR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, 98, or 100% identical to SEQ ID NO: 3. In some embodiments, the E/I binder comprises an amino acid sequence at least 80, 85, 90, 95, 98, or 100% identical to SEQ ID NOs: 26, 27, 29, 30, 89, 91, 100 or 102.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising a) an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 82, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 82, and an FG loop having the amino acid sequence set forth in amino acids 76-87 of SEQ ID NO: 82; covalently or non-covalently linked to b) an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 3, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 3, and an FG loop having the amino acid sequence set forth in amino acids 76-83 of SEQ ID NO: 3. In some embodiments, the EGFR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, 98, or 100% identical to SEQ ID NO: 82. In some embodiments, the IGFIR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, 98, or 100% identical to SEQ ID NO: 3. In some embodiments, the E/I

binder comprises an amino acid sequence at least 80, 85, 90, 95, 98, or 100% identical to SEQ ID NOs: 53, 54, 87, 88, 98, 99, 104 or 105.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising a) an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 106, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 106, and an FG loop having the amino acid sequence set forth in amino acids 76-92 of SEQ ID NO: 106; covalently or non-covalently linked to b) an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 3, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 3, and an FG loop having the amino acid sequence set forth in amino acids 76-83 of SEQ ID NO: 3. In some embodiments, the EGFR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, 98, or 100% identical to SEQ ID NO: 106. In some embodiments, the IGFIR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, 98, or 100% identical to SEQ ID NO: 3. In some embodiments, the E/I binder comprises an amino acid sequence at least 80, 85, 90, 95, 98, or 100% identical to SEQ ID NOs: 118-125.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising a) an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 112, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 112, and an FG loop having the amino acid sequence set forth in amino acids 76-92 of SEQ ID NO: 112; covalently or non-covalently linked to b) an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 3, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 3, and an FG loop having the amino acid sequence set forth in amino acids 76-83 of SEQ ID NO: 3. In some embodiments, the EGFR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, 98, or 100% identical to SEQ ID NO: 112. In some embodiments, the IGFIR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, 98, or 100% identical to SEQ ID NO: 3. In some embodiments, the E/I binder comprises an amino acid sequence at least 80, 85, 90, 95, 98, or 100% identical to SEQ ID NOs: 126-133.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising a) an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 13-22 of SEQ ID NO: 141, a DE loop having the amino acid sequence set forth in amino acids 43-48 of SEQ ID NO: 141, and an FG loop having the amino acid sequence set forth in amino acids 68-84 of SEQ ID NO: 141; covalently or non-covalently linked to b) an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 3, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 3, and an FG loop having the amino acid sequence set forth in amino acids 76-83 of SEQ ID NO: 3. In some embodiments, the EGFR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, 98, or 100% identical to SEQ ID NO: 140, 141, 142 or 300. In some embodiments, the IGFIR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, 98, or 100% identical to SEQ ID NO: 3. In some embodiments, the E/I binder comprises an amino acid sequence at least 80, 85, 90, 95, 98, or 100% identical to SEQ ID NOs: 149-154.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising a) an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 13-22 of SEQ ID NO: 156, a DE loop having the amino acid sequence set forth in amino acids 43-48 of SEQ ID NO: 156, and an FG loop having the amino acid sequence set forth in amino acids 68-84 of SEQ ID NO: 156; covalently or non-covalently linked to b) an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 3, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 3, and an FG loop having the amino acid sequence set forth in amino acids 76-83 of SEQ ID NO: 3. In some embodiments, the EGFR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, 98, or 100% identical to SEQ ID NO: 155, 156, 157 or 305. In some embodiments, the IGFIR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, 98, or 100% identical to SEQ ID NO: 3. In some embodiments, the E/I binder comprises an amino acid sequence at least 80, 85, 90, 95, 98, or 100% identical to SEQ ID NOs: 158-166.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising a) an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid

sequence set forth in amino acids 13-22 of SEQ ID NO: 171, a DE loop having the amino acid sequence set forth in amino acids 43-48 of SEQ ID NO: 171, and an FG loop having the amino acid sequence set forth in amino acids 68-84 of SEQ ID NO: 171; covalently or non-covalently linked to b) an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 3, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 3, and an FG loop having the amino acid sequence set forth in amino acids 76-83 of SEQ ID NO: 3. In some embodiments, the EGFR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, 98, or 100% identical to SEQ ID NO: 170, 171, 172 or 311. In some embodiments, the IGFIR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, 98, or 100% identical to SEQ ID NO: 3. In some embodiments, the E/I binder comprises an amino acid sequence at least 80, 85, 90, 95, 98, or 100% identical to SEQ ID NOs: 179-184.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising a) an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 13-22 of SEQ ID NO: 186, a DE loop having the amino acid sequence set forth in amino acids 43-48 of SEQ ID NO: 186, and an FG loop having the amino acid sequence set forth in amino acids 68-84 of SEQ ID NO: 186; covalently or non-covalently linked to b) an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 3, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 3, and an FG loop having the amino acid sequence set forth in amino acids 76-83 of SEQ ID NO: 3. In some embodiments, the EGFR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, 98, or 100% identical to SEQ ID NO: 185, 186, 187 or 320. In some embodiments, the IGFIR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, 98, or 100% identical to SEQ ID NO: 3. In some embodiments, the E/I binder comprises an amino acid sequence at least 80, 85, 90, 95, 98, or 100% identical to SEQ ID NOs: 192-197.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising a) an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 13-22 of SEQ ID NO: 199, a DE loop having the amino acid sequence set forth in amino acids 43-48 of SEQ ID NO: 199, and an FG

loop having the amino acid sequence set forth in amino acids 68-84 of SEQ ID NO: 199; covalently or non-covalently linked to b) an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 3, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 3, and an FG loop having the amino acid sequence set forth in amino acids 76-83 of SEQ ID NO: 3. In some embodiments, the EGFR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, 98, or 100% identical to SEQ ID NO: 198, 199, 200 or 327. In some embodiments, the IGFIR binding ^{10}Fn3 has an amino acid sequence at least 80, 90, 95, 98, or 100% identical to SEQ ID NO: 3. In some embodiments, the E/I binder comprises an amino acid sequence at least 80, 85, 90, 95, 98, or 100% identical to SEQ ID NOs: 205-210.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence $X_aSARLKVAX_b$ (SEQ ID NO: 46), a DE loop having the amino acid sequence X_cKNVYX_d (SEQ ID NO: 48), and an FG loop having the amino acid sequence $X_eRFRDYQX_f$ (SEQ ID NO: 50), covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence $X_gDSGRGSYQX_h$ (SEQ ID NO: 40), a DE loop having the amino acid sequence X_iGPVHX_j (SEQ ID NO: 42), and an FG loop having the amino acid sequence $X_kDHKPHADGPHTYHEX_l$ (SEQ ID NO: 44); wherein X is any amino acid and a, b, c, d, e, f, g, h, i, j, k, and l are integers independently selected from 0 to 5. In some embodiments, a, g, and l are 2; b-f and i-k are 1; and h is zero. In some embodiments, a-l are zero.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWSARLKVAR (SEQ ID NO: 45), a DE loop having the amino acid sequence PKNVYT (SEQ ID NO: 47), and an FG loop having the amino acid sequence TRFRDYQP (SEQ ID NO: 49); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWDSGRGSYQ (SEQ ID NO: 39), a DE loop having the amino acid sequence PGPVHT (SEQ ID NO: 41), and an FG loop having the amino acid sequence TDHKPHADGPHTYHESP (SEQ ID NO: 43).

In some embodiments, an E/I binder is an antibody-like protein dimer comprising an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence $X_aSARLKVAX_b$ (SEQ ID NO: 46), a DE loop having the amino acid sequence X_cKNVYX_d (SEQ ID NO: 48), and an FG loop having the amino acid sequence $X_eRFRDYQX_f$ (SEQ ID NO: 50); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence $X_mVAGAEDYQX_n$ (SEQ ID NO: 34), a DE loop having the amino acid sequence X_oHDLVX_p (SEQ ID NO: 36), and an FG loop having the amino acid sequence $X_qDMMHVEYTEHX_r$ (SEQ ID NO: 38); wherein X is any amino acid and a, b, c, d, e, f, m, n, o, p, q, and r are integers from 0 to 5, independently. In some embodiments, a and m are 2; b-f and o-r are 1; and n is zero. In some embodiments, a-f and m-r are zero.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWSARLKVAR (SEQ ID NO: 45), a DE loop having the amino acid sequence PKNVYT (SEQ ID NO: 47), and an FG loop having the amino acid sequence TRFRDYQP (SEQ ID NO: 49); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWVAGAEDYQ (SEQ ID NO: 33), a DE loop having the amino acid sequence PHDLVT (SEQ ID NO: 35), and an FG loop having the amino acid sequence TDMMHVEYTEHP (SEQ ID NO: 37).

In some embodiments, an E/I binder is an antibody-like protein dimer comprising an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence $X_aSARLKVAX_b$ (SEQ ID NO: 46), a DE loop having the amino acid sequence X_cKNVYX_d (SEQ ID NO: 48), and an FG loop having the amino acid sequence $X_eRFRDYQX_f$ (SEQ ID NO: 50); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence $X_sLPGKLRYQX_t$ (SEQ ID NO: 60), a DE loop having the amino acid sequence X_uHDLRX_w (SEQ ID NO: 62), and an FG loop having the amino acid sequence $X_yNMMHVEYSEYX_z$ (SEQ ID NO: 64); wherein X is any amino acid and a, b, c, d, e, f, s, t, u, w, y, and z are integers from 0 to 5, independently. In some

embodiments, a and s are 2; b-f, u, w, y and z are 1; and t is zero. In some embodiments, a-f, s-u, w, y and z are zero.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWSARLKVAR (SEQ ID NO: 45), a DE loop having the amino acid sequence PKNVYT (SEQ ID NO: 47), and an FG loop having the amino acid sequence TRFRDYQP (SEQ ID NO: 49); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWLPGKLRYQ (SEQ ID NO: 59), a DE loop having the amino acid sequence PHDLRT (SEQ ID NO: 61), and an FG loop having the amino acid sequence TNMMHVEYSEYP (SEQ ID NO: 63).

In some embodiments, an E/I binder is an antibody-like protein dimer comprising an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence X_aSARLKVAX_b (SEQ ID NO: 46), a DE loop having the amino acid sequence X_cKNVYX_d (SEQ ID NO: 48), and an FG loop having the amino acid sequence X_eRFRDYQX_f (SEQ ID NO: 50); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence X_gHERDGSRQX_h (SEQ ID NO: 134), a DE loop having the amino acid sequence X_iGGVRX_j (SEQ ID NO: 135), and an FG loop having the amino acid sequence X_kDYFNPTTHEYIYQTTX_l (SEQ ID NO: 136); wherein X is any amino acid and a, b, c, d, e, f, g, h, i, j, k and l are integers from 0 to 5, independently. In some embodiments, a and g are 2; b-f and i-l are 1; and h is zero. In some embodiments, a-l are zero.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWSARLKVAR (SEQ ID NO: 45), a DE loop having the amino acid sequence PKNVYT (SEQ ID NO: 47), and an FG loop having the amino acid sequence TRFRDYQP (SEQ ID NO: 49); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWHERDGSRQ (SEQ ID NO: 109), a DE loop having the amino acid sequence PGGVRT (SEQ ID NO: 110), and an FG loop having the amino acid sequence TDYFNPTTHEYIYQTTP (SEQ ID NO: 111).

In some embodiments, an E/I binder is an antibody-like protein dimer comprising an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence $X_aSARLKVAX_b$ (SEQ ID NO: 46), a DE loop having the amino acid sequence X_cKNVYX_d (SEQ ID NO: 48), and an FG loop having the amino acid sequence $X_eRFRDYQX_f$ (SEQ ID NO: 50); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence $X_gWAPVDRYQX_h$ (SEQ ID NO: 137), a DE loop having the amino acid sequence X_iRDVYX_j (SEQ ID NO: 138), and an FG loop having the amino acid sequence $X_kDYKPHADGPHTYHESX_l$ (SEQ ID NO: 139); wherein X is any amino acid and a, b, c, d, e, f, g, h, i, j, k and l are integers from 0 to 5, independently. In some embodiments, a and g are 2; b-f and i-l are 1; and h is zero. In some embodiments, a-l are zero.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWSARLKVAR (SEQ ID NO: 45), a DE loop having the amino acid sequence PKNVYT (SEQ ID NO: 47), and an FG loop having the amino acid sequence TRFRDYQP (SEQ ID NO: 49); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWWAPVDRYQ (SEQ ID NO: 115), a DE loop having the amino acid sequence PRDVYT (SEQ ID NO: 116), and an FG loop having the amino acid sequence TDYKPHADGPHTYHESP (SEQ ID NO: 117).

In some embodiments, an E/I binder is an antibody-like protein dimer comprising an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence $X_aSARLKVAX_b$ (SEQ ID NO: 46), a DE loop having the amino acid sequence X_cKNVYX_d (SEQ ID NO: 48), and an FG loop having the amino acid sequence $X_eRFRDYQX_f$ (SEQ ID NO: 50); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence $X_gTQGSTHYQX_h$ (SEQ ID NO: 146), a DE loop having the amino acid sequence X_iGMVYX_j (SEQ ID NO: 147), and an FG loop having the amino acid sequence $X_kDYFDRSTHEYKYRTTX_l$ (SEQ ID NO: 148); wherein X is any amino acid and a, b, c, d, e, f, g, h, i, j, k and l are integers from 0 to 5, independently. In some

embodiments, a and g are 2; b-f and i-l are 1; and h is zero. In some embodiments, a-l are zero.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWSARLKVAR (SEQ ID NO: 45), a DE loop having the amino acid sequence PKNVYT (SEQ ID NO: 47), and an FG loop having the amino acid sequence TRFRDYQP (SEQ ID NO: 49); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWTQGSTHYQ (SEQ ID NO: 143), a DE loop having the amino acid sequence PGMVYT (SEQ ID NO: 144), and an FG loop having the amino acid sequence TDYFDRSTHEYKYRTTP (SEQ ID NO: 145).

In some embodiments, an E/I binder is an antibody-like protein dimer comprising an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence X_aSARLKVAX_b (SEQ ID NO: 46), a DE loop having the amino acid sequence X_cKNVYX_d (SEQ ID NO: 48), and an FG loop having the amino acid sequence X_eRFRDYQX_f (SEQ ID NO: 50); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence X_gYWEGLPYQX_h (SEQ ID NO: 161), a DE loop having the amino acid sequence X_iRDVNX_j (SEQ ID NO: 162), and an FG loop having the amino acid sequence X_kDWYNPDTHEYIYHTIX_l (SEQ ID NO: 163); wherein X is any amino acid and a, b, c, d, e, f, g, h, i, j, k and l are integers from 0 to 5, independently. In some embodiments, a and g are 2; b-f and i-l are 1; and h is zero. In some embodiments, a-l are zero.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWSARLKVAR (SEQ ID NO: 45), a DE loop having the amino acid sequence PKNVYT (SEQ ID NO: 47), and an FG loop having the amino acid sequence TRFRDYQP (SEQ ID NO: 49); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWYWEGLPYQ (SEQ ID NO: 158), a DE loop having the amino acid sequence PRDVNT (SEQ ID NO: 159), and an FG loop having the amino acid sequence TDWYNPDTHEYIYHTIP (SEQ ID NO: 160).

In some embodiments, an E/I binder is an antibody-like protein dimer comprising an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence $X_aSARLKVAX_b$ (SEQ ID NO: 46), a DE loop having the amino acid sequence X_cKNVYX_d (SEQ ID NO: 48), and an FG loop having the amino acid sequence $X_eRFRDYQX_f$ (SEQ ID NO: 50); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence $X_gASNRGTYQX_h$ (SEQ ID NO: 176), a DE loop having the amino acid sequence X_iGGVSX_j (SEQ ID NO: 177), and an FG loop having the amino acid sequence $X_kDAFNPTTHEYNYFTTX_l$ (SEQ ID NO: 178); wherein X is any amino acid and a, b, c, d, e, f, g, h, i, j, k and l are integers from 0 to 5, independently. In some embodiments, a and g are 2; b-f and i-l are 1; and h is zero. In some embodiments, a-l are zero.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWSARLKVAR (SEQ ID NO: 45), a DE loop having the amino acid sequence PKNVYT (SEQ ID NO: 47), and an FG loop having the amino acid sequence TRFRDYQP (SEQ ID NO: 49); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWASNRGTYQ (SEQ ID NO: 173), a DE loop having the amino acid sequence PGGVST (SEQ ID NO: 174), and an FG loop having the amino acid sequence TDAFNPTTHEYNYFTTP (SEQ ID NO: 175).

In some embodiments, an E/I binder is an antibody-like protein dimer comprising an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence $X_aSARLKVAX_b$ (SEQ ID NO: 46), a DE loop having the amino acid sequence X_cKNVYX_d (SEQ ID NO: 48), and an FG loop having the amino acid sequence $X_eRFRDYQX_f$ (SEQ ID NO: 50); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence $X_gDAPTSRYQX_h$ (SEQ ID NO: 190), a DE loop having the amino acid sequence X_iGGLSX_j (SEQ ID NO: 191), and an FG loop having the amino acid sequence $X_kDYKPHADGPHTYHESX_l$ (SEQ ID NO: 139); wherein X is any amino acid and a, b, c, d, e, f, g, h, i, j, k and l are integers from 0 to 5, independently. In some

embodiments, a and g are 2; b-f and i-l are 1; and h is zero. In some embodiments, a-l are zero.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWSARLKVAR (SEQ ID NO: 45), a DE loop having the amino acid sequence PKNVYT (SEQ ID NO: 47), and an FG loop having the amino acid sequence TRFRDYQP (SEQ ID NO: 49); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWDAPTSRYQ (SEQ ID NO: 188), a DE loop having the amino acid sequence PGGLST (SEQ ID NO: 189), and an FG loop having the amino acid sequence TDYKPHADGPHTYHESP (SEQ ID NO: 117).

In some embodiments, an E/I binder is an antibody-like protein dimer comprising an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence X_aSARLKVAX$_b$ (SEQ ID NO: 46), a DE loop having the amino acid sequence X_cKNVYX$_d$ (SEQ ID NO: 48), and an FG loop having the amino acid sequence X_eRFRDYQX$_f$ (SEQ ID NO: 50); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence X_gDAGAVTYQX$_h$ (SEQ ID NO: 203), a DE loop having the amino acid sequence X_iGGVRX$_j$ (SEQ ID NO: 135), and an FG loop having the amino acid sequence X_kDYKPHADGPHTYHEYX$_l$ (SEQ ID NO: 204); wherein X is any amino acid and a, b, c, d, e, f, g, h, i, j, k and l are integers from 0 to 5, independently. In some embodiments, a and g are 2; b-f and i-l are 1; and h is zero. In some embodiments, a-l are zero.

In some embodiments, an E/I binder is an antibody-like protein dimer comprising an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWSARLKVAR (SEQ ID NO: 45), a DE loop having the amino acid sequence PKNVYT (SEQ ID NO: 47), and an FG loop having the amino acid sequence TRFRDYQP (SEQ ID NO: 49); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWDAGAVTYQ (SEQ ID NO: 201), a DE loop having the amino acid sequence PGGVRT (SEQ ID NO: 110), and an FG loop having the amino acid sequence TDYKPHADGPHTYHEYP (SEQ ID NO: 202).

In some embodiments, an E/I binder is an antibody-like protein dimer comprising a) an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 23-29 of SEQ ID NO: 3, a DE loop having the amino acid sequence set forth in amino acids 52-55 of SEQ ID NO: 3, and an FG loop having the amino acid sequence set forth in amino acids 77-82 of SEQ ID NO: 3; covalently or non-covalently linked to b) an EGFR binding ^{10}Fn3 comprising a BC, DE and FG loop as set forth in any one of SEQ ID NOs: 219-327 (see e.g., Figure 45 wherein the BC, DE and FG loop sequences for each EGFR binding ^{10}Fn3 are underlined). In some embodiments, an E/I binder is an antibody-like protein dimer comprising a) an IGFIR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids 23-29 of SEQ ID NO: 3, a DE loop having the amino acid sequence set forth in amino acids 52-55 of SEQ ID NO: 3, and an FG loop having the amino acid sequence set forth in amino acids 77-82 of SEQ ID NO: 3; covalently or non-covalently linked to b) an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence set forth in amino acids corresponding to amino acid residues 23-30 of SEQ ID NO: 1 of any one of SEQ ID NOs: 5-8, 52, 66-68, 106-108, 112-114, 140-142, 155-157, 170-172, 182, 185-187, 198-200, or 219-327, a DE loop having the amino acid sequence set forth in amino acids corresponding to amino acid residues 52-55 of SEQ ID NO: 1 of any one of SEQ ID NOs: 5-8, 52, 66-68, 106-108, 112-114, 140-142, 155-157, 170-172, 182, 185-187, 198-200, or 219-327, and an FG loop having the amino acid sequence set forth in amino acids corresponding to amino acid residues 77-86 of SEQ ID NO: 15-8, 52, 66-68, 106-108, 112-114, 140-142, 155-157, 170-172, 182, 185-187, 198-200, or 219-327. In some embodiments, the EGFR binding ^{10}Fn3 of the antibody-like protein dimer comprises an amino acid sequence at least 80, 90, 95, or 100% identical to the amino acid sequence of amino acid residues corresponding to E9 of SEQ ID NO: 1 through T94 of SEQ ID NO: 1 of any one of SEQ ID NOs: 5-8, 52, 66-68, 106-108, 112-114, 140-142, 155-157, 170-172, 182, 185-187, 198-200, or 219-327. In some embodiments, the IGFIR binding ^{10}Fn3 of the antibody-like protein dimer has an amino acid sequence at least 80, 90, 95, 98, 99, or 100% identical to the amino acid sequence of amino acid residues corresponding to E9 of SEQ ID NO: 1 through T94 of SEQ ID NO: 1 of SEQ ID NO: 3. In some

embodiments, the E/I binder comprises an amino acid sequence at least 80, 85, 90, 95, 98, 99, or 100% identical to any one of SEQ ID NOs: 20-31, 53-58, 87-92, 98-105, 118-133, 149-154, 164-169, 179-184, 192-197, 205-210 and 211-216.

Preferably, X as defined herein is a naturally occurring amino acid.

In certain embodiments, the E binders, or the E and/or I monomers of the E/I binders described herein may contain a Ser to Cys amino acid substitution at a position corresponding to serine 62 or serine 91 of SEQ ID NO: 1.

In certain aspects, the disclosure provides short peptide sequences that mediate EGFR binding. Examples of such sequences include the amino acid residues that correspond to the BC, DE, and FG loops from SEQ ID NOs: 5, 7, 82, 106, 112, 141, 156, 171, 186 and 199. Other examples of such sequences include the amino acid residues that correspond to the BC, DE, and FG loops from SEQ ID NOs: 219-327. In some embodiments, the peptides bind to their respective ligand with a dissociation constant (K_D) of less than 500nM, 100nM, 50nM, 5nM or less. Such sequences may mediate ligand binding in an isolated form or when inserted into a particular protein structure, such as an immunoglobulin or immunoglobulin-like domain.

In one embodiment, an antibody-like protein dimer comprises a polypeptide having the structure A-B-C, wherein A is a polypeptide comprising, consisting essentially of, or consisting of a ^{10}Fn3 domain that binds to EGFR, B is a polypeptide linker, and C is a polypeptide comprising, consisting essentially of, or consisting of a ^{10}Fn3 domain that binds to IGF-IR. In another embodiment, a antibody-like protein dimer comprises a polypeptide having the structure A-B-C, wherein A is a polypeptide comprising, consisting essentially of, or consisting of a ^{10}Fn3 domain that binds to IGF-IR, B is a polypeptide linker, and C is a polypeptide comprising, consisting essentially of, or consisting of a ^{10}Fn3 domain that binds to EGFR. Specific examples of antibody-like protein dimers having the structure A-B-C are polypeptides comprising (i) a polypeptide having an amino acid sequence set forth in any one of SEQ ID NOs: 20-31, 53-58, 87-92, 98-105, 118-133, 149-154, 164-169, 179-184, 192-197, 205-210 and 211-216, or (ii) a polypeptide comprising an amino acid sequence at least 85%, 90%, 95%, 97%, 98%, or 99% identical to any

one of the amino acid sequences set forth in SEQ ID NOs: 20-31, 53-58, 87-92, 98-105, 118-133, 149-154, 164-169, 179-184, 192-197, 205-210 and 211-216.

In certain embodiments, the A or C region is a polypeptide comprising a ^{10}Fn3 domain that binds to EGFR; wherein the ^{10}Fn3 domain has the structure from N-terminus to C-terminus: beta strand A, loop AB, beta strand B, loop BC, beta strand C, loop CD, beta strand D, loop DE, beta strand E, loop EF, beta strand F, loop FG, beta strand G; wherein: (i) the BC loop has the amino acid sequence of SEQ ID NO: 33 or 34, the DE loop has the amino acid sequence of SEQ ID NO: 35 or 36, and the FG loop has the amino acid sequence of SEQ ID NO: 37 or 38, (ii) the BC loop has the amino acid sequence of SEQ ID NO: 39 or 40, the DE loop has the amino acid sequence of SEQ ID NO: 41 or 42, and the FG loop has the amino acid sequence of SEQ ID NO: 43 or 44, (iii) the BC loop has the amino acid sequence of SEQ ID NO: 59 or 60, the DE loop has the amino acid sequence of SEQ ID NO: 61 or 62, and the FG loop has the amino acid sequence of SEQ ID NO: 63 or 64, (iv) the BC loop has the amino acid sequence of SEQ ID NO: 109 or 134, the DE loop has the amino acid sequence of SEQ ID NO: 110 or 135, and the FG loop has the amino acid sequence of SEQ ID NO: 111 or 136, (v) the BC loop has the amino acid sequence of SEQ ID NO: 115 or 137, the DE loop has the amino acid sequence of SEQ ID NO: 116 or 138, and the FG loop has the amino acid sequence of SEQ ID NO: 117 or 139, (vi) the BC loop has the amino acid sequence of SEQ ID NO: 143 or 146, the DE loop has the amino acid sequence of SEQ ID NO: 144 or 147, and the FG loop has the amino acid sequence of SEQ ID NO: 145 or 148, (vii) the BC loop has the amino acid sequence of SEQ ID NO: 158 or 161, the DE loop has the amino acid sequence of SEQ ID NO: 159 or 162, and the FG loop has the amino acid sequence of SEQ ID NO: 160 or 163, (viii) the BC loop has the amino acid sequence of SEQ ID NO: 173 or 176, the DE loop has the amino acid sequence of SEQ ID NO: 174 or 177, and the FG loop has the amino acid sequence of SEQ ID NO: 175 or 178, (ix) the BC loop has the amino acid sequence of SEQ ID NO: 188 or 190, the DE loop has the amino acid sequence of SEQ ID NO: 189 or 191, and the FG loop has the amino acid sequence of SEQ ID NO: 117 or 139, (x) the BC loop has the amino acid sequence of SEQ ID NO: 201 or 203, the DE loop has the amino acid sequence of SEQ ID NO: 110 or 135, and the FG loop has the amino

acid sequence of SEQ ID NO: 202 or 204, or (xi) the BC, DE and FG loops have the amino acid sequences as set forth in any one of SEQ ID NOs: 219-327 (see e.g., Figure 45 wherein the BC, DE and FG loops for each of SEQ ID NOs: 219-327 are underlined); wherein the ^{10}Fn3 domain folds into an antibody heavy chain variable region-like structure; and wherein the polypeptide binds to EGFR with a K_D of less than 100 nM. The ^{10}Fn3 domain that binds to EGFR preferably folds into a structure wherein the 7 beta strands are distributed between two beta sheets that pack against each other forming a stable core and wherein the beta strands are connected by the six loops which are solvent exposed. In exemplary embodiments, the ^{10}Fn3 domain is from 80-150 amino acids in length.

In exemplary embodiments, the A or C region is a ^{10}Fn3 domain that binds to EGFR with a K_D of less than 100 nM having a sequence selected from the group consisting of SEQ ID NO: 83-85 and 466-472 as set forth below:

EVVAATX$_{n1}$SLLIX$_{a1}$**SWVAGAEDYQ**X$_{a2}$YYRITYGEX$_{n2}$QEFTVX$_{a3}$**PHDLVT**X$_{a4}$ATIX$_{n3}$DYTITVYAVX$_{a5}$**TDMMHVEYTEHP**X$_{a6}$ISINYRT (SEQ ID NO: 83);

EVVAATX$_{n1}$SLLIX$_{a1}$**SWDSGRGSYQ**X$_{a2}$YYRITYGEX$_{n2}$QEFTVX$_{a3}$**PGPVHT**X$_{a4}$ATIX$_{n3}$DYTITVYAVX$_{a5}$**TDHKPHADGPHTYHESP**X$_{a6}$ISINYRT (SEQ ID NO: 84); or

EVVAATX$_{n1}$SLLIX$_{a1}$**SWLPGKLRYQ**X$_{a2}$YYRITYGEX$_{n2}$QEFTVX$_{a3}$**PHDLRT**X$_{a4}$ATIX$_{n3}$DYTITVYAVX$_{a5}$**TNMMHVEYSEYP**X$_{a6}$ISINYRT (SEQ ID NO: 85).

EVVAATX$_{n1}$SLLIX$_{a1}$**SWHERDGSRQ**X$_{a2}$YYRITYGEX$_{n2}$QEFTVX$_{a3}$**PGGVRT**X$_{a4}$ATIX$_{n3}$DYTITVYAVX$_{a5}$**TDYFNPTTHEYIYQTTP**X$_{a6}$ISINYRT (SEQ ID NO: 466).

EVVAATX$_{n1}$SLLIX$_{a1}$**SWWAPVDRYQ**X$_{a2}$YYRITYGEX$_{n2}$QEFTVX$_{a3}$**PRDVYT**X$_{a4}$ATIX$_{n3}$DYTITVYAVX$_{a5}$**TDYKPHADGPHTYHESP**X$_{a6}$ISINYRT (SEQ ID NO: 467).

EVVAATX$_{n1}$SLLIX$_{a1}$**SWTQGSTHYQ**X$_{a2}$YYRITYGEX$_{n2}$QEFTVX$_{a3}$**PGMVYT**X$_{a4}$ATIX$_{n3}$DYTITVYAVX$_{a5}$**TDYFDRSTHEYKYRTTP**X$_{a6}$ISINYRT (SEQ ID NO: 468).

EVVAATX$_{n1}$SLLIX$_{a1}$**SWYWEGLPYQ**X$_{a2}$YYRITYGEX$_{n2}$QEFTVX$_{a3}$**PRDVNT**X$_{a4}$ATIX$_{n3}$DYTITVYAVX$_{a5}$**TDWYNPDTHEYIYHTIP**X$_{a6}$ISINYRT (SEQ ID NO: 469).

EVVAATX$_{n1}$SLLIX$_{a1}$**SWASNRGTYQ**X$_{a2}$YYRITYGEX$_{n2}$QEFTVX$_{a3}$**PGGVST**X$_{a4}$ATIX$_{n3}$DYTITVYAVX$_{a5}$**TDAFNPTTHEYNYFTTP**X$_{a6}$ISINYRT (SEQ ID NO: 470).

EVVAATX$_{n1}$SLLIX$_{a1}$**SWDAPTSRYQ**X$_{a2}$YYRITYGEX$_{n2}$QEFTVX$_{a3}$**PGGLST**X$_{a4}$ATIX$_{n3}$DYTITVYAVX$_{a5}$**TDYKPHADGPHTYHESP**X$_{a6}$ISINYRT (SEQ ID NO: 471).

EVVAATX$_{n1}$SLLIX$_{a1}$**SWDAGAVTYQ**X$_{a2}$YYRITYGEX$_{n2}$QEFTVX$_{a3}$**PGGVRT**X$_{a4}$ATIX$_{n3}$DYTITVYAVX$_{a5}$**TDYKPHADGPHTYHEYP**X$_{a6}$ISINYRT (SEQ ID NO: 472).

In SEQ ID NOs: 83-85 and 466-472, the BC, DE and FG loops have a fixed sequence as shown in bold, or a sequence at least 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% identical to the sequences shown in bold, the AB loop is represented by X$_{n1}$, the CD is represented by X$_{n2}$, and EF loop is represented by X$_{n3}$, and the beta strands A-G are underlined. X represents any amino acid and the subscript following the X represents an integer of the number of amino acids. In particular, n1 may be anywhere from 1-15, 2-15, 1-10, 2-10, 1-8, 2-8, 1-5, 2-5, 1-4, 2-4, 1-3, 2-3, or 1-2 amino acids; n2 and n3 may each independently be anywhere from 2-20, 2-15, 2-10, 2-8, 5-20, 5-15, 5-10, 5-8, 6-20, 6-15, 6-10, 6-8, 2-7, 5-7, or 6-7 amino acids; and a1-a6 may each independently comprise from 0-10, 0-5, 1-10, 1-5, or 2-5 amino acids. In preferred embodiments, n1 is 2 amino acids, n2 is 7 amino acids, n3 is 7 amino acids, and a1-a6 is 0 amino acids. The sequences of the beta strands may

have anywhere from 0 to 10, from 0 to 8, from 0 to 6, from 0 to 5, from 0 to 4, from 0 to 3, from 0 to 2, or from 0 to 1 substitutions, deletions or additions across all 7 scaffold regions relative to the corresponding amino acids shown in SEQ ID NO: 1. In an exemplary embodiment, the sequences of the beta strands may have anywhere from 0 to 10, from 0 to 8, from 0 to 6, from 0 to 5, from 0 to 4, from 0 to 3, from 0 to 2, or from 0 to 1 conservative substitutions across all 7 scaffold regions relative to the corresponding amino acids shown in SEQ ID NO: 1. In certain embodiments, the core amino acid residues are fixed and any substitutions, conservative substitutions, deletions or additions occur at residues other than the core amino acid residues. In certain embodiments, the EGFR binder is represented by one of the following amino acid sequences:

EVVAATPTSLLISW**VAGAEDYQ**YYRITYGETGGNSPVQEFTVP**HDLV**TATIS GLKPGVDYTITVYAVT**DMMHVEYTEH**PISINYRT (SEQ ID NO: 66);

EVVAATPTSLLISW**DSGRGSYQ**YYRITYGETGGNSPVQEFTVP**GPVH**TATIS GLKPGVDYTITVYAVT**DHKPHADGPHTYHES**PISINYRT (SEQ ID NO: 67);

EVVAATPTSLLISW**LPGKLRYQ**YYRITYGETGGNSPVQEFTVP**HDLR**TATIS GLKPGVDYTITVYAVT**NMMHVEYSEY**PISINYRT (SEQ ID NO: 68);

EVVAATPTSLLISW**HERDGSRQ**YYRITYGETGGNSPVQEFTVP**GGVR**TATIS GLKPGVDYTITVYAVT**DYFNPTTHEYIYQTT**PISINYRT (SEQ ID NO: 108); or

EVVAATPTSLLISW**WAPVDRYQ**YYRITYGETGGNSPVQEFTVP**RDVY**TATI SGLKPGVDYTITVYAVT**DYKPHADGPHTYHES**PISINYRT (SEQ ID NO: 114).

EVVAATPTSLLISW**TQGSTHYQ**YYRITYGETGGNSPVQEFTVP**GMVY**TATI SGLKPGVDYTITVYAVT**DYFDRSTHEYKYRTT**PISINYRT (SEQ ID NO: 141)

EVVAATPTSLLISW**YWEGLPYQ**YYRITYGETGGNSPVQEFTVP**RDVN**TATI SGLKPGVDYTITVYAVT**DWYNPDTHEYIYHTI**PISINYRT (SEQ ID NO: 156)

EVVAATPTSLLISW**ASNRGTYQ**YYRITYGETGGNSPVQEFTVP**GGVS**TATIS GLKPGVDYTITVYAVT**DAFNPTTHEYNYFTT**PISINYRT (SEQ ID NO: 171)

EVVAATPTSLLISW**DAPTSRYQ**YYRITYGETGGNSPVQEFTVP**GGLS**TATIS GLKPGVDYTITVYAVT**DYKPHADGPHTYHES**PISINYRT (SEQ ID NO: 186)
E112
EVVAATPTSLLISW**DAGAVTYQ**YYRITYGETGGNSPVQEFTVP**GGVR**TATI SGLKPGVDYTITVYAVT**DYKPHADGPHTYHEY**PISINYRT (SEQ ID NO: 199)

In SEQ ID NOs: 66-68, 108, 114, 141, 156, 171, 186 and 199, the sequence of the BC, DE and FG loops have a fixed sequence as shown in bold, or a sequence at least 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% identical to the sequences shown in bold, and the remaining sequence which is underlined (e.g., the sequence of the 7 beta strands and the AB, CD and EF loops) has anywhere from 0 to 20, from 0 to 15, from 0 to 10, from 0 to 8, from 0 to 6, from 0 to 5, from 0 to 4, from 0 to 3, from 0 to 2, or from 0 to 1 substitutions, conservative substitutions, deletions or additions relative to the corresponding amino acids shown in SEQ ID NO: 66-68, 108, 114, 141, 156, 171, 186 and 199. In certain embodiments, the core amino acid residues are fixed and any substitutions, conservative substitutions, deletions or additions occur at residues other than the core amino acid residues. The ^{10}Fn3 domain that binds to EGFR may optionally comprise an N-terminal extension of from 1-20, 1-15, 1-10, 1-8, 1-5, 1-4, 1-3, 1-2, or 1 amino acids in length. Exemplary N-terminal extensions include (represented by the single letter amino acid code) M, MG, G, MGVSDVPRDL (SEQ ID NO: 69), GVSDVPRDL (SEQ ID NO: 70), and VSDVPRDL (SEQ ID NO: 71), or N-terminal truncations of any one of SEQ ID NOs: 69, 70, or 71. Other suitable N-terminal extensions include, for example, X_nSDVPRDL (SEQ ID NO: 72), X_nDVPRDL (SEQ ID NO: 73), X_nVPRDL (SEQ ID NO: 74), X_nPRDL (SEQ ID NO: 75), X_nRDL (SEQ ID NO: 76), X_nDL (SEQ ID

NO: 77), or X_nL, wherein n = 0, 1 or 2 amino acids, wherein when n = 1, X is Met or Gly, and when n = 2, X is Met-Gly. The ^{10}Fn3 domain that binds to EGFR may optionally comprise a C-terminal tail. Exemplary C-terminal tails include polypeptides that are from 1-20, 1-15, 1-10, 1-8, 1-5, 1-4, 1-3, 1-2, or 1 amino acids in length. Specific examples of C-terminal tails include EIDKPSQ (SEQ ID NO: 9), EIDKPCQ (SEQ ID NO: 10), and EIDK (SEQ ID NO: 78). In other embodiments, suitable C-terminal tails may be a C-terminally truncated fragment of SEQ ID NOs: 9, 10 or 78, including, for example, one of the following amino acid sequences (represented by the single letter amino acid code): E, EI, EID, EIDKP (SEQ ID NO: 79), EIDKPS (SEQ ID NO: 80), or EIDKPC (SEQ ID NO: 81). Other suitable C-terminal tails include, for example, ES, EC, EGS, EGC, EGSGS (SEQ ID NO: 96), EGSGC (SEQ ID NO: 97), or EIEK (SEQ ID NO: 217). In certain embodiments, the ^{10}Fn3 domain that binds to EGFR comprises both an N-terminal extension and a C-terminal tail. In exemplary embodiments, the A region comprises an N-terminal extension beginning with Gly or Met-Gly and a C-terminal extension that does not contain a cysteine residue and the B region comprises an N-terminal extension that does not start with a Met and a C-terminal extension that comprises a cysteine residue. Specific examples of ^{10}Fn3 domains that bind to EGFR are polypeptides comprising (i) a polypeptide having an amino acid sequence set forth in any one of SEQ ID NOs: 5-8, 52, 66-68, 82-85, 106-108, 112-114, 140-142, 155-157, 170-172, 185-187, 198-200, and 219-327, or (ii) a polypeptide comprising an amino acid sequence at least 85%, 90%, 95%, 97%, 98%, or 99% identical to the amino acid sequence set forth in any one of SEQ ID NOs: 5-8, 52, 66-68, 82-85, 106-108, 112-114, 140-142, 155-157, 170-172, 185-187, 198-200, and 219-327.

In certain embodiments, the A or C region is a polypeptide comprising a ^{10}Fn3 domain that binds to IGF-IR, wherein the ^{10}Fn3 domain has the structure from N-terminus to C-terminus: beta strand A, loop AB, beta strand B, loop BC, beta strand C, loop CD, beta strand D, loop DE, beta strand E, loop EF, beta strand F, loop FG, beta strand G, wherein the BC loop has the amino acid sequence of SEQ ID NO: 45 or 46, the DE loop has the amino acid sequence of SEQ ID NO: 47 or 48, and the FG loop has the amino acid sequence of SEQ ID NO: 49 or 50, wherein the ^{10}Fn3 domain folds into an antibody heavy chain variable region-like structure, and

wherein the polypeptide binds to IGF-IR with a K_D of less than 100 nM. The ^{10}Fn3 domain that binds to IGF-IR preferably folds into a structure wherein the 7 beta strands are distributed between two beta sheets that pack against each other forming a stable core and wherein the beta strands are connected by the six loops which are solvent exposed. In exemplary embodiments, the ^{10}Fn3 domain is from 80-150 amino acids in length.

In exemplary embodiments, the A or C region is a ^{10}Fn3 domain that binds to IGF-IR with a K_D of less than 100 nM having the sequence set forth below:

EVVAATX$_{n1}$SLLIX$_{a1}$**SWSARLKVAR**X$_{a2}$YYRITYGEX$_{n2}$QEFTVX$_{a3}$**PKNVYT**X$_{a4}$ATIX$_{n3}$DYTITVYAVX$_{a5}$**TRFRDYQP**X$_{a6}$ISINYRT (SEQ ID NO: 86).

In SEQ ID NO: 86, the BC, DE and FG loops have a fixed sequence as shown in bold, or a sequence at least 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% identical to the sequences shown in bold, the AB loop is represented by X_{n1}, the CD loop is represented by X_{n2}, and the EF loop is represented by X_{n3}, and the beta strands A-G are underlined. X represents any amino acid and the subscript following the X represents an integer of the number of amino acids. In particular, n1 may be anywhere from 1-15, 2-15, 1-10, 2-10, 1-8, 2-8, 1-5, 2-5, 1-4, 2-4, 1-3, 2-3, or 1-2 amino acids; n2 and n3 may each independently be anywhere from 2-20, 2-15, 2-10, 2-8, 5-20, 5-15, 5-10, 5-8, 6-20, 6-15, 6-10, 6-8, 2-7, 5-7, or 6-7 amino acids; and a1-a6 may each independently comprise from 0-10, 0-5, 1-10, 1-5, or 2-5 amino acids. In preferred embodiments, n1 is 2 amino acids, n2 is 7 amino acids, n3 is 7 amino acids, and a1-a6 is 0 amino acids. The sequences of the beta strands may have anywhere from 0 to 10, from 0 to 8, from 0 to 6, from 0 to 5, from 0 to 4, from 0 to 3, from 0 to 2, or from 0 to 1 substitutions, deletions or additions across all 7 scaffold regions relative to the corresponding amino acids shown in SEQ ID NO: 1. In an exemplary embodiment, the sequences of the beta strands may have anywhere from 0 to 10, from 0 to 8, from 0 to 6, from 0 to 5, from 0 to 4, from 0 to 3, from 0 to 2, or from 0 to 1 conservative substitutions across all 7 scaffold regions relative to the corresponding amino acids shown in SEQ ID NO: 1. In certain embodiments, the core amino acid residues are fixed and any substitutions, conservative

substitutions, deletions or additions occur at residues other than the core amino acid residues. In certain embodiments, the IGF-IR binder is represented by the following amino acid sequence:

EVVAATPTSLLISW**SARLKVA**RYYRITYGETGGNSPVQEFTVP**KNVY**TATIS GLKPGVDYTITVYAVT**RFRDYQ**PISINYRT (SEQ ID NO: 65).

In SEQ ID NO: 65, the sequence of the BC, DE and FG loops have a fixed sequence as shown in bold, or a sequence at least 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% identical to the sequences shown in bold, and the remaining sequence which is underlined (e.g., the sequence of the 7 beta strands and the AB, CD and EF loops) has anywhere from 0 to 20, from 0 to 15, from 0 to 10, from 0 to 8, from 0 to 6, from 0 to 5, from 0 to 4, from 0 to 3, from 0 to 2, or from 0 to 1 substitutions, conservative substitutions, deletions or additions relative to the corresponding amino acids shown in SEQ ID NO: 65. In certain embodiments, the core amino acid residues are fixed and any substitutions, conservative substitutions, deletions or additions occur at residues other than the core amino acid residues. The ^{10}Fn3 domain that binds to IGF-IR may optionally comprise an N-terminal extension of from 1-20, 1-15, 1-10, 1-8, 1-5, 1-4, 1-3, 1-2, or 1 amino acids in length. Exemplary N-terminal extensions include (represented by the single letter amino acid code) M, MG, G, MGVSDVPRDL (SEQ ID NO: 69), GVSDVPRDL (SEQ ID NO: 70), and VSDVPRDL (SEQ ID NO: 71), or N-terminal truncations of any one of SEQ ID NOs: 69, 70, or 71. Other suitable N-terminal extensions include, for example, X_nSDVPRDL (SEQ ID NO: 72), X_nDVPRDL (SEQ ID NO: 73), X_nVPRDL (SEQ ID NO: 74), X_nPRDL (SEQ ID NO: 75), X_nRDL (SEQ ID NO: 76), X_nDL (SEQ ID NO: 77), or X_nL, wherein n = 0, 1 or 2 amino acids, wherein when n = 1, X is Met or Gly, and when n = 2, X is Met-Gly. The ^{10}Fn3 domain that binds to IGF-IR may optionally comprise a C-terminal tail. Exemplary C-terminal tails include polypeptides that are from 1-20, 1-15, 1-10, 1-8, 1-5, 1-4, 1-3, 1-2, or 1 amino acids in length. Specific examples of C-terminal tails include EIDKPSQ (SEQ ID NO: 9), EIDKPCQ (SEQ ID NO: 10), and EIDK (SEQ ID NO: 78). In other embodiments, suitable C-terminal tails may be a C-terminally truncated fragment of SEQ ID NOs:

9, 10 or 78, including, for example, one of the following amino acid sequences (represented by the single letter amino acid code): E, EI, EID, EIDKP (SEQ ID NO: 79), EIDKPS (SEQ ID NO: 80), or EIDKPC (SEQ ID NO: 81). Other suitable C-terminal tails include, for example, ES, EC, EGS, EGC, EGSGS (SEQ ID NO: 96), EGSGC (SEQ ID NO: 97), or EIEK (SEQ ID NO: 217). In certain embodiments, the ^{10}Fn3 domain that binds to IGF-IR comprises both an N-terminal extension and a C-terminal tail. In exemplary embodiments, the A region comprises an N-terminal extension beginning with Gly or Met-Gly and a C-terminal extension that does not contain a cysteine residue and the B region comprises an N-terminal extension that does not start with a Met and a C-terminal extension that comprises a cysteine residue. Specific examples of ^{10}Fn3 domains that bind to IGF-IR are polypeptides comprising (i) a polypeptide having an amino acid sequence set forth in any one of SEQ ID NOs: 3, 4, 65 or 86, or (ii) a polypeptide comprising an amino acid sequence at least 85%, 90%, 95%, 97%, 98%, or 99% identical to the amino acid sequence set forth in any one of SEQ ID NOs: 3, 4, 65 or 86.

The B region is a linker as described further herein. In exemplary embodiments, the B region is a polypeptide linker. Exemplary polypeptide linkers include polypeptides having from 1-20, 1-15, 1-10, 1-8, 1-5, 1-4, 1-3, or 1-2 amino acids. Specific examples of suitable polypeptide linkers are described further herein and include, for example, linkers having a sequence selected from the group consisting of SEQ ID NOs: 11-19, 51, 93-95 and 218. In certain embodiments, the linker may be a C-terminal tail polypeptide as described herein, an N-terminal extension polypeptide as described herein, or a combination thereof.

In one embodiment, an antibody-like protein dimer comprises a polypeptide having the structure X_1-A-X_2-B-X_3-C-X_4, wherein X_1 is an optional N-terminal extension, A is a ^{10}Fn3 domain that binds to EGFR, X_2 is an optional C-terminal tail, B is a polypeptide linker, X_3 is an optional N-terminal extension, C is a ^{10}Fn3 domain that binds to IGF-IR, and X_4 is an optional C-terminal tail. In another embodiment, an antibody-like protein dimer comprises a polypeptide having the structure X_1-A-X_2-B-X_3-C-X_4, wherein X_1 is an optional N-terminal extension, A is a ^{10}Fn3 domain that binds to IGF-IR, X_2 is an optional C-terminal tail, B is a polypeptide linker, X_3 is an optional N-terminal extension, C is a ^{10}Fn3 domain that

binds to EGFR, and X_4 is an optional C-terminal tail. Specific examples of suitable N-terminal extensions and C-terminal tails are described above. In certain embodiments, one or more of X_1, X_2, B, X_3 or X_4 may comprise an amino acid residue suitable for pegylation, such as a cysteine or lysine residue. In exemplary embodiments, X_4 comprises at least one amino acid suitable for pegylation, such as a cysteine or lysine residue. Specific examples of suitable polypeptide linkers are described further below. Specific examples of antibody-like protein dimers having the structure X_1-A-X_2-B-X_3-C-X_4 are polypeptides comprising (i) a polypeptide having the amino acid sequence set forth in any one of SEQ ID NOs: 20-31, 53-58, 87-92, 98-105, 118-133, 149-154, 164-169, 179-184, 192-197, 205-210 and 211-216, or (ii) a polypeptide comprising an amino acid sequence at least 85%, 90%, 95%, 97%, 98%, or 99% identical to the amino acid sequence set forth in any one of SEQ ID NOs: 20-31, 53-58, 87-92, 98-105, 118-133, 149-154, 164-169, 179-184, 192-197, 205-210 and 211-216.

In certain embodiments, it may be desirable to tune the potency of one ^{10}Fn3 binding domain relative to the other ^{10}Fn3 binding domain in the antibody-like protein dimers described herein. For example, if the binding affinity of the first ^{10}Fn3 domain is significantly higher than the binding affinity of the second ^{10}Fn3 domain, the biological effect of the first ^{10}Fn3 domain could overwhelm the effects of the second of second ^{10}Fn3 domain. Accordingly, in certain embodiments, it may be desirable for the binding affinities of the first and second ^{10}Fn3 domains of an antibody-like protein dimer to be similar to each other, e.g., binding affinities within 100-fold, 30-fold, 10-fold, 3-fold, 1-fold, 0.3-fold or 0.1-fold, of each other, or binding affinities within 0.1-fold to 10-fold, within 0.3-fold to 10-fold, within 0.1-fold to 3-fold, within 0.3-fold to 3-fold, within 0.1-fold to 1-fold, within 0.3-fold to 1-fold, within 1-fold to 10-fold, within 3-fold to 10-fold, within 3-fold to 30-fold, or within 1-fold to 3-fold of each other.

Conjugation

Multimers of antibody-like proteins may be covalently or non-covalently linked. In some embodiments, an EGFR binding ^{10}Fn3 may be directly or indirectly linked to an IGFIR binding ^{10}Fn3 via a polypeptide linker. Suitable linkers for joining Fn3 are those which allow the separate domains to fold independently of

each other forming a three dimensional structure that permits high affinity binding to a target molecule.

The disclosure provides a number of suitable linkers that meet these requirements, including glycine-serine based linkers, glycine-proline based linkers, as well as the linker having the amino acid sequence PSTSTST (SEQ ID NO: 12). The Examples described herein demonstrate that Fn3 domains joined via polypeptide linkers retain their target binding function. In some embodiments, the linker is a glycine-serine based linker. These linkers comprise glycine and serine residues and may be between 8 and 50, 10 and 30, and 10 and 20 amino acids in length. Examples include linkers having an amino acid sequence GSGSGSGSGSGSGSGSGSGS (SEQ ID NO: 11), GSGSGSGSGS (SEQ ID NO: 13), GGGGS GGGGS GGGGS (SEQ ID NO: 14), GGGGS GGGGS GGGGS GGGGS (SEQ ID NO: 15), GGGGS GGGGS GGGGS GGGGS GGGGS (SEQ ID NO: 16), or GGGGSGGGGSGGGSG (SEQ ID NO: 17). In some embodiments, the linker is a glycine-proline based linker. These linkers comprise glycine and proline residues and may be between 3 and 30, 10 and 30, and 3 and 20 amino acids in length. Examples include linkers having an amino acid sequence GPGPGPG (SEQ ID NO: 18), GPGPGPGPGPG (SEQ ID NO: 19), and GPG (SEQ ID NO: 51). In some embodiments, the linker is a proline-alanine based linker. These linkers comprise proline and alanine residues and may be between 3 and 30, 10 and 30, 3 and 20 and 6 and 18 amino acids in length. Examples of such linkers include SEQ ID NOs: 93, 94 and 95. It is contemplated, that the optimal linker length and amino acid composition may be determined by routine experimentation by methods well known in the art.

In some embodiments, multimers of antibody-like proteins are linked via a polypeptide linker having a protease site that is cleavable by a protease in the blood or target tissue. Such embodiments can be used to release two or more therapeutic proteins for better delivery or therapeutic properties or more efficient production compared to separately producing such proteins.

Additional linkers or spacers, e.g., SEQ ID NOs: 9 and 10, may be introduced at the C-terminus of a Fn3 domain between the Fn3 domain and the

polypeptide linker. Additional linkers or spacers may be introduced at the N-terminus of a Fn3 domain between the Fn3 domain and the polypeptide linker.

In some embodiments, multimers of antibody-like proteins may be directly or indirectly linked via a polymeric linker. Polymeric linkers can be used to optimally vary the distance between each protein moiety to create a protein with one or more of the following characteristics: 1) reduced or increased steric hindrance of binding of one or more protein domain when binding to a protein of interest, 2) increased protein stability or solubility, 3) decreased protein aggregation, and 4) increased overall avidity or affinity of the protein.

In some embodiments, multimers of antibody-like proteins are linked via a biocompatible polymer such as a polymeric sugar. The polymeric sugar can include an enzymatic cleavage site that is cleavable by an enzyme in the blood or target tissue. Such embodiments can be used to release two or more therapeutic proteins for better delivery or therapeutic properties or more efficient production compared to separately producing such proteins

In some embodiments, multimers of antibody-like proteins are linked via a polyoxyalkylene, in particular a polyethylene glycol (PEG) moiety. Antibody-like proteins may comprise a cysteine containing linker, such as the linker set forth in SEQ ID NO: 10, 81, 97 or 218. PEG may be conjugated to the cysteine moiety in the linker sequence and may operably link the two domains.

Pharmacokinetic moieties

In one aspect, the disclosure provides E binders and E/I binders further comprising a pharmacokinetic (PK) moiety. In some embodiments, the E/I binder is a multimer of antibody-like proteins, in particular, a dimer of an EGFR binding ^{10}Fn3 and an IGFIR binding ^{10}Fn3. Improved pharmacokinetics may be assessed according to the perceived therapeutic need. Often it is desirable to increase bioavailability and/or increase the time between doses, possibly by increasing the time that a protein remains available in the serum after dosing. In some instances, it is desirable to improve the continuity of the serum concentration of the protein over time (e.g., decrease the difference in serum concentration of the protein shortly after administration and shortly before the next administration). E binders and E/I binders may be attached to a moiety that reduces the clearance rate of the polypeptide in a

mammal (e.g., mouse, rat, or human) by greater than three-fold relative to the unmodified polypeptide. Other measures of improved pharmacokinetics may include serum half-life, which is often divided into an alpha phase and a beta phase. Either or both phases may be improved significantly by addition of an appropriate moiety.

Moieties that tend to slow clearance of a protein from the blood include polyoxyalkylene moieties (e.g., polyethylene glycol); sugars (e.g., sialic acid); and well-tolerated protein moieties (e.g., Fc, Fc fragments, transferrin, or serum albumin).

In some embodiments, the PK moiety is a serum albumin binding protein such as those described in U.S. Publication Nos. 2007/0178082 and 2007/0269422.

In some embodiments, the PK moiety is a serum immunoglobulin binding protein such as those described in U.S. Publication No. 2007/0178082.

In some embodiments, the PK moiety is polyethylene glycol (PEG).

The serum clearance rate of a PK-modified antibody-like protein multimer may be decreased by about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or even 90%, relative to the clearance rate of the unmodified E/I binders. The PK-modified multimer may have a half-life ($t_{1/2}$) which is enhanced relative to the half-life of the unmodified multimer. The half-life of PK- binding polypeptide may be enhanced by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 125%, 150%, 175%, 200%, 250%, 300%, 400% or 500%, or even by 1000% relative to the half-life of the unmodified multimer. In some embodiments, the multimer half-life is determined in vitro, such as in a buffered saline solution or in serum. In other embodiments, the multimer half-life is an in vivo half life, such as the half-life of the multimer in the serum or other bodily fluid of an animal.

In some embodiments, a PK moiety is linked to an antibody-like protein multimer via at least one disulfide bond, a peptide bond, a polypeptide, a polymeric sugar, or a polyethylene glycol moiety. Exemplary polypeptide linkers include PSTSTST (SEQ ID NO: 12), EIDKPSQ (SEQ ID NO: 9), and GS linkers, such as GSGSGSGSGS (SEQ ID NO: 13) and multimers thereof.

Binding/Screening

The disclosure provides E binders and E/I binders, in particular, antibody-like protein multimers such as a dimer of an EGFR binding ^{10}Fn3 and an IGFIR binding ^{10}Fn3. Binding to EGFR or IGFIR may be assessed in terms of equilibrium constants (e.g., dissociation, K_D) and in terms of kinetic constants (e.g., on rate constant, k_{on} and off rate constant, k_{off}). In some embodiments, an antibody-like protein monomer or multimer will bind to EGFR with a K_D of less than 500nM, 100nM, 50nM, 5nM or less. In some embodiments, an antibody-like protein multimer will bind to IGFIR with a K_D of less than 500nM, 100nM, 50nM, 5nM or less. Higher K_D values may be tolerated where the k_{off} is sufficiently low or the k_{on} is sufficiently high.

E binders and E/I binders may bind to any part of EGFR, including the extracellular domain of a EGFR, in particular the ligand binding domain of EGFR. Binding of E binders and E/I binders to EGFR may disrupt the interaction of EGFR with one or more ligands, including TGF-alpha and EGF, and/or disrupt receptor dimerization. In some embodiments, E binders and E/I binders compete with an anti-EGFR antibody for binding to EGFR. The anti-EGFR antibody may be selected from any known anti-EGFR antibody including panitumumab (Amgen), nimotuzumab (YM Biosciences), zalutumumab (Genmab), EMD72000 (Merck KGaA), and cetuximab (ImClone Systems).

In some embodiments, E binders and E/I binders inhibit downstream signaling of EGFR. EGFR ligand binding leads to homo- or heterodimeric receptor dimerization with EGFR or another HER family member. Dimerization promotes receptor autophosphorylation, which in turn leads to the activation of several signaling pathways.

E/I binders may bind to any part of IGFIR, including the extracellular domain of a IGFIR, in particular the ligand binding domain of IGFIR. Binding of E/I binders to IGFIR may disrupt the interaction of IGFIR with one or more ligands, e.g., IGF-I and IGF-II; and/or disrupt assembly of receptor heterotetramers. In some embodiments, E/I binders compete with an anti-IGFIR antibody for binding to IGFIR. The anti-IGFIR antibody may be selected from any known anti-IGFIR antibody.

In some embodiments, E/I binders inhibit downstream signaling of IGFIR. The IGF-I receptor is composed of two types of subunits: an alpha subunit (a 130-135 kDa protein that is entirely extracellular and functions in ligand binding) and a beta subunit (a 95-kDa transmembrane protein, with transmembrane and cytoplasmic domains). IGFIR is initially synthesized as a single chain proreceptor polypeptide that is processed by glycosylation, proteolytic cleavage, and covalent bonding to assemble into a mature 460-kDa heterotetramer comprising two alpha-subunits and two beta-subunits. The beta subunit(s) possesses ligand-activated tyrosine kinase activity.

EGFR and IGFIR receptor signaling independently activates the MAPK pathway, including the phosphorylation of MEK. Another activated pathway is the phosphatidylinositol 3-kinase (PI3K) pathway, including phosphorylation of AKT. Receptor signaling is transduced to the nucleus, resulting in the activation of various transcription factors.

Screening assays may be designed to identify and characterize E binders and E/I binders. Binding assays, such as surface plasmon resonance and ELISA, and assays that detect activated signaling pathways are well-known in the art, see e.g., Example 5. Various antibodies, including many that are commercially available, have been produced which specifically bind to phosphorylated, activated isoforms of EGFR and IGFIR, see e.g., Examples 6 and 7. Downstream signaling events may also be used as an indicator of receptor inhibition, such as by measuring levels of AKT phosphorylation, see e.g., Example 8. Cell proliferation assays are also a useful method for characterizing the ability of candidate E/I binders to bind and inhibit EGFR and IGFIR signaling, see e.g., Example 9.

Polymer conjugation

Conjugation to a biocompatible polymer may be used to link antibody-like protein multimers and/or to improve the pharmacokinetics of the proteins. The identity, size and structure of the polymer is selected so as to improve the circulation half-life of the multimer or decrease the antigenicity of the multimer without an unacceptable decrease in activity.

Examples of polymers useful in the invention include, but are not limited to, poly(alkylene glycols) such as polyethylene glycol (PEG). The polymer is not

limited to a particular structure and can be linear (e.g., alkoxy PEG or bifunctional PEG), or non-linear such as branched, forked, multi-armed (e.g., PEGs attached to a polyol core), and dendritic.

Typically, PEG and other water-soluble polymers (i.e., polymeric reagents) are activated with a suitable activating group appropriate for coupling to a desired site on the polypeptide. Thus, a polymeric reagent will possess a reactive group for reaction with the polypeptide. Representative polymeric reagents and methods for conjugating these polymers to an active moiety are well-known in the art and further described in Zalipsky, S., et al., "Use of Functionalized Poly(Ethylene Glycols) for Modification of Polypeptides" in Polyethylene Glycol Chemistry: Biotechnical and Biomedical Applications, J. M. Harris, Plenus Press, New York (1992), and in Zalipsky (1995) Advanced Drug Reviews 16: 157-182.

Typically, the weight-average molecular weight of the polymer is from about 100 Daltons to about 150,000 Daltons. Exemplary weight-average molecular weights for the biocompatible polymer include about 20,000 Daltons, about 40,000 Daltons, about 60,000 Daltons and about 80,000 Daltons. Branched versions of the biocompatible polymer having a total molecular weight of any of the foregoing can also be used.

In some embodiments, the polymer is PEG. PEG is a well-known, water soluble polymer that is commercially available or can be prepared by ring-opening polymerization of ethylene glycol according to methods well known in the art (Sandler and Karo, Polymer Synthesis, Academic Press, New York, Vol. 3, pages 138-161). The term "PEG" is used broadly to encompass any polyethylene glycol molecule, without regard to size or to modification at an end of the PEG, and can be represented by the formula: $X—O(CH_2CH_2O)_{n-1}CH_2CH_2OH$, where n is 20 to 2300 and X is H or a terminal modification, e.g., a C_{1-4} alkyl. PEG can contain further chemical groups which are necessary for binding reactions, which result from the chemical synthesis of the molecule; or which act as a spacer for optimal distance of parts of the molecule. In addition, such a PEG can consist of one or more PEG side-chains which are linked together. PEGs with more than one PEG chain are called multiarmed or branched PEGs. Branched PEG are described in, for example, European Published Application No. 473084A and U.S. Patent No. 5,932,462.

To effect covalent attachment of the polymer molecule(s) to a polypeptide, the hydroxyl end groups of the polymer molecule must be provided in activated form, i.e. with reactive functional groups. Suitably activated polymer molecules are commercially available, e.g. from Nektar Therapeutics, Inc., Huntsville, Ala., USA; PolyMASC Pharmaceuticals plc, UK; or SunBio Corporation, Anyang City, South Korea. Alternatively, the polymer molecules can be activated by conventional methods known in the art, e.g. as disclosed in WO 90/13540. Specific examples of activated PEG polymers include the following linear PEGs: NHS-PEG, SPA-PEG, SSPA-PEG, SBA-PEG, SS-PEG, SSA-PEG, SC-PEG, SG-PEG, SCM-PEG, NOR-PEG, BTC-PEG, EPOX-PEG, NCO-PEG, NPC-PEG, CDI-PEG, ALD-PEG, TRES-PEG, VS-PEG, OPSS-PEG, IODO-PEG, and MAL-PEG, and branched PEGs, such as PEG2-NHS, PEG2-MAL, and those disclosed in U.S. Pat. No. 5,932,462 and U.S. Pat. No. 5,643,575, both of which are incorporated herein by reference.

In some embodiments where PEG molecules are conjugated to cysteine residues on an antibody-like protein multimer, the cysteine residues are native to the protein, whereas in other embodiments, one or more cysteine residues are engineered into the protein. Mutations may be introduced into a protein coding sequence to generate cysteine residues. This might be achieved, for example, by mutating one or more amino acid residues to cysteine. Preferred amino acids for mutating to a cysteine residue include serine, threonine, alanine and other hydrophilic residues. Preferably, the residue to be mutated to cysteine is a surface-exposed residue. Algorithms are well-known in the art for predicting surface accessibility of residues based on primary sequence or a protein. Alternatively, surface residues may be predicted by comparing the amino acid sequences of binding polypeptides, given that the crystal structure of the framework based on which binding polypeptides are designed and evolved has been solved (see Himanen et al., Nature. (2001) 20-27;414(6866):933-8) and thus the surface-exposed residues identified. In some embodiments, cysteine residues are introduced into antibody-like protein multimers at or near the N- and/or C-terminus, or within loop regions. Pegylation of cysteine residues may be carried out using, for example, PEG-maleiminde, PEG-vinylsulfone, PEG-iodoacetamide, or PEG-orthopyridyl disulfide.

In some embodiments, the pegylated antibody-like protein multimer

comprises a PEG molecule covalently attached to the alpha amino group of the N-terminal amino acid. Site specific N-terminal reductive amination is described in Pepinsky et al., (2001) JPET, 297,1059, and U.S. Patent No. 5,824,784. The use of a PEG-aldehyde for the reductive amination of a protein utilizing other available nucleophilic amino groups is described in U.S. Patent No. 4,002,531, in Wieder et al., (1979) J. Biol. Chem. 254,12579, and in Chamow et al., (1994) Bioconjugate Chem. 5, 133.

In another embodiment, pegylated antibody-like protein multimer comprises one or more PEG molecules covalently attached to a linker, which in turn is attached to the alpha amino group of the amino acid residue at the N-terminus of the binding polypeptide. Such an approach is disclosed in U.S. Publication No. 2002/0044921 and PCT Publication No. WO 94/01451.

In some embodiments, an antibody-like protein multimer is pegylated at the C-terminus. A protein may be pegylated at the C-terminus by the introduction of C-terminal azido-methionine and the subsequent conjugation of a methyl-PEG-triarylphosphine compound via the Staudinger reaction. This C-terminal conjugation method is described in Cazalis et al., C-Terminal Site-Specific PEGylation of a Truncated Thrombomodulin Mutant with Retention of Full Bioactivity, *Bioconjug Chem.* 2004;15(5):1005-1009.

Conventional separation and purification techniques known in the art can be used to purify PEGylated antibody-like protein multimers, such as size exclusion (e.g., gel filtration) and ion exchange chromatography. Products may also be separated using SDS-PAGE. Products that may be separated include mono-, di-, tri-, poly- and un- pegylated binding polypeptide, as well as free PEG. The percentage of mono-PEG conjugates can be controlled by pooling broader fractions around the elution peak to increase the percentage of mono-PEG in the composition. About ninety percent mono-PEG conjugates represents a good balance of yield and activity.

In some embodiments, the pegylated antibody-like protein multimers will preferably retain at least about 25%, 50%, 60%, 70%, 80%, 85%, 90%, 95% or 100% of the biological activity associated with the unmodified protein. In some embodiments, biological activity refers to its ability to bind to EGFR and IGFIR, as

assessed by K_D, k_{on} or k_{off}. In some embodiments, the pegylated antibody-like protein multimer shows an increase in binding to EGFR and/or IGFIR relative to unpegylated protein.

Deimmunization of binding polypeptides

The amino acid sequences of E binders and E/I binders, in particular, antibody-like protein multimers, such as a dimer of an EGFR binding ^{10}Fn3 and an IGFIR binding ^{10}Fn3, may be altered to eliminate one or more B- or T-cell epitopes. A protein, or a multimer of proteins, may be deimmunized to render it non-immunogenic, or less immunogenic, to a given species. Deimmunization can be achieved through structural alterations to the protein. Any deimmunization technique known to those skilled in the art can be employed, see e.g., WO 00/34317, the disclosure of which is incorporated herein in its entirety.

In one embodiment, the sequences of the E binders and E/I binders can be analyzed for the presence of MHC class II binding motifs. For example, a comparison may be made with databases of MHC-binding motifs such as, for example by searching the "motifs" database on the worldwide web at sitewehil.wehi.edu.au. Alternatively, MHC class II binding peptides may be identified using computational threading methods such as those devised by Altuvia et al. (J. Mol. Biol. 249 244-250 (1995)) whereby consecutive overlapping peptides from the polypeptide are testing for their binding energies to MHC class II proteins. Computational binding prediction algorithms include iTope™, Tepitope, SYFPEITHI, EpiMatrix (EpiVax), and MHCpred. In order to assist the identificationof MHC class II-binding peptides, associated sequence features which relate to successfully presented peptides such as amphipathicity and Rothbard motifs, and cleavage sites for cathepsin B and other processing enzymes can be searched for.

Having identified potential (e.g. human) T-cell epitopes, these epitopes are then eliminated by alteration of one or more amino acids, as required to eliminate the T-cell epitope. Usually, this will involve alteration of one or more amino acids within the T-cell epitope itself. This could involve altering an amino acid adjacent the epitope in terms of the primary structure of the protein or one which is not adjacent in the primary structure but is adjacent in the secondary structure of the

molecule. The usual alteration contemplated will be amino acid substitution, but it is possible that in certain circumstances amino acid addition or deletion will be appropriate. All alterations can be accomplished by recombinant DNA technology, so that the final molecule may be prepared by expression from a recombinant host, for example by well established methods, but the use of protein chemistry or any other means of molecular alteration may also be used.

Once identified T-cell epitopes are removed, the deimmunized sequence may be analyzed again to ensure that new T-cell epitopes have not been created and, if they have, the epitope(s) can be deleted.

Not all T-cell epitopes identified computationally need to be removed. A person skilled in the art will appreciate the significance of the "strength" or rather potential immunogenicity of particular epitopes. The various computational methods generate scores for potential epitopes. A person skilled in the art will recognize that only the high scoring epitopes may need to be removed. A skilled person will also recognize that there is a balance between removing potential epitopes and maintaining binding affinity of the protein. Therefore, one strategy is to sequentially introduce substitutions into the protein and then test for antigen binding and immunogenicity.

In one aspect, the deimmunized protein is less immunogenic (or rather, elicits a reduced HAMA response) than the original protein in a human subject. Assays to determine immunogenicity are well within the knowledge of the skilled person. Art-recognized methods of determining immune response can be performed to monitor a HAMA response in a particular subject or during clinical trials. Subjects administered deimmunized protein can be given an immunogenicity assessment at the beginning and throughout the administration of said therapy. The HAMA response is measured, for example, by detecting antibodies to the deimmunized protein in serum samples from the subject using a method known to one in the art, including surface plasmon resonance technology (BIAcore) and/or solid-phase ELISA analysis. Alternatively, in vitro assays designed to measure a T-cell activation event are also indicative of immunogenicity.

Additional Modifications

In certain embodiments, E binders and E/I binders, in particular, antibody-like protein multimers such as a dimer of an EGFR binding ^{10}Fn3 and an IGFIR binding ^{10}Fn3, may further comprise post-translational modifications. Exemplary post-translational protein modification include phosphorylation, acetylation, methylation, ADP-ribosylation, ubiquitination, glycosylation, carbonylation, sumoylation, biotinylation or addition of a polypeptide side chain or of a hydrophobic group. As a result, the modified E binders and E/I binders may contain non-amino acid elements, such as lipids, poly- or mono-saccharide, and phosphates. A preferred form of glycosylation is sialylation, which conjugates one or more sialic acid moieties to the polypeptide. Sialic acid moieties improve solubility and serum half-life while also reducing the possible immunogenicity of the protein. See, e.g., Raju et al. Biochemistry. 2001 Jul 31;40(30):8868-76. Effects of such non-amino acid elements on the functionality of an E binder or E/I binder may be tested for its antagonizing role in EGFR and IGFIR signaling function.

In some embodiments, E binders and E/I binders are modified to enhance antigen-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC). In some embodiments, the E/I binder is a dimer of an EGFR binding ^{10}Fn3 and an IGFIR binding ^{10}Fn3, further comprising an Fc region. In some embodiments, the Fc region is a variant that enhances ADCC or CDC. The Fc region variant may comprise a human Fc region sequence (e.g., a human IgG1, IgG2, IgG3 or IgG4 Fc region) comprising an amino acid modification (e.g., a substitution) at one or more amino acid positions, including positions 256, 290, 298, 312, 326, 330, 333, 334, 360, 378 or 430, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

Vectors & Polynucleotides Embodiments

Also included in the present disclosure are nucleic acid sequences encoding any of the proteins described herein. As appreciated by those skilled in the art, because of third base degeneracy, almost every amino acid can be represented by more than one triplet codon in a coding nucleotide sequence. In addition, minor base pair changes may result in a conservative substitution in the amino acid sequence encoded but are not expected to substantially alter the biological activity of

the gene product. Therefore, a nucleic acid sequence encoding a protein described herein may be modified slightly in sequence and yet still encode its respective gene product.

Exemplary nucleic acids encoding the E/I binders described herein include nucleic acids having SEQ ID NOs: 442-465 or nucleic acids having a sequence at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to any one of SEQ ID NOs: 442-465. Isolated nucleic acids which differ from the nucleic acids as set forth in SEQ ID NOs: 442-465 due to degeneracy in the genetic code are also within the scope of the invention. Also provided are E/I binders comprising an I monomer encoded by a nucleotide sequence at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 328 and/or E/I binders comprising an E monomer encoded by a nucleotide sequence at 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to any one of SEQ ID NOs: 329-441 or 495. Also provided are E binders encoded by a nucleotide sequence at 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to any one of SEQ ID NOs: 329-441 or 495. In certain embodiments, the nucleotide sequences encoding the E/I binders, an E monomer, or an I monomer do not contain a sequence encoding a 6XHis tag (SEQ ID NO: 487).

Nucleic acids encoding any of the various proteins or polypeptides disclosed herein may be synthesized chemically. Codon usage may be selected so as to improve expression in a cell. Such codon usage will depend on the cell type selected. Specialized codon usage patterns have been developed for E. coli and other bacteria, as well as mammalian cells, plant cells, yeast cells and insect cells. See for example: Mayfield et al., Proc Natl Acad Sci U S A. 2003 100(2):438-42; Sinclair et al. Protein Expr Purif. 2002 (1):96-105; Connell ND. Curr Opin Biotechnol. 2001 (5):446-9; Makrides et al. Microbiol Rev. 1996 60(3):512-38; and Sharp et al. Yeast. 1991 7(7):657-78.

General techniques for nucleic acid manipulation are within the purview of one skilled in the art and are also described for example in Sambrook et al., Molecular Cloning: A Laboratory Manual, Vols. 1-3, Cold Spring Harbor Laboratory Press, 2 ed., 1989, or F. Ausubel et al., Current Protocols in Molecular Biology (Green Publishing and Wiley-Interscience: New York, 1987) and periodic updates, herein incorporated by reference. The DNA encoding a protein is operably

linked to suitable transcriptional or translational regulatory elements derived from mammalian, viral, or insect genes. Such regulatory elements include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences that control the termination of transcription and translation. The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants are additionally incorporated. Suitable regulatory elements are well-known in the art.

The proteins described herein may be produced as a fusion protein with a heterologous polypeptide, which is preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process a native signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion, the native signal sequence may be substituted by, e.g., the yeast invertase leader, a factor leader (including Saccharomyces and Kluyveromyces alpha-factor leaders), or acid phosphatase leader, the C. albicans glucoamylase leader, or the signal described in PCT Publication No. WO 90/13646. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available. The DNA for such precursor regions may be ligated in reading frame to DNA encoding the protein.

Expression vectors used in eukaryotic host cells (e.g., yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the multivalent antibody. One useful transcription termination component is the bovine growth hormone polyadenylation

region. See PCT Publication No. WO 94/11026 and the expression vector disclosed therein.

The recombinant DNA can also include any type of protein tag sequence that may be useful for purifying the protein. Examples of protein tags include but are not limited to a histidine tag, a FLAG tag, a myc tag, an HA tag, or a GST tag. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts can be found in Cloning Vectors: A Laboratory Manual, (Elsevier, New York, 1985), the relevant disclosure of which is hereby incorporated by reference.

The expression construct is introduced into the host cell using a method appropriate to the host cell, as will be apparent to one of skill in the art. A variety of methods for introducing nucleic acids into host cells are known in the art, including, but not limited to, electroporation; transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; and infection (where the vector is an infectious agent).

Suitable host cells include prokaryotes, yeast, mammalian cells, or bacterial cells. Suitable bacteria include gram negative or gram positive organisms, for example, E. coli or Bacillus spp. Yeast, preferably from the Saccharomyces species, such as S. cerevisiae, may also be used for production of polypeptides. Various mammalian or insect cell culture systems can also be employed to express recombinant proteins. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, (Bio/Technology, 6:47, 1988). In some instance it will be desired to produce proteins in vertebrate cells, such as for glycosylation, and the propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of suitable mammalian host cell lines include endothelial cells, COS-7 monkey kidney cells, CV-1, L cells, C127, 3T3, Chinese hamster ovary (CHO), human embryonic kidney cells, HeLa, 293, 293T, and BHK cell lines. For many applications, the small size of the protein multimers described herein would make E. coli the preferred method for expression.

Protein Production

Host cells are transformed with the herein-described expression or cloning vectors for protein production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The host cells used to produce the proteins of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., Meth. Enz. 58:44 (1979), Barnes et al., Anal. Biochem.102:255 (1980), U.S. Patent Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent No. Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN^{TM} drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

Proteins disclosed herein can also be produced using cell-translation systems. For such purposes, the nucleic acids encoding the proteins must be modified to allow in vitro transcription to produce mRNA and to allow cell-free translation of the mRNA in the particular cell-free system being utilized. Exemplary eukaryotic cell-free translation systems include, for example, mammalian or yeast cell-free translation systems, and exemplary prokaryotic cell-free translation systems include, for example, bacterial cell-free translation systems.

Proteins disclosed herein can also be produced by chemical synthesis (e.g., by the methods described in Solid Phase Peptide Synthesis, 2nd ed., 1984, The Pierce Chemical Co., Rockford, IL). Modifications to the protein can also be produced by chemical synthesis.

The proteins disclosed herein can be purified by isolation/purification methods for proteins generally known in the field of protein chemistry. Non-limiting examples include extraction, recrystallization, salting out (e.g., with ammonium sulfate or sodium sulfate), centrifugation, dialysis, ultrafiltration, adsorption chromatography, ion exchange chromatography, hydrophobic chromatography, normal phase chromatography, reversed-phase chromatography, gel filtration, gel permeation chromatography, affinity chromatography, electrophoresis, countercurrent distribution or any combinations of these. After purification, proteins may be exchanged into different buffers and/or concentrated by any of a variety of methods known to the art, including, but not limited to, filtration and dialysis.

The purified proteins are preferably at least 85% pure, more preferably at least 95% pure, and most preferably at least 98% pure. Regardless of the exact numerical value of the purity, the proteins are sufficiently pure for use as a pharmaceutical product.

Imaging, Diagnostic and Other Applications

The E binders described herein can be detectably labeled and used to contact cells expressing EGFR for imaging or diagnostic applications. The E/I binders described herein can be detectably labeled and used to contact cells expressing EGFR and/or IGFIR for imaging or diagnostic applications. Any method known in the art for conjugating a protein to the detectable moiety may be employed, including those methods described by Hunter, et al., Nature 144:945 (1962); David, et al., Biochemistry 13:1014 (1974); Pain, et al., J. Immunol. Meth. 40:219 (1981); and Nygren, J. Histochem. and Cytochem. 30:407 (1982).

In certain embodiments, the E binders and E/I binders described herein are further attached to a label that is able to be detected (e.g., the label can be a radioisotope, fluorescent compound, enzyme or enzyme co-factor). The label may be a radioactive agent, such as: radioactive heavy metals such as iron chelates,

radioactive chelates of gadolinium or manganese, positron emitters of oxygen, nitrogen, iron, carbon, or gallium, ^{43}K, ^{52}Fe, ^{57}Co, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{123}I, ^{125}I, ^{131}I, ^{132}I, or ^{99}Tc. An E binder or E/I binder affixed to such a moiety may be used as an imaging agent and is administered in an amount effective for diagnostic use in a mammal such as a human and the localization and accumulation of the imaging agent is then detected. The localization and accumulation of the imaging agent may be detected by radioscintigraphy, nuclear magnetic resonance imaging, computed tomography or positron emission tomography. As will be evident to the skilled artisan, the amount of radioisotope to be administered is dependent upon the radioisotope. Those having ordinary skill in the art can readily formulate the amount of the imaging agent to be administered based upon the specific activity and energy of a given radionuclide used as the active moiety.

E binders and E/I binders also are useful as affinity purification agents. In this process, the proteins are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The proteins can be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays (Zola, Monoclonal Antibodies: A Manual of Techniques, pp. 147-158 (CRC Press, Inc., 1987)).

E binders are useful in methods for detecting EGFR in a sample. E/I binders also are useful in methods for detecting EGFR and/or IGFIR in a sample. The sample will often by a biological sample, such as a biopsy, and particularly a biopsy of a tumor, a suspected tumor. The sample may be from a human or other mammal. The E binder or E/I binder may be labeled with a labeling moiety, such as a radioactive moiety, a fluorescent moiety, a chromogenic moiety, a chemiluminescent moiety, or a hapten moiety; and may be immobilized on a solid support. Detection may be carried out using any technique known in the art, such as, for example, radiography, immunological assay, fluorescence detection, mass spectroscopy, or surface plasmon resonance.

Therapeutic/in vivo uses

The E binders described herein are also useful in methods for treating conditions which respond to an inhibition of EGFR biological activity. The E/I binders described herein are also useful in methods for treating conditions which

respond to an inhibition of EGFR and/or IGFIR biological activity. EGFR and IGFIR are involved either directly or indirectly in the signal transduction pathways of various cell activities, including proliferation, adhesion and migration, as well as differentiation.

In one aspect, the application provides methods for treating a subject afflicted with a hyperproliferative disorder with a therapeutically effective amount of an E binder or an E/I binder. In particular, E binders and E/I binders are useful for the treatment and/or prophylaxis of tumors and/or tumor metastases. In exemplary embodiments, the E/I binder is an antibody-like protein multimer such as a dimer of an EGFR binding ^{10}Fn3 and an IGFIR binding ^{10}Fn3.

In some embodiments, pharmaceutical compositions comprising E binders or E/I binders are administered to a subject afflicted with a tumor, including but not limited to, a brain tumor, tumor of the urogenital tract, tumor of the lymphatic system, stomach tumor, laryngeal tumor, monocytic leukemia, lung adenocarcinoma, small-cell lung carcinoma, pancreatic cancer, glioblastoma and breast carcinoma; or a cancerous disease, including but not limited to, squamous cell carcinoma, bladder cancer, stomach cancer, liver cancer, kidney cancer, colorectal cancer, breast cancer, head cancer, neck cancer, oesophageal cancer, gynecological cancer, thyroid cancer, lymphoma, chronic leukemia and acute leukemia.

An E binder or an E/I binder can be administered alone or in combination with one or more additional therapies such as chemotherapy radiotherapy, immunotherapy, surgical intervention, or any combination of these. Long-term therapy is equally possible as is adjuvant therapy in the context of other treatment strategies, as described herein. Techniques and dosages for administration vary depending on the type of specific polypeptide and the specific condition being treated but can be readily determined by the skilled artisan.

Additional Agents That May be Used with E/I Binders

One aspect of the application provides combinations of E binder or E/I binders and an additional therapeutic agent, such as a cytotoxic agent. In some embodiments, an E binder or E/I binder is linked to a cytotoxic agent. Such embodiments can be prepared by in vitro or in vivo methods as appropriate. In vitro methods include conjugation chemistry well know in the art, such as conjugation to

cysteine and lysine residues. In order to link a cytotoxic agent to a polypeptide, a linking group or reactive group is used. Suitable linking groups are well known in the art and include disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups and esterase labile groups. Cytotoxic agents can also be linked to E binders or E/I binders through an intermediary carrier molecule such as serum albumin

Exemplary cytotoxic agents that may be linked to E binders or E/I binders, include maytansinoids, taxanes, analogs of CC-1065, bacterial toxin, plant toxin, ricin, abrin, a ribonuclease (RNase), DNase I, a protease, Staphylococcal enterotoxin-A, pokeweed antiviral protein, gelonin, diphtherin toxin, Pseudomonas exotoxin, Pseudomonas endotoxin, Ranpimase (Rap), Rap (N69Q), methotrexate, daunorubicin, doxorubicin, vincristine, vinblastine, melphalan, mitomycin C, chlorambucil, and calicheamicin.

In other therapeutic treatments or compositions, E binders or E/I binders are co-administered, or administered sequentially, with one or more additional therapeutic agents. Suitable therapeutic agents include, but are not limited to, cytotoxic or cytostatic agents, such as cancer therapeutic agents.

Cancer therapeutic agents are those agents that seek to kill or limit the growth of cancer cells while having minimal effects on the patient. Thus, such agents may exploit any difference in cancer cell properties (e.g., metabolism, vascularization or cell-surface antigen presentation) from healthy host cells. Therapeutic agents that can be combined with E/I binders for improved anti-cancer efficacy include diverse agents used in oncology practice (Reference: Cancer, Principles & Practice of Oncology, DeVita, V. T., Hellman, S., Rosenberg, S. A., 6th edition, Lippincott-Raven, Philadelphia, 2001), such as doxorubicin, epirubicin, cyclophosphamide, trastuzumab, capecitabine, tamoxifen, toremifene, letrozole, anastrozole, fulvestrant, exemestane, goserelin, oxaliplatin, carboplatin, cisplatin, dexamethasone, antide, bevacizumab, 5-fluorouracil, leucovorin, levamisole, irinotecan, etoposide, topotecan, gemcitabine, vinorelbine, estramustine, mitoxantrone, abarelix, zoledronate, streptozocin, rituximab, idarubicin, busulfan, chlorambucil, fludarabine, imatinib, cytarabine, ibritumomab, tositumomab, interferon alpha-2b, melphalam, bortezomib, altretamine, asparaginase, gefitinib,

erlonitib, anti-EGF receptor antibody (e.g., cetuximab or panitumab), ixabepilone, epothilones or derivatives thereof, platinum agents (such as carboplatin, oxaliplatin, cisplatin), taxanes (such as paclitaxel, docetaxel), and camptothecin.

Therapeutic Formulations and Modes of Administration

The present application provides methods for treating conditions which respond to an inhibition of EGFR and/or IGFIR biological activity. Techniques and dosages for administration vary depending on the type of specific polypeptide and the specific condition being treated but can be readily determined by the skilled artisan. In general, regulatory agencies require that a protein reagent to be used as a therapeutic is formulated so as to have acceptably low levels of pyrogens. Accordingly, therapeutic formulations will generally be distinguished from other formulations in that they are substantially pyrogen free, or at least contain no more than acceptable levels of pyrogen as determined by the appropriate regulatory agency (e.g., FDA).

In some embodiments, the E binders and E/I binders are are pharmaceutically acceptable to a mammal, in particular a human. A "pharmaceutically acceptable" polypeptide refers to a polypeptide that is administered to an animal without significant adverse medical consequences. Examples of pharmaceutically acceptable E binders and E/I binders include ^{10}Fn3 domains that lack the integrin-binding domain (RGD) and ^{10}Fn3 domains that are essentially endotoxin free or have very low endotoxin levels.

Therapeutic compositions may be administered with a pharmaceutically acceptable diluent, carrier, or excipient, in unit dosage form. Administration may be parenteral (e.g., intravenous, subcutaneous), oral, or topical, as non-limiting examples. In addition, any gene therapy technique using nucleic acids encoding E binders or E/I binders, may be employed, such as naked DNA delivery, recombinant genes and vectors, cell-based delivery, including ex vivo manipulation of patients' cells, and the like.

The composition can be in the form of a pill, tablet, capsule, liquid, or sustained release tablet for oral administration; a liquid for intravenous, subcutaneous or parenteral administration; or a gel, lotion, ointment, cream, or a polymer or other sustained release vehicle for local administration.

Methods well known in the art for making formulations are found, for example, in "Remington: The Science and Practice of Pharmacy" (20th ed., ed. A.R. Gennaro AR., 2000, Lippincott Williams & Wilkins, Philadelphia, PA). Formulations for parenteral administration may, for example, contain excipients, sterile water, saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Nanoparticulate formulations (e.g., biodegradable nanoparticles, solid lipid nanoparticles, liposomes) may be used to control the biodistribution of the compounds. Other potentially useful parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. The concentration of the compound in the formulation varies depending upon a number of factors, including the dosage of the drug to be administered, and the route of administration.

The polypeptide may be optionally administered as a pharmaceutically acceptable salt, such as non-toxic acid addition salts or metal complexes that are commonly used in the pharmaceutical industry. Examples of acid addition salts include organic acids such as acetic, lactic, pamoic, maleic, citric, malic, ascorbic, succinic, benzoic, palmitic, suberic, salicylic, tartaric, methanesulfonic, toluenesulfonic, or trifluoroacetic acids or the like; polymeric acids such as tannic acid, carboxymethyl cellulose, or the like; and inorganic acid such as hydrochloric acid, hydrobromic acid, sulfuric acid phosphoric acid, or the like. Metal complexes include zinc, iron, and the like. In one example, the polypeptide is formulated in the presence of sodium acetate to increase thermal stability.

Formulations for oral use include tablets containing the active ingredient(s) in a mixture with non-toxic pharmaceutically acceptable excipients. These excipients may be, for example, inert diluents or fillers (e.g., sucrose and sorbitol), lubricating agents, glidants, and anti-adhesives (e.g., magnesium stearate, zinc stearate, stearic acid, silicas, hydrogenated vegetable oils, or talc).

Formulations for oral use may also be provided as chewable tablets, or as hard gelatin capsules wherein the active ingredient is mixed with an inert solid

diluent, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium.

A therapeutically effective dose refers to a dose that produces the therapeutic effects for which it is administered. The exact dose will depend on the disorder to be treated, and may be ascertained by one skilled in the art using known techniques. In general, the E binder or E/I binder is administered at about 0.01 μg/kg to about 50 mg/kg per day, preferably 0.01 mg/kg to about 30 mg/kg per day, most preferably 0.1 mg/kg to about 20 mg/kg per day. The polypeptide may be given daily (e.g., once, twice, three times, or four times daily) or less frequently (e.g., once every other day, once or twice weekly, or monthly). In addition, as is known in the art, adjustments for age as well as the body weight, general health, sex, diet, time of administration, drug interaction, and the severity of the disease may be necessary, and will be ascertainable with routine experimentation by those skilled in the art.

EXEMPLIFICATION

The invention now being generally described will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention in any way.

Summary of Sequences

Many of the sequences referenced in this application are summarized in the table below. Unless otherwise specified, N-terminal extensions are indicated with a single underline, C-terminal tails are indicated with a double underline, and linker sequences are indicated in bold.

SEQ ID NO:	Description	Sequence
1	WT human ^{10}Fn3 domain	VSDVPRDLEVVAATPTSLLISWDAPAVT VRYYRITYGETGGNSPVQEFTVPGSKST ATISGLKPGVDYTITVYAVTGRGDSPAS SKPISINYRT
2	Variant human ^{10}Fn3 with the integrin binding motif removed (RGD changed to SGE; changes from SEQ ID NO: 1 are	VSDVPRDLEVVAATPTSLLISWDAPAVT VRYYRITYGETGGNSPVQEFTVPGSKST ATISGLKPGVDYTITVYAVTGSGESPASS

SEQ ID NO:	Description	Sequence
	underlined)	KPISINYRT
3	I1 IGF-IR monomer with N-terminal extension (N+8) and no tail	VSDVPRDLEVVAATPTSLLISWSARLKV ARYYRITYGETGGNSPVQEFTVPKNVY TATISGLKPGVDYTITVYAVTRFRDYQPI SINYRT
4	I1 IGF-IR monomer with N-terminal extension (N+10) and Ser tail with His tag	MGVSDVPRDLEVVAATPTSLLISWSARL KVARYYRITYGETGGNSPVQEFTVPKN VYTATISGLKPGVDYTITVYAVTRFRDY QPISINYRTEIDKPSQHHHHHH
5	E2 EGFR monomer with N-terminal extension (N+8) and no tail	VSDVPRDLEVVAATPTSLLISWDSGRGS YQYYRITYGETGGNSPVQEFTVPGPVHT ATISGLKPGVDYTITVYAVTDHKPHAD GPHTYHESPISINYRT
6	E2 EGFR monomer with N-terminal extension (N+10) and Ser tail with his tag	MGVSDVPRDLEVVAATPTSLLISWDSG RGSYQYYRITYGETGGNSPVQEFTVPGP VHTATISGLKPGVDYTITVYAVTDHKPH ADGPHTYHESPISINYRTEIDKPSQHHHH HH
7	E1 EGFR monomer with N-terminal extension (N+8) and no tail	VSDVPRDLEVVAATPTSLLISWVAGAE DYQYYRITYGETGGNSPVQEFTVPHDL VTATISGLKPGVDYTITVYAVTDMMHV EYTEHPISINYRT
8	E1 EGFR monomer with N-terminal extension (N+10) and Ser tail with his tag	MGVSDVPRDLEVVAATPTSLLISWVAG AEDYQYYRITYGETGGNSPVQEFTVPH DLVTATISGLKPGVDYTITVYAVTDMM HVEYTEHPISINYRTEIDKPSQHHHHHH
9	Ser tail	EIDKPSQ
10	Cys tail	EIDKPCQ
11	$(GS)_{10}$ Linker	GSGSGSGSGSGSGSGSGS
12	Fn Based Linker	PSTSTST
13	$(GS)_5$ Linker	GSGSGSGSGS
14	$(GGGGS)_3$ Linker	GGGGS GGGGS GGGGS
15	$(GGGGS)_4$ Linker	GGGGS GGGGS GGGGS GGGGS
16	$(GGGGS)_5$ Linker	GGGGS GGGGS GGGGS GGGGS GGGGS
17	$G_4SG_4SG_3SG$ Linker	GGGGS GGGGS GGGSG
18	Linker	GPGPGPG
19	Linker	GPGPGPGPGPG
20	I1-GS10-E2: I/E tandem having I1 (with N-terminal extension (N+8) and short tail) fused via	VSDVPRDLEVVAATPTSLLISWSARLKV ARYYRITYGETGGNSPVQEFTVPKNVY TATISGLKPGVDYTITVYAVTRFRDYQPI

SEQ ID NO:	Description	Sequence
	GS_{10} linker (GS10 is SEQ ID NO: 11) to E2 (with N-terminal extension (N+8) and no tail)	SINYRTEIDK**GSGSGSGSGSGSGSGSGS GS**VSDVPRDLEVVAATPTSLLISWDSGR GSYQYYRITYGETGGNSPVQEFTVPGPV HTATISGLKPGVDYTITVYAVTDHKPHA DGPHTYHESPISINYRT
21	I1-GS10-E2: I/E tandem having I1 (with N-terminal extension (N+10) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to E2 (with N-terminal extension (N+8) and Ser tail)	MGVSDVPRDLEVVAATPTSLLISWSARL KVARYYRITYGETGGNSPVQEFTVPKN VYTATISGLKPGVDYTITVYAVTRFRDY QPISINYRTEIDK**GSGSGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWDS GRGSYQYYRITYGETGGNSPVQEFTVPG PVHTATISGLKPGVDYTITVYAVTDHKP HADGPHTYHESPISINYRTEIDKPSQ
22	I1-GS10-E2: I/E tandem I1 (with N-terminal extension (N+10) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to E2 (with N-terminal extension (N+8) and Ser tail with his tag)	MGVSDVPRDLEVVAATPTSLLISWSARL KVARYYRITYGETGGNSPVQEFTVPKN VYTATISGLKPGVDYTITVYAVTRFRDY QPISINYRTEIDK**GSGSGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWDS GRGSYQYYRITYGETGGNSPVQEFTVPG PVHTATISGLKPGVDYTITVYAVTDHKP HADGPHTYHESPISINYRTEIDKPSQHHH HHH
23	E2-GS10-I1: E/I tandem having E2 (with N-terminal extension (N+8) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and Ser tail)	VSDVPRDLEVVAATPTSLLISWDSGRGS YQYYRITYGETGGNSPVQEFTVPGPVHT ATISGLKPGVDYTITVYAVTDHKPHAD GPHTYHESPISINYRTEIDK**GSGSGSGSG SGSGSGSGSGS**VSDVPRDLEVVAATPTS LLISWSARLKVARYYRITYGETGGNSPV QEFTVPKNVYTATISGLKPGVDYTITVY AVTRFRDYQPISINYRTEIDKPSQ
24	E2-GS10-I1: E/I tandem having E2 (with N-terminal extension (N+10) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and Ser tail)	MGVSDVPRDLEVVAATPTSLLISWDSG RGSYQYYRITYGETGGNSPVQEFTVPGP VHTATISGLKPGVDYTITVYAVTDHKPH ADGPHTYHESPISINYRTEIDK**GSGSGSG SGSGSGSGSGSGS**VSDVPRDLEVVAAT PTSLLISWSARLKVARYYRITYGETGGN SPVQEFTVPKNVYTATISGLKPGVDYTI TVYAVTRFRDYQPISINYRTEIDKPSQ
25	E2-GS10-I1: E/I tandem having E2 (with N-terminal extension (N+10) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and Ser tail with his tag)	MGVSDVPRDLEVVAATPTSLLISWDSG RGSYQYYRITYGETGGNSPVQEFTVPGP VHTATISGLKPGVDYTITVYAVTDHKPH ADGPHTYHESPISINYRTEIDK**GSGSGSG SGSGSGSGSGSGS**VSDVPRDLEVVAAT PTSLLISWSARLKVARYYRITYGETGGN SPVQEFTVPKNVYTATISGLKPGVDYTI TVYAVTRFRDYQPISINYRTEIDKPSQHH HHHH

SEQ ID NO:	Description	Sequence
26	I1-GS10-E1: I/E tandem having I1 (with N-terminal extension (N+8) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to E1 (with N-terminal extension (N+8) and no tail)	VSDVPRDLEVVAATPTSLLISWSARLKV ARYYRITYGETGGNSPVQEFTVPKNVY TATISGLKPGVDYTITVYAVTRFRDYQPI SINYRTEIDK**GSGSGSGSGSGSGSGSGS GS**VSDVPRDLEVVAATPTSLLISWVAG AEDYQYYRITYGETGGNSPVQEFTVPH DLVTATISGLKPGVDYTITVYAVTDMM HVEYTEHPISINYRT
27	I1-GS10-E1: I/E tandem having I1 (with N-terminal extension (N+10) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to E1 (with N-terminal extension (N+8) and Ser tail)	MGVSDVPRDLEVVAATPTSLLISWSARL KVARYYRITYGETGGNSPVQEFTVPKN VYTATISGLKPGVDYTITVYAVTRFRDY QPISINYRTEIDK**GSGSGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWVA GAEDYQYYRITYGETGGNSPVQEFTVP HDLVTATISGLKPGVDYTITVYAVTDM MHVEYTEHPISINYRTEIDKPSQ
28	I1-GS10-E1: I/E tandem having I1 (with N-terminal extension (N+10) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to E1 (with N-terminal extension (N+8) and Ser tail with his tag)	MGVSDVPRDLEVVAATPTSLLISWSARL KVARYYRITYGETGGNSPVQEFTVPKN VYTATISGLKPGVDYTITVYAVTRFRDY QPISINYRTEIDK**GSGSGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWVA GAEDYQYYRITYGETGGNSPVQEFTVP HDLVTATISGLKPGVDYTITVYAVTDM MHVEYTEHPISINYRTEIDKPSQHHHHH H
29	E1-GS10-I1: E/I tandem having E1 (with N-terminal extension (N+8) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and no tail)	VSDVPRDLEVVAATPTSLLISWVAGAE DYQYYRITYGETGGNSPVQEFTVPHDL VTATISGLKPGVDYTITVYAVTDMMHV EYTEHPISINYRTEIDK**GSGSGSGSGSGS GSGSGSGS**VSDVPRDLEVVAATPTSLLI SWSARLKVARYYRITYGETGGNSPVQE FTVPKNVYTATISGLKPGVDYTITVYAV TRFRDYQPISINYRT
30	E1-GS10-I1: E/I tandem having E1 (with N-terminal extension (N+10) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and Ser tail)	MGVSDVPRDLEVVAATPTSLLISWVAG AEDYQYYRITYGETGGNSPVQEFTVPH DLVTATISGLKPGVDYTITVYAVTDMM HVEYTEHPISINYRTEIDK**GSGSGSGSGS GSGSGSGSGS**VSDVPRDLEVVAATPTS LLISWSARLKVARYYRITYGETGGNSPV QEFTVPKNVYTATISGLKPGVDYTITVY AVTRFRDYQPISINYRTEIDKPSQ
31	E1-GS10-I1: E/I tandem having E1 (with N-terminal extension (N+10) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and Ser tail with	MGVSDVPRDLEVVAATPTSLLISWVAG AEDYQYYRITYGETGGNSPVQEFTVPH DLVTATISGLKPGVDYTITVYAVTDMM HVEYTEHPISINYRTEIDK**GSGSGSGSGS GSGSGSGSGS**VSDVPRDLEVVAATPTS LLISWSARLKVARYYRITYGETGGNSPV

SEQ ID NO:	Description	Sequence
	his tag)	QEFTVPKNVYTATISGLKPGVDYTITVY AVTRFRDYQPISINYRTEIDKPSQHHHH HH
32	^{10}Fn3 scaffold, wherein the BC, DE, and FG loops are represented by $(X)_n$, $(X)_o$, and $(X)_p$, respectively, and n is an integer from 1-20, o is an integer from 1-20, and p is an integer from 1-40	VSDVPRDLEVVAATPTSLLI $(X)_n$YYRITYGETGGNSPVQEFTV$(X)_o$ATI SGLKPGVDYTITVYAV$(X)_p$ISINYRT
33	BC loop sequence from EGFR binder E1	SWVAGAEDYQ
34	BC loop sequence from EGFR binder E1, wherein X is any amino acid and m and n are independently selected from 0 to 5 amino acids	X_mVAGAEDYQX_n
35	DE loop sequence from EGFR binder E1	PHDLVT
36	DE loop sequence from EGFR binder E1, wherein X is any amino acid and o and p are independently selected from 0 to 5 amino acids	X_oHDLVX_p
37	FG loop sequence from EGFR binder E1	TDMMHVEYTEHP
38	FG loop sequence from EGFR binder E1, wherein X is any amino acid and q and r are independently selected from 0 to 5 amino acids	X_qDMMHVEYTEHX_r
39	BC loop sequence from EGFR binder E2	SWDSGRGSYQ
40	BC loop sequence from EGFR binder E2, wherein X is any amino acid and g and h are independently selected from 0 to 5 amino acids	X_gDSGRGSYQX_h
41	DE loop sequence from EGFR binder E2	PGPVHT
42	DE loop sequence from EGFR binder E2, wherein X is any amino acid and i and j are independently selected from 0 to	X_iGPVHX_j

SEQ ID NO:	Description	Sequence
	5 amino acids	
43	FG loop sequence from EGFR binder E2	TDHKPHADGPHTYHESP
44	FG loop sequence from EGFR binder E2, wherein X is any amino acid and k and l are independently selected from 0 to 5 amino acids	X_kDHKPHADGPHTYHEX_l
45	BC loop sequence from IGF-IR binder I1	SWSARLKVAR
46	BC loop sequence from IGF-IR binder I1, wherein X is any amino acid and a and b are independently selected from 0 to 5 amino acids	X_aSARLKVAX_b
47	DE loop sequence from IGF-IR binder I1	PKNVYT
48	DE loop sequence from IGF-IR binder I1, wherein X is any amino acid and c and d are independently selected from 0 to 5 amino acids	X_cKNVYX_d
49	FG loop sequence from IGF-IR binder I1	TRFRDYQP
50	FG loop sequence from IGF-IR binder I1, wherein X is any amino acid and e and f are independently selected from 0 to 5 amino acids	X_eRFRDYQX_f
51	Linker	GPG
52	E3 EGFR monomer with N-terminal extension (N+10), Ser tail and his tag	MGVSDVPRDLEVVAATPTSLLISWLPG KLRYQYYRITYGETGGNSPVQEFTVPH DLRTATISGLKPGVDYTITVYAVTNMM HVEYSEYPISINYRTEIDKPSQHHHHHH
53	E3-GS10-I1: E/I tandem having E3 (with N-terminal extension (N+10) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and Cys tail with his tag)	MGVSDVPRDLEVVAATPTSLLISWLPG KLRYQYYRITYGETGGNSPVQEFTVPH DLRTATISGLKPGVDYTITVYAVTNMM HVEYSEYPISINYRTEIDK**GSGSGSGSGS GSGSGSGSGS**VSDVPRDLEVVAATPTS LLISWSARLKVARYYRITYGETGGNSPV QEFTVPKNVYTATISGLKPGVDYTITVY AVTRFRDYQPISINYRTEIDKPCQHHHH HH
54	I1-GS10-E3: I/E tandem having	MGVSDVPRDLEVVAATPTSLLISWSARL

SEQ ID NO:	Description	Sequence
	I1 with N-terminal extension (N+10) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to E3 (with N-terminal extension (N+8) and Cys tail with his tag)	KVARYYRITYGETGGNSPVQEFTVPKN VYTATISGLKPGVDYTITVYAVTRFRDY QPISINYRTEIDK**GSGSGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWLP GKLRYQYYRITYGETGGNSPVQEFTVP HDLRTATISGLKPGVDYTITVYAVTNM MHVEYSEYPISINYRTEIDKPCQHHHHH H
55	E1-GS10-I1: E/I tandem having E1 (with N-terminal extension (N+10) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and Cys tail with his tag)	MGVSDVPRDLEVVAATPTSLLISWVAG AEDYQYYRITYGETGGNSPVQEFTVPH DLVTATISGLKPGVDYTITVYAVTDMM HVEYTEHPISINYRTEIDK**GSGSGSGSGS GSGSGSGSGS**VSDVPRDLEVVAATPTS LLISWSARLKVARYYRITYGETGGNSPV QEFTVPKNVYTATISGLKPGVDYTITVY AVTRFRDYQPISINYRTEIDKPCQHHHH HH
56	E2-GS10-I1: E/I tandem having E2 (with N-terminal extension (N+10) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and Cys tail with his tag)	MGVSDVPRDLEVVAATPTSLLISWDSG RGSYQYYRITYGETGGNSPVQEFTVPGP VHTATISGLKPGVDYTITVYAVTDHKPH ADGPHTYHESPISINYRTEIDK**GSGSGSG SGSGSGSGSGSGS**VSDVPRDLEVVAAT PTSLLISWSARLKVARYYRITYGETGGN SPVQEFTVPKNVYTATISGLKPGVDYTI TVYAVTRFRDYQPISINYRTEIDKPCQH HHHHH
57	I1-GS10-E1: I/E tandem having I1 (with N-terminal extension (N+10) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to E1 (with N-terminal extension (N+8) and Cys tail with his tag)	MGVSDVPRDLEVVAATPTSLLISWSARL KVARYYRITYGETGGNSPVQEFTVPKN VYTATISGLKPGVDYTITVYAVTRFRDY QPISINYRTEIDK**GSGSGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWVA GAEDYQYYRITYGETGGNSPVQEFTVP HDLVTATISGLKPGVDYTITVYAVTDM MHVEYTEHPISINYRTEIDKPCQHHHHH H
58	I1-GS10-E2: I/E tandem having I1 (with N-terminal extension (N+10) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to E2 (with N-terminal extension (N+8) and Cys tail with his tag)	MGVSDVPRDLEVVAATPTSLLISWSARL KVARYYRITYGETGGNSPVQEFTVPKN VYTATISGLKPGVDYTITVYAVTRFRDY QPISINYRTEIDK**GSGSGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWDS GRGSYQYYRITYGETGGNSPVQEFTVPG PVHTATISGLKPGVDYTITVYAVTDHKP HADGPHTYHESPISINYRTEIDKPCQHH HHHH
59	BC loop sequence from EGFR binder E3	SWLPGKLRYQ

SEQ ID NO:	Description	Sequence
60	BC loop sequence from EGFR binder E3, wherein X is any amino acid and s and t are independently selected from 0 to 5 amino acids	$X_sLPGKLRYQX_t$
61	DE loop sequence from EGFR binder E3	PHDLRT
62	DE loop sequence from EGFR binder E3, wherein X is any amino acid and u and w are independently selected from 0 to 5 amino acids	X_uHDLRX_w
63	FG loop sequence from EGFR binder E3	TNMMHVEYSEYP
64	DE loop sequence from EGFR binder E3, wherein X is any amino acid and y and z are independently selected from 0 to 5 amino acids	$X_yNMMHVEYSEYX_z$
65	I1 IGF-IR monomer core sequence: I1 without N-terminal extension or C-terminal tail	EVVAATPTSLLISWSARLKVARYYRITY GETGGNSPVQEFTVPKNVYTATISGLKP GVDYTITVYAVTRFRDYQPISINYRT
66	E1 EGFR monomer core sequence: E1 without N-terminal extension or C-terminal tail	EVVAATPTSLLISWVAGAEDYQYYRITY GETGGNSPVQEFTVPHDLVTATISGLKP GVDYTITVYAVTDMMHVEYTEHPISIN YRT
67	E2 EGFR monomer core sequence: E2 without N-terminal extension or C-terminal tail	EVVAATPTSLLISWDSGRGSYQYYRITY GETGGNSPVQEFTVPGPVHTATISGLKP GVDYTITVYAVTDHKPHADGPHTYHES PISINYRT
68	E3 EGFR monomer core sequence: SEQ ID NO: 82 without N-terminal extension or C-terminal tail	EVVAATPTSLLISWLPGKLRYQYYRITY GETGGNSPVQEFTVPHDLRTATISGLKP GVDYTITVYAVTNMMHVEYSEYPISINY RT
69	Exemplary N-terminal extension (N+10)	MGVSDVPRDL
70	Exemplary N-terminal extension	GVSDVPRDL
71	Exemplary N-terminal extension (N+8)	VSDVPRDL
72	Exemplary N-terminal extension, wherein X is any amino acid and n is 0, 1 or 2, preferably when n=1, X is Met or Gly and when	$X_nSDVPRDL$

SEQ ID NO:	Description	Sequence
	n=2, X is Met-Gly	
73	Exemplary N-terminal extension, wherein X is any amino acid and n is 0, 1 or 2, preferably when n=1, X is Met or Gly and when n=2, X is Met-Gly	X_nDVPRDL
74	Exemplary N-terminal extension, wherein X is any amino acid and n is 0, 1 or 2, preferably when n=1, X is Met or Gly and when n=2, X is Met-Gly	X_nVPRDL
75	Exemplary N-terminal extension, wherein X is any amino acid and n is 0, 1 or 2, preferably when n=1, X is Met or Gly and when n=2, X is Met-Gly	X_nPRDL
76	Exemplary N-terminal extension, wherein X is any amino acid and n is 0, 1 or 2, preferably when n=1, X is Met or Gly and when n=2, X is Met-Gly	X_nRDL
77	Exemplary N-terminal extension, wherein X is any amino acid and n is 0, 1 or 2, preferably when n=1, X is Met or Gly and when n=2, X is Met-Gly	X_nDL
78	Short tail	EIDK
79	Exemplary C-terminal tail	EIDKP
80	Exemplary C-terminal tail	EIDKPS
81	Exemplary C-terminal tail	EIDKPC
82	E3 EGFR monomer with N-terminal extension (N+8) and no tail	VSDVPRDLEVVAATPTSLLISWLPGKLR YQYYRITYGETGGNSPVQEFTVPHDLRT ATISGLKPGVDYTITVYAVTNMMHVEY SEYPISINYRT
87	E3-GS10-I1: E/I tandem having E3 (with N-terminal extension (N+8) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and Cys tail)	VSDVPRDLEVVAATPTSLLISWLPGKLR YQYYRITYGETGGNSPVQEFTVPHDLRT ATISGLKPGVDYTITVYAVTNMMHVEY SEYPISINYRTEIDK**GSGSGSGSGSGSGS GSGSGS**VSDVPRDLEVVAATPTSLLISW SARLKVARYYRITYGETGGNSPVQEFTV PKNVYTATISGLKPGVDYTITVYAVTRF RDYQPISINYRTEIDKPCQ
88	I1-GS10-E3: I/E tandem having	VSDVPRDLEVVAATPTSLLISWSARLKV

SEQ ID NO:	Description	Sequence
	I1 (with N-terminal extension (N+8) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to E3 (with N-terminal extension (N+8) and Cys tail)	ARYYRITYGETGGNSPVQEFTVPKNVY TATISGLKPGVDYTITVYAVTRFRDYQPI SINYRTEIDK**GSGSGSGSGSGSGSGSGS GS**VSDVPRDLEVVAATPTSLLISWLPGK LRYQYYRITYGETGGNSPVQEFTVPHDL RTATISGLKPGVDYTITVYAVTNMMHV EYSEYPISINYRTEIDKPCQ
89	E1-GS10-I1: E/I tandem having E1 (with N-terminal extension (N+8) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and Cys tail)	VSDVPRDLEVVAATPTSLLISWVAGAE DYQYYRITYGETGGNSPVQEFTVPHDL VTATISGLKPGVDYTITVYAVTDMMHV EYTEHPISINYRTEIDK**GSGSGSGSGSGS GSGSGSGS**VSDVPRDLEVVAATPTSLLI SWSARLKVARYYRITYGETGGNSPVQE FTVPKNVYTATISGLKPGVDYTITVYAV TRFRDYQPISINYRTEIDKPCQ
90	E2-GS10-I1: E/I tandem having E2 (with N-terminal extension (N+8) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and Cys tail)	VSDVPRDLEVVAATPTSLLISWDSGRGS YQYYRITYGETGGNSPVQEFTVPGPVHT ATISGLKPGVDYTITVYAVTDHKPHAD GPHTYHESPISINYRTEIDK**GSGSGSGSG SGSGSGSGSGS**VSDVPRDLEVVAATPTS LLISWSARLKVARYYRITYGETGGNSPV QEFTVPKNVYTATISGLKPGVDYTITVY AVTRFRDYQPISINYRTEIDKPCQ
91	I1-GS10-E1: I/E tandem having I1 (with N-terminal extension (N+8) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to E1 (with N-terminal extension (N+8) and Cys tail)	VSDVPRDLEVVAATPTSLLISWSARLKV ARYYRITYGETGGNSPVQEFTVPKNVY TATISGLKPGVDYTITVYAVTRFRDYQPI SINYRTEIDK**GSGSGSGSGSGSGSGSGS GS**VSDVPRDLEVVAATPTSLLISWVAG AEDYQYYRITYGETGGNSPVQEFTVPH DLVTATISGLKPGVDYTITVYAVTDMM HVEYTEHPISINYRTEIDKPCQ
92	I1-GS10-E2: I/E tandem having I1 (with N-terminal extension (N+8) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to E2 (with N-terminal extension (N+8) and Cys tail)	VSDVPRDLEVVAATPTSLLISWSARLKV ARYYRITYGETGGNSPVQEFTVPKNVY TATISGLKPGVDYTITVYAVTRFRDYQPI SINYRTEIDK**GSGSGSGSGSGSGSGSGS GS**VSDVPRDLEVVAATPTSLLISWDSGR GSYQYYRITYGETGGNSPVQEFTVPGPV HTATISGLKPGVDYTITVYAVTDHKPHA DGPHTYHESPISINYRTEIDKPCQ
93	PA3 Linker	PAPAPA
94	PA6 Linker	PAPAPAPAPAPA
95	PA9 Linker	PAPAPAPAPAPAPAPAPA
96	Modified Ser tail	EGSGS
97	Modified Cys tail	EGSGC

SEQ ID NO:	Description	Sequence
98	E3-$(PA)_n$-I1: E/I tandem having E3 (with N-terminal extension (N+8) and an E tail) fused via $(PA)_n$ linker ($(PA)_n$ is SEQ ID NO: 488) to I1 (with N-terminal extension (N+8) and a modified Ser or Cys tail), wherein n = 3, 6 or 9, and X = Ser or Cys	VSDVPRDLEVVAATPTSLLISWLPGKLR YQYYRITYGETGGNSPVQEFTVPHDLRT ATISGLKPGVDYTITVYAVTNMMHVEY SEYPISINYRTE**(PA)$_n$**VSDVPRDLEVVAA TPTSLLISWSARLKVARYYRITYGETGG NSPVQEFTVPKNVYTATISGLKPGVDYT ITVYAVTRFRDYQPISINYRTEGSGX
99	I1-$(PA)_n$-E3: I/E tandem having I1 (with N-terminal extension (N+8) and an E tail) fused via $(PA)_n$ linker ($(PA)_n$ is SEQ ID NO: 488) to E3 (with N-terminal extension (N+8) and a modified Ser or Cys tail), wherein n = 3, 6 or 9, and X = Ser or Cys	VSDVPRDLEVVAATPTSLLISWSARLKV ARYYRITYGETGGNSPVQEFTVPKNVY TATISGLKPGVDYTITVYAVTRFRDYQPI SINYRTE**(PA)$_n$**VSDVPRDLEVVAATPTSL LISWLPGKLRYQYYRITYGETGGNSPVQ EFTVPHDLRTATISGLKPGVDYTITVYA VTNMMHVEYSEYPISINYRTEGSGX
100	E1-$(PA)_n$-I1: E/I tandem having E1 (with N-terminal extension (N+8) and an E tail) fused via $(PA)_n$ linker ($(PA)_n$ is SEQ ID NO: 488) to I1 (with N-terminal extension (N+8) and a modified Ser or Cys tail), wherein n = 3, 6 or 9, and X = Ser or Cys	VSDVPRDLEVVAATPTSLLISWVAGAE DYQYYRITYGETGGNSPVQEFTVPHDL VTATISGLKPGVDYTITVYAVTDMMHV EYTEHPISINYRTE**(PA)$_n$**VSDVPRDLEVV AATPTSLLISWSARLKVARYYRITYGET GGNSPVQEFTVPKNVYTATISGLKPGVD YTITVYAVTRFRDYQPISINYRTEGSGX
101	E2-$(PA)_n$-I1: E/I tandem having E2 (with N-terminal extension (N+8) and an E tail) fused via $(PA)_n$ linker ($(PA)_n$ is SEQ ID NO: 488) to I1 (with N-terminal extension (N+8) and a modified Ser or Cys tail), wherein n = 3, 6 or 9, and X = Ser or Cys	VSDVPRDLEVVAATPTSLLISWDSGRGS YQYYRITYGETGGNSPVQEFTVPGPVHT ATISGLKPGVDYTITVYAVTDHKPHAD GPHTYHESPISINYRTE**(PA)$_n$**VSDVPRDL EVVAATPTSLLISWSARLKVARYYRITY GETGGNSPVQEFTVPKNVYTATISGLKP GVDYTITVYAVTRFRDYQPISINYRTEG SGX
102	I1-$(PA)_n$-E1: I/E tandem having I1 (with N-terminal extension (N+8) and an E tail) fused via $(PA)_n$ linker ($(PA)_n$ is SEQ ID NO: 488) to E1 (with N-terminal extension (N+8) and a modified Ser or Cys tail), wherein n = 3, 6 or 9, and X = Ser or Cys	VSDVPRDLEVVAATPTSLLISWSARLKV ARYYRITYGETGGNSPVQEFTVPKNVY TATISGLKPGVDYTITVYAVTRFRDYQPI SINYRTE**(PA)$_n$**VSDVPRDLEVVAATPTSL LISWVAGAEDYQYYRITYGETGGNSPV QEFTVPHDLVTATISGLKPGVDYTITVY AVTDMMHVEYTEHPISINYRTEGSGX
103	I1-$(PA)_n$-E2: I/E tandem having I1 (with N-terminal extension (N+8) and an E tail) fused via $(PA)_n$ linker ($(PA)_n$ is SEQ ID NO: 488) to E2 (with N-terminal extension (N+8) and a modified Ser or Cys tail), wherein n = 3, 6	VSDVPRDLEVVAATPTSLLISWSARLKV ARYYRITYGETGGNSPVQEFTVPKNVY TATISGLKPGVDYTITVYAVTRFRDYQPI SINYRTE**(PA)$_n$**VSDVPRDLEVVAATPTSL LISWDSGRGSYQYYRITYGETGGNSPVQ EFTVPGPVHTATISGLKPGVDYTITVYA VTDHKPHADGPHTYHESPISINYRTEGS

SEQ ID NO:	Description	Sequence
	or 9, and X = Ser or Cys	GX
104	E3-GS10-I1: E/I tandem having E3 (with N-terminal extension (N+8) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and Ser tail)	VSDVPRDLEVVAATPTSLLISWLPGKLR YQYYRITYGETGGNSPVQEFTVPHDLRT ATISGLKPGVDYTITVYAVTNMMHVEY SEYPISINYRTEIDK**GSGSGSGSGSGSGS GSGSGS**VSDVPRDLEVVAATPTSLLISW SARLKVARYYRITYGETGGNSPVQEFTV PKNVYTATISGLKPGVDYTITVYAVTRF RDYQPISINYRTEIDKPSQ
105	I1-GS10-E3: I/E tandem having I1 with N-terminal extension (N+8) and short tail) fused via GS_{10} linker (GS10 is SEQ ID NO: 11) to E3 (with N-terminal extension (N+8) and Ser tail)	VSDVPRDLEVVAATPTSLLISWSARLKV ARYYRITYGETGGNSPVQEFTVPKNVY TATISGLKPGVDYTITVYAVTRFRDYQPI SINYRTEIDK**GSGSGSGSGSGSGSGSGS GS**VSDVPRDLEVVAATPTSLLISWLPGK LRYQYYRITYGETGGNSPVQEFTVPHDL RTATISGLKPGVDYTITVYAVTNMMHV EYSEYPISINYRTEIDKPSQ
106	E4 EGFR monomer with N-terminal extension (N+8) and no tail	VSDVPRDLEVVAATPTSLLISWHERDGS RQYYRITYGETGGNSPVQEFTVPGGVRT ATISGLKPGVDYTITVYAVTDYFNPTTH EYIYQTTPISINYRT
107	E4 EGFR monomer with N-terminal extension (N+10) and a Ser with His tag	MGVSDVPRDLEVVAATPTSLLISWHER DGSRQYYRITYGETGGNSPVQEFTVPGG VRTATISGLKPGVDYTITVYAVTDYFNP TTHEYIYQTTPISINYRTEIDKPSQHHHH HH
108	E4 EGFR monomer core sequence: E4 without N-terminal extension or C-terminal tail	EVVAATPTSLLISWHERDGSRQYYRITY GETGGNSPVQEFTVPGGVRTATISGLKP GVDYTITVYAVTDYFNPTTHEYIYQTTP ISINYRT
109	BC loop sequence from EGFR binder E4	SWHERDGSRQ
110	DE loop sequence from EGFR binder E4	PGGVRT
111	FG loop sequence from EGFR binder E4	TDYFNPTTHEYIYQTTP
112	E5 EGFR monomer with N-terminal extension (N+8) and no tail	VSDVPRDLEVVAATPTSLLISWWAPVD RYQYYRITYGETGGNSPVQEFTVPRDV YTATISGLKPGVDYTITVYAVTDYKPHA DGPHTYHESPISINYRT
113	E5 EGFR monomer with N-terminal extension (N+10) and a modified Ser or Cys tail, wherein X = Ser or Cys; may optionally	MGVSDVPRDLEVVAATPTSLLISWWAP VDRYQYYRITYGETGGNSPVQEFTVPR DVYTATISGLKPGVDYTITVYAVTDYKP HADGPHTYHESPISINYRTEIDKPXQ

SEQ ID NO:	Description	Sequence
	comprise a 6X His tag (SEQ ID NO: 487)	
114	E5 EGFR monomer core sequence: E5 without N-terminal extension or C-terminal tail	EVVAATPTSLLISWWAPVDRYQYYRIT YGETGGNSPVQEFTVPRDVYTATISGLK PGVDYTITVYAVTDYKPHADGPHTYHE SPISINYRT
115	BC loop sequence from EGFR binder E5	SWWAPVDRYQ
116	DE loop sequence from EGFR binder E5	PRDVYT
117	FG loop sequence from EGFR binder E5	TDYKPHADGPHTYHESP
118	E4-GS10-I1: E/I tandem having E4 (with N-terminal extension (N+8) and short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and no tail)	VSDVPRDLEVVAATPTSLLISWHERDGS RQYYRITYGETGGNSPVQEFTVPGGVRT ATISGLKPGVDYTITVYAVTDYFNPTTH EYIYQTTPISINYRTEIDK**GSGSGSGSGS GSGSGSGSGS**VSDVPRDLEVVAATPTS LLISWSARLKVARYYRITYGETGGNSPV QEFTVPKNVYTATISGLKPGVDYTITVY AVTRFRDYQPISINYRT
119	E4-GS10-I1: E/I tandem having E4 (with N-terminal extension (N+8) and short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and modified Ser or Cys tail), wherein X = Ser or Cys; may optionally comprise a 6X His tag (SEQ ID NO: 487)	VSDVPRDLEVVAATPTSLLISWHERDGS RQYYRITYGETGGNSPVQEFTVPGGVRT ATISGLKPGVDYTITVYAVTDYFNPTTH EYIYQTTPISINYRTEIDK**GSGSGSGSGS GSGSGSGSGS**VSDVPRDLEVVAATPTS LLISWSARLKVARYYRITYGETGGNSPV QEFTVPKNVYTATISGLKPGVDYTITVY AVTRFRDYQPISINYRTEIDKPXQ
120	E4-GS10-I1: E/I tandem having E4 (with N-terminal extension (N+10) and short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and Cys tail) with his tag	MGVSDVPRDLEVVAATPTSLLISWHER DGSRQYYRITYGETGGNSPVQEFTVPGG VRTATISGLKPGVDYTITVYAVTDYFNP TTHEYIYQTTPISINYRTEIDK**GSGSGSG SGSGSGSGSGSGS**VSDVPRDLEVVAAT PTSLLISWSARLKVARYYRITYGETGGN SPVQEFTVPKNVYTATISGLKPGVDYTI TVYAVTRFRDYQPISINYRTEIDKPCQH HHHHH
121	E4-$(PA)_n$-I1: E/I tandem having E4 (with N-terminal extension (N+8) and an E tail) fused via $(PA)_n$ linker ($(PA)_n$ is SEQ ID NO: 488) to I1 (with N-terminal extension (N+8) and modified Ser or Cys tail), wherein n = 3, 6 or 9,	VSDVPRDLEVVAATPTSLLISWHERDGS RQYYRITYGETGGNSPVQEFTVPGGVRT ATISGLKPGVDYTITVYAVTDYFNPTTH EYIYQTTPISINYRTE**(PA)$_n$**VSDVPRDLE VVAATPTSLLISWSARLKVARYYRITYG ETGGNSPVQEFTVPKNVYTATISGLKPG VDYTITVYAVTRFRDYQPISINYRTEIDK

SEQ ID NO:	Description	Sequence
	and X = Ser or Cys; may optionally comprise a 6X His tag (SEQ ID NO: 487)	PCQHHHHHH
122	I1-GS10-E4: I/E tandem having I1 (with N-terminal extension (N+8) and short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to E4 (having N-terminal extension (N+8) and no tail)	VSDVPRDLEVVAATPTSLLISWSARLKV ARYYRITYGETGGNSPVQEFTVPKNVY TATISGLKPGVDYTITVYAVTRFRDYQPI SINYRTEIDK**GSGSGSGSGSGSGSGSGS GS**VSDVPRDLEVVAATPTSLLISWHERD GSRQYYRITYGETGGNSPVQEFTVPGGV RTATISGLKPGVDYTITVYAVTDYFNPT THEYIYQTTPISINYRT
123	I1-GS10-E4: I/E tandem having I1 (with N-terminal extension (N+8) and short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to E4 (with N-terminal extension (N+8) and modified Ser or Cys tail), wherein X = Ser or Cys; may optionally comprise a 6X His tag (SEQ ID NO: 487)	VSDVPRDLEVVAATPTSLLISWSARLKV ARYYRITYGETGGNSPVQEFTVPKNVY TATISGLKPGVDYTITVYAVTRFRDYQPI SINYRTEIDK**GSGSGSGSGSGSGSGSGS GS**VSDVPRDLEVVAATPTSLLISWHERD GSRQYYRITYGETGGNSPVQEFTVPGGV RTATISGLKPGVDYTITVYAVTDYFNPT THEYIYQTTPISINYRTEIDKPXQ
124	I1-GS10-E4: I/E tandem having I1 (with N-terminal extension (N+10) and short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to E4 (with N-terminal extension (N+8) and a Cys tail) with his tag	MGVSDVPRDLEVVAATPTSLLISWSARL KVARYYRITYGETGGNSPVQEFTVPKN VYTATISGLKPGVDYTITVYAVTRFRDY QPISINYRTEIDK**GSGSGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWHE RDGSRQYYRITYGETGGNSPVQEFTVPG GVRTATISGLKPGVDYTITVYAVTDYFN PTTHEYIYQTTPISINYRTEIDKPCQHHH HHH
125	I1-$(PA)_n$-E4: I/E tandem having I1 (with N-terminal extension (N+8) and an E tail) fused via $(PA)_n$ linker ($(PA)_n$ is SEQ ID NO: 488) to E4 (with N-terminal extension (N+8) and modified Ser or Cys tail), wherein n = 3, 6 or 9, and X = Ser or Cys; may optionally comprise a 6X His tag (SEQ ID NO: 487)	VSDVPRDLEVVAATPTSLLISWSARLKV ARYYRITYGETGGNSPVQEFTVPKNVY TATISGLKPGVDYTITVYAVTRFRDYQPI SINYRTE**(PA)$_n$**VSDVPRDLEVVAATPTSL LISWHERDGSRQYYRITYGETGGNSPVQ EFTVPGGVRTATISGLKPGVDYTITVYA VTDYFNPTTHEYIYQTTPISINYRTEIDKP XQ
126	E5-GS10-I1: E/I tandem having E5 (with N-terminal extension (N+8) and short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and no tail)	VSDVPRDLEVVAATPTSLLISWWAPVD RYQYYRITYGETGGNSPVQEFTVPRDV YTATISGLKPGVDYTITVYAVTDYKPHA DGPHTYHESPISINYRTEIDK**GSGSGSGS GSGSGSGSGSGS**VSDVPRDLEVVAATP TSLLISWSARLKVARYYRITYGETGGNS PVQEFTVPKNVYTATISGLKPGVDYTIT VYAVTRFRDYQPISINYRT

SEQ ID NO:	Description	Sequence
127	E5-GS10-I1: E/I tandem having E5 (with N-terminal extension (N+8) and short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and modified Ser or Cys tail), wherein X = Ser or Cys; may optionally comprise a 6X His tag (SEQ ID NO: 487)	VSDVPRDLEVVAATPTSLLISWWAPVD RYQYYRITYGETGGNSPVQEFTVPRDV YTATISGLKPGVDYTITVYAVTDYKPHA DGPHTYHESPISINYRTEIDK**GSGSGSGS GSGSGSGSGSGS**VSDVPRDLEVVAATP TSLLISWSARLKVARYYRITYGETGGNS PVQEFTVPKNVYTATISGLKPGVDYTIT VYAVTRFRDYQPISINYRTEIDKPXQ
128	E5-GS10-I1: E/I tandem having E5 (with N-terminal extension (N+10) and short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and a Cys tail), with a His tag	MGVSDVPRDLEVVAATPTSLLISWWAP VDRYQYYRITYGETGGNSPVQEFTVPR DVYTATISGLKPGVDYTITVYAVTDYKP HADGPHTYHESPISINYRTEIDK**GSGSGS GSGSGSGSGSGSGS**VSDVPRDLEVVAA TPTSLLISWSARLKVARYYRITYGETGG NSPVQEFTVPKNVYTATISGLKPGVDYT ITVYAVTRFRDYQPISINYRTEIDKPCQH HHHHH
129	E5-$(PA)_n$-I1: E/I tandem having E5 (with N-terminal extension (N+8) and an E tail) fused via $(PA)_n$ linker ($(PA)_n$ is SEQ ID NO: 488) to I1 (with N-terminal extension (N+8) and modified Ser or Cys tail), wherein n = 3, 6 or 9, and X = Ser or Cys; may optionally comprise a 6X His tag (SEQ ID NO: 487)	VSDVPRDLEVVAATPTSLLISWWAPVD RYQYYRITYGETGGNSPVQEFTVPRDV YTATISGLKPGVDYTITVYAVTDYKPHA DGPHTYHESPISINYRTE**(PA)$_n$**VSDVPRD LEVVAATPTSLLISWSARLKVARYYRIT YGETGGNSPVQEFTVPKNVYTATISGLK PGVDYTITVYAVTRFRDYQPISINYRTEI DKPXQ
130	I1-GS10-E5: I/E tandem having I1 (with N-terminal extension (N+8) and short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to E5 (with N-terminal extension (N+8) and no tail)	VSDVPRDLEVVAATPTSLLISWSARLKV ARYYRITYGETGGNSPVQEFTVPKNVY TATISGLKPGVDYTITVYAVTRFRDYQPI SINYRTEIDK**GSGSGSGSGSGSGSGSGS GS**VSDVPRDLEVVAATPTSLLISWWAP VDRYQYYRITYGETGGNSPVQEFTVPR DVYTATISGLKPGVDYTITVYAVTDYKP HADGPHTYHESPISINYRT
131	I1-GS10-E5: I/E tandem having I1 (with N-terminal extension (N+8) and short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to E5 (with N-terminal extension (N+8) and modified Ser or Cys tail), wherein X = Ser or Cys; may optionally comprise a 6X His tag (SEQ ID NO: 487)	VSDVPRDLEVVAATPTSLLISWSARLKV ARYYRITYGETGGNSPVQEFTVPKNVY TATISGLKPGVDYTITVYAVTRFRDYQPI SINYRTEIDK**GSGSGSGSGSGSGSGSGS GS**VSDVPRDLEVVAATPTSLLISWWAP VDRYQYYRITYGETGGNSPVQEFTVPR DVYTATISGLKPGVDYTITVYAVTDYKP HADGPHTYHESPISINYRTEIDKPXQ
132	I1-GS10-E5: I/E tandem having I1 (with N-terminal extension	MGVSDVPRDLEVVAATPTSLLISWSARL KVARYYRITYGETGGNSPVQEFTVPKN

SEQ ID NO:	Description	Sequence
	(N+10) and short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to E5 (with N-terminal extension (N+8) and a Cys tail) with a His tag	VYTATISGLKPGVDYTITVYAVTRFRDY QPISINYRTEIDK**GSGSGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWW APVDRYQYYRITYGETGGNSPVQEFTVP RDVYTATISGLKPGVDYTITVYAVTDY KPHADGPHTYHESPISINYRTEIDKPCQH HHHHH
133	I1-$(PA)_n$-E5: I/E tandem having I1 (with N-terminal extension (N+8) and an E tail) fused via $(PA)_n$ linker ($(PA)_n$ is SEQ ID NO: 488) to E5 (with N-terminal extension (N+8) and modified Ser or Cys tail), wherein n = 3, 6 or 9, and X = Ser or Cys; may optionally comprise a 6X His tag (SEQ ID NO: 487)	MGVSDVPRDLEVVAATPTSLLISWSARL KVARYYRITYGETGGNSPVQEFTVPKN VYTATISGLKPGVDYTITVYAVTRFRDY QPISINYRTE**(PA)$_n$**VSDVPRDLEVVAATP TSLLISWWAPVDRYQYYRITYGETGGN SPVQEFTVPRDVYTATISGLKPGVDYTIT VYAVTDYKPHADGPHTYHESPISINYRT EIDKPXQ
134	BC loop sequence from EGFR binder E4, wherein X is any amino acid and g and h are independently selected from 0 to 5 amino acids	X_gHERDGSRQX_h
135	DE loop sequence from EGFR binder E4, wherein X is any amino acid and i and j are independently selected from 0 to 5 amino acids	X_iGGVRX_j
136	FG loop sequence from EGFR binder E4, wherein X is any amino acid and k and l are independently selected from 0 to 5 amino acids	X_kDYFNPTTHEYIYQTTX_l
137	BC loop sequence from EGFR binder E5, wherein X is any amino acid and g and h are independently selected from 0 to 5 amino acids	X_gWAPVDRYQX_h
138	DE loop sequence from EGFR binder E5, wherein X is any amino acid and i and j are independently selected from 0 to 5 amino acids	X_iRDVYX_j
139	FG loop sequence from EGFR binder E5, wherein X is any amino acid and k and l are independently selected from 0 to	X_kDYKPHADGPHTYHESX_l

SEQ ID NO:	Description	Sequence
	5 amino acids	
140	E85 EGFR monomer with N-terminal extension (N+10) and Ser tail with his tag	MGVSDVPRDLEVVAATPTSLLISWTQG STHYQYYRITYGETGGNSPVQEFTVPG MVYTATISGLKPGVDYTITVYAVTDYF DRSTHEYKYRTTPISINYRTEIDKPSQHH HHHH
141	E85 EGFR monomer core: E85 monomer without N-terminal extension or C-terminal tail	EVVAATPTSLLISWTQGSTHYQYYRITY GETGGNSPVQEFTVPGMVYTATISGLKP GVDYTITVYAVTDYFDRSTHEYKYRTT PISINYRT
142	E85 EGFR monomer, wherein X_1 is selected from the group consisting of SEQ ID NOs: 69-77 and X_2 is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81; in exemplary emobidments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10; may optionally comprise a his tag	X_1EVVAATPTSLLISWTQGSTHYQYYRIT YGETGGNSPVQEFTVPGMVYTATISGL KPGVDYTITVYAVTDYFDRSTHEYKYR TTPISINYRTX_2
143	BC loop sequence from EGFR binder E85	SWTQGSTHYQ
144	DE loop sequence from EGFR binder E85	PGMVYT
145	FG loop sequence from EGFR binder E85	TDYFDRSTHEYKYRTTP
146	BC loop sequence from EGFR binder E85, wherein X is any amino acid and g and h are independently selected from 0 to 5 amino acids	X_gTQGSTHYQX_h
147	DE loop sequence from EGFR binder E85, wherein X is any amino acid and i and j are independently selected from 0 to 5 amino acids	X_iGMVYX_j
148	FG loop sequence from EGFR binder E85, wherein X is any amino acid and k and l are independently selected from 0 to 5 amino acids	X_kDYFDRSTHEYKYRTTX_l
149	E85-GS10-I1: E/I tandem having E85 (with N-terminal extension (N+10) and a short tail) fused via	MGVSDVPRDLEVVAATPTSLLISWTQG STHYQYYRITYGETGGNSPVQEFTVPG MVYTATISGLKPGVDYTITVYAVTDYF

SEQ ID NO:	Description	Sequence
	GS10 linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and Cys tail) with a 6X His tag (SEQ ID NO: 487)	DRSTHEYKYRTTPISINYRTEIDK**GSGSGSGSGSGSGSGSGS**VSDVPRDLEVVAATPTSLLISWSARLKVARYYRITYGETGGNSPVQEFTVPKNVYTATISGLKPGVDYTITVYAVTRFRDYQPISINYRTEIDKPCQHHHHHH
150	E85-GS10-I1 core, wherein X_1 is optional and when present is selected from the group consisting of SEQ ID NOs: 69-77, X_2 is optional and when present is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81, and n = 3, 6 or 9; in exemplary embodiments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10	X_1EVVAATPTSLLISWTQGSTHYQYYRITYGETGGNSPVQEFTVPGMVYTATISGLKPGVDYTITVYAVTDYFDRSTHEYKYRTTPISINYRTEIDK**GSGSGSGSGSGSGSGSGSGS**VSDVPRDLEVVAATPTSLLISWSARLKVARYYRITYGETGGNSPVQEFTVPKNVYTATISGLKPGVDYTITVYAVTRFRDYQPISINYRTX_2
151	E85-$(PA)_n$-I1 core, wherein X_1 is optional and when present is selected from the group consisting of SEQ ID NOs: 69-77, X_2 is optional and when present is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81, and n = 3, 6 or 9; in exemplary embodiments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10	X_1EVVAATPTSLLISWTQGSTHYQYYRITYGETGGNSPVQEFTVPGMVYTATISGLKPGVDYTITVYAVTDYFDRSTHEYKYRTTPISINYRTE**(PA)$_n$**VSDVPRDLEVVAATPTSLLISWSARLKVARYYRITYGETGGNSPVQEFTVPKNVYTATISGLKPGVDYTITVYAVTRFRDYQPISINYRTX_2
152	I1-GS10-E85: I/E tandem having I1 (with N-terminal extension (N+10) and a short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to E85 (with N-terminal extension (N+8) and Cys tail) with a 6X His tag (SEQ ID NO: 487)	MGVSDVPRDLEVVAATPTSLLISWSARLKVARYYRITYGETGGNSPVQEFTVPKNVYTATISGLKPGVDYTITVYAVTRFRDYQPISINYRTEIDK**GSGSGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWTQGSTHYQYYRITYGETGGNSPVQEFTVPGMVYTATISGLKPGVDYTITVYAVTDYFDRSTHEYKYRTTPISINYRTEIDKPCQHHHHHH
153	I1-GS10-E85 core, wherein X_1 is optional and when present is selected from the group consisting of SEQ ID NOs: 69-77, X_2 is optional and when present is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81, and n = 3, 6 or 9; in exemplary embodiments, X_1 is SEQ ID NO: 69 or 71 and X_2 is	X_1EVVAATPTSLLISWSARLKVARYYRITYGETGGNSPVQEFTVPKNVYTATISGLKPGVDYTITVYAVTRFRDYQPISINYRTEIDK**GSGSGSGSGSGSGSGSGSGS**VSDVPRDLEVVAATPTSLLISWTQGSTHYQYYRITYGETGGNSPVQEFTVPGMVYTATISGLKPGVDYTITVYAVTDYFDRSTHEYKYRTTPISINYRTX_2

SEQ ID NO:	Description	Sequence
	SEQ ID NO: 9 or 10	
154	I1-$(PA)_n$-E85 core, wherein X_1 is optional and when present is selected from the group consisting of SEQ ID NOs: 69-77, X_2 is optional and when present is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81, and n = 3, 6 or 9; in exemplary embodiments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10	X_1EVVAATPTSLLISWSARLKVARYYRIT YGETGGNSPVQEFTVPKNVYTATISGLK PGVDYTITVYAVTRFRDYQPISINYRTE(**PA)$_n$**VSDVPRDLEVVAATPTSLLISWTQG STHYQYYRITYGETGGNSPVQEFTVPG MVYTATISGLKPGVDYTITVYAVTDYF DRSTHEYKYRTTPISINYRTX_2
155	E90 EGFR monomer with N-terminal extension (N+10) and Ser tail with his tag	MGVSDVPRDLEVVAATPTSLLISWYWE GLPYQYYRITYGETGGNSPVQEFTVPRD VNTATISGLKPGVDYTITVYAVTDWYN PDTHEYIYHTIPISINYRTEIDKPSQHHHH HH
156	E90 EGFR monomer core: E90 monomer without N-terminal extension or C-terminal tail	EVVAATPTSLLISWYWEGLPYQYYRITY GETGGNSPVQEFTVPRDVNTATISGLKP GVDYTITVYAVTDWYNPDTHEYIYHTIP ISINYRT
157	E90 EGFR monomer, wherein X_1 is selected from the group consisting of SEQ ID NOs: 69-77 and X_2 is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81; in exemplary emobidments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10; may optionally comprise a his tag	X_1EVVAATPTSLLISWYWEGLPYQYYRI TYGETGGNSPVQEFTVPRDVNTATISGL KPGVDYTITVYAVTDWYNPDTHEYIYH TIPISINYRTX_2
158	BC loop sequence from EGFR binder E90	SWYWEGLPYQ
159	DE loop sequence from EGFR binder E90	PRDVNT
160	FG loop sequence from EGFR binder E90	TDWYNPDTHEYIYHTIP
161	BC loop sequence from EGFR binder E90, wherein X is any amino acid and g and h are independently selected from 0 to 5 amino acids	X_gYWEGLPYQX_h
162	DE loop sequence from EGFR binder E90, wherein X is any amino acid and i and j are	X_iRDVNX_j

SEQ ID NO:	Description	Sequence
	independently selected from 0 to 5 amino acids	
163	FG loop sequence from EGFR binder E90, wherein X is any amino acid and k and l are independently selected from 0 to 5 amino acids	X_kDWYNPDTHEYIYHTIX_l
164	E90-GS10-I1: E/I tandem having E90 (with N-terminal extension (N+10) and a short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and Cys tail) with a 6X His tag (SEQ ID NO: 487)	MGVSDVPRDLEVVAATPTSLLISWYWE GLPYQYYRITYGETGGNSPVQEFTVPRD VNTATISGLKPGVDYTITVYAVTDWYN PDTHEYIYHTIPISINYRTEIDK**GSGSGSG SGSGSGSGSGSGS**VSDVPRDLEVVAAT PTSLLISWSARLKVARYYRITYGETGGN SPVQEFTVPKNVYTATISGLKPGVDYTI TVYAVTRFRDYQPISINYRTEIDKPCQH HHHHH
165	E90-GS10-I1 core, wherein X_1 is optional and when present is selected from the group consisting of SEQ ID NOs: 69-77, X_2 is optional and when present is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81, and n = 3, 6 or 9; in exemplary embodiments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10	X_1EVVAATPTSLLISWYWEGLPYQYYRI TYGETGGNSPVQEFTVPRDVNTATISGL KPGVDYTITVYAVTDWYNPDTHEYIYH TIPISINYRTEIDK**GSGSGSGSGSGSGSG SGSGS**VSDVPRDLEVVAATPTSLLISWS ARLKVARYYRITYGETGGNSPVQEFTVP KNVYTATISGLKPGVDYTITVYAVTRFR DYQPISINYRTX_2
166	E90-$(PA)_n$-I1 core, wherein X1 is optional and when present is selected from the group consisting of SEQ ID NOs: 69-77, X_2 is optional and when present is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81, and n = 3, 6 or 9; in exemplary embodiments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10	X_1EVVAATPTSLLISWYWEGLPYQYYRI TYGETGGNSPVQEFTVPRDVNTATISGL KPGVDYTITVYAVTDWYNPDTHEYIYH TIPISINYRTE**(PA)$_n$**VSDVPRDLEVVAATP TSLLISWSARLKVARYYRITYGETGGNS PVQEFTVPKNVYTATISGLKPGVDYTIT VYAVTRFRDYQPISINYRTX_2
167	I1-GS10-E90: I/E tandem having I1 (with N-terminal extension (N+10) and a short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to E90 (with N-terminal extension (N+8) and Cys tail) with a 6X His tag (SEQ ID NO: 487)	MGVSDVPRDLEVVAATPTSLLISWSARL KVARYYRITYGETGGNSPVQEFTVPKN VYTATISGLKPGVDYTITVYAVTRFRDY QPISINYRTEIDK**GSGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWY WEGLPYQYYRITYGETGGNSPVQEFTV PRDVNTATISGLKPGVDYTITVYAVTD WYNPDTHEYIYHTIPISINYRTEIDKPCQ HHHHHH

SEQ ID NO:	Description	Sequence
168	I1-GS10-E90 core, wherein X_1 is optional and when present is selected from the group consisting of SEQ ID NOs: 69-77, X_2 is optional and when present is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81, and n = 3, 6 or 9; in exemplary embodiments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10	X_1EVVAATPTSLLISWSARLKVARYYRITYGETGGNSPVQEFTVPKNVYTATISGLKPGVDYTITVYAVTRFRDYQPISINYRTEIDK**GSGSGSGSGSGSGSGSGSGS**VSDVPRDLEVVAATPTSLLISWYWEGLPYQYYRITYGETGGNSPVQEFTVPRDVNTATISGLKPGVDYTITVYAVTDWYNPDTHEYIYHTIPISINYRTX_2
169	I1-$(PA)_n$-E90 core, wherein X_1 is optional and when present is selected from the group consisting of SEQ ID NOs: 69-77, X_2 is optional and when present is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81, and n = 3, 6 or 9; in exemplary embodiments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10	X_1EVVAATPTSLLISWSARLKVARYYRITYGETGGNSPVQEFTVPKNVYTATISGLKPGVDYTITVYAVTRFRDYQPISINYRTE**(PA)$_n$**VSDVPRDLEVVAATPTSLLISWYWEGLPYQYYRITYGETGGNSPVQEFTVPRDVNTATISGLKPGVDYTITVYAVTDWYNPDTHEYIYHTIPISINYRTX_2
170	E96 EGFR monomer with N-terminal extension (N+10) and Ser tail with his tag	MGVSDVPRDLEVVAATPTSLLISWASNRGTYQYYRITYGETGGNSPVQEFTVPGGVSTATISGLKPGVDYTITVYAVTDAFNPTTHEYNYFTTPISINYRTEIDKPSQHHHHHH
171	E96 EGFR monomer core: E96 monomer without N-terminal extension or C-terminal tail	EVVAATPTSLLISWASNRGTYQYYRITYGETGGNSPVQEFTVPGGVSTATISGLKPGVDYTITVYAVTDAFNPTTHEYNYFTTPISINYRT
172	E96 EGFR monomer, wherein X_1 is selected from the group consisting of SEQ ID NOs: 69-77 and X_2 is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81; in exemplary emobidments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10; may optionally comprise a his tag	X_1EVVAATPTSLLISWASNRGTYQYYRITYGETGGNSPVQEFTVPGGVSTATISGLKPGVDYTITVYAVTDAFNPTTHEYNYFTTPISINYRTX_2
173	BC loop sequence from EGFR binder E96	SWASNRGTYQ
174	DE loop sequence from EGFR binder E96	PGGVST

SEQ ID NO:	Description	Sequence
175	FG loop sequence from EGFR binder E96	TDAFNPTTHEYNYFTTP
176	BC loop sequence from EGFR binder E96, wherein X is any amino acid and g and h are independently selected from 0 to 5 amino acids	X_gASNRGTYQX_h
177	DE loop sequence from EGFR binder E96, wherein X is any amino acid and i and j are independently selected from 0 to 5 amino acids	X_iGGVSX_j
178	FG loop sequence from EGFR binder E96, wherein X is any amino acid and k and l are independently selected from 0 to 5 amino acids	X_kDAFNPTTHEYNYFTTX_l
179	E96-GS10-I1: E/I tandem having E96 (with N-terminal extension (N+10) and a short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and Cys tail) with a 6X His tag (SEQ ID NO: 487)	MGVSDVPRDLEVVAATPTSLLISWASN RGTYQYYRITYGETGGNSPVQEFTVPG GVSTATISGLKPGVDYTITVYAVTDAFN PTTHEYNYFTTPISINYRTEIDK**GSGSGS GSGSGSGSGSGSGS**VSDVPRDLEVVAA TPTSLLISWSARLKVARYYRITYGETGG NSPVQEFTVPKNVYTATISGLKPGVDYT ITVYAVTRFRDYQPISINYRTEIDKPCQH HHHHH
180	E96-GS10-I1 core, wherein X_1 is optional and when present is selected from the group consisting of SEQ ID NOs: 69-77, X_2 is optional and when present is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81, and n = 3, 6 or 9; in exemplary embodiments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10	X_1EVVAATPTSLLISWASNRGTYQYYRI TYGETGGNSPVQEFTVPGGVSTATISGL KPGVDYTITVYAVTDAFNPTTHEYNYF TTPISINYRTEIDK**GSGSGSGSGSGSGSG SGSGS**VSDVPRDLEVVAATPTSLLISWS ARLKVARYYRITYGETGGNSPVQEFTVP KNVYTATISGLKPGVDYTITVYAVTRFR DYQPISINYRTX_2
181	E96-$(PA)_n$-I1 core, wherein X1 is optional and when present is selected from the group consisting of SEQ ID NOs: 69-77, X_2 is optional and when present is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81, and n = 3, 6 or 9; in exemplary embodiments, X_1 is	X_1EVVAATPTSLLISWASNRGTYQYYRI TYGETGGNSPVQEFTVPGGVSTATISGL KPGVDYTITVYAVTDAFNPTTHEYNYF TTPISINYRTE**$(PA)_n$**VSDVPRDLEVVAAT PTSLLISWSARLKVARYYRITYGETGGN SPVQEFTVPKNVYTATISGLKPGVDYTI TVYAVTRFRDYQPISINYRTX_2

SEQ ID NO:	Description	Sequence
	SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10	
182	I1-GS10-E96: I/E tandem having I1 (with N-terminal extension (N+10) and a short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to E96 (with N-terminal extension (N+8) and Cys tail) with a 6X His tag (SEQ ID NO: 487)	MGVSDVPRDLEVVAATPTSLLISWSARL KVARYYRITYGETGGNSPVQEFTVPKN VYTATISGLKPGVDYTITVYAVTRFRDY QPISINYRTEIDK**GSGSGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWAS NRGTYQYYRITYGETGGNSPVQEFTVP GGVSTATISGLKPGVDYTITVYAVTDAF NPTTHEYNYFTTPISINYRTEIDKPCQHH HHHH
183	I1-GS10-E96 core, wherein X_1 is optional and when present is selected from the group consisting of SEQ ID NOs: 69-77, X_2 is optional and when present is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81, and n = 3, 6 or 9; in exemplary embodiments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10	X_1EVVAATPTSLLISWSARLKVARYYRIT YGETGGNSPVQEFTVPKNVYTATISGLK PGVDYTITVYAVTRFRDYQPISINYRTEI DK**GSGSGSGSGSGSGSGSGS**VSDVP RDLEVVAATPTSLLISWASNRGTYQYY RITYGETGGNSPVQEFTVPGGVSTATISG LKPGVDYTITVYAVTDAFNPTTHEYNY FTTPISINYRTX_2
184	I1-$(PA)_n$-E96 core, wherein X_1 is optional and when present is selected from the group consisting of SEQ ID NOs: 69-77, X_2 is optional and when present is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81, and n = 3, 6 or 9; in exemplary embodiments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10	X_1EVVAATPTSLLISWSARLKVARYYRIT YGETGGNSPVQEFTVPKNVYTATISGLK PGVDYTITVYAVTRFRDYQPISINYRTE**(PA)$_n$**VSDVPRDLEVVAATPTSLLISWASN RGTYQYYRITYGETGGNSPVQEFTVPG GVSTATISGLKPGVDYTITVYAVTDAFN PTTHEYNYFTTPISINYRTX_2
185	E105 EGFR monomer with N-terminal extension (N+10) and Ser tail with his tag	MGVSDVPRDLEVVAATPTSLLISWDAP TSRYQYYRITYGETGGNSPVQEFTVPGG LSTATISGLKPGVDYTITVYAVTDYKPH ADGPHTYHESPISINYRTEIDKPSQHHHH HH
186	E105 EGFR monomer core: E105 monomer without N-terminal extension or C-terminal tail	EVVAATPTSLLISWDAPTSRYQYYRITY GETGGNSPVQEFTVPGGLSTATISGLKP GVDYTITVYAVTDYKPHADGPHTYHES PISINYRT
187	E105 EGFR monomer, wherein X_1 is selected from the group consisting of SEQ ID NOs: 69-77 and X_2 is selected from the group	X_1EVVAATPTSLLISWDAPTSRYQYYRIT YGETGGNSPVQEFTVPGGLSTATISGLK PGVDYTITVYAVTDYKPHADGPHTYHE SPISINYRTX_2

SEQ ID NO:	Description	Sequence
	consisting of SEQ ID NOs: 9, 10, or 78-81; in exemplary emobidments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10; may optionally comprise a his tag	
188	BC loop sequence from EGFR binder E105	SWDAPTSRYQ
189	DE loop sequence from EGFR binder E105	PGGLST
117	FG loop sequence from EGFR binder E105	TDYKPHADGPHTYHESP
190	BC loop sequence from EGFR binder E105, wherein X is any amino acid and g and h are independently selected from 0 to 5 amino acids	X_gDAPTSRYQX_h
191	DE loop sequence from EGFR binder E105, wherein X is any amino acid and i and j are independently selected from 0 to 5 amino acids	X_iGGLSX_j
139	FG loop sequence from EGFR binder E105, wherein X is any amino acid and k and l are independently selected from 0 to 5 amino acids	X_kDYKPHADGPHTYHESX_l
192	E105-GS10-I1: E/I tandem having E105 (with N-terminal extension (N+10) and a short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and Cys tail) with a 6X His tag (SEQ ID NO: 487)	MGVSDVPRDLEVVAATPTSLLISWDAP TSRYQYYRITYGETGGNSPVQEFTVPGG LSTATISGLKPGVDYTITVYAVTDYKPH ADGPHTYHESPISINYRTEIDK**GSGSGSG SGSGSGSGSGSGS**VSDVPRDLEVVAAT PTSLLISWSARLKVARYYRITYGETGGN SPVQEFTVPKNVYTATISGLKPGVDYTI TVYAVTRFRDYQPISINYRTEIDKPCQH HHHHH
193	E105-GS10-I1 core, wherein X_1 is optional and when present is selected from the group consisting of SEQ ID NOs: 69-77, X_2 is optional and when present is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81, and n = 3, 6 or 9; in exemplary embodiments, X_1 is	X_1EVVAATPTSLLISWDAPTSRYQYYRIT YGETGGNSPVQEFTVPGGLSTATISGLK PGVDYTITVYAVTDYKPHADGPHTYHE SPISINYRTEIDK**GSGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWSA RLKVARYYRITYGETGGNSPVQEFTVPK NVYTATISGLKPGVDYTITVYAVTRFRD YQPISINYRTX_2

SEQ ID NO:	Description	Sequence
	SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10	
194	E105-$(PA)_n$-I1 core, wherein X1 is optional and when present is selected from the group consisting of SEQ ID NOs: 69-77, X_2 is optional and when present is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81, and n = 3, 6 or 9; in exemplary embodiments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10	X_1EVVAATPTSLLISWDAPTSRYQYYRIT YGETGGNSPVQEFTVPGGLSTATISGLK PGVDYTITVYAVTDYKPHADGPHTYHE SPISINYRTE**(PA)$_n$**VSDVPRDLEVVAATP TSLLISWSARLKVARYYRITYGETGGNS PVQEFTVPKNVYTATISGLKPGVDYTIT VYAVTRFRDYQPISINYRTX_2
195	I1-GS10-E105: I/E tandem havingI1 (with N-terminal extension (N+10) and a short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to E105 (with N-terminal extension (N+8) and Cys tail) with a 6X His tag (SEQ ID NO: 487)	MGVSDVPRDLEVVAATPTSLLISWSARL KVARYYRITYGETGGNSPVQEFTVPKN VYTATISGLKPGVDYTITVYAVTRFRDY QPISINYRTEIDK**GSGSGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWDA PTSRYQYYRITYGETGGNSPVQEFTVPG GLSTATISGLKPGVDYTITVYAVTDYKP HADGPHTYHESPISINYRTEIDKPCQHH HHHH
196	I1-GS10-E105 core, wherein X_1 is optional and when present is selected from the group consisting of SEQ ID NOs: 69-77, X_2 is optional and when present is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81, and n = 3, 6 or 9; in exemplary embodiments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10	X_1EVVAATPTSLLISWSARLKVARYYRIT YGETGGNSPVQEFTVPKNVYTATISGLK PGVDYTITVYAVTRFRDYQPISINYRTEI DK**GSGSGSGSGSGSGSGSGS**VSDVP RDLEVVAATPTSLLISWDAPTSRYQYYR ITYGETGGNSPVQEFTVPGGLSTATISGL KPGVDYTITVYAVTDYKPHADGPHTYH ESPISINYRTX_2
197	I1-$(PA)_n$-E105 core, wherein X_1 is optional and when present is selected from the group consisting of SEQ ID NOs: 69-77, X_2 is optional and when present is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81, and n = 3, 6 or 9; in exemplary embodiments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10	X_1EVVAATPTSLLISWSARLKVARYYRIT YGETGGNSPVQEFTVPKNVYTATISGLK PGVDYTITVYAVTRFRDYQPISINYRTE**(PA)$_n$**VSDVPRDLEVVAATPTSLLISWDAP TSRYQYYRITYGETGGNSPVQEFTVPGG LSTATISGLKPGVDYTITVYAVTDYKPH ADGPHTYHESPISINYRTX_2
198	E112 EGFR monomer with N-terminal extension (N+10) and Ser tail with his tag	MGVSDVPRDLEVVAATPTSLLISWDAG AVTYQYYRITYGETGGNSPVQEFTVPG GVRTATISGLKPGVDYTITVYAVTDYKP

SEQ ID NO:	Description	Sequence
		HADGPHTYHEYPISINYRTEIDKPSQHH HHHH
199	E112 EGFR monomer core: E112 monomer without N-terminal extension or C-terminal tail	EVVAATPTSLLISWDAGAVTYQYYRITY GETGGNSPVQEFTVPGGVRTATISGLKP GVDYTITVYAVTDYKPHADGPHTYHEY PISINYRT
200	E112 EGFR monomer, wherein X_1 is selected from the group consisting of SEQ ID NOs: 69-77 and X_2 is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81; in exemplary emobidments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10; may optionally comprise a his tag	X_1EVVAATPTSLLISWDAGAVTYQYYRI TYGETGGNSPVQEFTVPGGVRTATISGL KPGVDYTITVYAVTDYKPHADGPHTYH EYPISINYRTX_2
201	BC loop sequence from EGFR binder E112	SWDAGAVTYQ
110	DE loop sequence from EGFR binder E112	PGGVRT
202	FG loop sequence from EGFR binder E112	TDYKPHADGPHTYHEYP
203	BC loop sequence from EGFR binder E112, wherein X is any amino acid and g and h are independently selected from 0 to 5 amino acids	X_gDAGAVTYQX_h
135	DE loop sequence from EGFR binder E112, wherein X is any amino acid and i and j are independently selected from 0 to 5 amino acids	X_iGGVRX_j
204	FG loop sequence from EGFR binder E112, wherein X is any amino acid and k and l are independently selected from 0 to 5 amino acids	X_kDYKPHADGPHTYHEYX_l
205	E112-GS10-I1: E/I tandem having E112 (with N-terminal extension (N+10) and a short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to I1 (with N-terminal extension (N+8) and Cys tail) with a 6X His tag (SEQ ID	MGVSDVPRDLEVVAATPTSLLISWDAG AVTYQYYRITYGETGGNSPVQEFTVPG GVRTATISGLKPGVDYTITVYAVTDYKP HADGPHTYHEYPISINYRTEIDK**GSGSG SGSGSGSGSGSGSGS**VSDVPRDLEVVA ATPTSLLISWSARLKVARYYRITYGETG GNSPVQEFTVPKNVYTATISGLKPGVDY TITVYAVTRFRDYQPISINYRTEIDKPCQ

SEQ ID NO:	Description	Sequence
	NO: 487)	HHHHHH
206	E112-GS10-I1 core, wherein X_1 is optional and when present is selected from the group consisting of SEQ ID NOs: 69-77, X_2 is optional and when present is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81, and n = 3, 6 or 9; in exemplary embodiments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10	X_1EVVAATPTSLLISWDAGAVTYQYYRI TYGETGGNSPVQEFTVPGGVRTATISGL KPGVDYTITVYAVTDYKPHADGPHTYH EYPISINYRTEIDK**GSGSGSGSGSGSGSG SGSGS**VSDVPRDLEVVAATPTSLLISWS ARLKVARYYRITYGETGGNSPVQEFTVP KNVYTATISGLKPGVDYTITVYAVTRFR DYQPISINYRTX_2
207	E112-$(PA)_n$-I1 core, wherein X1 is optional and when present is selected from the group consisting of SEQ ID NOs: 69-77, X_2 is optional and when present is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81, and n = 3, 6 or 9; in exemplary embodiments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10	X_1EVVAATPTSLLISWDAGAVTYQYYRI TYGETGGNSPVQEFTVPGGVRTATISGL KPGVDYTITVYAVTDYKPHADGPHTYH EYPISINYRTE**(PA)$_n$**VSDVPRDLEVVAAT PTSLLISWSARLKVARYYRITYGETGGN SPVQEFTVPKNVYTATISGLKPGVDYTI TVYAVTRFRDYQPISINYRTX_2
208	I1-GS10-E112: I/E tandem having I1 (with N-terminal extension (N+10) and a short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to E112 (with N-terminal extension (N+8) and Cys tail) with a 6X His tag (SEQ ID NO: 487)	MGVSDVPRDLEVVAATPTSLLISWSARL KVARYYRITYGETGGNSPVQEFTVPKN VYTATISGLKPGVDYTITVYAVTRFRDY QPISINYRTEIDK**GSGSGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWDA GAVTYQYYRITYGETGGNSPVQEFTVP GGVRTATISGLKPGVDYTITVYAVTDY KPHADGPHTYHEYPISINYRTEIDKPCQ HHHHHH
209	I1-GS10-E112 core, wherein X_1 is optional and when present is selected from the group consisting of SEQ ID NOs: 69-77, X_2 is optional and when present is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81, and n = 3, 6 or 9; in exemplary embodiments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10	X_1EVVAATPTSLLISWSARLKVARYYRIT YGETGGNSPVQEFTVPKNVYTATISGLK PGVDYTITVYAVTRFRDYQPISINYRTEI DK**GSGSGSGSGSGSGSGSGSGS**VSDVP RDLEVVAATPTSLLISWDAGAVTYQYY RITYGETGGNSPVQEFTVPGGVRTATIS GLKPGVDYTITVYAVTDYKPHADGPHT YHEYPISINYRTX_2
210	I1-$(PA)_n$-E112 core, wherein X_1 is optional and when present is selected from the group consisting of SEQ ID NOs: 69-	X_1EVVAATPTSLLISWSARLKVARYYRIT YGETGGNSPVQEFTVPKNVYTATISGLK PGVDYTITVYAVTRFRDYQPISINYRTE(**PA)$_n$**VSDVPRDLEVVAATPTSLLISWDAG

SEQ ID NO:	Description	Sequence
	77, X_2 is optional and when present is selected from the group consisting of SEQ ID NOs: 9, 10, or 78-81, and n = 3, 6 or 9; in exemplary embodiments, X_1 is SEQ ID NO: 69 or 71 and X_2 is SEQ ID NO: 9 or 10	AVTYQYYRITYGETGGNSPVQEFTVPG GVRTATISGLKPGVDYTITVYAVTDYKP HADGPHTYHEYPISINYRTX_2
211	I1-GSGCGS8-E5: I/E tandem having I1 (with N-terminal extension (N+10) and a modified short tail) fused via GSGCGS8 linker (GSGCGS8 is SEQ ID NO: 218) to E5 (with N-terminal extension (N+8) and an E tail) with an optional 6X His tag (SEQ ID NO: 487)	MGVSDVPRDLEVVAATPTSLLISWSARL KVARYYRITYGETGGNSPVQEFTVPKN VYTATISGLKPGVDYTITVYAVTRFRDY QPISINYRTEIEK**GSGCGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWW APVDRYQYYRITYGETGGNSPVQEFTVP RDVYTATISGLKPGVDYTITVYAVTDY KPHADGPHTYHESPISINYRTEHHHHHH
212	I1-GS10-E5-GSGC: I/E tandem having I1 (with N-terminal extension (N+10) and a modified short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to E5 (with N-terminal extension (N+8) and a modified Cys tail) with an optional 6X His tag (SEQ ID NO: 487)	MGVSDVPRDLEVVAATPTSLLISWSARL KVARYYRITYGETGGNSPVQEFTVPKN VYTATISGLKPGVDYTITVYAVTRFRDY QPISINYRTEIEK**GSGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWW APVDRYQYYRITYGETGGNSPVQEFTVP RDVYTATISGLKPGVDYTITVYAVTDY KPHADGPHTYHESPISINYRTEGSGCHH HHHH
213	I1(S62C)-GS10-E5: I/E tandem having I1 (with N-terminal extension (N+10), an S62C substitution (boxed), and a modified short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to E5 (with N-terminal extension (N+8) and an E tail) with an optional 6X His tag (SEQ ID NO: 487); position 62 refers to the amino acid corresponding to position 62 of SEQ ID NO:1	MGVSDVPRDLEVVAATPTSLLISWSARL KVARYYRITYGETGGNSPVQEFTVPKN VYTATICGLKPGVDYTITVYAVTRFRDY QPISINYRTEIEK**GSGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWW APVDRYQYYRITYGETGGNSPVQEFTVP RDVYTATISGLKPGVDYTITVYAVTDY KPHADGPHTYHESPISINYRTEHHHHHH
214	I1-GS10-E5(S62C): I/E tandem having I1 (with N-terminal extension (N+10) and a modified short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to E5 (with N-terminal extension (N+8), an S62C substitution (boxed), and an E tail) with an optional 6X His tag (SEQ ID NO: 487); position 62 refers to the	MGVSDVPRDLEVVAATPTSLLISWSARL KVARYYRITYGETGGNSPVQEFTVPKN VYTATISGLKPGVDYTITVYAVTRFRDY QPISINYRTEIEK**GSGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWW APVDRYQYYRITYGETGGNSPVQEFTVP RDVYTATICGLKPGVDYTITVYAVTDY KPHADGPHTYHESPISINYRTEHHHHHH

SEQ ID NO:	Description	Sequence
	amino acid corresponding to position 62 of SEQ ID NO:1	
215	I1(S91C)-GS10-E5: I/E tandem having I1 (with N-terminal extension (N+10), an S91C substitution (boxed), and a modified short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to E5 (with N-terminal extension (N+8) and an E tail) with an optional 6X His tag (SEQ ID NO: 487); position 91 refers to the amino acid corresponding to position 91 of SEQ ID NO:1	MGVSDVPRDLEVVAATPTSLLISWSARL KVARYYRITYGETGGNSPVQEFTVPKN VYTATISGLKPGVDYTITVYAVTRFRDY QPI[C]INYRTEIEK**GSGSGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWW APVDRYQYYRITYGETGGNSPVQEFTVP RDVYTATISGLKPGVDYTITVYAVTDY KPHADGPHTYHESPISINYRTEHHHHHH
216	I1-GS10-E5(S91C): I/E tandem having I1 (with N-terminal extension (N+10) and a modified short tail) fused via GS10 linker (GS10 is SEQ ID NO: 11) to E5 (with N-terminal extension (N+8), an S91C substitution (boxed), and an E tail) with an optional 6X His tag (SEQ ID NO: 487); position 91 refers to the amino acid corresponding to position 91 of SEQ ID NO:1	MGVSDVPRDLEVVAATPTSLLISWSARL KVARYYRITYGETGGNSPVQEFTVPKN VYTATISGLKPGVDYTITVYAVTRFRDY QPISINYRTEIEK**GSGSGSGSGSGSGSGS GSGS**VSDVPRDLEVVAATPTSLLISWW APVDRYQYYRITYGETGGNSPVQEFTVP RDVYTATISGLKPGVDYTITVYAVTDY KPHADGPHTYHESPI[C]INYRTEHHHHHH
217	Modified short tail	EIEK
218	GSGCGS8 Linker	GSGCGSGSGSGSGSGSGS

Example 1: In Cell Western Assay to Screen for EGFR Activity

In Cell Western assays were developed to screen various single ^{10}Fn3 clones for the ability to inhibit EGFR activity in order to identify those that could be linked with IGF1R ^{10}Fn3 binders to construct E/I binders. In Cell Western assays were also used to screen and determine relative potency of specific E/I ^{10}Fn3 binders. Two In Cell Western assays were developed to measure 1) inhibition of EGF-stimulated EGFR phosphorylation or 2) inhibition of EGF-stimulated ERK phosphorylation. Cells were seeded into poly-D-lysine coated 96-well microtiter plates (Becton Dickinson, Franklin Lakes, NJ) at 24,000 cells/well for A431 epidermoid carcinoma or FaDu head & neck carcinoma cells and allowed to adhere overnight. Cells were washed once and then incubated for 24 hours in serum free

media. Serial dilutions of the ^{10}Fn3-based binders were next applied to the cells and incubated for 2-3 hours prior to stimulation with 100 ng/ml EGF for 10 minutes. Following stimulation, cells were fixed for 20 minutes in PBS containing 3.7% formaldehyde and then permeabilized in PBS containing 0.1% triton-X-100 for 15 minutes. Cells were blocked for one hour in Odyssey blocker (Li-Cor Biosciences, Lincoln, Nebraska) and incubated with antibodies to detect either EGFR phosphorylated on tyrosine 1068 (Cell Signaling, Beverly, MA) and β-actin (Sigma, St. Louis, MO) or pERK (MAP kinase phosphorylated on tyrosine 202/threonine 204) and total ERK (Santa Cruz Biotechnology, Santa Cruz, CA). After washing three times in PBS containing 0.1% tween-20, secondary antibodies were added (Invitrogen, Carlsbad, CA or Rockland, Gilbertsville, PA). Cells were washed three times in PBS containing 0.1% tween-20 and imaged on a Li-Cor Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, Nebraska). Each clone was assayed in duplicate or triplicate and values were normalized to β-actin for the pEGFR assay and total ERK for the pERK assay. IC50 values were calculated from linear regression analysis of percent inhibition of maximum signal minus background.

Results yielded various ^{10}Fn3 clones that had ability to inhibit activity of EGFR, and showed that certain specific E/I ^{10}Fn3 binders possessed similar activity to the example shown in Figure 9.

Example 2: Expression of ^{10}Fn3-based binders

E/I binders were produced by covalently linking an EGFR-binding ^{10}Fn3 to an IGFIR-binding ^{10}Fn3 using a glycine-serine linker, thereby generating ^{10}Fn3 dimers, wherein each ^{10}Fn3 domain binds to a different target. The IGFIR-binding ^{10}Fn3 (I1) was previously described as SEQ ID NO: 226 in PCT Publication No. WO 2008/066752. Two novel EGFR-binding ^{10}Fn3 (E2 and E1) were identified by screening an RNA-protein fusion library, as described in PCT Publication No. WO 2008/066752, for binders to EGFR-Fc (R&D Systems, Minneapolis, MN). The following examples describe results using a variety of His-tagged E/I ^{10}Fn3-based binders (non-pegylated): E2-GS10-I1 (SEQ ID NO: 25), E1-GS10-I1 (SEQ ID NO: 31), I1-GS10-E1 (SEQ ID NO: 28), and I1-GS10-E2 (SEQ ID NO: 22).

The following examples also describe results with the following pegylated, His-tagged E/I ^{10}Fn3-based binders: E1-GS10-I1 (SEQ ID NO: 55), E2-GS10-I1 (SEQ ID NO: 56), E3-GS10-I1 (SEQ ID NO: 53), I1-GS10-E1 (SEQ ID NO: 57), I1-GS10-E2 (SEQ ID NO: 58), I1-GS10-E3 (SEQ ID NO: 54), E4-GS10-I1 (SEQ ID NO: 120), I1-GS10-E4 (SEQ ID NO: 124), E5-GS10-I1 (SEQ ID NO: 128), I1-GS10-E5 (SEQ ID NO: 132), E85-GS10-I1 (SEQ ID NO: 149), I1-GS10-E85 (SEQ ID NO: 152), E90-GS10-I1 (SEQ ID NO: 164), E96-GS10-I1 (SEQ ID NO: 179), E105-GS10-I1 (SEQ ID NO: 192), I1-GS10-E105 (SEQ ID NO: 195), E112-GS10-I1 (SEQ ID NO: 205), I1-GS10-E112 (SEQ ID NO: 208), I1-GSGCGS8-E5 (SEQ ID NO: 211), I1-GS10-E5-GSGC (SEQ ID NO: 212), I1(S62C)-GS10-E5 (SEQ ID NO: 213), I1-GS10-E5(S62C) (SEQ ID NO: 214), I1(S91C)-GS10-E5 (SEQ ID NO: 215), and I1-GS10-E5(S91C) (SEQ ID NO: 216).

The examples also describe results using a His-tagged IGFRIR ^{10}Fn3-based binder, I1 (SEQ ID NO: 4), and ten His-tagged EGFR ^{10}Fn3-based binders, E2 (SEQ ID NO: 6), E1 (SEQ ID NO: 8), E3 (SEQ ID NO: 52), E4 (SEQ ID NO: 107), E5 (SEQ ID NO: 113, wherein X = Ser and with a His tag at the C-terminus), E5 pegylated (SEQ ID NO: 113, wherein X = Cys and with a His tag at the C-terminus), E85 (SEQ ID NO: 140), E90 (SEQ ID NO: 155), E96 (SEQ ID NO: 170), E105 (SEQ ID NO: 185), and E112 (SEQ ID NO: 198). Examples 32 also describes a variety of E monomers having the sequences set forth in Figure 45 and including a His tag at the C-terminus.

The various ^{10}Fn3-based binders were purified using a high throughput protein production process (HTPP). Selected binders were cloned into the pET9d vector in order to generate His_6 tag (SEQ ID NO: 487) fusions. DNA was transformed into *E. coli* HMS174(DE3), and cells were inoculated in 5 ml LB medium containing 50 μg/mL kanamycin in a 24-well format and grown at 37 °C overnight. Fresh 5 ml LB medium (50 μg/mL kanamycin) cultures were prepared for inducible expression by aspirating 200 μl from the overnight culture and dispensing it into the appropriate well. The cultures were grown at 37 °C until A_{600} 0.6-0.9. After induction with 1 mM isopropyl-β-thiogalactoside (IPTG), the culture was grown for another 6 hours at 30 °C and harvested by centrifugation for 10 minutes at 3220 x g at 4 °C. Cell pellets were frozen at 80 °C.

Cell pellets (in 24-well format) were lysed by resuspension in 450 µl of Lysis buffer (50 mM NaH_2PO_4, 0.5 M NaCl, 1x Complete™ Protease Inhibitor Cocktail-EDTA free (Roche), 1 mM PMSF, 10 mM CHAPS, 40 mM imidazole, 1 mg/ml lysozyme, 30 ug/ml DNAse, 2 µg/ml aprotonin, pH 8.0) and shaken at room temperature for 1 hour. Lysates were clarified and re-racked into a 96-well format by transfer into a 96-well Whatman GF/D Unifilter fitted with a 96-well, 650 µl catch plate and centrifuged for 5 minutes at 200 x g. The clarified lysates were transferred to a 96-well Ni-Chelating Plate that had been equilibrated with equilibration buffer (50 mM NaH_2PO_4, 0.5 M NaCl,10 mM CHAPS, 40 mM imidazole, pH 8.0) and incubated for 5 minutes. Unbound material was removed by vacuum. The resin was washed 2 x 0.3 ml/well with Wash buffer #1 (50 mM NaH_2PO_4, 0.5 M NaCl, 5 mM CHAPS, 40 mM imidazole, pH 8.0) with each wash removed by vacuum. Next, the resin was washed with 3 x 0.3 ml/well with PBS with each wash step removed by vacuum. Prior to elution, each well was washed with 50 µl Elution buffer (PBS + 20 mM EDTA), incubated for 5 minutes, and the wash discarded by vacuum. Protein was eluted by applying an additional 100 µl of Elution buffer to each well. After a 30 minute incubation at room temperature, the plate(s) were centrifuged for 5 minutes at 200 x g and eluted protein collected in 96-well catch plates containing 5 µl of 0.5 M $MgCl_2$ affixed to the bottom of the Ni-plates. Eluted protein was quantified using a BCA Protein assay with SEQ ID NO: 2 as the protein standard.

HTPP yielded active ^{10}Fn3-based binders that were expressed in a soluble form and purified from the soluble fraction of the bacterial cytosol. Figure 1 depicts an exemplary SDS-PAGE analysis from one of the E/I ^{10}Fn3-based binders. SEC analysis on a Superdex 200 5/150 GL in a mobile phase of 100 mM NaPO4, 100 mM NaSO4, 150 mM NaCl, pH 6.8 (GE Healthcare) demonstrated predominantly monomeric proteins (see Example 4).

In addition, midscale expression and purification of select ^{10}Fn3-based binders was performed. The selected binders, fused to a His_6 tag (SEQ ID NO: 487), were cloned into a pET9d or pET29 vector and expressed in *E. coli* HMS174(DE3) or BL212(DE3) (EMD Biosciences, San Diego, CA) cells. 20 ml of an inoculum culture (generated from a single plated colony) was used to inoculate 1 liter of LB

medium containing 50 μg/mL kanamycin. The culture was grown at 37 °C until A_{600} 0.6-1.0. After induction with 1 mM isopropyl-β-thiogalactoside (IPTG), the culture was grown for another 6 hours at 30 °C. Alternatively, expression was carried out at 18 °C after initial growth at 37 °C using autoinduction media ("ONE" medium, EMD Biosciences, San Diego, CA). Cell pellets were harvested by centrifugation for 30 minutes at ≥10,000 x g at 4 °C and frozen at 80 °C. The cell pellet was resuspended in 25 mL of lysis buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 1x Complete™ Protease Inhibitor Cocktail-EDTA free (Roche), pH 7.4) using an Ultra-turrax homgenizer on ice. Cell lysis was achieved by high pressure homogenization (≥18,000psi) using a Model M-110S Microfluidizer (Microfluidics). The soluble fraction was separated by centrifugation for 30 minutes at 23,300 x g at 4 °C. The supernatant was clarified via 0.45 μm filter. The clarified lysate was loaded onto a HisTrap column (GE) pre-equilibrated with 20 mM NaH_2PO_4, 0.5 M NaCl, pH 7.4. The column was then washed with 25 column volumes of 20 mM NaH_2PO_4, 0.5 M NaCl, pH 7.4, followed by 20 column volumes of 20 mM NaH_2PO_4, 0.5 M NaCl, 25 mM imidazole, pH 7.4, and then 35 column volumes of 20 mM NaH_2PO_4, 0.5 M NaCl, 40 mM imidazole, pH 7.4. Protein was eluted with 15 column volumes of 20 mM NaH_2PO_4, 0.5 M NaCl, 500 mM imidazole, pH 7.4, fractions pooled based on absorbance at A_{280} and dialyzed against 1x PBS, 50 mM Tris, 150 mM NaCl, pH 8.5 or 50 mM NaOAc, 150 mM NaCl, pH4.5. Any precipitate was removed by filtering at 0.22 μm .

Midscale expression and purification yielded highly pure and active proteins that were expressed in a soluble form and purified from the soluble fraction of the bacterial cytosol. SEC analysis on a Superdex 200 10/30GL in a mobile phase of 100 mM $NaPO_4$, 100 mM $NaSO_4$, 150 mM NaCl, pH 6.8 (GE Healthcare) demonstrated predominantly monomeric proteins (see Example 4).

Example 3: Pegylation of E/I ^{10}Fn3-Based Binders

Multi-valent fibronectin based scaffold proteins, such as E/I ^{10}Fn3-based binders, can be pegylated with various sizes and types of PEG. To allow for pegylation, the protein is typically modified near the C-terminus by a single point mutation of an amino acid, typically a serine, to a cysteine. PEGylation of the

protein at the single cysteine residue is accomplished through conjugation with various maleimide-derivatized PEG forms by combining the derivitized-PEG reagent with the protein solution and incubating. Progress and confirmation of the PEGylation conjugation reaction can be confirmed by SDS-PAGE and/or SE-HPLC methods that separate the non-PEGylated protein from the PEGylated protein.

For example, the construct E2-GS10-I1 (SEQ ID NO: 25) was pegylated by replacing a serine that was at position 221 with a cysteine. The resulting construct, SEQ ID NO: 56, was then conjugated with a maleimide-derivatized 40 kDa branched PEG (NOF America Corporation, White Plains, NY). The derivatized PEG reagent was mixed with the protein construct in solution and incubated at pH 7.40 at Room temperature until the reaction was complete, typically 30 minutes or overnight at 4 °C. The pH was lowered to pH 4.5 or pH 5.0 by dialysis or rapid desalting using size exclusion column chromotography into in 50 NaOAc, 150 mM NaCl buffer. The mixture of products and excess reactants from the PEGylation reaction were then loaded onto a cation exchange chromotography column at the lowered pH and eluted with a 150 mM to 1 M NaCl gradient. Studies to confirm the pegylation were also conducted as described in the paragraph above. The conjugations can be performed with the His tagged or the His-Tag free versions of the protein.

On occasions in which *E coli* endotoxin contamination needed to be depleted in the sample, two methods used either separately or in conjunction with one another were empoloyed. The first was to wash the cation exchange column with typically 5 column volumes NaOAc buffer supplemented with 0.5% Triton X-100, followed by 20 column volumes (or more) of the same buffer without Triton X-100. Additionally or in place of this procedure, the protein was passed very slowly through a Sartorius Sartobind® Q filter (Sartorius Stedim Biotech Bohemia, New York).

Two of the E/I ^{10}Fn3-based binders, E2-GS10-I1-cys (with his) (SEQ ID NO: 56) and E3-GS10-I1-Cys (with his) (SEQ ID NO: 53), were pegylated using an alternative procedure. Five ml of an inoculum culture of BL21(DE3) *E. coli* cells containing a T7 ploymerase driven pET29 plasmid encoding either E2-GS10-I1-cys (with his) or E3-GS10-I1-Cys (with his), were generated from a single plated colony

and used to inoculate 1 liter of auto-induction media ("ONE" medium, EMD Biosciences, San Diego, CA) containing 50 μg/mL kanamycin. Expression was carried out at 18 °C after initial growth at 37 °C and harvested by centrifugation for 10 minutes at ~10,000 x g at 4 °C. Cell pellets were frozen at 80 °C. The cell pellet was resuspended in 10 mL of lysis buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 5 mM Immidazole, pH 7.4) and mechanically lysed using an Avestin homgenizer. The soluble fraction was separated by centrifugation for 15 minutes at 23,300 x g at 4 °C. The supernatant was decanted and the pellet was solubilized in Lysis buffer (above) supplemented with 4 M to 6 M guanidine hydrochloride (GdnHCl). Solubilized protein was then purified on a suitably sized NiNTA column (Qiagen, Inc.) pre-equilibrated with the GdnHCL supplemented Lysis Buffer. The column was then washed with 5 to 10 column volumes of the same buffer, followed by elution with the same buffer supplemented with 300 mM Immidazole. The fractions eluted off the column containing the protein of interest were diluted to 2-3 mgs/mL protein and then combined with a 1.2-1.5 molar excess of solid NEM-PEG (40 kDa branched or other). The mixture was allowed to react at room temperature for 30 minutes or until the reaction was complete. The entire reaction volume was then placed into a dialysis bag (5,000 Da Molecular Weight cutoff) and the mixture was subjected to a dialysis refolding process. For example, this process may consist of two 10-16 hour 500:1 (buffer: dialysate) dialysis exchanges against 50mM NaOAc, 150 mm NaCl, pH 4.5. The dialysate from this procedure contains properly folded, PEGylated materials plus excess reactants. The mixture of products and excess reactants from the PEGylation reaction were clarified via centrifugation or filtration prior to loading them onto a cation exchange chromotography column (SP Sepharose or Resource S, GE Healthcare). The column was developed with 150 mM to 1 M NaCl gradient in the NaOAc background buffer. Studies to confirm the pegylation were conducted as described above.

Example 4: Biophysical Characterization of ^{10}Fn3-Based Binders

Standard size exclusion chromatography (SEC) was performed on the proteins purified from the HTPP and the midscale processes (0.1 to 1 μg of protein for HTPP and 10-50 ug for midscale). SEC of HTPP derived material was

performed using a Superdex 200 5/150 column (GE Healthcare) or on a Superdex 200 10/30 column (GE Healthcare) for midscaled material on an Agilent 1100 or 1200 HPLC system with UV detection at A_{214}nm and A_{280}nm and with fluorescence detection (excitation = $_{280}$nm, emission = $_{350}$nm). A buffer of 100 mM sodium sulfate, 100 mM sodium phosphate, 150 mM sodium chloride, pH 6.8 at appropriate flow rate of the SEC column employed. Gel filtration standards (Bio-Rad Laboratories, Hercules, CA) were used for molecular weight calibration.

The results of the SEC on the HTPP purified ^{10}Fn3-based binders showed predominantly monomeric proteins and elution in the approximate range of 25 kDa vs. globular Gel Filtration standards (BioRad).

The results of the SEC on the midscaled purified ^{10}Fn3-based binders showed predominantly monomeric proteins and elution in the approximate range of 25 kDa vs. globular Gel Filtration standards (BioRad). Figure 2 depicts exemplary SEC profiles for E/I ^{10}Fn3-based binders (I1-GS10-E2 in Figure 2A and E2-GS10-Il in Figure 2B).

Select midscale ^{10}Fn3-based binders were further analyzed by LC-MS (Water's 2695 liquid chromatography HPLC system coupled with Waters Q-TOF API mass spectrometer, Waters Corporation, Milford, MA). Samples were diluted to approximately 0.5 mg/ml with HPLC grade water. Approximately 5 µl of diluted sample was injected onto a Jupiter C18 column (Catalog number 00G-4053-80, Phenomenex). Buffer A: 0.02%TFA+0.08% formic acid in HPLC grade water. Buffer B: 0.02% TFA + 0.08 % formic acid in HPLC grade acetonitrile. Sample was eluted with gradient (Table 1) at flow rate 0.2 ml/minutes.

Table 1

Time	%A	B%
0	95	5
5.00	75	25
25.00	55	45
30.00	5	95
32.00	95	5
45.00	95	5

HPLC elution was split at approximately to 1:1 ratio and half sent to UV detector and the other half to mass spectrometer. Mass spectrometer had the following instrument settings: capillary voltage 3.5 KV, cone voltage 40, source

temperature 80 °C, desolvation temperature 250 °C, desolvation gas flow 450 and multi channel photo detector voltage 2200. Raw spectra were deconvoluted with MaxEn1 (Waters Corporation).

The molecular weight of I1-GS10-E2 (SEQ ID NO: 22) as measured by LC-MS is 24,445 Dalton, which is within 1 Dalton from the molecular weight calculated from the amino acid composition. This indicates that the protein has the correct amino acid composition and the N terminal methionine is processed. There is no other post translational modification on the protein.

Differential Scanning Calorimetry (DSC) analysis of the midscaled I1-GS10-E2 was performed to determine the T_m. A 1 mg/ml solution was scanned in a N-DSC II calorimeter (Calorimetry Sciences Corp) by ramping the temperature from 5 °C to 95 °C at a rate of 1 degree per minute under 3 atm pressure. The data was analyzed versus a control run of the appropriate buffer using a best fit using Orgin Software (OrginLab Corp). The results of this assay demonstrate that the E/I binder has a T_m of 50.69 °C (see Figure 3A). Using the same methods, the T_m of E2-GS10-I1 (with Peg) was determined to be 50.72 °C and the T_m of E2-GS10-Il (without Peg) was determined to be 56.82 °C (see Figure 3B).

Example 5: Determination of Binding Affinity

Surface plasmon resonance (BIAcore) analysis was performed on solution-phase ^{10}Fn3-based binders in order to determine off-rates and/or binding affinities using captured EGFR-Fc and IGF1R-Fc. The extracellular domain of human IGF1R (aa 1-932) was cloned into a mammalian expression vector containing the hinge and constant regions of human IgG1. Transient transfection of the plasmid produced a fusion protein, IGF1R-Fc which was subsequently purified by Protein A chromatography. Recombinant human EGFR-Fc (aa 1-645 of the extracellular domain of human EGFR fused to human Fc) was purchased from R&D systems (Minneapolis, MN). IGF1R-Fc was captured on immobilized Protein A whereas EGFR-Fc was captured on immobilized anti-human antibody.

In a typical experiment, anti-human IgG was immobilized on flow cells 1 and 2 of a CM5 chip following the manufacturer's recommendations (GE Healthcare, Piscataway, NJ). EGFR-Fc (50 nM) was injected at 5 uL for 2 minutes

on flow cell 2 (Fc2). Two 30 second injections of 3 M $MgCl_2$ were used for regeneration of the bound EGFR-Fc from the anti-human IgG surface. Protein A was diluted to 80 ug/mL in acetate pH 4.5 and immobilized to ~3000 RU on flow cells 3 and 4 of a CM5 chip surface. Approximately 1300 RU of IGF1R-Fc was captured on Fc 4. Two 30 second injections of 50 mM glycine pH 1.5 were used to regenerate the surface between samples.

A concentration series of 100 nM to 1 nM of HTPP purified protein (three data points collected) or 300 nM to 0.05 nM of midscale purified protein (eleven data points collected) was evaluated for binding to EGFR-Fc or IGF1R-Fc. Sensorgrams were obtained at each concentration and were evaluated using Biacore T100 Evaluation Software, Version 1.1.1 (GE healthcare/Biacore) to determine the rate constants k_a (k_{on}) and k_d (k_{off}). For the HTPP evaluation the off-rate was fitted from the 3 point curves. The affinity K_D was calculated from the ratio of rate constants k_{off}/k_{on}.

The EGFR ^{10}Fn3-based binders were evaluated for specificity in a similar format using anti-human IgG to capture HER2-Fc. The ^{10}Fn3-based binders did not show any discernible binding to captured HER2-Fc under conditions where robust binding was seen for EGFR-Fc.

As shown in Table 2, both domains of the E/I ^{10}Fn3-based binders are functional, retaining their binding properties to the respective targets. The off rates shown in Table 2 are from midscale material and are similar to the qualitative results obtained with the HTPP material.

Table 2: Summary of binding constants for ^{10}Fn3-based binders

Target	Protein	ka (1/Ms)	kd (1/s)	K_D (nM)
EGFR-Fc	E1	1.19E+05 1.43E+05	1.18E-03 1.89E-03	9.92 13.2
	E1-GS10-I1	6.29E+04 3.82E+04	4.74E-04 3.89E-04	7.53 10.17
	I1-GS10-E1	1.26E+05 4.13E+04	6.03E-04 4.25E-04	4.8 10.28
	E2	3.73E+05 3.27E+05	2.72E-04 3.2E-04	0.73 0.98
	E2-GS10-I1	3.93E+05 3.75E+05	1.75E-04 1.67E-04	0.45 0.45
	I1-GS10-E2	6.47E+05 3.90E+05	1.42E-04 1.14E-04	0.22 0.29

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Target	Protein	ka (1/Ms)	kd (1/s)	K_D (nM)
	E3	2.83E+05	3.98E-04	3.4 1.4
	E3-GS10-I1	3.49E+05	2.29E-04	0.66
	I1-GS10-E3	1.17E+05	2.91E-04	2.48
IGF1R-Fc	I1	3.84E+06	4.34E-04	0.11
	E1-GS10-I1	5.13E+05	3.38E-04	0.66
	I1-GS10-E1	1.47E+06	3.98E-04	0.27
	E2-GS10-I1	1.24E+06	3.95E-04	0.32
	I1-GS10-E2	3.82E+06	4.79E-04	0.13
	E3-GS10-I1	1.8E+06	2.09E-04	0.12
	I1-GS10-E3	1.37E+06	4.54E-05	0.03

Example 6: Inhibition of IGFR activity in H292 Cells

The ability of E/I ^{10}Fn3-based binders to inhibit phosphorylation of IGF1R on tyrosine 1131 was determined using an H292 cell in vitro assay. Briefly, 65 X 10^3 H292 cells were plated in 96-well microplates (Biocoat Poly-D-Lysine coated 96-well plate, cat#356640, Becton Dickinson, Franklin Lakes, NJ) in RPMI-1640 culture medium containing 10 mM Hepes pH 7.4 and 10% fetal bovine serum. Cells were allowed to adhere for 24 hours at 37 °C, 5% CO_2. The next day cells were washed once with 200 microliters per well of serum free RPMI-1640 and incubated overnight in 100 µL per well of serum free RPMI-1640. Serial dilutions of HTPP material was added and cells were incubated for an additional 3 hours. Cells were stimulated with 100 ng/ml of IGF-1 (cat#500-P11, PeproTech, Rocky Hill, NJ) for 10 minutes at 37 °C. Media was dumped from the plate and 100 µL of cell lysis buffer (Cell Signaling cat#9803, Beverly, MA) was added to each well. Cells were incubated at room temperature for 15 minutes to allow lysis and lysate was transferred to a phospho-IGFR ELISA (cat#7302, Cell Signaling, Beverly, MA). The manufacturer's procedure was followed to carry out the ELISA.

As demonstrated in Figure 4, His tagged E1-GS10-I1 inhibited IGF1-stimulated phosphorylation of the IGF1R (IC_{50} = 0.004 uM) with comparable potency to the isolated IGF1R binder, I1 (IC_{50} = 0.018uM). The EGFR binder, E1, alone had very little effect on IGF1R phosphorylation (IC_{50} > 3.5uM). As shown in Figure 9, additional E/I binders demonstrated ability to inhibit IGF1R-stimulated phosphorylation with an IC50 in the range of 0.1 nM to 19 nM, including several

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pegylated E/I binders that were tested . In particular, for the pegylated E/I binders E1-GS10-I1, and I1-GS10-E1, inhibition of pIGFR was shown at 0.9 nM and 4 nM, respectively. For pegylated E/I binders E2-GS10-I1 and I1-GS10-E2, inhibition of pIGFR was shown at 0.3 nM and 0.8 nM, respectively.

Example 7: Inhibition of EGFR activity in H292 Cells

The ability of E/I ^{10}Fn3-based binders to inhibit phosphorylation of the EGFR on tyrosine 1068 was determined using an H292 cell in vitro assay. The assay was carried out as described in Example 6, except that cells were stimulated with 100 ng/ml of EGF (cat#236-EG-200, R & D Systems, Minneapolis, MN) and a phospho-EGFR ELISA was performed (cat#7240, Cell Signaling, Beverly, MA). The manufacturer's procedure was followed to carry out the ELISA.

As demonstrated in Figure 5, His-tagged E1-GS10-I1 inhibited EGF-stimulated phosphorylation of the EGFR (IC_{50} = 0.020uM) with comparable potency to the isolated EGFR binder, E1 (IC_{50} = 0.007uM). The IGF1R binder, I1 alone had very little effect on EGFR phosphorylation (IC_{50} > 6.21uM). As shown in Figure 9, additional E/I binders demonstrated ability to inhibit EGF-stimulated phosphorylation with an IC50 in the range of 7 nM to 127 nM, including several pegylated E/I binders that were tested. In particular, for pegylated E2-GS10-I1 and I1-GS10-E2, inhibition of pEGFR was shown at 32 nM and 47 nM, respectively. Similar data is shown in Figure 11 for the pegylated E/I binders E2-GS10-I1, and I1-GS10-E2.

Example 8: Inhibition of EGF+IGF1-induced pAKT in H292 Cells

The ability of E/I ^{10}Fn3-based binders to inhibit phosphorylation of AKT on serine 473 was determined using an H292 cell in vitro assay. The assay was carried out as described in Example 6, except that cells were simultaneously stimulated with both EGF and IGF1 as described above and lysates were analyzed with a phospho-AKT ELISA (cat#7160, Cell Signaling, Beverly, MA). The manufacturer's procedure was followed to carry out the ELISA.

Signal transduction at EGFR and IGF1R feeds into the PI3K-AKT signaling pathway and stimulates phosphorylation of AKT. As demonstrated in Figure 6, E1-

GS10-I1 inhibited EGF and IGF1-stimulated phosphorylation of AKT in H292 cells. The E/I ^{10}Fn3-based binder was slightly more potent in its ability to block AKT activation (IC_{50} = 0.004 uM) than the IGF1R binder, I1, by itself (IC_{50} = 0.031 uM). The EGFR binder, E1, exhibited only modest activity in its ability to block AKT activation by both ligands (IC_{50} = 1.28 uM). As shown in Figure 9, additional E/I binders demonstrated ability to inhibit EGF and IGF1-stimulated phosphorylation of AKT with an IC50 in the range of 0.1 nM to 26 nM, including several pegylated E/I binders that were tested.

Example 9: Inhibition of cell proliferation in RH41 and H292 cells

E/I ^{10}Fn3-based binders were evaluated for antiproliferative activity in the H292 non-small cell lung carcinoma cell line, which depends on EGFR signaling for growth, or the RH41 Ewing sarcoma cell line, which depends on IGF1R signaling for growth. Antiproliferative activity of binders was assessed in monolayer cultures by staining cellular DNA with the CyQuantNF fluorescent stain (cat#C35006, Invitrogen, Carlsbad, CA). Briefly, 2 X 10^3 H292 or 5 X 10^3 RH41 cells were plated into 96-well microplates (View Plates 96F cat#6005225, Perkin-Elmer, Waltham, MA) in RPMI-1640 culture medium containing 10 mM Hepes pH 7.4 and 10% fetal bovine serum and allowed to adhere for 24 hours at 37 °C, 5% CO_2. Cells were maintained as exponentially growing monolayers and remained in logarithmic growth phase during the period of the assay without reaching confluence during the course of the assay. Twenty-four hours after plating, serial dilutions of midscale material was added and cells were incubated for an additional 72 hours. Following this incubation, cells were treated with CyQuantNF reagent and allowed to incorporate dye into cellular DNA for 1 hour at 37 °C. Total DNA was quantified by reading fluorescence at 485 nm excitation and 530 nm emission on a CytoFluor 4000 instrument (Applied Biosystems, Framingham, MA). Total time that cells were exposed to drug was 72 hours. Standard compounds were included in each experiment to verify assay performance and reproducibility. Linear regression analysis of the percent of inhibition by test compound was used to determine IC_{50} values.

As demonstrated in Figure 7, in RH41 cells, His-tagged E1-GS10-I1 inhibited proliferation with comparable potency (IC_{50} = 0.009 uM) to the IGFR binder, I1 (IC_{50} = 0.028 uM). The EGFR binder, E1, by itself had very little effect on the proliferation in this cell line (IC_{50} > 12.5 uM).

As demonstrated in Figure 8, in H292 cells, His-tagged E2-GS10-I1 inhibited proliferation with greater potency (IC_{50} = 0.329 uM) than the IGFR binder, I1, (IC_{50} = 0.699 uM) or the EGFR binder, E2 (IC_{50} = 0.553 uM). See Table 4 below for the IC50 values for the E and I monomers.

Example 10: Competitive EGF ligand binding Assay

The E/I binders E1-GS10-I1, I1-GS10-E1, E2-GS10-I1 and I1-GS10-E2 (HTPP material) were tested in an EGF ligand binding cell-based competition assay in A431 cells and compared to EGFR ^{10}Fn3-based binders E1 and E2 (midscale material). A431 cells were plated at 15000 cells/well in 96-well plates in DMEM + 10% FBS and incubated 48 hours. Cells were washed with starvation media (DMEM + 0.1% BSA) and incubated in starvation media for 1 hour. Starvation media was removed and replaced with ^{10}Fn3-based binders that were diluted in starvation media and cells were pre-incubated for 30 minutes at 37 °C to allow proteins to bind to EGF receptors on cell surfaces. 10 nM final concentration of Europium (Eu)-labeled EGF (Perkin Elmer, Boston, MA) diluted in starvation media was added to pre-incubated cells and plates were incubated for 3 hours at 4 °C in the dark. Plates were washed twice with cold PBS and 50 ul/well of Enhancement solution (Perkin Elmer, Boston, MA) was added to plates and incubated 1 hour at 37 °C. Plates were read on the Flexstation II (Molecular Devices). The data was plotted with Softmax plus software and IC50 values, i.e., the concentration of ^{10}Fn3-based binders required to inhibit 50% of the Eu-EGF ligand from binding to the EGF receptor on the cell surfaces, were calculated.

The results for E2 and E1 compared with E2-GS10-I1, I1-GS10-E2, E1-GS10-I1 and I1-GS10-E1 are summarized in Table 3. This data indicates that the E/I ^{10}Fn3-based binders compete with, and inhibit the binding of, EGF to the EGFR receptor on A431 cells with similar potency to the EGFR ^{10}Fn3-based binders. See Table 4 below for the IC50 values for the E and I monomers.

Table 3: Summary of IC50 values for inhibition of EGF Binding to EGFR on A431 cell surfaces

Protein	IC50 (nM)
E2	7
E1	14
E2-GS10-I1	1.8
I1-GS10-E2	1.4
E1-GS10-I1	14.6
I1-GS10-E1	7

Example 11: Activation and Signaling Activity in Cell-Based Assays

Target effects of the various E/I ^{10}Fn3-based binders were evaluated in DiFi colon carcinoma cells by immunoblotting. Cells were seeded at 4 x 10^5 cells in each 25 cm^2 flask and incubated overnight at 37 ^oC in 5% CO_2. The next day, treatments were initiated and cells were further incubated for various times from 1.5 to 120 hours. Cells were then lysed in HNTG (50 mM Hepes, 150 mM NaCl, 0.5% triton-X-100, 8% glycerol, 2 mM Na_3VO_4, 1.5 mM $MgCl_2$, 1 mM EDTA containing the protease inhibitors AEBSF, aprotinin, leupeptin, bestatin, pepstatin-A and E64) and total protein was quantified with the BCA protein assay (Pierce, Waltham, MA). Levels of total EGFR, total IGF1R and the phosphorylation state of the EGFR, MAP kinase protein ERK1/2 isoforms, was detected by SDS-PAGE analysis of 20 micrograms of total protein followed by transfer of proteins to nitrocellulose and immunoblotting with specific antibodies. Blots were also probed with β-actin to demonstrate equal loading of each sample.

The pegylated E/I ^{10}Fn3-based binders E1-GS10-I1 (SEQ ID NO: 55), E2-GS10-I1 (SEQ ID NO: 56), and E3-GS10-I1 (SEQ ID NO: 53), demonstrated the ability to degrade EGFR in this assay. In addition, for E3-GS10-I1 (SEQ ID NO: 53), degradation of IGF1R was also observed. The effect on EGFR degradation for the pegylated binder E2-GS10-I1 is shown in Figure 10, as are other effects on signaling molecules. Additionally, the non-pegylated version of the binder E2-GS10-I1 demonstrated similar EGFR degradation (data not shown). Figure 10 shows that for the pegylated binder I1-GS10-E2, there was no EGFR degradation. Table 4 below summarizes various properties of the E monomers.

Table 4. Summary of properties of E monomers.

EGFR					
Monomer	BIAcore KD IC50	Neutralizes EGF Binding IC50	Inhibition of pEGFR IC50	Inhibition of pERK IC50	Inhibition of H292 Proliferation IC50
E1	14.6 nM	0.53 nM	18 nM	17 nM	18 nM
E2	1.4 nM	1.46 nM	20 nM	40 nM	30 nM
E3	0.72 nM	0.87 nM	11 nM	97 nM	26 nM

Example 12: Evaluation of Certain E/I ^{10}Fn3-Based Binders on H292 Tumor Xenografts Grown In Nude Mice

The pegylated E/I binders E2-GS10-I1 and E3-GS10-I1 as well as the monoclonal antibody panitumumab were evaluated in an H292 tumor xenograft model. For *in vivo* models, panitumumab was obtained as the marketed drug and E/I binders were purified as described above. In vitro activity of all E/I binders was validated prior to administration in animals by testing functionality of each end in the EGF-stimulated pEGFR and the IGF1-stimulated pIGFR assay in H292 cells. E/I binders were diluted in phosphate buffered saline (PBS) at the beginning of the experiment and stored at 2-4 °C for the duration of each study. Both compounds were administered i.p. in a total volume of 500 µl/inj/mouse and were equilibrated to room temperature prior to administration.

Mice and tumor propagation. Female athymic (nude) mice 5-6 weeks of age were obtained from Harlan Sprague-Dawley Co. (Indianapolis, IN). and were quarantined for approximately 3 weeks prior to their use for tumor propagation or drug efficacy testing. The animals were provided food and water *ad libitum*. Animal care was performed in keeping with AAALAC and Bristol-Myers Squibb guidelines. Tumors were propagated by subcutaneous (s.c.) implantation in nude mice. Tumor passages occurred approximately every two to four weeks.

In vivo antitumor testing. Estimated tumor weight was calculated using the formula: Tumor weight (mg) = (w^2 * l)/2; where w = width and l = length in mm. Antitumor activity was evaluated in terms of % tumor growth inhibition (TGI) where a %TGI of >50% was considered active. Relative % tumor growth inhibition was calculated as %TGI=[(C_t-T_t)/(C_t-C_0)] x 100 where C_t=median tumor weight of control mice at time t in days after tumor implant, T_t=median tumor weight of treated mice at time t, C_0= median tumor weight of control mice at time 0. %TGI

value was calculated at various time points beginning after 1.5 tumor volume doubling times and sustained over a time period of 3 tumor volume doubling times (TVDT) where possible. Where, TVDT = median time (days) for control tumors to reach target size – median time (days) for control tumors to reach half the target size. The definition of a cured mouse was one whose tumor was undetectable, or <35 mg, when assessed more than 10 TVDTs post-treatment. The dose of a compound which yielded the maximum therapeutic effect, was termed the optimal dose (OD). Treatment groups (typically 8 mice) with more than one death attributable to drug toxicity were considered to have had excessively toxic treatments and their data were not used in the evaluation of antitumor activity. The maximum tolerated dose (MTD) is defined as the dose level immediately below which excessive toxicity (i.e. more than one death) occurred. Treated mice dying prior to having their tumors reach target size were considered to have died from drug toxicity. Statistical evaluations of data were performed using Gehan's generalized Wilcoxon test (Gehan, EA, A Generalized Wilcoxon Test for Comparing Arbitrarily Slightly-Censored Samples, Biometrika 52:203-223, 1965).

Measurement of pharmacodynamic endpoints in tumors. Tumors were harvested from untreated or drug treated mice and snap frozen in liquid nitrogen. Samples were weighed and homogenized in 10 µl of lysis buffer (50 mM Hepes, 150 mM NaCl, 0.5% triton-X-100, 8% glycerol, 2 mM Na_3VO_4, 1.5 mM $MgCl_2$, 1mM EDTA containing one complete mini protease inhibitor tablet Sigma #S8820 per 15 ml buffer and phosphatase inhibitor cocktail Sigma #P5726) for each mg of tissue. Tissues were minced in a 100 mm petri dish with two scalpels, transferred to Falcon#2059 polypropylene round bottom tubes and macerated with a hand held homogenizer for 30 seconds. Homogenate was transferred to 1.5 ml eppendorf tubes and centrifuged at 15000 x g for 2 minutes in a microfuge. Clarified supernatant was transferred to a new tube and total protein concentration was determined with the Pierce BCA protein assay (Pierce Biotechnology). Samples were analyzed by immunoblotting or on a Meso scale MSD Sector Imager 6000 multi spot assay system as recommended by the manufacturer (Meso Scale Discovery, Gaithersburg, MD).

The pegylated E/I binders E2-GS10-I1 and E3-GS10-I1 were tested in an H292 NSCLC in athymic mice. Tumors were implanted subcutaneously with 1 mm^3 H292 tumor fragments in the hind flank and allowed to establish to a size of 50-150 mg prior to initiation of treatment on Day 6 post-tumor implant. The pegylated E/I binders were administered i.p. at a dose of 100 mg/kg on a TIWX3 schedule to assess antitumor activity. Panitumumab was obtained as marketed drug and administered i.p. at its optimal dose of 1 mg/mouse and at a lower dose of 0.1 mg/mouse on a Q3DX5 schedule. Mean tumor sizes calculated from groups of 8 mice are shown in Figure 12A. The 1 mg/mouse and 0.1 mg/mouse doses of panitumumab were both active by %TGI with values of 101% and 100%, respectively and these values were significantly different from control animals (p=0.0002, Table 5). Pegylated E2-GS10-I1 was also significantly active by %TGI with a value of 96% (p=0.0005). Pegylated E3-GS10-I1 was not active in this study with a %TGI value of 31% that was not statistically different from the control group (p=0.416). Post dosing analysis indicated that approximately two thirds of the pegylated E3-GS10-I1 was aggregated (66.64% aggregation/33.36% monomer for one batch and 72.53% aggregation/27.47% monomer for another batch) which could account for the poor activity of pegylated E3-GS10-I1 in this assay. In contrast, the pegylated E2-GS10-I1 showed only a small percentage of aggregation in post dosing studies (1.79% aggregation/98.21% monomer).

All treatments were well tolerated with no treatment related deaths or excessive weight loss over the course of the study. Clinical observations revealed no evidence of toxicity and the average weight change over the course of therapy was within acceptable limits (Figure 12B).

Table 5. Results of the H292 human tumor xenograft study

Group	Compound	Schedule, Route	Dose (mg/kg)	AVE weight change (g)	%TGI	p value for %TGI	Outcome by %TGI
1	Control (untreated)	---	---	5.3	---	1.0	---
2	panitumumab	q3dx5; 6 ip[a]	1mg/mse	9.6	101	0.0002	A
3	panitumumab	q3dx5;	0.1mg/mse	6.3	100	0.0002	A

Group	Compound	Schedule, Route	Dose (mg/kg)	AVE weight change (g)	%TGI	p value for %TGI	Outcome by %TGI
		6 ip[a]					
4	E2-GS10-I1 (w/ PEG)	TIWx3; 6 ip[a]	100	-0.1	94	0.0005	A
5	E3-GS10-I1 (w/ PEG)	TIWx3; 6 ip[a]	100	9.5	28	0.416	I

[a]Vehicle was phosphate buffered saline. Abbreviations used are as follows: ip, intraperitoneal route; %TGI, relative % tumor growth inhibition calculated as %TGI=[(Ct-Tt)/(Ct-C0)] x 100 where Ct=median tumor weight of control mice at time t in days after tumor implant, Tt=median tumor weight of treated mice at time t, C0= median tumor weight of control mice at time 0. %TGI value was calculated at two points as the average inhibition of Day 12 and Day20. Outcome, a treatment regimen was considered active if it produced a statistically significant %TGI value of >50%; q3dx5;6, compound was administered every three days for five doses starting on the sixth day after tumor implant; TIWx3;6, compound was administered three times a week for three weeks starting on the sixth day after tumor implant. p values were calculated on Day 20 relative to the control group in a two tailed paired analysis with 8 measurements per group. Outcome by %TGI, A = active and I = inactive.

Pharmacodynamic endpoints from the H292 tumor study. Samples of tumors from untreated control, panitumumab and E/I binder treated groups were analyzed for levels of phosphorylated EGFR, ErbB2 and IGFR that would indicate target suppression. Tumors were also analyzed for levels of total EGFR to determine if EGF receptor degradation occurred. On day 20, a final treatment was administered and tumors were removed from 2 animals at 1 hour after dosing, 3 animals at 4 hours after dosing and 3 animals at 24 hours after dosing. All treatments showed marked suppression of phosphorylated EGFR and ErbB2 while the basal levels of phosphorylated IGFR were too low to discern a difference in this study (Figure 13). All treatments showed a reduction in the amount of total EGFR indicating degradation of the receptor had occurred.

Example 13: Selection and Characterization of MCF7 Cells Resistant to IGF1R Inhibitor

MCF7 cells (American Type Culture Collection, Cat No. HTB-22, Manassas, VA) were cultured in RPMI medium containing 10 mM hepes and 10% FBS at 37 °C in the presence of 5% CO_2. The small molecule IGF1R inhibitor BMS-754807 was added to the culture medium and the concentration increased at

stepwise increments over a period of 10 months until the cells exhibited continued proliferation in the presence of 200 mM BMS-754807. The resistant cells were designated MCF7r and the IC50 for BMS-754807 was 1239 nM compared to 120 nM for the parental MCF7 cells as measured in a proliferation assay carried out as previously described (Carboni et al., Cancer Res. 69: 161-170 (2009)). The drug was then removed from the culture medium and the MCF7r cells were passaged in complete medium for an additional 20 or 60 days to remove all traces of residual BMS-754807. Analysis of the MCF7r cells by immunoblotting revealed that EGFR was significantly overexpressed in the resistant cells compared to the parental MCF7 cells (Figure 14). In addition, when MCF7 and MCF7r cells were serum starved and then stimulated with EGF for 7 minutes, phosphorylated EGFR could not be detected in the parental MCF7 cells (probably due to low levels of EGFR) but was strongly visible in MCF7r cells. In serum starved cells stimulated with IGF ligand, phosphorylated IGFR was seen in the parental MCF7 cells but despite the slightly higher levels of total IGFR present in the MCF7r cells almost no pIGFR was observed. This shows that the IGFR in the resistant MCF7r cells lost the ability to activate IGFR in response to IGF1 stimulation (Figure 14). Activation of the MAP kinase pathway in response to EGF stimulation was stronger in the MCF7r cells as measured by pERK activation.

Example 14: Antitumor Studies in MCF7 and MCF7r Xenografts

MCF7r cells were scaled up in T75 flasks and isolated by centrifugation. Viable cell numbers were measured by trypan blue exclusion with a Vi-CELL XR (Beckman Coulter, Fullerton, CA), resuspended in PBS to 5 x 10^6 viable cells/ml and implanted subcutaneously in the hind flank of athymic mice in a volume of 0.2 ml. For MCF7 and MCF7r tumor growth, all mice were supplemented with 0.25 mg 90 day release pellets of 17-β-estradiol (Innovative Research of America, Sarasota, FL, Cat. No. NE-121). Tumors were propagated until they reached a median size of 500-1000 mg when they were excised and 1 mm^3 fragments were reimplanted in the hind flank of new athymic mice. Tumors were adapted for solid tumor growth by serial trocar passage in mice through at least four rounds of growth during which tumor volume doubling time and take rate were monitored for each passage.

Growth characteristics were observed to determine if the xenografts exhibited acceptable properties to serve as a reliable, reproducible model. The MCF7r tumor type demonstrated an acceptable take rate and doubling time and therefore satisfied the criteria for use as a xenograft model. The MCF7 parental tumor model had been previously established using the same techniques. For the MCF7 parental xenograft, 1 mm^3 tumor fragments were implanted subcutaneously in the hind flank and allowed to establish to a size of 50-150 mg prior to initiation of treatment on Day 13 post-tumor implant. Cetuximab was obtained as marketed drug and administered i.p. at its optimal dose of 1 mg/mouse and at a lower dose of 0.1 mg/mouse on a Q3DX5 schedule (doses administered on Day 13, 16, 19, 22, 25). Mean tumor sizes calculated from groups of 8 mice are shown in Figure 15A. In the MCF7 xenograft model, neither the 1 mg/mouse or the 0.1 mg/mouse dose of cetuximab was active by %TGI with values of -9% and 3.2%, respectively and the tumor sizes were not statistically different from the control group (Table 6).

For the MCF7r resistant xenograft, 1 mm^3 tumor fragments were implanted subcutaneously in the hind flank and allowed to establish to a size of 50-150 mg prior to initiation of treatment on Day 6 post-tumor implant. Cetuximab was obtained as marketed drug and administered i.p. at its optimal dose of 1 mg/mouse and at a lower dose of 0.1 mg/mouse on a Q3DX5 schedule (doses administered on Day 6, 9, 12, 15, 18). Mean tumor sizes calculated from groups of 8 mice are shown in Figure 15B. In the MCF7r xenograft model, doses of cetuximab were active by %TGI with values of 105% and 75%, respectively. The high dose of cetuximab had a TGI value over 100% which indicates that it caused tumor regression below the starting size at the initiation of treatment. Both doses resulted in a statistically significant difference in tumor size compared to the control group (Table 7).

Table 6. Results of the MCF7 human breast carcinoma tumor xenograft study.

Group	Compound	Schedule, Route	Dose (mg/mouse)	%TGI	p value for %TGI	Outcome by %TGI
1	Control (untreated)	---	---	---	1.0	---
2	cetuximab	q3dx5; 13 ip[a]	1mg/mse	-9	0.223	I

3	cetuximab	q3dx5; 13 ip[a]	0.1mg/mse	3.2	0.220	I

[a]Vehicle was phosphate buffered saline. Abbreviations used are as follows: ip, intraperitoneal route; %TGI, relative % tumor growth inhibition calculated as %TGI=[(Ct-Tt)/(Ct-C0)] x 100 where Ct=median tumor weight of control mice at time t in days after tumor implant, Tt=median tumor weight of treated mice at time t, C0= median tumor weight of control mice at time 0. %TGI value was calculated at three points as the average inhibition of Day 20, 24 and Day 27. Outcome, a treatment regimen was considered active if it produced a statistically significant %TGI value of >50%; q3dx5;13, compound was administered every three days for six doses starting on the thirteenth day after tumor implant. p values were calculated on Day 24 relative to the control group in a two tailed paired analysis with 8 measurements per group. Outcome by %TGI, A = active and I = inactive.

Table 7. Results of the MCF7r human breast carcinoma tumor xenograft study.

Group	Compound	Schedule, Route	Dose (mg/kg)	%TGI	p value for %TGI	Outcome by %TGI
1	Control (untreated)	---	---	---	1.0	---
2	cetuximab	q3dx5; 6 ip[a]	1mg/mse	105	0.001	A
3	cetuximab	q3dx5; 6 ip[a]	0.1mg/mse	75	0.024	A

See footnotes to Table 6. p values were calculated on Day 19 relative to the control group in a two tailed paired analysis with 8 measurements per group.

Example 15: Antitumor Studies in GEO Xenografts

GEO tumors were established by implanting 1 mm^3 tumor fragments subcutaneously in the hind flank of athymic mice and allowing them to reach a size of 50-150 mg prior to initiation of treatment on Day 18 post-tumor implant. Cetuximab was administered ip at 0.25 mg/mouse on a Q3DX5 schedule (doses administered on Day 18, 21, 24, 27, 30). The IGFR kinase inhibitor BMS-754807 was administered at 25 mg/kg on a QDX21 schedule. Mean tumor sizes calculated from groups of 8 mice are shown in Figure 16. Cetuximab was active at 0.25 mg/mouse with a %TGI value of 67%. BMS-754807 was active with a %TGI of 80% and the combination of the two was considerably more active then either agent alone with a %TGI of 94% (Table 8). All treatment groups were statistically different from the control group on Day 26 (Table 8).

Table 8. Results of the GEO human colon carcinoma tumor xenograft study.

	Cetuximab		BMS-754807					
Group	Schedule, Route	Dose (mg/ mouse)	Schedule, Route	Dose (mg/kg)	%TGI	p value	Out come by %TGI	Synergy
1	Control (untreated)	---	---	---	---	---	---	---
2	q3dx5;6 ip[a]	0.25 mg/ mse	---		80		A	---
3	---	---	qdx21;18[b]	25	67		A	---
4	q3dx5;6 ip[a]	0.25 mg/ mse	qdx21;18[b]	25	94		A	YES

[a]Vehicle for cetuximab was phosphate buffered saline. Vehicle for BMS-754807 was 50% polyethylene glycol 400, 50% water. Abbreviations used are as described in Table 6 and synergy is defined as statistically significant activity that is better than either agent in the combination demonstrated on its own. Outcome by %TGI, A = active and I = inactive.

Example 16: Antitumor studies in H292 xenografts

H292 cells were implanted subcutaneously in the hind flank of athymic mice as 1 mm^3 fragments and allowed to establish to a size of 50-150 mg prior to initiation of treatment on Day 12 post-tumor implant. Cetuximab was administered ip at 0.1 mg/mouse on a Q3DX5 schedule. MAB391 is an antibody to IGF1R (R&D Systems, Minneapolis, MN, Cat. No. MAB391) and was administered at a dose of 40 mg/kg on a BIWX3 schedule. Mean tumor sizes calculated from groups of 8 mice are shown in Figure 17. Cetuximab was active at 0.1 mg/mouse with a %TGI value of 95.1% and MAB391 was inactive at 40 mg/kg with a %TGI value of 10.5% (Table 9). Mice dosed with the combination of cetuximab and MAB391 exhibited a %TGI value of 109.2% indicating tumor regression in the combination group (Table 9). After dosing ceased, tumors regrew in the cetuximab treated group more rapidly than in the group treated with the combination of cetuximab and MAB391 (Figure 17).

Table 9. Results of the H292 human NSCLC tumor xenograft study.

	Cetuximab		MAB391					
Group	Schedule, Route	Dose (mg/ mouse)	Schedule, Route	Dose (mg/kg)	%TGI	p value	Out come by %TGI	Synergy
1	Control (untreated)	---	---	---	---	---	---	---
2	q3dx5;12 ip[a]	0.1 mg/ mse	---		95.1		A	---
3	---	---	BIWx3;12[a]	40	10.5		I	---
4	q3dx5;12 ip[a]	0.1 mg/ mse	BIWx3;12[a]	40	109.2		A	YES

[a]Vehicle for cetuximab and MAB391 was phosphate buffered saline. Abbreviations used are as described in Table 6 and 8.

Example 17: Colony Formation Assay

To determine the effects of test compounds on the ability to inhibit colony formation of H292 cells, 400 cells were seeded into 24-well plates (Becton-Dickinson, Franklin Lakes, NJ, Cat. No. 351143) in complete medium and allowed to adhere overnight. The next day medium was removed and replaced with medium containing 2% FBS. Test compound was diluted into medium containing 2% FBS and added to cells in serial dilutions. Cells were incubated at 37 °C for 14 days. After 14 days, media was discarded and wells rinsed once with 2 ml PBS. Cells were stained with 0.5 ml Coomassie Stain Solution (Bio-Rad, Hercules, CA, Cat. No. 161-0436) for 20 min. The stain was aspirated and wells were washed quickly with 1X Destain Solution Coomassie R-250 (Bio-Rad, Cat. No. 161-0438). A final rinse with 1 ml water per well was carried out and plates were inverted and allowed to dry. Colonies consisting of (at least) 50 cells or larger were counted by eye under low power magnification (10X-20X). All samples were tested in triplicate and IC50 values were calculated from linear regression of the percent inhibition of control. Representative results for a PEGylated E/I binder are shown in Figure 18 and IC50 values for various E/I ^{10}Fn3-based binders, monospecific IGF1R ^{10}Fn3-based binder, and EGFR antibody is shown in Table 10.

Table 10. IC50 values of various E/I ^{10}Fn3-based binders, monospecific IGF1R ^{10}Fn3-based binder, and EGFR antibody in the colony formation assay.

SAMPLE	IC50 (nM)
E4-GS10-I1 (with Peg)	5
I1-GS10-E5 (with Peg)	1
I1-GS10-E4	6
E2-GS10-I1 (with Peg)	560
I1 monomer (with Peg)	15,510
panitumumab	140

Example 18: Epitope mapping assay

An epitope mapping assay was developed utilizing commercially available antibodies where the binding site on the EGFR extracellular domain is roughly known according to various literature reports. The antibodies used in this assay are listed in Table 11 and Figure 19A depicts how antibodies were localized to approximate binding domains on EGFR . The assay is a variation of the In Cell Western assay previously described and assesses the ability of EGFR ^{10}Fn3-based binders preincubated with A431 or other cells expressing EGFR to block binding of the detection antibodies from the panel listed in Table 11. The assay was carried out as follows: A431 cells in log phase growth were harvested by trypsinization and seeded in a 96 well plate at 24,000 cells/well in a total volume of 100 µl/well. The next day, media was dumped and the EGFR ^{10}Fn3-based binders diluted in cold DMEM base media were added to the plate and allowed to bind for 1 hour at 4°C to prevent internalization of EGFR. After binding, cells were washed with 0.2 ml PBS+0.1% Tween-20 and fixed for 20 minutes in PBS+3.7% formaldehyde at room temp. Cells were blocked in 0.2 ml of Odyssey blocking buffer for 1 hour at room temp. Next, primary antibodies were diluted in 50 µl of Odyssey blocker per well and incubated for 1-2 hours at room temp. Primary antibodies were dumped by inverting the plate, and each well washed 3X with 200µl of PBS+0.1% Tween-20. Secondary antibodies are the same ones used in the In Cell Western assay and were appropriate for the species of antibody being detected. These secondary antibodies were diluted (1:800) in Odyssey Blocker+0.2% Tween-20 and added in a volume of 50µl per well along with TOPRO3 (Invitrogen, Carlsbad, CA, cat#T3605) diluted at

(1:3000) to counterstain cells for normalization. Cells were incubated on bench for 1 hour at room temp. Secondary antibody was dumped out and each well washed 4X with 200µl of PBS+0.1% Tween-20 for 5 minutes at room temp. Plates were imaged on a Licor instrument at 160µm resolution, medium quality, focus offset of 3mm, intensity of 5. This assay was also carried out with the marketed drug antibodies cetuximab, panitumumab and nimotuzumab to determine if the EGFR^{10}Fn3-based binders were interfering with their binding to EGFR on A431 cells. Representative results are shown in Figure 19B.

Table 11. Commercially available antibodies to the extracellular domain of EGFR.

	Clone	SUPPLIER and cat#	SPECIES	BINDS	EPITOPE	Binding motif
1		Abcam ab38165	Rab	h	Peptide AA 42-58	linear
2	E234	Abcam ab32198	Rab	h,mu,rat	Peptide AA 40-80 (No ICC)	linear
3	N-20	Santa Cruz#31155	Goat IgG	h	AA 110-160	linear
4	ICR10	Abcam ab231 Santa Cruz #57095	Rat IgG2a	h(HN5)	AA 124-176[b], neutralizing[e]	conf
5	EGFR 1	Abcam ab30 Chemicon MAB88910 Labvision MS-311	Mu IgG1	h(A431)	AA 176-294, neutralizing[b] ab30&MAB88910 @(1mg/ml)	conf
6	199.12	Labvision MS-396-P	Mu IgG2a	h	AA 124-176, non-neutralizing[b]	conf
7	LA22	Upstate 05-104	Mu IgG2a	h(A431)	AA 351-364, neutralizing[a]	linear
8		Abcam ab15669	Rab	Mu,rat	Peptide AA376-394[d]	linear
9	225	Sigma E2156 Labvision MS-269-P	Mu IgG1	h(A431)	AA 294-475, neutralizing[b,c]	conf
10	528	Abcam ab3103 Santa Cruz#120 Labvision MS-268-P	Mu IgG2a	h(A431)	AA 294-475, neutralizing[b,c]	conf
11	B1D8	Labvision MS-666-P	Mu IgG2a	h(A431)	AA 294-475[b]	conf
12	LA1	Upstate 05-101	Mu IgG1	h	neutralizing	
13	H11	Labvision MS-316-P	Mu IgG1	h	AA 294-475, non-neutralizing[b]	linear
14	111.6	Labvision	Mu IgG1	h	AA 294-475,	linear

	Clone	SUPPLIER and cat#	SPECIES	BINDS	EPITOPE	Binding motif
		MS-378-P Imgenex IMG-80179			neutralizing[b]	
15	29.1	Sigma E2760 Abcam ab10414	Mu IgG1	h(A431)	External carbohydrate non-neutralizing	

Abbreviations: conf: epitope conformationally specific; linear: epitope independent of conformation. [a]JBC 264(1989)17469 Ala351-Asp364, [b]J Immunological Methods 287(2004)147, [c]Mol Biol Med1(1983)511, [d]Raised against a peptide to mouse EGFR [FKGDSFTRTPPLDPRELEI (SEQ ID NO: 491)], [e]Int J Oncol 4(1994)277. [f][EEKKVCQGTSNKLTQLGTFEDHFLSLQRMFNNCEVVLGNLEITYVQRNYDLSFLK TIQEVAGYVLIALNTVERIPLENLQIIRGNMYYENSYALAVLSNY (SEQ ID NO: 492)], [g]Ile-Gln-Cys-Ala-His-Tyr-Ile-Asp-Gly-Pro-His-Cys (SEQ ID NO: 493) (amino acids 580-591). [h]Cancer Cell 7(2005)301.

Using various approaches, we have confirmed that the EGFR monomer E3 binds to domain I of EGFR. Since other E monomers have similar properties in various experiments, it is thought that the other E monomers also bind to domain I of EGFR.

Example 19: Properties of I Monomers

BIAcore analysis of the soluble fibronectin-based scaffold proteins

The kinetics of I monomers binding to the target was measured using BIAcore 2000 or 3000 biosensors (Pharmacia Biosensor). A capture assay was developed utilizing an IGF-IR-Fc fusion. A similar reagent had been described by Forbes et al. (Forbes et al. 2002, European J. Biochemistry, 269, 961-968). The extracellular domain of human IGF-IR (aa 1-932) was cloned into a mammalian expression vector containing the hinge and constant regions of human IgG1. Transient transfection of the plasmid produced a fusion protein, IGF-IR-Fc which was subsequently purified by Protein A chromatography and captured on Protein A immobilized on Biasensor CM5 chips by amine coupling. The kinetic analysis involved the capture of IGF-IR-Fc on Protein A followed by injection of the fibronectin-based scaffold protein in solution and regeneration of the Protein A surface by glycine pH 2.0. Sensorgrams were obtained at each concentration and were evaluated using a program Biaevaluation, BIA Evaluation 2.0 (BIAcore), to determine the rate constants k_a (k_{on}) and k_d (k_{off}) The dissociation constant, K_D was calculated from the ratio of rate constants k_{off}/k_{on}. Typically, a concentration series

(2 uM to 0 uM) of purified fibronectin-based scaffold protein was evaluated for binding to protein A captured human IGF-IR-Fc fusion protein.

For experiments determining binding to human insulin receptor, recombinant human insulin receptor (IR) and recombinant human VEGF-R2-Fc were directly coupled to a CM5 Biasensor chip by amine group linkage following standard procedures recommended by Biacore (Uppsala, Sweden). In brief, 60 ug/mL of IR diluted in acetate 4.5 was coupled/immobilized to a level of 8300 RU and 11.9 ug/mL of VEGF-R2-Fc diluted in acetate 5.0 was immobilized to a level of 9700 RU on flow cells 2 and 3. A blank reference surface was prepared on FC1. Specific binding to either IR or VEGF-R2-Fc was calculated by subtracting the binding observed to the blank reference flow cell 1. Fibronectin-based scaffold proteins were diluted to 10 uM in HBS-EP (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.05% Surfactant P20) and injected at 20 uL/min for 3 minutes over the flow cells at 25 °C and dissociation was observed over 10 mins.

Cell-based Receptor Blocking Assay

The human breast adenocarcinoma MCF-7 (ATCC, Manassas, VA) was plated in 24 well plates at a concentration of 50,000 cells per well in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Hyclone, Logan, UT). The following day, cells were washed in binding buffer consisting of RPMI 1640 containing 0.1% BSA (Sigma, St. Louis, MO), and then pre-incubated for 30 minutes on ice in 200 μL binding buffer containing IGF-IR competitor. After the pre-incubation period, 40 pM [^{125}I]-IGF-I (Perkin Elmer, Wellesley, MA), equivalent to approximately 60000 counts per minute, was added to each well and allowed to incubate for an additional three hours on ice with gentle agitation. The wells were then washed with ice cold PBS containing 0.1% BSA. Cells were lysed with 500 μL buffer consisting of 0.1% SDS + 0.5 N NaOH. Radioactivity of the lysates was measured using a Wallac 1470 Gamma Counter (Perkin Elmer, Wellesley, MA), and the data were analyzed using SigmaPlot (Systat Software, Point Richmond, CA).

pIGFR Assay

Fibronectin-based scaffold proteins fused to Fc were evaluated for their ability to inhibit IGF-1R phosphorylation in Rh41 human rhabdomyosarcoma cells.

A Western Blot was employed to assess the ability of the I monomer to inhibit IGF-1R phosphorylation in Rh41 human rhabdomyosarcoma cells. Cells were stimulated with IGF-I, IGF-II, insulin ligands (50ng/ml), or no stimulation (NS) and then treated with various concentrations of the I monomer. Membranes were probed with phospho-specific antibodies.

Cellular Proliferation in Rh41 (human Rhabdomyosarcoma and H929 human Multiple Myeloma)

Proliferation was evaluated by incorporation of [^{3}H]-thymidine into DNA after a 72 hour exposure to reagents. Rh41 cells were plated at a density of 3500 cells/well in 96-well microtiter plates and 24 hours later they were exposed to a range of I monomer concentrations. After 72 hours incubation at 37°C, cells were pulsed with 4μCi/ml [^{3}H] thymidine (Amersham Pharmacia Biotech, UK) for 3 hours, trypsinized, harvested onto UniFilter-96, GF/B plates (PerkinElmer, Boston, MA) and scintillation was measured on a TopCount NXT (Packard, CT). Results are expressed as an IC50, which is the drug concentration required to inhibit cell proliferation by 50% to that of untreated control cells Data represents the average of triplicate wells with standard deviations shown.

Results of the characterization of the I monomer are shown below in Table 12.

Table 12. Properties of I monomers.

IGFR				
Monomer	BIAcore KD IC50	Neutralizes IGF Binding IC50	Inhibition of pIGFR IC50	Inhibition of RH41 Proliferation IC50
I1	0.11 nM	8 nM	0.2 nM	28 nM

Example 20: Additional Characteristics of Monospecific and Bispecific EGFR and IGF-IR ^{10}Fn3-based binders

^{10}Fn3-based binders that bound either EGFR or IGF-IR were identified using the biochemical selection technique of mRNA display in which a protein is covalently attached to its coding nucleic acid sequences. ^{10}Fn3-based proteins-mRNA fusion populations that bound either IGF-IR or EGFR when the receptors

were presented at concentrations from 1 to 10 nM were cloned into *E. coli* and expressed as ^{10}Fn3-based proteins. A subset of target binders that blocked EGFR or IGF-IR signaling and had suitable biophysical properties were identified (Table 13). These initial clones were optimized for target binding affinity and cellular potency with additional mRNA selection at increasingly lower target concentrations and selection for lower dissociation rate constants. IC_{50} values obtained during the selection procedures ranged from 9 to 304 nM, illustrating the opportunity for choosing molecules from a wide range of potency values for the construction of bi-specific ^{10}Fn3-based binders. EGFR ^{10}Fn3-based binders were tested by In-Cell Western screening assays for the blockade of phosphorylation of EGFR and ERK, a downstream signaling molecule of EGFR activation (methods similar to Example 1). Analogous studies were performed on optimized IGF-IR binders. Optimized EGFR-binding clones (E3, E1, and E2) inhibited EGFR phosphorylation on Y1068 and downstream phosphorylation of ERK on Y204 of p42/p44 *in vitro* with IC_{50} values ranging from 9 to 40 nM, potencies that were more than 100-fold higher than the parental EGFR clone (Table 13, methods similar to Example 1).

I1 bound to IGF-IR with a K_D value of 0.11 nM and inhibited IGF-I-stimulated IGF-IR phosphorylation with an IC_{50} of 0.2 nM (Table 13, methods similar to Example 6). The optimized IGF-IR and EGFR single-domain ^{10}Fn3-based binders were >95% monomeric based on size exclusion chromatography, had melting temperatures >56°C (Table 13, methods similar to Example 4), and exhibited minimal immunogenic potential as predicted from EpiMatrix (<7 for five out of six loops), a matrix-based algorithm for T-cell epitope mapping (De Groot AS, Moise L (2007) Prediction of immunogenicity for therapeutic proteins: state of the art. Curr Opin Drug Discovery Devel 10:332-340). The ^{10}Fn3-based binders E1, E2, and E3 were selected for further development, and had EGFR binding constants in the range of 0.7 to 10 nM as determined from Biacore assay (Table 13, methods similar to Example 5). EGFR-binding of these ^{10}Fn3-based binders was competitive for EGF binding to EGFR (Table 13) as measured by a displacement assay using Europium labeled EGF (methods similar to Example 10). Similarly, IGF-I binding to IGF-IR was inhibited by I1 (Table 13, methods similar to Example19).

Biophysical Characterization of Bi-Specific ^{10}Fn3-based binders. T_m values of selected E/I ^{10}Fn3-based binders ranged from 49-58°C and their SEC profiles indicated the protein was >90% monomer (Table 14, methods similar to Example 4). Monospecific ^{10}Fn3-based binders and E/I ^{10}Fn3-based binders showed comparable binding affinities, although T_m values decreased slightly when the single domain ^{10}Fn3-based binders were linked together (Tables 13 and 14). To increase serum half life for *in vivo* applications, E/I ^{10}Fn3-based binders were PEGylated with a 40 kDa branched PEG (methods similar to Example 3). PEGylation of E/I ^{10}Fn3-based binders resulted in a 10- to 20-fold reduction of binding affinity relative to the un-PEGylated constructs due to decreased association rate constants but did not decrease T_m. Furthermore, PEGylation did not markedly reduce inhibition of EGFR/IGF-IR phosphorylation in cells. The PEGylated E-I orientation (wherein the EGFR binder is at the N terminus, and IGF1R is at the C terminus) exhibited slightly lower IC_{50} values for the inhibition of EGFR and IGF-IR phosphorylation by ELISA compared to the I-E orientation. While minor differences in the K_D values and biological activity were found between PEGylated E-I orientation, vs the I-E orientation, there were no consistent trends.

Table 13. Properties of Monospecific ^{10}Fn3-based binders Relevant to the Construction of E/I ^{10}Fn3-based binders.

Name	T_m °C	SEC Monomer %	EGFR KD nM	IGF-IR KD nM	A431 pEGFR IC_{50}, nM	A431 pERK IC_{50}, nM	H292 pEGFR IC_{50}, nM	H292 pIGF-IR IC_{50}, nM	Competition EGFR/IGF-IR IC_{50}, nM
E-parent	56	ND	42.5		2580	2370	1148 ± 21	ND	29 ± 12.73
E3	60	>80	3.4	NA	15 ± 8	11 ± 7	22 ± 1	>7000	4.75 ± 1.77
E1	64	>95	9.92	NA	24 ± 7	13 ± 3	9 ± 2	>3400	15.9 ± 2.97
E2	72	>95	0.7	NA	38 ± 15	40 ± 9	31 ± 1	>3400	9.4 ± 3.68
I-parent	ND	ND	NA	1.8	ND	ND	ND	ND	13**
I1	61.5	>95	>6210	0.11	NA	NA	NA	0.2	8**

ND, not done; NA, not applicable; SEC, size exclusion chromatography. *IC_{50} values for EGFR and

ERK phosphorylation levles in A431 cells were determined by In-Cell Western assay (ICW). Phosphorylation levels
of EGFR and IGF-IR in H292 cells were determined by Enzyme-linked immunosorbent assay (ELISA).
** Competition for IGF-IR binding. Standard deviations are from 3 - 6 experiments.

Table 14. Properties of the E/I ^{10}Fn3-based binders.

Name	T_m °C	EGFR K_D nM	IGF-IR K_D nM	H292 pEGFR IC_{50}, nM	H292 pIGF-IR IC_{50}, nM	A431 pERK IC_{50}, nM	A431 pEGFR IC_{50}, nM	EGF-EGFR Competition IC_{50}, nM
E3-GS10-I1	52	0.7	0.1	7	6	12	14	25 ± 6.5
E3-GS10-I1-PEG	52.5	10.4	0.74	10	6	40	42	80.5 ± 12.02
E1-GS10-I1	48	3.8	0.8	30	1	51	36	51
E1-GS10-I1-PEG	49	57.9	2.4	123	4	295	297	396 ± 223
E2-GS10-I1	56	0.5	0.2	8	0.1	20	19	2.1 ± 0.57
E2-GS10-I1-PEG	57.5	10.1	1.17	32	0.3	78	77	56.5 ± 24.5
I1-GS10-E2-PEG	60	3.6	0.46	47	0.8	118	97	128 ± 4.95

T_m measurements are from thermal scanning flurometry. K_D values are from Biacore binding assays using recombinant EGFR or IGF-IR domains adsorbed on the chip. In-Cell Western assays (ICW) were conducted to determine the ability of EI-Tandems to inhibit the phosphorylation of EGFR or ERK in A431 cells. Enzyme-linked immunosorbent assays (ELISA) were used to determine the phosphorylation of EGFR or IGF-IR in H292 cells.

Example 21: Species Cross-Reactivity of E/I ^{10}Fn3-based binders

Pegylated E/I ^{10}Fn3-based binders were analyzed for their binding affinities to EGFR from mouse, rat and monkey using surface plasmon resonance (BIAcore) analysis (methods identical to Example 5). Mouse EGFR was purchased from R&D systems (Minneapolis, MN), rat EGFR was produced in house, and monkey EGFR was purchased from KEMP (Frederick, MD)

As shown in Table 15, all pegylated E/I ^{10}Fn3-based binders bound to mouse, rat and monkey EGFR with low nanomolar affinities indicating that all pegylated E/I binders are cross-reactive with human, mouse, rat and monkey EGFR.

Table 15

Analyte	KD (nM) (mouse EGFR)	KD (nM) (rat EGFR)	KD (nM) (monkey EGFR)
I1-GS10-E105 (pegylated)	2.7	2.9	4.4
I1-GS10-E5 (pegylated)	3.4	3.6	5.1
I1-GS10-E4 (pegylated)	5.5	3.7	3.9
E4-GS10-I1 (pegylated)	6.9	5.6	5.7
E2-GS10-I1 (pegylated)	9.6	9.6	18.0
I1-GS10-E85 (pegylated)	13.9	10.7	7.0

Example 22: Characterization of Additional E/I ^{10}Fn3-based binders

Figure 43 summarizes various characteristics of additional E/I ^{10}Fn3-based binders.

The pegylated E/I ^{10}Fn3-based binders were tested to determine inhibition of EGF induced EGFR and ERK phosphorylation in A431, using methods as previously described in Example 1. Results demonstrated that the pegylated E/I ^{10}Fn3-based binders inhibited EGF induced EGFR phosphorylation with IC50's ranging from 12 nM -297 nM and phosphorylation of ERK with IC50's ranging from 12 nM -295 nM (Figure 43, columns a and b).

The ability of the pegylated E/I ^{10}Fn3-based binders to inhibit IGFR and EGFR activity was also examined in H292 cells using methods previously described in Examples 6 and 7. Results indicated that the pegylated E/I ^{10}Fn3-based binders inhibited IGFR activity with IC50's ranging from 0.2 nM – 6 nM (Figure 43, column d) and inhibited EGFR activity with IC50's ranging from 1.3 nM – 123 nM (Figure 43, columns c).

The pegylated E/I ^{10}Fn3-based binders were tested to determine if they could induce degradation of EGFR and IGFR in Difi cells as shown in columns e and f of Table 16. Cells were treated with 1uM of pegylated E/I ^{10}Fn3-based binders and

harvested at time points starting at 7 hrs and ending at 120 hrs and levels of EGFR and IGF1R were determine by Western blot analysis. The strength of degradation was scored as either (+) indicating the tandem degraded that receptor but the degrdation was not sustained and receptor expression reappeared during the time course or (++) which indicates the tandem degraded the receptor and sustained that degradation throughout the time course. Results (Figure 43, column e and f) demonstrated that the pegylated E/I ^{10}Fn3-based binders displayed various patterns of EGFR and IGF1R degradation; degradation of only IGFR, degradation of both EGFR and IGFR or no degradation of either receptor. No tandem tested displayed the ability to degrade only EGFR.

The binding affinity of the pegylated E/I ^{10}Fn3-based binders for EGFR and IGF1R was assessed by surface Plasmon resonance (BIAcore) analysis as previously described in Example 5. Results demonstrated that the pegylated E/I ^{10}Fn3-based binders bound to EGFR with affinities ranging between 3.35 nM- 57.9 nM and bound to IGF1R with affinities ranging between 0.37 nM- 2.43 nM (Figure 43, columns g and h).

The pegylated E/I ^{10}Fn3-based binders were tested to determine their potency for blocking EGF binding to EGFR on the surface of A431 cells using methods previously described in Example 10. The the pegylated E/I ^{10}Fn3-based binders blocked EGF binding to A431 cells with IC50's ranging from 19.5 nM to 238nM (Figure 43, column i).

The pegylated E/I ^{10}Fn3-based binders were assessed for their ability to inhibit colony formation of H292 cells using methods described in Example 17. As shown in Figure 43, column j, the pegylated E/I ^{10}Fn3-based binders inhibited colony formation with IC50 values ranging from 1 nM – 560 nM and three of the four pegylated E/I ^{10}Fn3-based binders tested were 23-140 fold more potent than the anti-EGFR monoclonal antibody panitumumab. The fourth pegylated E/I ^{10}Fn3-based binders was 4 fold less potent than panitumuab. The pegylated I1 monomer was only marginally active in inhibiting colony formation in H292 with an IC50 >15uM and this is expected since H292 cell growth is predominantly driven by EGFR signaling and not IGF1R signaling.

The melting temperature was assessed for pegylated E/I ^{10}Fn3-based binders by DSC (as previously described in Example 4) or thermal dye melt methodology. For thermal dye melt assessment, the pegylated E/I ^{10}Fn3-based binders were diluted to 0.2 mg/mL in 50mM NaAc buffer pH 4.5. Each sample was spiked with 1uL of the 200x Sypro Orange in DMSO buffer for a final concentration of 0.5% dye. Each sample was loaded into the 96 well tray and coated with 5uL of silicone oil. The tray was spun down at 1,000 RPM and loaded onto the Bio-Rad CFX96 system and the following method was selected: 25°C for 10 minutes + Plate Read 25°C to 95°C @ 0.5°C increments for 15 minutes + Plate Read. Data analysis was performed for the inflection point with the CFX software. As shown in Figure 43, column k, all pegylated E/I ^{10}Fn3-based binders had similar Tm measurements, ranging from 49-62.5 degrees celsius. Tm measurements for the pegylated E/I ^{10}Fn3-based binders were independent of concentration and remained consistent at all concentrations tested. DSC analysis of an exemplary binder, I1-GS10-E5 pegylated, measured with a scan range of 15-95°C at 1mg/ml protein concentration in PBS, resulted in a Tm measurement of 55.2°C as shown in Figure 20.

Size exclusion chromatography (SEC) was performed on the pegylated E/I ^{10}Fn3-based binders as previously described in Example 4. SEC analysis revealed that all of the pegylated E/I ^{10}Fn3-based binders were >95% monomeric as shown in Figure 43 (column l of Table).

Example 23: Biochemical and biophysical properties of E/I ^{10}Fn3-based binder I1-GS10-E5 pegylated with selected amino acid changes

I1-GS10-E5 pegylated was constructed without the 6HIS tag (SEQ ID NO: 487) and also with various alterations to the linker region. In addition, a global change was made to all the constructs wherein the C-terminal tail of the first monomer had a single point change of the aspartic acid to glutamic acid (D to an E). Several clones were made with selected serine residues mutated to cysteines (S to C) to provide for alternate PEGylation sites. The effect of these changes on biochemical and biophysical properties of the molecule were compared and are summarized in Table 17. Methods for measuring inhibition of pEGFR are described in Example 7, pIGFR in Example 6, pERK in Example 1, Tm in Example 4, EGFR

and IGFR KD in Example 5. Detailed analysis of the binding kinetics were also carried out on these clones and are presented in Tables 18 and 19 (using methods similar to those described in Example 5).

Table 17.

CLONE NAME	pEGFR IC50 (nM)	pIGFR IC50 (nM)	pERK IC50 (nM)	Tm (°C)	EGFR KD (nM)	IGFR KD (nM)	SEC % mono
I1-GS10-E5 pegylated	28	2.2	12	56	2.7	0.25	96
I1-GS10-E5 pegylated(1)	30	1.2	11	56.8	Sticky(9)	0.23	94.1
I1-GSGCGS8-E5(3)	19.8	1.4	8	54.8	4	0.29	95.2
I1-GS10-E5-GSGC(4)	28.7	1.2	19	55	1.4	0.25	92.7
I1 (S62C)-GS10-E5(5)	21	1.9	10	55.5	8.7	0.7	97.45
I1-GS10-E5 (S62C) (6)	68.4	2.2	30	56	1.7	0.26	96.12
I1 (S91C)-GS10-E5(7)	22.7	6.2	15	52	17	7.16	95.98
I1-GS10-E5(S91C)(8)	22.6	2.1	29	50.5	17.9	0.28	93.39

(1) No His Tag was used for this construct. (2) a global change was made to all the alternative constructs of I1-GS10-E5 pegylated, wherein the C-terminal tail of the first monomer had a single point change of aspartic acid to glutamic acid (D to an E). (3) The I1 mononer linked with GSGC (SEQ ID NO: 489) plus GS8 (SEQ ID NO: 494), to E5. (4) I1 linked with GS10 to E5 with GSGC (SEQ ID NO: 489) at the tail of E5. (5) I1 linked with GS10 to E5, wherein the I1 has a single point change of serine to cysteine at position 62. (6) I1 linked with GS10 to E5, wherein the E5 has a single point change of serine to cysteine at position 62. (7) I1 linked with GS10 to E5, wherein the I1 has a single point change of serine to cysteine at position 91. (8) I1 linked with GS10 to E5, wherein the E5 has a single point change of serine to cysteine at position 91. (9) This construct demonstrated non-specific binding to the flow cell so an accurate determination of affinity was not possible in this experiment.

Table 18. Biacore binding of altered I1-GS10-E5 Pegylated clones to EGFR645-Fc.

Description	ka (1/Ms)	kd (1/s)	Kd (nm)	Δka (fold)	Δkd (fold)	ΔKd (fold)
I1-GS10-E5 Pegylated	2.93 ± 0.67 E+04	7.24 ± 3.14 E-05	2.69 ± 1.53	---	---	---
I1-GS10-E5 pegylated	2.27E+04	1.49E-04	6.6	0.8	0.5	0.4
I1-GS10-E5 pegylated(1)	Non-specific binding to reference cell surface at higher analyte concentrations (600nM, 200nM prohibited kinetic value determination)					
ALTERNATIVE CLONES(2)						
I1-GSGCGS8-E5(3)	2.94E+04	1.18E-04	4.0	1.0	0.6	0.7

I1-GS10-E5-GSGC[4]	3.34E+04	4.52E-05	1.4	1.1	1.6	2.0
I1(S62C)-GS10-E5[5]	2.28E+04	1.99E-04	8.7	0.8	0.4	0.3
I1-GS10-E5(S62C)[6]	1.78E+04	3.04E-05	1.7	0.6	2.4	1.6
I1(S91C)-GS10-E5[7]	1.96E+04	3.34E-04	17.0	0.7	0.2	0.2
I1-GS10-E5(S91C)[8]	1.08E+04	1.93E-04	17.9	0.4	0.4	0.2

(1) No His Tag was used for this construct. (2) a global change was made to all the alternative constructs of I1-GS10-E5 pegylated, wherein the C-terminal tail of the first monomer had a single point change of aspartic acid to glutamic acid (D to an E). (3) The I1 mononer linked with GSGC (SEQ ID NO: 489) plus GS8 (SEQ ID NO: 494), to E5. (4) I1 linked with GS10 to E5 with GSGC (SEQ ID NO: 489) at the tail of E5. (5) I1 linked with GS10 to E5, wherein the I1 has a single point change of serine to cysteine at position 62. (6) I1 linked with GS10 to E5, wherein the E5 has a single point change of serine to cysteine at position 62. (7) I1 linked with GS10 to E5, wherein the I1 has a single point change of serine to cysteine at position 91. (8) I1 linked with GS10 to E5, wherein the E5 has a single point change of serine to cysteine at position 91.

Table 19. Biacore binding of altered I1-GS10-E5 Pegylated clones to IGF1R-Fc.

Description	ka (1/Ms)	kd (1/s)	Kd (nm)	Δka (fold)	Δkd (fold)	ΔKd (fold)
I1-GS10-E5 pegylated	1.04 ± 0.04 E+06	2.62 ± 0.21 E-04	0.25 ± 0.01	---	---	---
I1-GS10-E5 pegylated	1.10E+06	2.78E-04	0.25	1.1	0.9	1.0
I1-GS10-E5 pegylated[1]	1.28E+06, 1.22E+06	2.88E-04, 2.76E-04	0.22, 0.23	1.2	0.9	1.1
ALTERNATIVE CLONES[2]						
I1-GSGCGS8-E5[3]	8.52E+05	2.45E-04	0.29	0.8	1.1	0.9
I1-GS10-E5-GSGC[4]	1.07E+06	2.65E-04	0.25	1.0	1.0	1.0
I1(S62C)-GS10-E5[5]	3.34E+05	2.34E-04	0.70	0.3	1.1	0.4
I1-GS10-E5(S62C)[6]	1.07E+06	2.79E-04	0.26	1.0	0.9	1.0
I1(S91C)-GS10-E5[7]	8.22E+04	5.89E-04	7.16	0.1	0.4	0.04
I1-GS10-E5(S91C)[8]	9.86E+05	2.81E-04	0.28	0.9	0.9	0.9

(1) No His Tag was used for this construct. (2) a global change was made to all the alternative constructs of I1-GS10-E5 pegylated, wherein the C-terminal tail of the first monomer had a single point change of aspartic acid to glutamic acid (D to an E). (3) The I1 mononer linked with GSGC (SEQ ID NO: 489) plus GS8 (SEQ ID NO: 494), to E5. (4) I1 linked with GS10 to E5 with GSGC (SEQ ID NO: 489) at the tail of E5. (5) I1 linked with GS10 to E5, wherein the I1 has a single point change of serine to cysteine at position 62. (6) I1 linked with GS10 to E5, wherein the E5 has a single point change of serine to cysteine at position 62. (7) I1 linked with GS10 to E5, wherein the I1 has a single point change of serine to cysteine at position 91. (8) I1 linked with GS10 to E5, wherein the E5 has a single point change of serine to cysteine at position 91.

Example 24: Inhibition of shared downstream signaling pathways of EGFR and IGFR

Inhibition of downstream signaling pathways were analyzed with a pAKT ELISA identical to those previously described in Example 8. Results of this study demonstrate that I1-GS10-E5 pegylated is more potent than I1 pegylated alone at

blocking IGF1-stimulated AKT activation in H292 cells. E5 pegylated, the EGFR monospecific binder alone did not efficiently prevent activation of AKT by IGF1 stimulation (Figure 21).

Example 25: Inhibition of cell proliferation by ^{10}Fn3-based binders and comparator antibody

H292 and RH41 cell proliferation experiments were conducted as described in Example 9. The EGFR monospecific ^{10}Fn3-based binder E5-pegylated inhibited proliferation of H292 cells with an IC50 value of 0.016 μM. The IGFR monospecific ^{10}Fn3-based binder I1-pegylated had an IC50 value of >8.4 μM while the E/I ^{10}Fn3-based binder I1-GS10-E5 pegylated was slightly more potent with an IC50 value of 0.006 μM (Figure 22). The H292 cell line is of lung cancer origin and sensitive to inhibition of IGFR and EGFR ((Akashi Y, et al. (2008) Enhancement of the antitumor activity of ionising radiation by nimotuzumab, a humanised monoclonal antibody to the epidermal growth factor receptor, in non-small cell lung cancer cell lines of differing epidermal growth factor receptor status. Br. J. Cancer 98:749-755; and Buck E, et al. (2008) Feedback mechanisms promote cooperativity for small molecule inhibitors of epidermal and insulin-like growth factor receptors. Cancer Res. 68:8322-8332.)) In contrast, only the I1-GS10-E5 pegylated binder and the I1-pegylated binder inhibited the proliferation of RH41 cells (IC50 values were 0.0002 and 0.0004 μM, respectively, Figure 23). This was expected, since RH41 is a pediatric rhabdomyosarcoma cell line that is known to be driven predominantly by IGFR signaling ((Huang F, et al. (2009). The mechanisms of differential sensitivity to an insulin-like growth factor-1 receptor inhibitor (BMS-536924) and rationale for combining with EGFR/HER2 inhibitors. Cancer Res. 69:161-170)) and thus not sensitive to EGFR blockade.

Example 26: Inhibition of receptor activation and downstream signaling in vitro by pegylated and non-pegylated ^{10}Fn3-based binders

In order to understand the dynamics of EGFR/IGFR signaling and its inhibition by I1-GS10-E5 pegylated, DiFi, H292 or BxPC3 cells were serum-starved, exposed to 1 μM or 0.1 μM E5 pegylated, I1 pegylated, or I1-GS10-E5

pegylated, or vehicle control for 2 hours, then stimulated with either EGF, IGF-I, or EGF + IGF-I for 10 min.

Cells were cultured in vitro, serum starved overnight and then exposed to ^{10}Fn3-based binders for 2 hours prior to stimulation with 100ng/ml of EGF or IGF. Cell lysates were prepared in lysis buffer (1% Triton X-100, 5% glycerol, 0.15 M NaCl, 20 mM Tris-HCl pH 7.6, Complete Protease Inhibitor Cocktail Tablets [Roche, Indianapolis, IN] and Phosphatase Inhibitor Cocktail 2 [Sigma-Aldrich Corp.]). Lysates (30 μg) were resolved by SDS-PAGE, transferred to membranes, and immunoblotted with antibodies to phospho-EGFR and total EGFR (Santa Cruz Biotechnology, Carlsbad, CA), phospho-AKT (Ser 473), phospho-p44/42 MAPK (Thr202/Tyr204) (Cell Signaling Technology, Beverly, MA), or total actin (Chemicon International, Temecula, CA) in Odyssey Blocking Buffer with 0.1% Tween 20 (LI-COR Biosciences, Lincoln, NE). Membranes were incubated with the appropriate secondary antibodies. Protein visualization was performed using a LI-COR Biosciences Odyssey infrared imaging system.

As shown in Figure 24, the basal levels of phosphorylated EGFR, IGF-IR, and AKT were nearly undetectable after serum deprivation. In DiFi cells, neither I1-GS10-E5 pegylated or E5 pegylated (monospecific EGFR binder) are able to completely suppress EGF-stimulated EGFR phosphorylation. In H292 and BxPC3 cells there is strong inhibition of EGFR phosphorylation by both I1-GS10-E5 pegylated and E5 pegylated. In DiFi and BxPC3 cells, I1-GS10-E5 pegylated blocks IGF-stimulated IGFR phosphorylation more than I1 pegylated (monospeciific IGFR binder) by itself. In H292 cells, IGF-stimulation cross activates the EGFR only when EGFR is blocked. I1-GS10-E5 pegylated inhibited EGF-stimulated pAKT in DiFi; increased pAKT in EGF-stimulated H292 and in BxPC3 EGF did not activate pAKT. In DiFi, H292 and BxPC3 cells I1-GS10-E5 pegylated inhibited IGF-stimulated pIGFR more than the individual E5 pegylated and I1 pegylated by themselves. I1-GS10-E5 pegylated had very little if any effect on EGF-stimulated pERK in DiFi, H292 or BxPC3. IGF-stimulation did not induce pERK in any cell line examined.

In another experiment with unPEGylated_^{10}Fn3-based binders, H292 cells were serum-starved, exposed to 1 μM unPEGylated monospecific EGFR binder E2,

IGFR binder I1, or E2-GS10-I1, or vehicle control for 1 hour, then stimulated with either EGF, IGF-I, or EGF + IGF-I for 10 min. The basal levels of phosphorylated EGFR, IGFR, and AKT were nearly undetectable after serum deprivation (Figure 25). Stimulation with EGF induced EGFR phosphorylation, but did not transactivate IGFR. EGFR phosphorylation was blocked by the E2, and E2-GS10-I1, but not I1. Similarly, stimulation with IGF-I induced strong phosphorylation of IGFR that was blocked by I1and E2-GS10-I1, but not by E2. EGF stimulation only slightly increased AKT phosphorylation, but IGF-I or EGF + IGF-I strongly induced phosphorylation of AKT that was suppressed to basal levels by both I1 and E2-GS10-I1. The combination of IGF-I and EGF induced AKT phosphorylation more than either growth factor alone. E2 partially reduced pAKT induced by the combination of EGF and IGF-I. However, I1 showed the most dramatic reduction in pAKT, suggesting that stimulation with EGF + IGF-I led to strong AKT phosphorylation through the IGFR pathway. Surprisingly, blockade of the EGFR pathway by E2 followed by stimulation with EGF ligand actually increased the phosphorylation of AKT, perhaps as a result of EGFR-independent activation of AKT ((Dobashi Y, et al. (2009) EGFR-dependent and independent activation of Akt/mTOR cascade in bone and soft tissue tumors. Mod Pathol (Epub Ahead of Print)). These results illustrate the complex cross-talk between the EGFR and IGFR pathways and feed-back mechanisms.

Example 27: Competition binding studies with E/I ^{10}Fn3-based binders

For Biacore competition experiments, EGFR-Fc (3μg/mL in Na-acetate pH 5.0) was immobilized on the Biacore CM5 chip surface using standard EDC/NHS amide coupling chemistry to a surface density of 300 RU. EGFR antibodies were obtained as a marketed drug and competition between monospecific EGFR binder E2 and antibodies for binding to EGFR-Fc was assessed by binding 450 nM E2 (30μL/min, 200s contact time), immediately followed by 450 nM E2 alone, or a mixture of 450 nM E2 plus 450 nM cetuximab, panitumumab, or nimotuzumab (30μL/min, 200 sec contact time). The surface was successfully regenerated between cycles using two 10 sec pulses of 50 mM NaOH at a flow rate of 30μL/min. Initial injection of E2 shows binding to EGFR on the surface of the chip. A second

injection of E2 mixed with an equal amount of cetuximab, panitumumab, or nimotuzumab shows no competition for binding of antibodies to EGFR by E2 (Figure 26A).

Surface plasmon resonance (BIAcore) analysis was utilized to demonstrate simultaneous engagement of captured EGFR-Fc and solution phase IGF1R by E/I ^{10}Fn3-based binders. Recombinant human EGFR-Fc (aa 1-645 of the extracellular domain of human EGFR fused to human Fc) was purchased from R&D systems (Minneapolis, MN). Recombinant IGF1R (aa 1-932 of human IGF1R propeptide, proteolytically cleaved and disulfide linked) was purchased from R&D systems (Minneapolis, MN). To demonstrate simultaneous engagement, anti-human IgG was immobilized on flow cells 1 and 2 of a CM5 chip following the manufacturer's recommendations (GE Healthcare, Piscataway, NJ). EGFR-Fc (50 nM) was captured on flow cell 2 at 10uL/min for 2 minutes. Binding of E/I ^{10}Fn3-based binders to EGFR-Fc was achieved by injecting ^{10}Fn3-based protein samples (100nM) over both flow cells at 10uL/min for 2 minutes. Simultaneous engagement of EGFR-Fc and IGF1R was probed by subsequently injecting IGF1R (0,100nM) over both flow cells at 30uL/min for 2 minutes. Dissociation of the complex was monitored for 300 seconds. Two 30 second injections of 3 M $MgCl_2$ were used for regeneration of the bound complex from the anti-human IgG surface. Biacore T100 Evaluation Software, Version 2.0.1 (GE healthcare/Biacore) was utilized to overlay sensograms and remove airspikes. As shown in Figure 26B, both domains of the E/I ^{10}Fn3-based binder are functional and able to bind to EGFR-Fc and IGF1R simultaneously.

Binding specificity of E2-GS10-I1 pegylated to HER family receptors was assessed by Biacore as described in Example 5. HER-2-Fc, HER-3-Fc and HER-4-Fc (R&D Systems) was captured on the surface of the CM5 chip with anti-human IgG. E2-GS10-I1 pegylated did not show any discernible binding to other HER family members under conditions where robust binding was seen for EGFR-Fc (HER-1) (Table 20).

Table 20. Binding affinity of E2-GS10-I1 pegylated to extracellular domains of HER family of receptors.

Name	EGFR-Fc K_D, nM*	HER-2-Fc K_D, nM	HER-3-Fc K_D, nM	HER-4-Fc K_D, nM
E2-GS10-I1 pegylated	10.1	>1000	>1000	>1000

Example 28: Measurement of plasma biomarkers

Levels of soluble biomarkers TGFα and mIGF1 were measured in mouse plasma at the end of xenograft studies or in non tumor bearing mice at various times following treatment. Blood was obtained by terminal cardiac puncture into tubes containing EDTA as an anticoagulant. Plasma was prepared by centrifuging blood at 1300 xg for 10 minutes at 4 degrees C and removing the clarified supernatant to a separate tube. TGFα levels were measured in 0.1ml of plasma, mIGF1 levels were measured in 0.02 ml plasma with an ELISA assay as recommended by manufacturer (R&D Systems, Minneapolis, MN). Plasma levels of TGFα were increased in mice treated with I1-GS10-E5 pegylated or the monospecific EGFR binder E5 pegylated but not cetuximab (Figure 27A-C). The TGFα could be secreted from the human tumor or may represent endogenous mouse TGFα. Due to the high homology between human and mouse TGFα (93% amino acid identity) the ELISA may cross react with mouse TGFα. Furthermore, human TGFα secreted by the implanted tumor can bind to the mouse EGFR. Because I1-GS10-E5 pegylated and E5 pegylated can bind both human and mouse EGFR, all host and tumor EGFR binding sites are blocked by these ^{10}Fn3-based binders while cetuximab does not bind mouse EGFR. To determine if these ^{10}Fn3-based binders cause increases in endogenouse mouse TGFα and if the ELISA cross reacts with mouse TGFα, non-tumor bearing nude mice were dosed with I1-GS10-E5 pegylated at 100mg/kg and plasma samples were taken at 4, 24, 48, 72 hours post dose. Increases in mouse TGFα were in fact observed that persisted out past 72 hours (Figure 28A). Plasma samples from non-tumored mice were also tested for mIGF1 with a mouse specific ELISA and increases in this ligand were also observed (Figure 28B).

Example 29: Results of *in vivo* human tumor xenograft studies for various E/I ^{10}Fn3-based binders

Several E/I ^{10}Fn3-based binders were evaluated in a head-to-head H292 NSCLC study (methods described in Example 12) at a lower dose than previously used so that differences in relative activity could be ascertained. Efficacy of the E/I ^{10}Fn3-based binders E2-GS10-I1 pegylated, E4-GS10-I1 pegylated, I1-GS10-E5 pegylated, I1-GS10-E85 pegylated, I1-GS10-E4 pegylated, I1-GS10-E105 pegylated at a single dose of 0.625mg/mouse and panitumumab at two doses (1 mg/mouse and 0.1 mg/mouse) were compared.

Both doses of panitumumab and all E/I ^{10}Fn3-based binders evaluated in this study were active by a tumor growth inhibition (TGI) endpoint. During the dosing phase, E4-GS10-I1 pegylated, I1-GS10-E5 pegylated, I1-GS10-E4 pegylated and panitumumab all caused tumor regression (Table 21, TGI values greater than 100%) while E2-GS10-I1 pegylated, I1-GS10-E85 pegylated and I1-GS10-E105 pegylated caused tumor growth inhibition (Table 21, TGI values up to 100%). Differences in activity were statistically significant when compared to the control group. All treatments were well tolerated with no treatment related deaths or excessive weight loss over the course of the study. Comparison of the efficacy of the E/I ^{10}Fn3-based binders and panitumumab are presented in Table 21 below and in Figure 29. In Figure 29A, measurements out to day 43 shows the pattern of regrowth of the tumors after dosing ceased. Figure 29B shows measurements out to day 27 and the y-axis is expanded to illustrate the relative differences in activity among the treatment groups.

Table 21. In vivo antitumor activity in the H292 NSCLC study

Group	Compound	Schedule, Route	Dose (mg/kg)	AVE weight change (g)	%TGI	p value for %TGI	Outcome by %TGI
1	Control (untreated)	---	---	3.36	---	1.0	---
2	panitumumab	q3dx5; 6 ip[a]	1mg/ mse	5.19	107	0.0023	A
3	panitumumab	q3dx5; 6 ip[a]	0.1mg/ mse	5.9	105	0.0029	A

4	E2-GS10-I1 pegylated	TIWX3; 6 ip[a]	0.625mg /mse	-1.4	93	0.0067	A
5	E4-GS10-I1 pegylated	TIWX3; 6 ip[a]	0.625mg /mse	-0.23	105	0.0023	A
6	I1-GS10-E5 pegylated	TIWX3; 6 ip[a]	0.625mg /mse	-2.92	103	0.0033	A
7	I1-GS10-E85 pegylated	TIWX3; 6 ip[a]	0.625mg /mse	1.08	86	0.0114	A
8	I1-GS10-E4 pegylated	TIWX3; 6 ip[a]	0.625mg /mse	-1.21	103	0.0034	A
9	I1-GS10-E105 pegylated	TIWX3; 6 ip[a]	0.625mg /mse	-1.54	95	0.0035	A

[a]Vehicle was phosphate buffered saline. Abbreviations used are as follows: ip, intraperitoneal route; %TGI, relative % tumor growth inhibition calculated as $\%TGI=[(C_t-T_t)/(C_t-C_0)] \times 100$ where C_t=median tumor weight of control mice at time t in days after tumor implant, T_t=median tumor weight of treated mice at time t, C_0= median tumor weight of control mice at time 0. %TGI value was calculated at two points as the average inhibition of Day 20, Day 24 and Day 27. Outcome, a treatment regimen was considered active if it produced a statistically significant %TGI value of >50%; q3dx5;6, compound was administered every three days for six doses starting on the sixth day after tumor implant; 6 on/1 off; 6, compound was administered once a day for 6 days then no treatment for 1 day and this regimen started on the sixth day after tumor implant. p values were calculated on Day 20 relative to the control group in a two tailed paired analysis with 8 measurements per group.

Further in vivo studies were carried out with selected E/I ^{10}Fn3-based binders below, in various xenograft models using the methods described in Example 12. A description of the various xenograft models is as follows: H292 is a non-small cell lung carcinoma (NSCLC) and is described in more detail Example 12; MCF7r breast carcinoma is described in Example 14; and GEO colon carcinoma is described in Example 15. The DiFi human colon carcinoma expresses high levels of activated EGFR and also expresses IGFR; RH41 is a pediatric rhabdomyosarcoma cell line that is known to be driven predominantly by IGFR signaling (Huang F, et al. ((2009)) The mechanisms of differential sensitivity to an insulin-like growth factor-1 receptor inhibitor (BMS-536924) and rationale for combining with EGFR/HER2 inhibitors. Cancer Res. 69:161-170) and thus is not sensitive to EGFR blockade; Cal27 is a human head and neck carcinoma expressing high levels of EGFR and moderate levels of IGFR; BxPC3 is a human pancreatic carcinoma; and H441 is a NSCLC.

Comparison of the efficacy of selected E/I ^{10}Fn3-based binders are presented in Table 22. In these efficacy studies, all of the E/I ^{10}Fn3-based binders showed

equivalent activity to panitumumab and all treatments were able to regress H292 tumors below their starting size as indicated by %TGI values over 100%. In the DiFi study, panitumumab regressed tumors at the 1mg/mouse dose and was active at the 0.1mg/mouse dose while all of the E/I ^{10}Fn3-based binders were inactive although the I1-GS10-E5 pegylated showed some inhibition of tumor growth (TGI = 43.8%). In the RH41 study, panitumumab was not active at either dose, the E2-pegylated construct was not active while the E/I ^{10}Fn3-based binders and the I1-pegylated construct were all active. Figure 32 shows antitumor efficacy in the RH41 model for a representative construct E2-GS10-I1 pegylated (data also shown in Table 23). In the Cal27 study panitumumab regressed tumors at the 1mg/mouse dose and was active at the 0.1mg/mouse dose but among the E/I ^{10}Fn3-based binders only the I1-GS10-E5 pegylated E/I ^{10}Fn3-construct was active.

Results of human tumor xenograft studies with I1-GS10-E5 pegylated and individual I1 and E5 components designed to assess synergy are presented in Table 23. These combination (synergy) studies were structured such that the individual pieces of the E/I ^{10}Fn3-based binders (ie., IGFR and EGFR monospecific pegylated versions) were included so antitumor effects beyond the contribution of isolated ends could be discerned. In the MCF7r study, I1 pegylated was not active while the E5 pegylated, (E5 pegylated + I1 pegylated) and the I1-GS10-E5 pegylated clones were all active and exhibited similar activity meaning that all of the antitumor activity likely comes from inhibition of EGFR and blocking the IGFR pathway did not provide any enhancement. Cetuximab regressed tumors at the 1mg/mouse dose and was not active at the 0.1mg/mouse dose. BMS-754807 was also not active showing that blocking the IGFR pathway with a small molecule inhibitor did not result in efficacy in this model.

In the BxPC3 study, I1 pegylated was not active while the E5 pegylated and (E5 pegylated + I1 pegylated) clones were active (TGI = 61.2% and 68.8%, respectively). The I1-GS10-E5 pegylated clone was more active (TGI = 78%) than the individual pieces it is made from and the difference was statistically significant by a two tailed paired t-test showing that it has synergistic activity in this model. Cetuximab was active at all doses studied but adding in IGFR inhibition by combining it with the I1-pegylated did not result in synergy.

In the GEO study, I1 pegylated was not active while the E5 pegylated and (E5 pegylated + I1 pegylated) and I1-GS10-E5 pegylated clones were active (TGI = 83.5%, 92.1 and 92.1%, respectively). While there may have been some enhancement provided by combining EGFR and IGFR inhibition together in this model, the difference was not significantly better than the E5 pegylated by itself. Cetuximab was active at both doses studied but adding in IGFR inhibition by combining it with the I1-pegylated did not result in synergy.

In the H441 study, I1 pegylated and E5 pegylated were not active on their own but (E5 pegylated + I1 pegylated) was active (TGI = 54.5%). The I1-GS10-E5 pegylated clone was more active (TGI = 69.2%) than the individual pieces it is made from but the differences were not statistically significant showing that it provides enhanced activity but not synergy in this model. Cetuximab was active at the 1mg/mouse dose and was not active at the 0.1mg/mouse dose. Adding in IGFR inhibition by combining it with the I1-pegylated did not result in any enhancement in this model.

Table 22. In vivo results of selected E/I ^{10}Fn3-based binders

Group	Compound	Schedule	Dose (mg/kg)[a]	AVE weight change (g)	%TGI	p value for %TGI	Outcome by %TGI
In vivo antitumor activity in the H292 study							
1	Control (untreated)	---	---	5.7	---	1.0	---
2	panitumumab	Q3dx5;6 ip[a]	1mg/mse	5.0	104	0.0006	A
3	panitumumab	Q3dx5;6 ip[a]	0.1mg/mse	1.5	102	0.0005	A
4	E4-GS10-I1 pegylated	TIWX3;6	2mg/mse	-4.86	105	0.0004	A
5	I1-GS10-E5 pegylated	TIWX3;6	2mg/mse	-10.0	102	0.0006	A
6	I1-GS10-E4 pegylated	TIWX3;6	2mg/mse	-1.41	105	0.0005	A
In vivo antitumor activity in the DiFi study							
1	Control (untreated)	---	---	-0.5	---	1.0	---
2	panitumumab	Q3dx5;6 ip[a]	1mg/mse	5.3	109.7	0.006	A
3	panitumumab	Q3dx5;6 ip[a]	0.1mg/mse	2.6	99.9	0.005	A
4	E4-GS10-I1 pegylated	TIWX3;6	3mg/mse	-10.8	-1.1	0.815	I
5	I1-GS10-E5 pegylated	TIWX3;6	3mg/mse	-16.4	43.8	0.310	I
6	I1-GS10-E4 pegylated	TIWX3;6	3mg/mse	-8.5	1.4	0.977	I
In vivo antitumor activity in the RH41study							
1	Control (untreated)	---	---	7.2	---	1.0	---
2	panitumumab	q3dx5;6 ip[a]	1mg/mse	10.7	16.5	0.721	I
3	panitumumab	q3dx5;6 ip[a]	0.1mg/mse	8.6	38.4	0.563	I
4	E4-GS10-I1 pegylated	TIWX3;6	2.5mg/mse	-5.3	72.7	0.02	A
5	I1-GS10-E5 pegylated	TIWX3;6	2.5mg/mse	-7.8	68	0.019	A
6	I1-GS10-E4 pegylated	TIWX3;6	2.5mg/mse	-2.9	64.5	0.018	A
7	Control (untreated)	---	---	12.3	---	1.0	---
8	E2-GS10-I1 pegylated	TIWX3;18	2.5mg/mse	-1.8	58.6	0.044	A
9	E2-pegylated	TIWX3;18	1.25mg/mse	5.9	20.2	0.530	I
10	I1 pegylated	TIWX3;18	1.25mg/mse	7.1	58.6	0.025	A
In vivo antitumor activity in the Cal27 study							

Group	Compound	Schedule	Dose (mg/kg)[a]	AVE weight change (g)	%TGI	p value for %TGI	Outcome by %TGI
1	Control (untreated)	---	---	9.4	---	1.0	---
2	Panitumumab	q3dx5;6 ip[a]	1mg/mse	6.1	109.8	0.0006	A
3	panitumumab	q3dx5;6 ip[a]	0.1mg/mse	5.8	72.9	0.003	A
4	E4-GS10-I1 pegylated	TIWX3;6	2mg/mse	-1.2	-11.4	0.587	I
5	I1-GS10-E5 pegylated	TIWX3;6	2mg/mse	-11.6	57.6	0.037	A
6	I1-GS10-E4 pegylated	TIWX3;6	2mg/mse	-2.2	-9.2	0.177	I

[a]Vehicle was phosphate buffered saline for all treatments. Abbreviations used are as follows: ip, intraperitoneal route; po, oral route; %TGI, relative % tumor growth inhibition calculated as %TGI=[(Ct-Tt)/(Ct-C0)] x 100 where Ct=median tumor weight of control mice at time t in days after tumor implant, Tt=median tumor weight of treated mice at time t, C0= median tumor weight of control mice at time 0. %TGI value was calculated at two points as the average inhibition on Day 19 and 23 for H292, Day 39 and 41 for DiFi, Day 34 and 37 for RH41 for groups 1-6 and Day 35, 36 and 39 for groups 7-10, Day 18 and 20 for Cal27. Outcome, a treatment regimen was considered active if it produced a statistically significant %TGI value of >50%; q3dx5;6, compound was administered every three days for six doses starting on the sixth day after tumor implant; TIWX3;6, compound was administered three times a week for 3 weeks and this regimen started on the sixth day after tumor implant. p values were calculated relative to the control group in a two tailed paired analysis with 8 measurements per group on Day 23 for H292, Day 39 for DiFi, Day 37 for RH41 for groups 1-6 and Day 39 for groups 7-10 and Day 20 for Cal27.

Table 23. Summary of in vivo experiments with ^{10}Fn3-based binders and comparators

Group	Compound	Schedule	Dose (mg/kg)[a]	AVE weight change (g)	%TGI	p value for %TGI	Outcome by %TGI
In vivo antitumor activity in the MCF7r study							
1	Control (untreated)	---	---	6.1	---	1.0	---
2	I1 pegylated[a]	TIWX3;7	50mg/kg, ip	17.1	-40.8	0.195	I
3	E5 pegylated[a]	TIWX3;7	50mg/kg, ip	5.1	75.8	0.007	A
4	E5 pegylated[a] + I1 pegylated[a]	TIWX3;7	50mg/kg, ip	-3.0	81.8	<0.0001	A
5	I1-GS10-E5 pegylated[a]	TIWX3;7	100mg/kg, ip	-4.5	78	0.009	A
6	cetuximab[a]	Q3DX5;7	1mg/mse, ip	11.7	105.4	0.0009	A
7	cetuximab[a]	Q3DX5;7	0.1mg/mse, ip	7.5	34.3	0.031	I
8	BMS-754807[b]	QDX14;7	50mg/kg, po	-4.0	44.5	0.146	I
In vivo antitumor activity in the BxPC3 study							
1	Control (untreated)	---	---	3.1	---	1.0	---
2	I1 pegylated	TIWX3;9	50mg/kg, ip	4.3	14.3	0.315	I
3	E5 pegylated	TIWX3;9	50mg/kg, ip	-5.3	61.2	0.0003	A
4	E5 pegylated + I1 pegylated	TIWX3;9	50mg/kg, ip	-4.9	68.8	0.0019	A
5	I1-GS10-E5 pegylated	TIWX3;9	100mg/kg, ip	-14.0	78.0	0.0002	A
6	cetuximab	Q3DX5;9	1mg/mse, ip	5.2	62.6	0.0026	A
7	cetuximab	Q3DX5;9	0.25mg/mse, ip	2.5	62.8	0.0005	A
8	Cetuximab +I1 pegylated	Q3DX5;9 TIWX3;9	1mg/mse, ip 50mg/kg, ip	3.6	62.1	0.0005	A
In vivo antitumor activity in the GEO study							
1	Control (untreated)	---	---	7.5	---	1.0	---
2	I1 pegylated	TIWX3;9	50mg/kg, ip	-7.2	26.8	0.594	I
3	E5 pegylated	TIWX3;9	50mg/kg, ip	9.7	83.5	0.0028	A
4	E5 pegylated + I1 pegylated	TIWX3;9	50mg/kg, ip	5.4	92.1	0.0005	A

Group	Compound	Schedule	Dose (mg/kg)[a]	AVE weight change (g)	%TGI	p value for %TGI	Outcome by %TGI
5	I1-GS10-E5 pegylated	TIWX3;9	100mg/kg, ip	-7.3	92.1	0.0006	A
6	cetuximab	Q3DX5;9	1mg/mse, ip	7.7	91.8	0.0008	A
7	cetuximab	Q3DX5;9	0.25mg/mse, ip	7.8	92.0	0.0007	A
8	Cetuximab + I1 pegylated	Q3DX5;9 TIWX3;9	1mg/mse, ip 50mg/kg, ip	7.1	91.3	0.0006	A
In vivo antitumor activity in the H441 study							
1	Control (untreated)	---	---	12.4	---	1.0	---
2	I1 pegylated	TIWX3;9	50mg/kg, ip	11.5	30.8	0.701	I
3	E5 pegylated	TIWX3;9	50mg/kg, ip	-8.8	43.1	0.292	I
4	E5 pegyalted + I1 pegylated	TIWX3;9	50mg/kg, ip	-0.8	54.5	0.011	A
5	I1-GS10-E5 pegylated	TIWX3;9	100mg/kg, ip	-3.9	69.2	0.022	A
6	cetuximab	Q3DX5;9	1mg/mse, ip	12.6	65.2	0.002	A
7	cetuximab	Q3DX5;9	0.25mg/mse, ip	13.7	43.9	0.110	I
8	cetuximab +I1 pegylated	Q3DX5;9 TIWX3;9	1mg/mse, ip 50mg/kg, ip	10.2	66.7	0.060	I

[a]Vehicle was phosphate buffered saline for all treatments. Abbreviations used are as follows: ip, intraperitoneal route; po, oral route; %TGI, relative % tumor growth inhibition calculated as %TGI=[(Ct-Tt)/(Ct-C0)] x 100 where Ct=median tumor weight of control mice at time t in days after tumor implant, Tt=median tumor weight of treated mice at time t, C0= median tumor weight of control mice at time 0. %TGI value was calculated at two points as the average inhibition on Day 22 and 26 for MCF7r, Day 23 and 27 for BxPC3, Day 29 and 31 for GEO and Day 17 and for H441. Outcome, a treatment regimen was considered active if it produced a statistically significant %TGI value of >50%; q3dx5;6, compound was administered every three days for six doses starting on the sixth day after tumor implant; TIWX3;6, compound was administered three times a week for 3 weeks and this regimen started on the sixth day after tumor implant. p values were calculated relative to the control group in a two tailed paired analysis with 8 measurements per group on Day 26 for MCF7r, Day 27 for BxPC3, Day 29 for GEO and Day17 for H441.

Example 30: Pharmacokinetic profile of various E/I ^{10}Fn3-based binders in mice

The pharmacokinetic profiles of the pegylated E/I ^{10}Fn3-based binder, E2-GS10-I1, were assessed in mice via intraperitoneal injection. Three nude mice per dose group were dosed with E2-GS10-I1, formulated in PBS, at 10 and 100 mg/kg, ip and plasma samples were collected in citrate phosphate dextrose solution at pre dosing, 0.5, 2, 4, 8, 12, 24, 48, 72, 96, 144, and 168 hours post dosing. Plasma samples were assessed for pegylated E2-GS10-I1 Fn3-based binder levels using a quantitative electrochemiluminescence (ECL) assay developed to detect and quantitate the pegylated E/I ^{10}Fn3-based binder in plasma samples. In this assay, a mouse monoclonal antibody with specificity toward the EGFR binding region was adsorbed to Meso Scale Discovery plates overnight at 4°C to allow capture of the pegylated E/I ^{10}Fn3-based binder in the plasma samples. The plasma samples were added to the plates and incubated at 22°C for 1 h. The captured pegylated E/I ^{10}Fn3-based binder was detected by a rabbit polyclonal antibody specific to the scaffold region of the E/I ^{10}Fn3-based binder, mixed with a goat anti-rabbit antibody linked with a SULFO-TAG. Following a wash to remove unbound SULFO-TAG reagent, a read buffer was added and ECL detection was used. The level of pegylated E2-GS10-I1 in plasma samples was calculated based on comparison to a 4-parameter fit of a standard curve of the pegylated E2-GS10-I1 Fn3-based binder.

Mice administered 10 or 100 mg/kg interperitoneally (ip) of pegylated E2-GS10-I1 resulted in peak levels of approximately 200 and 1700 μg/mL, respectively, indicating dose-proportional pharmacokinetics (Figure 30). Pharmacokinetic parameters for Figure 30 were calculated in a similar fashion to those described in the paragraph below (note that "T 1/2" is interchangeable with "HL_lambda_z"and AUC is interchangeable with "AUCINF_obs". The half-life of pegylated E2-GS10-I1 in mice was 15.75 ± 1.52 h (Figure 30). Based on these pharmacokinetic parameters, administration of 100 mg/kg three times weekly (TIW) in human tumor xenograft studies was able to maintain drug levels 10- to 100-fold higher than the in vitro IC50 value.

Additional pharmacokinetic experiments were conducted on several pegylated E/I ^{10}Fn3-based binders, where mice were administered 10 or 100 mg/kg

interperitoneally (ip) and, for the pegylated I1-GS10-E5, 10 or 64 mg/kg sub-cutaneously (sc), plasma was collected and analyzed as described above to measure the levels of pegylated E/I ^{10}Fn3-based binders. The pharmacokinetic parameters of these various E/I ^{10}Fn3-based binders were obtained by non-compartmental analysis of plasma (serum) concentration vs. time data. WinNonlin software (version 5.1, Pharsight Corp. Mountain View CA) was used to calculate the terminal half-life (HL_lambda_z), maximum observed concentration (Cmax), the area under the curve from time zero extrapolated to infinity (AUCINF_obs), clearance (CL_F_obs), volume of distribution based on the terminal phase (Vz_F_obs) and the mean residence time extrapolated to infinity (MRTINF_obs). Results showed that the half life for the pegylated E/I ^{10}Fn3-based binders were between 12.1-20.9 hours, as shown in Figure 44 and Figure 31.

Example 31: Pharmacodynamics

Samples were taken from the H292 and the DiFi xenograft models described in Table 22 at the end of the study and processed as outlined under *Measurement of pharmacodynamic endpoints in tumors* in Example 12 for analysis of total levels of EGFR and IGFR protein and phosphorylated EGFR and IGFR. Target effects of I1-GS10-E5-pegylated and panitumumab were evaluated by immunoblotting as described in Example 11. In Figure 33A, levels of total EGFR, pEGFR and total IGFR were lower in I1-GS10-E5-pegylated treated tumors than in untreated tumors at the end of the DiFi xenograft model. In Figure 33B, levels of pEGFR were lower in tumors treated with panitumumab and I1-GS10-E5-pegylated. Levels of total EGFR were lower only in I1-GS10-E5-pegylated treated tumors but not in panitumumab treated tumors. Levels of total IGFR were lower in both I1-GS10-E5-pegylated treated tumors and in one panitumumab treated tumor but not the other. The amount of pIGFR in these models was too low to detect differences following treatment. Immunoblots were probed with GAPDH to demonstrate equal loading of protein.

Example 32: EGFR ^{10}Fn3-based binders Optimization and Consensus Sequence Analysis

The ^{10}Fn3-based binder 679F09 (as described in PCT WO 2009/102421) (Figure 34) was identified as a binder to EGFR ectodomain-Fc fusion protein (R&D Systems). Binding activity was selected using a bead coated with EGFR-Fc and ^{10}Fn3-based binders coupled to their nucleic acid coding sequence (see e.g., Xu et al., Directed Evolution of High-Affinity Antibody Mimics Using MRNA Display, Chem. Biol. 9: 933-942 (2002)). More potent variants of the parental EGFR binder 679F09 having alterations to the amino acid sequences in the BC, DE and FG loops were also identified.

Sequence Analysis I: All ^{10}Fn3-based binders selected for high-affinity binding to EGFR

In order to reveal sequence patterns that defined strong affinity for EGFR, all unique EGFR binding sequences (1044) were analyzed using several methods. First, the sequences were analyzed by the frequency of amino acids at each position in the loops (Figures 35-38). Only unique sequences for each loop were analyzed.

From the above sequence analysis, the following broad sequence motif was defined:

Sequence Motif #1

(a) BC loop: "YQ" in positions 7-8 (i.e., corresponding to positions 29 and 30 of SEQ ID NO: 1)

(b) DE loop: aliphatic residue ("V/I/L/M/A") in position 3 (i.e., corresponding to position 54 of SEQ ID NO: 1)

(c) FG loop: "D/N" in position 1 (i.e., corresponding to position 77 of SEQ ID NO: 1)

All 1044 sequences analyzed, except one, follow the FG loop sequence pattern (c). Of all unique sequences analyzed, 90% follow pattern (a) for the BC loop, and 95% follow pattern (b) for the DE loop. All sequences analyzed, except four, follow at least two of the three patterns above. In addition, the 15-amino acid FG loop length is a noteworthy sequence feature.

In addition to the broad Sequence Motif #1 defined above, the data in Figures 35-38 were used to define a second sequence motif based on the dominant residues at each position. Residues were included in this motif if the sum of the top 3 most frequent amino acids had a greater than 50% frequency.

Sequence Motif #2

(a) BC loop: XXXXXXYQ (same as Motif #1), wherein X is any amino acid

(b) DE loop: (G/Y/H)(D/M/G)(V/L/I)X, wherein X is any amino acid

(c) FG loop, 10 amino acid length:
(D/N)(Y/M)(Y/A/M)(Y/H/F)(K/Q/V)(E/P/R)(Y/T/K)X(E/Y/Q)(Y/G/H), wherein X is any amino acid

(d) FG loop, 15 amino acid length:
D(Y/F/W)(Y/F/K)(N/D/P)(P/H/L)(A/T/V)(T/D/S)(H/Y/G)(E/P/V)(Y/H)(T/K/I)(Y/F)(H/N/Q)(T/Q/E)(T/S/I)

The analysis methods used to define Sequence Motifs #1 and #2 evaluate each residue position within a loop separately. To reveal any sequence motifs spanning multiple residues within a loop, the ^{10}Fn3-based binders were subjected to further analysis. In this analysis, the loop sequences were aligned using ClustalW (Thompson JD *et al.* CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673–4680, 1994). From this alignment, families of sequences were grouped using manual inspection. For the BC and DE loops, sequence patterns similar to Sequence Motifs #1 and #2 were observed. However, additional sequence motifs could be defined for the 10 and 15 amino acid long FG loops.

Sequence Motif #3

(a) FG loop, 10 amino acid length

(1) DY(A/Y)GKPYXEY (SEQ ID NO: 473), wherein X is any amino acid

(2) DY(A/Y)Y(K/R/Q/T)PYXEY (SEQ ID NO: 474), wherein X is any amino acid

(3) (D/N)Y(A/Y)(Y/F)(K/R/Q/T)EYXE(Y/H) (SEQ ID NO: 475), wherein X is any amino acid

(4) DYY(H/Y)X(R/K)X(E/T)YX (SEQ ID NO: 476), wherein X is any amino acid

(5) DYY(H/Y)(K/H/Q)(R/K)T(E/T)Y(G/P) (SEQ ID NO: 477)

(6) (D/N)MMHV(E/D)YXEY (SEQ ID NO: 478), wherein X is any amino acid

(7) DYMHXXYXEY (SEQ ID NO: 479) (like FG loop of 679F09), wherein X is any amino acid

(8) D(M/Y)YHX(K/R)X(V/I/L/M)YG (SEQ ID NO: 480), wherein X is any amino acid

(b) FG loop, 15 amino acid length

(1) D(Y/F)(Y/F)NPXTHEYXYXXX (SEQ ID NO: 481), wherein X is any amino acid

(2) D(Y/F)(Y/F)D(P/L)X(T/S)HXYXYXXX (SEQ ID NO: 482), wherein X is any amino acid

(3) D(Y/F)(K/R)PHXDGPH(T/I)YXE(S/Y) (SEQ ID NO: 483), wherein X is any amino acid

Sequence Analysis II: ^{10}Fn3-based binders showing more potent inhibition of EGFR phosphorylation

Another overall sequence analysis was performed on the subset of ^{10}Fn3-based binders that showed the most potent activity in a cell-based assay (as opposed to Sequence Analysis I, which was performed on all binders selected for high-affinity binding to EGFR through Profusion). Because many of the binders were only run through single-point cell-based assays, binders that showed greater than 75% inhibition of EGFR phosphorylation at a fixed concentration of 100 nM were included in this analysis. The percent inhibition at a given concentration is related to the IC50 by: % inhibition = 100 x concentration/(concentration + IC50).

Normally, an IC50 is calculated by fitting the data for % inhibition at various concentrations. However, given that only a single data point is available for each binder, it is inappropriate to use this single data point to calculate an IC50.

Therefore, the percent inhibition of EGFR signaling at a single concentration point was used as an approximation of the potency of the binder. Although a binder may show 75% inhibition at a concentration of 100 nM, increasing the concentration will allow the clone to show 100% inhibition at a higher concentration. The % inhibition is inversely related to the IC50; i.e., the higher the % inhibition, the lower the IC50 and the more potent the binder. If a binder showed 75% inhibition at a concentration of 100 nM, we considered this to be a "potent" binder for the purposes of Sequence Analysis II. However, the binders which showed less than 75% inhibition at 100 nM concentration for the most part still bind to EGFR and still have an effect on EGFR signaling. For instance, the anti-EGFR monoclonal antibody Nimotuzumab (Friedlander E *et al.* ErbB-directed immunotherapy: antibodies in current practice and promising new agents. *Immunol Lett* **116**: 126–140, 2008) is currently under development as a therapeutic, but it shows <5% inhibition at a 100 nM concentration in the EGFR phosphorylation assay (data not shown). The sequences of all "potent" binders assayed and their % inhibition of EGFR phosphorylation at 100 nM concentration is shown in Figure 45.

The total number of unique ^{10}Fn3-based binders that showed >75% inhibition at 100 nM concentration was 111. As before, the sequences first were analyzed by the frequency of amino acids at each position in the loops (Figures 39-42). Since these binders are a subset of all the binders selected for high affinity binding to EGFR during Profusion, they also follow Sequence Motif #1 (see above). All "potent" sequences analyzed follow the FG loop sequence pattern ("D/N" in position 1). Of all unique "potent" sequences analyzed, 93% follow the pattern for the BC loop ("YQ" in positions 7-8), and 98% follow the pattern for the DE loop (aliphatic residue ("V/I/L/M/A") in position 3). All "potent" sequences analyzed follow at least two of the three patterns of Sequence Motif #1.

Of note, the 15-amino acid FG loop length also appears to be highly represented in the most "potent" binders. While 15-amino acid long FG loops represent only 55% of all binders selected for high affinity binding to EGFR (Sequence Analysis I), 15-amino acid FG loops represent 86% of the binders with >50% inhibition of EGFR phosphorylation at 100 nM concentration, and 91% of the binders with >75% inhibition ("potent" binders in Sequence Analysis II). Therefore,

the longer 15-amino acid FG loop appears to be a sequence pattern associated with greater potency.

Of the 111 "potent" sequences analyzed, only 10 contain 10-amino acid long FG loops, and 6 of those are unique. Therefore, a single sequence motif can encompass every "potent" 10-amino acid FG loop sequence. Sequence Motif #4 was defined based on these 6 sequences.

Sequence Motif #4

FG loop, 10-amino acid length, "potent" binders

(D/N)(M/Y)(M/A/W)(H/F/Y)(V/K)EY(A/Q/R/S/T)E(Y/H/D)

The sequence analysis of the "potent" binders with 15-amino acid FG loops also further illuminated which residue positions were most conserved, allowing Sequence Motif #5 to be defined. An "X" in this sequence motif denotes positions where there are no three dominant amino acids.

Sequence Motif #5

FG loop, 15-amino acid length, "potent" binders

D(Y/F/W)(Y/F/K)(N/P/D)(P/H/L)X(T/D/S)(H/G/Y)(E/P/Y)(Y/H)XYXXX,

wherein X is any amino acid

All of the EGFR binders that were analyzed are progeny of the parent 679F09 and constitute a sequence "family," i.e. they are all related in sequence according to the aforementioned sequence motifs. Various members of the 679F09 family of binders can tolerate a T51I scaffold mutation and retain binding activity. Therefore, a T51I scaffold mutation could be combined with any of the aforementioned sequence motifs to also yield a binder with high affinity binding to EGFR.

Finally, it should be noted that amino acids with similar properties can often be substituted into protein sequences with little or no effect on structure or function. This indeed is the case for ^{10}Fn3-based binders as well, where conservative amino acid substitutions in either the loop or scaffold regions can still lead to binders which

bind to EGFR. For instance, substituting "Y" for "H" in the second position of the FG loop of binder E98 yields binder E99, and both binders show similar potency in inhibiting EGFR phosphorylation (Figure 45).

CLAIMS:

1. An antibody-like protein dimer comprising a) a tenth fibronectin type III domain (^{10}Fn3) that binds IGF-IR with a K_D of less than 500 nM covalently or non-covalently linked to b) a ^{10}Fn3 that binds EGFR with a K_D of less than 500 nM;

wherein the IGF-IR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 3, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 3, and an FG loop having the amino acid sequence set forth in amino acids 76-83 of SEQ ID NO: 3, and

wherein the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 112, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 112, and an FG loop having the amino acid sequence set forth in amino acids 76-92 of SEQ ID NO: 112.

2. An antibody-like protein dimer comprising a tenth fibronectin type III domain (^{10}Fn3) that binds IGF-IR with a K_D of less than 500 nM covalently or non-covalently linked to a ^{10}Fn3 that binds EGFR with a K_D of less than 500 nM;

wherein the IGFIR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence $X_aSARLKVAX_b$ (SEQ ID NO: 46), a DE loop having the amino acid sequence X_cKNVYX_d (SEQ ID NO: 48), and an FG loop having the amino acid sequence $X_eRFRDYQX_f$ (SEQ ID NO: 50);

wherein the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence $X_gWAPVDRYQX_h$ (SEQ ID NO: 137), a DE loop having the amino acid sequence X_iRDVYX_j (SEQ ID NO: 138), and an FG loop having the amino acid sequence $X_kDYKPHADGPHTYHESX_l$ (SEQ ID NO: 139); and

wherein X is any amino acid and a, b, c, d, e, f, g, h, i, j, k, and l are integers independently selected from 0 to 5.

3. The antibody-like protein dimer of claim 2,

wherein the IGF-IR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence SWSARLKVAR (SEQ ID NO: 45), a DE loop having the amino acid sequence PKNVYT (SEQ ID NO: 47), and an FG loop having the amino acid sequence TRFRDYQP (SEQ ID NO: 49); and an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWWAPVDRYQ (SEQ ID NO: 115), a DE loop having the amino acid sequence PRDVYT (SEQ ID NO: 116), and an FG loop having the amino acid sequence TDYKPHADGPHTYHESP (SEQ ID NO: 117).

4. The antibody-like protein dimer of anyone of claims 1-3, wherein the IGF-IR binding ^{10}Fn3 has an amino acid sequence at least 90% identical to SEQ ID NO: 3 and the EGFR binding ^{10}Fn3 scaffold has an amino acid sequence at least 90% identical to SEQ ID NO: 112.

5. The antibody-like protein dimer of anyone of claims 1-4, wherein the IGF-IR binding ^{10}Fn3 is covalently linked to the EGFR binding ^{10}Fn3 via a polypeptide linker or a polyethylene glycol moiety.

6. The antibody-like protein dimer of claim 5 comprising an amino acid sequence at least 90% identical to any one of SEQ ID NOs: 126-132.

7. An antibody-like protein comprising a tenth fibronectin type III domain (^{10}Fn3) that binds EGFR with a K_D of less than 500 nM, wherein the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence set forth in amino acids 21-30 of SEQ ID NO: 112, a DE loop having the amino acid sequence set forth in amino acids 51-56 of SEQ ID NO: 112, and an FG loop having the amino acid sequence set forth in amino acids 76-92 of SEQ ID NO: 112.

8. An antibody-like protein comprising a tenth fibronectin type III domain (^{10}Fn3) that binds EGFR with a K_D of less than 500 nM, wherein the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence X_gWAPVDRYQX_h (SEQ ID NO: 137), a DE loop having the amino acid sequence X_iRDVYX_j (SEQ ID

NO: 138), and an FG loop having the amino acid sequence X_kDYKPHADGPHTYHESX$_l$ (SEQ ID NO: 139); and wherein X is any amino acid and a, b, c, d, e, f, g, h, i, j, k, and l are integers independently selected from 0 to 5.

9. The antibody-like protein of claim 8, wherein the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence SWWAPVDRYQ (SEQ ID NO: 115), a DE loop having the amino acid sequence PRDVYT (SEQ ID NO: 116), and an FG loop having the amino acid sequence TDYKPHADGPHTYHESP (SEQ ID NO: 117).

10. The antibody-like protein of anyone of claims 7-9, further comprising a second tenth fibronectin type III domain (^{10}Fn3).

11. The antibody-like protein of claim 10, wherein the second ^{10}Fn3 binds to IGF-IR.

12. The antibody-like protein of claims 10 or 11, wherein the second ^{10}Fn3 is covalently linked to the EGFR binding ^{10}Fn3 via a polypeptide linker or a polyethylene glycol moiety.

13. An antibody-like protein dimer comprising a tenth fibronectin type III domain (^{10}Fn3) that binds IGF-IR with a K_D of less than 500 nM covalently or non-covalently linked to a ^{10}Fn3 that binds EGFR with a K_D of less than 500 nM;

wherein the IGFIR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence X_aSARLKVAX$_b$ (SEQ ID NO: 46), a DE loop having the amino acid sequence X_cKNVYX$_d$ (SEQ ID NO: 48), and an FG loop having the amino acid sequence X_eRFRDYQX$_f$ (SEQ ID NO: 50);

wherein the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence X_gDSGRGSYQX$_h$ (SEQ ID NO: 40), a DE loop having the amino acid sequence X_iGPVHX$_j$ (SEQ ID NO: 42), and an FG loop having the amino acid sequence X_kDHKPHADGPHTYHEX$_l$ (SEQ ID NO: 44); and

wherein X is any amino acid and a, b, c, d, e, f, g, h, i, j, k, and l are integers independently selected from 0 to 5.

14. The antibody-like protein dimer of claim 13,

wherein the IGF-IR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence SWSARLKVAR (SEQ ID NO: 45), a DE loop having the amino acid sequence PKNVYT (SEQ ID NO: 47), and an FG loop having the amino acid sequence TRFRDYQP (SEQ ID NO: 49); and an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWDSGRGSYQ (SEQ ID NO: 39), a DE loop having the amino acid sequence PGPVHT (SEQ ID NO: 41), and an FG loop having the amino acid sequence TDHKPHADGPHTYHESP (SEQ ID NO: 43).

15. The antibody-like protein dimer of claims 13 or 14, wherein the IGF-IR binding ^{10}Fn3 has an amino acid sequence at least 90% identical to SEQ ID NO: 3 and the EGFR binding ^{10}Fn3 scaffold has an amino acid sequence at least 90% identical to SEQ ID NO: 5.

16. The antibody-like protein dimer of any one of claims 13-15, wherein the IGF-IR binding ^{10}Fn3 is covalently linked to the EGFR binding ^{10}Fn3 via a polypeptide linker or a polyethylene glycol moiety.

17. The antibody-like protein dimer of claim 16, comprising an amino acid sequence at least 90% identical to SEQ ID NOs: 20, 21, 23, or 24.

18. An antibody-like protein dimer comprising a tenth fibronectin type III domain (^{10}Fn3) that binds IGF-IR with a K_D of less than 500 nM covalently or non-covalently linked to a ^{10}Fn3 that binds EGFR with a K_D of less than 500 nM;

wherein the IGFIR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence X_aSARLKVAX$_b$ (SEQ ID NO: 46), a DE loop having the amino acid sequence X_cKNVYX$_d$ (SEQ ID NO: 48), and an FG loop having the amino acid sequence X_eRFRDYQX$_f$ (SEQ ID NO: 50);

wherein the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence $X_mVAGAEDYQX_n$ (SEQ ID NO: 34), a DE loop having the amino acid sequence X_oHDLVX_p (SEQ ID NO: 36), and an FG loop having the amino acid sequence $X_qDMMHVEYTEHX_r$ (SEQ ID NO: 38); and

wherein X is any amino acid and a, b, c, d, e, f, m, n, o, p, q, and r are integers independently selected from 0 to 5.

19. The antibody-like protein dimer of claim 18,

wherein the IGF-IR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence SWSARLKVAR (SEQ ID NO: 45), a DE loop having the amino acid sequence PKNVYT (SEQ ID NO: 47), and an FG loop having the amino acid sequence TRFRDYQP (SEQ ID NO: 49), covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWVAGAEDYQ (SEQ ID NO: 33), a DE loop having the amino acid sequence PHDLVT (SEQ ID NO: 35), and an FG loop having the amino acid sequence TDMMHVEYTEHP (SEQ ID NO: 37).

20. The antibody-like protein dimer of claims 18 or 19, wherein the IGF-IR binding ^{10}Fn3 has an amino acid sequence at least 90% identical to SEQ ID NO: 3 and the EGFR binding ^{10}Fn3 has an amino acid sequence at least 90% identical to SEQ ID NO: 7.

21. The antibody-like protein dimer of any one of claims 18-20, wherein the IGF-IR binding ^{10}Fn3 scaffold is covalently linked to the EGFR binding ^{10}Fn3 scaffold via a polypeptide linker or a polyethylene glycol moiety.

22. The antibody-like protein dimer of claim 21, comprising an amino acid sequence at least 90% identical to SEQ ID NOs: 26, 27, 29, or 30.

23. An antibody-like protein dimer comprising a tenth fibronectin type III domain (^{10}Fn3) that binds IGF-IR with a K_D of less than 500 nM covalently or non-covalently linked to a ^{10}Fn3 that binds EGFR with a K_D of less than 500 nM;

wherein the IGFIR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence $X_aSARLKVAX_b$ (SEQ ID NO: 46), a DE loop having the amino acid sequence X_cKNVYX_d (SEQ ID NO: 48), and an FG loop having the amino acid sequence $X_eRFRDYQX_f$ (SEQ ID NO: 50);

wherein the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence $X_sLPGKLRYQX_t$ (SEQ ID NO: 60), a DE loop having the amino acid sequence X_uHDLRX_w (SEQ ID NO: 62), and an FG loop having the amino acid sequence $X_yNMMHVEYSEYX_z$ (SEQ ID NO: 64); and

wherein X is any amino acid and a, b, c, d, e, f, s, t, u, w, y and z are integers independently selected from 0 to 5.

24. The antibody-like protein dimer of claim 23,

wherein the IGF-IR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence SWSARLKVAR (SEQ ID NO: 45), a DE loop having the amino acid sequence PKNVYT (SEQ ID NO: 47), and an FG loop having the amino acid sequence TRFRDYQP (SEQ ID NO: 49); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence LPGKLRYQ (residues 3-13 of SEQ ID NO: 59), a DE loop having the amino acid sequence PHDLR (residues 1-5 of SEQ ID NO: 61), and an FG loop having the amino acid sequence TNMMHVEYSEY (residues 1-11 of SEQ ID NO: 63).

25. The antibody-like protein dimer of claims 23 or 24, wherein the IGF-IR binding ^{10}Fn3 has an amino acid sequence at least 90% identical to SEQ ID NO: 3 and the EGFR binding ^{10}Fn3 has an amino acid sequence at least 90% identical to SEQ ID NO: 82.

26. The antibody-like protein dimer of any one of claims 23-25, wherein the IGF-IR binding ^{10}Fn3 scaffold is covalently linked to the EGFR binding ^{10}Fn3 scaffold via a polypeptide linker or a polyethylene glycol moiety.

27. The antibody-like protein dimer of claim 26, comprising an amino acid sequence at least 90% identical to any one of SEQ ID NOs: 53, 54, 87, 88, 98, 99, 104 or 105.

28. An antibody-like protein dimer comprising a tenth fibronectin type III domain (^{10}Fn3) that binds IGF-IR with a K_D of less than 500 nM covalently or non-covalently linked to a ^{10}Fn3 that binds EGFR with a K_D of less than 500 nM;

wherein the IGFIR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence $X_aSARLKVAX_b$ (SEQ ID NO: 46), a DE loop having the amino acid sequence X_cKNVYX_d (SEQ ID NO: 48), and an FG loop having the amino acid sequence $X_eRFRDYQX_f$ (SEQ ID NO: 50);

wherein the EGFR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence $X_gHERDGSRQX_h$ (SEQ ID NO: 134), a DE loop having the amino acid sequence X_iGGVRX_j (SEQ ID NO: 135), and an FG loop having the amino acid sequence $X_kDYFNPTTHEYIYQTTX_l$ (SEQ ID NO: 136); and

wherein X is any amino acid and a, b, c, d, e, f, g, h, i, j, k and l are integers independently selected from 0 to 5.

29. The antibody-like protein dimer of claim 28,

wherein the IGF-IR binding ^{10}Fn3 comprises a BC loop having the amino acid sequence SWSARLKVAR (SEQ ID NO: 45), a DE loop having the amino acid sequence PKNVYT (SEQ ID NO: 47), and an FG loop having the amino acid sequence TRFRDYQP (SEQ ID NO: 49); covalently or non-covalently linked to an EGFR binding ^{10}Fn3 comprising a BC loop having the amino acid sequence SWHERDGSRQ (SEQ ID NO: 109), a DE loop having the amino acid sequence PGGVRT (SEQ ID NO: 110), and an FG loop having the amino acid sequence TDYFNPTTHEYIYQTTP (SEQ ID NO: 111).

30. The antibody-like protein dimer of claims 28 or 29, wherein the IGF-IR binding ^{10}Fn3 has an amino acid sequence at least 90% identical to SEQ ID NO: 3 and the EGFR binding ^{10}Fn3 has an amino acid sequence at least 90% identical to SEQ ID NO: 107.

31. The antibody-like protein dimer of any one of claims 28-30, wherein the IGF-IR binding ^{10}Fn3 scaffold is covalently linked to the EGFR binding ^{10}Fn3 scaffold via a polypeptide linker or a polyethylene glycol moiety.

32. The antibody-like protein dimer of claim 31, comprising an amino acid sequence at least 90% identical to any one of SEQ ID NOs: 118-125.

33. A pharmaceutically acceptable composition comprising the antibody-like protein dimer or antibody-like protein of any one of claims 1-32, wherein the composition is essentially pyrogen free.

34. A method for treating a hyperproliferative disorder in a subject comprising administering to a subject in need thereof a therapeutically effective amount of the composition of claim 33.

35. An antibody-like protein comprising a tenth fibronectin type III domain (^{10}Fn3) that binds EGFR with a K_D of less than 500 nM, wherein the EGFR binding ^{10}Fn3 comprises a BC loop comprising the amino acid sequence XXXXXXYQ, a DE loop comprising the amino acid sequence XX(V/I/L/M/A)X, and an FG loop comprising the amino acid sequence (D/N)X_n, wherein X is any amino acid and n is 9-14 amino acids.

36. The antibody-like protein of claim 35, wherein n is 14.

37. The antibody-like protein of claims 35 or 36, further comprising a second tenth fibronectin type III domain (^{10}Fn3).

38. The antibody-like protein of claim 37, wherein the second ^{10}Fn3 binds to IGF-IR.

39. The antibody-like protein of claim 37 or 38, wherein the second ^{10}Fn3 is covalently linked to the EGFR binding ^{10}Fn3 via a polypeptide linker or a polyethylene glycol moiety.

40. A pharmaceutically acceptable composition comprising the antibody-like protein of any one of claims 35-39, wherein the composition is essentially pyrogen free.

41. A method for treating a hyperproliferative disorder in a subject comprising administering to a subject in need thereof a therapeutically effective amount of the composition of claim 40.