A fibronectin scaffold approach to bispecific inhibitors of epidermal growth factor receptor and insulin-like growth factor-I receptor

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Abbreviations: EGFR, epidermal growth factor receptor; IGF-IR, insulin-like growth factor-I receptor; EI-Tandem, EGFR+IGF-IR-tandem adnectin; HER, human epidermal growth factor receptor; VEGFR2, vascular endothelial growth factor receptor-2; Eu, europium; SEC, size exclusion chromatography; TGI, tumor growth inhibition; TIW, three times weekly; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay; ECL, electrochemiluminescence; IFNα2a, interferon α-2a

Engineered domains of human fibronectin (Adnectins™) were used to generate a bispecific Adnectin targeting epidermal growth factor receptor (EGFR) and insulin-like growth factor-I receptor (IGF-IR), two transmembrane receptors that mediate proliferative and survival cell signaling in cancer. Single-domain Adnectins that specifically bind EGFR or IGF-IR were generated using mRNA display with a library containing as many as 10¹⁵ Adnectin variants. mRNA display was also used to optimize lead Adnectin affinities, resulting in clones that inhibited EGFR phosphorylation at 7 to 38 nM compared to 2.6 μM for the parental clone. Individual optimized Adnectins specific for blocking either EGFR or IGF-IR signaling were engineered into a single protein (EI-Tandem Adnectin). The EI-Tandems inhibited phosphorylation of EGFR and IGF-IR, induced receptor degradation and inhibited downstream cell signaling and proliferation of human cancer cell lines (A431, H292, BxPC3 and RH41) with IC₅₀ values ranging from 0.1 to 113 nM. Although Adnectins bound to EGFR at a site distinct from those of anti-EGFR antibodies cetuximab, panitumumab and nimotuzumab, like the antibodies, the anti-EGFR Adnectins blocked the binding of EGF to EGFR. PEGylated EI-Tandem inhibited the growth of both EGFR and IGF-IR driven human tumor xenografts, induced degradation of EGFR and reduced EGFR phosphorylation in tumors. These results demonstrate efficient engineering of bispecific Adnectins with high potency and desired specificity. The bispecificity may improve biological activity compared to monospecific biologics as tumor growth is driven by multiple growth factors. Our results illustrate a technological advancement for constructing multi-specific biologics in cancer therapy.

Introduction

Poly-genetic diseases such as cancer often require combinations of targeted agents to obtain tumor growth suppression. The ability to combine multiple activities into a single molecular entity has the potential to improve therapeutic outcomes, simplifying drug development and improving patient compliance.1 Targeting multiple essential pathways that drive cell growth and survival may enhance the spectrum of treatable tumor types or increase treatment response by limiting the impact of resistance mechanisms.2 The design of a dual specific antibody targeting human epidermal growth factor receptor 2 (HER2 or Erb-B2) and vascular endothelial growth factor (VEGF) by the mutation of a monospecific antibody is a step in this direction,3 but this specific approach may be limited in that the individual antigen binding sites are not spatially separated. In targeting the growth factor receptors EGFR and IGF-IR, Lu et al.4 combined the variable regions of two antibodies and constructed a bispecific antibody-like IgG fusion protein.5 Another approach to the construction of bispecific biologics involves the linkage of single-chain variable fragments consisting of two separate antigen-binding domains.6 Bispecific or multi-specific single molecular entities could avoid the increased time and higher cost associated with clinical evaluation of separate biologics and provide equivalent or greater potencies at lower doses compared to antibody combinations.1,7

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In this report, a modular approach to generating bispecific targeted biologics using protein binding units called Adnectins is presented. Adnectins are a class of targeted biologics derived from a domain of human fibronectin, an abundant extracellular protein. This fibronectin-based protein is especially adaptable for the development of multi-specific targeted therapeutics due to its native structure as a polyfunctional multi-domain species. Adnectins can be rapidly developed that bind target proteins of interest with high affinities. The first Adnectin tested in a clinical trial, CT-322, targets vascular endothelial growth factor-2 (VEGFR-2). Phase I studies on CT-322 showed that it was well tolerated and produced pharmacological effects expected from the inhibition of the VEGFR-2 pathway.13

The first example of a bispecific Adnectin, which targets cell signaling through EGFR and IGF-IR, is described here. EGFR is validated as a target for cancer therapy and numerous anti-EGFR drugs, including cetuximab, panitumumab, erlotinib and lapatinib, are approved by the United States Food and Drug Administration. Several drugs targeting IGF-IR have advanced to Phase 2 or Phase 3 human trials. Current monoclonal antibody drugs that target EGFR and HER-2, as well as small-molecule inhibitors of EGFR and HER-2 kinases, provide modest improvements in overall survival, thereby validating the anti-tumor activity of EGFR blockade. Furthermore, overexpression of EGFR, IGF-IR, and/or their ligands is common in several types of cancers, including breast, prostate, lung, colorectal, head and neck, and brain. Overexpression of EGFR and IGF-IR in NSCLC patients led to shorter disease-free survival. It was also shown that dual silencing of IGF-IR and EGFR decreased proliferation and increased apoptosis in colon carcinoma cells. Additionally, resistance to treatment with the EGFR inhibitor AG1478 increased IGF-IR levels, and resistance to the EGFR inhibitor gefitinib was associated with a heterodimerization of EGFR with IGF-IR. Therefore, simultaneous blockade of both EGFR and IGF-IR signaling has the potential to improve clinical efficacy in tumors driven by both receptors, by limiting the development of EGFR or IGF-IR resistance from cross-talk between the two signaling pathways.

Our results demonstrate that the bispecific EI-Tandem blocked the function of EGFR and IGF-IR in vitro cell-based assays and exhibited antitumor activity in nude mouse models where growth is driven by EGFR or IGF-IR.

Results

Identification and optimization of EGFR- and IGF-IR-binding Adnectins. Adnectins that bind 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. Adnectin-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 Adnectin proteins. A subset of target binders that blocked EGFR or IGF-IR signaling and had suitable biophysical properties were identified (Table 1). 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. EGFR Adnectins were tested by In-Cell western screening assays for the blockade of phosphorylation of EGFR and ERK, a downstream signaling molecule of EGFR activation. IC_{50} values obtained during the selection procedures ranged from 9 to 250 nM, providing the opportunity for choosing molecules from a wide range of potency values for the construction of bispecific Adnectins. Analogous studies were performed on optimized IGF-IR binders. Optimized EGFR-binding clones (E#1, E#2, E#3 and E#4) inhibited EGFR phosphorylation on Y1068 and downstream phosphorylation of ERK on Y204 of p42/p44 in vitro with IC_{50} values ranging from 7 to 40 nM, potencies that were more than 100-fold higher than the parental lead clone (Table 1).

The lead IGF-IR-Adnectin (I-lead) 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 1). Lead IGF-IR and EGFR single-domain Adnectins were >95% monomeric in most cases by size exclusion chromatography, had melting temperatures >60°C, and exhibited minimal immunogenic potential as predicted from EpiMatrix (<7 score for five out of six loops), a matrix-based algorithm for T-cell epitope mapping. Current monoclonal antibody drugs that target EGFR and HER-2, as well as small-molecule inhibitors of EGFR and HER-2 kinases, provide modest improvements in overall survival, thereby validating the anti-tumor activity of EGFR blockade. Furthermore, overexpression of EGFR, IGF-IR, and/or their ligands is common in several types of cancers, including breast, prostate, lung, colorectal, head and neck, and brain. Overexpression of EGFR and IGF-IR in NSCLC patients led to shorter disease-free survival.

Our results demonstrate that the bispecific EI-Tandem blocked the function of EGFR and IGF-IR in vitro cell-based assays and exhibited antitumor activity in nude mouse models where growth is driven by EGFR or IGF-IR.
In the RH41 and BxPC3 in vivo studies (Figs. 3C and 4B) the I-GS10-E#4-PEG clone was used as supplies of the E#1-GS10-I-PEG and I-GS10-E#4-PEG clone were exhausted. However, the E#1-GS10-I-PEG and E#4-GS10-I-PEG clones have been run side-by-side in an H292 tumor xenograft model and demonstrated comparable activity (data not shown). Therefore, while these two clones differ slightly in the amino acid sequence in the binding loops, they have comparable functional activity in vitro and in vivo and can be used interchangeably to illustrate the enhanced activity obtained from parable functional activity in vitro and in vivo and can be used.

The EGFR monospecific Adnectin inhibited proliferation (IC50 values were 0.9 ± 0.3 and 0.7 ± 0.4 nM, respectively (Fig. 2A)). This was expected, since in the E#1-GS10-I-PEG tandem domain further away from the EGFR binding loops. In RH41 cells, only the EI-Tandem and the monospecific IGF-IR Adnectin inhibited proliferation (IC50 values were 0.9 ± 0.3 and 0.7 ± 0.4 nM, respectively, Fig. 2B). This was expected, since RH41 is a pediatric rhabdomyosarcoma cell line that is known to be driven predominantly by IGF-IR signaling2 and thus is not sensitive to EGFR blockade.

**Inhibition of cell proliferation by EI-Tandem Adnectins.** The EGFR monospecific Adnectin inhibited proliferation of H292 cells with an IC50 value of 979 ± 78 nM. The IGF-IR monospecific Adnectin had an IC50 value of >8,400 nM while the EI-Tandem was considerably more potent with an IC50 value of 93 ± 14 nM (Fig. 2A). The H292 lung cancer cell line was utilized due to its high level of EGFR and IGF-IR expression and its in vitro sensitivity to growth inhibition by anti-EGFR agents.10,31 H292 also responds to stimulation with EGF and IGF ligands by activating EGFR and IGF-IR and downstream pathways. The increased activity of EI-Tandem compared to the monospecific Adnectins might be, in part, due to the differences in the positioning of PEG end groups in these molecules resulting from steric hinderance for binding to the EGFR or IGF-IR target. The EGFR-Adnectin has a 40 kDa branched PEG attached to its C-terminal end, which could perturb its function but in the EI-Tandem, the PEG is attached to the C-terminal end of the IGF-IR-Adnectin, thus positioning the PEG moiety one tandem domain further away from the EGFR binding loops. In RH41 cells, only the EI-Tandem and the monospecific IGF-IR Adnectin inhibited proliferation (IC50 values were 0.9 ± 0.3 and 0.7 ± 0.4 nM, respectively, Fig. 2B). This was expected, since RH41 is a pediatric rhabdomyosarcoma cell line that is known to be driven predominantly by IGF-IR signaling2 and thus is not sensitive to EGFR blockade.

### Table 1. Properties of monospecific and bispecific adnectins

| Name          | Tm °C | SEC % Monomer† | EGFR KD nM | IGF-IR KD nM | A431 pEGFR IC50 nM†† | A431 pERK IC50 nM†† | H292 pEGFR IC50 nM†† | H292 pIGF-IR IC50 nM†† | Ligand binding competition to EGFR/IGF-IR IC50 nM†† |
|---------------|------|----------------|------------|--------------|----------------------|---------------------|----------------------|-----------------------|-----------------------------------------------|
| E-parent      | 56   | ND**           | 42.5       | >95          | 0.7                  | NA††                | 2580                 | 2370                  | 1148 ± 21                         | ND 29 ± 12.73            |
| E#1          | 72   | >95            | 0.7        | 3.4          | 0.9                  | NA††                | 38 ± 15              | 40 ± 9                | 32 ± 1                | >3400 9.4 ± 3.68               |
| E#2          | 60   | >80            | 0.4        | 2.4          | 0.7                  | NA††                | 15 ± 8               | 11 ± 7                | 22 ± 1                | >7000 4.75 ± 1.77             |
| E#3          | 64   | >95            | 0.4        | 9.9          | 9.2                  | NA††                | 24 ± 7               | 13 ± 3                | 9 ± 2                 | >3400 15.9 ± 2.97            |
| E#4          | 69.2 | >95            | 0.13       | NA           | 7 ± 3                | 7 ± 4               | 15 ± 2               | >3400                  | 2.5                            |
| I-parent      | ND   | ND             | 1.8        | NA           | 7.3 ± 0.4            | ND††                | ND                   | ND††                  | ND††                            |
| I-lead        | 61.5 | >95            | 0.11       | >6210        | 19                   | 20                 | 8                   | 0.1                   | 2.1 ± 0.57                   |
| E#1-GS10-I   | 56   | 95             | 0.5        | 0.2          | 21                   | 20                 | 8                   | 0.1                   | 2.1 ± 0.57                   |
| E#1-GS10-I-PEG | 57.5 | >95            | 10.1       | 1.17         | 77                   | 78                 | 31                  | 0.18                  | 56.5 ± 24.5                |
| E#2-GS10-I   | 52   | 84             | 0.7        | 0.1          | 12                   | 14                 | 7                   | 6                    | 25.6 ± 6.5                  |
| E#2-GS10-I-PEG | 52.5 | 97             | 10.4       | 0.74         | 42                   | 40                 | 10                  | 6                    | 80.5 ± 12                   |
| E#3-GS10-I   | 48   | 75             | 3.8        | 0.8          | 36                   | 51                 | 30                  | 1                    | 51                            |
| E#3-GS10-I-PEG | 49   | 99             | 57.9       | 2.4          | 297                  | 295                | 123                 | 4                    | 396 ± 223                   |
| I-GS10-E#1-PEG | 60   | 99             | 3.6        | 0.46         | 97                   | 118                | 47                  | 0.8                   | 128 ± 4.95                  |
| I-GS10-E#4-PEG | 56   | >98            | 7.66       | 0.4          | 53                   | 47                 | 10                  | 0.3                   | 1.5                           |

Adnectin sequences are as follows: E-parent (MGV SDV PRD LEV VAA TPT SLL ISW QVP RPM YQY YRI TYG EGS NVQ EFT VFG GVR TAT ISG LKP GVD YTI TVY AVT DYM HSE VQR YPI SIN YRT EID KPS QHH HHN HHN); remaining sequences are the same with underlined regions representing the binding loops (BC, DE, FG) replaced with the following: E#1 (DSGGRGYSQ, GPVH, DPKHDHAGPHYTHYES), E#2 (LPGKRYQ, HDLR, NMMHVEYSEY), E#3 (VAGAEDYQ, HDLV, DMMHVEYTHYE), E#4 (WAPVDRYQ, RDVY, DKYPHDHAGPHYTHYES), I-parent (SPYLRVAR, SSAR, PSNIIGRHY), I-lead (SARLKVAR, KNVY, RFRDYQ). Sequence for the E#1-GS10-I-PEG tandem is as follows with the PEG attached to the C-terminal cysteine: (MGV SDV PRD LEV VAA TPT SLL ISW DSG RGS YQY YRI TYG EGS NVQ EFT VFG GVR TAT ISG LKP GVD YTI TVY AVT DYM HSE VQR YPI SIN YRT EID KPS QHH HHN HHN); remaining sequences are the same with underlined regions representing the binding loops (BC, DE, FG) replaced with the following: E#1 (DSGGRGYSQ, GPVH, DPKHDHAGPHYTHYES), E#2 (LPGKRYQ, HDLR, NMMHVEYSEY), E#3 (VAGAEDYQ, HDLV, DMMHVEYTHYE), E#4 (WAPVDRYQ, RDVY, DKYPHDHAGPHYTHYES), I-parent (SPYLRVAR, SSAR, PSNIIGRHY), I-lead (SARLKVAR, KNVY, RFRDYQ).
Treatment with 1 μM of the EI-Tandem decreased total EGFR levels significantly by 24 h, comparable to the EGFR degradation induced by cetuximab (Fig. 2C). IGF-IR levels were not significantly reduced in the EI-Tandem treated cells, although tandems constructed from other EGFR-Adnectins were able to induce IGF-IR degradation. A decrease in total EGFR was also detectable by immunocytochemical staining of DiFi cells after 48 h of treatment (Fig. 2D).

Antitumor effects and pharmacokinetics. Mice administered 10 or 100 mg/kg of PEGylated EI-Tandem via intraperitoneal (i.p.) injection exhibited peak levels of approximately 200 and 1,200 μg/mL of Adnectin, respectively, indicating dose-proportional pharmacokinetics (Fig. 3A). Systemic clearance also supported linear pharmacokinetics between the two doses administered. The half-life of the PEGylated EI-Tandem in mice was 15.75 ± 1.52 h (Fig. 3A). Thus, administration of 100 mg/kg three times weekly (TIW) was able to maintain drug levels 10- to 100-fold higher than the in vitro IC₅₀ value.

To test the antitumor activity of the PEGylated EI-Tandem, H292 cell derived tumors were implanted in athymic mice and allowed to establish growth for six days prior to initiation of dosing. The PEGylated EI-Tandem was administered TIW at 100 mg/kg and, as a positive control, panitumumab was administered at 1 mg/mouse every three days. Treatments were formulated in PBS and administered by i.p. route. The EI-Tandem demonstrated significant tumor growth inhibition (TGI) relative to untreated animals (TGI = 94%, p = 0.0005) and the activity was comparable to panitumumab (TGI = 101%, p = 0.0002, Fig. 3B). The tumor inhibition was achieved without evidence of toxicity, based on <10% reduction in weight over the course of the study (data not shown). Efficacy was also evaluated in the RH41 xenograft where growth is driven by IGF-IR. RH41 tumor fragments were implanted and allowed to grow for 18 days until the desired size range of 50–150 mg was reached. The PEGylated EI-Tandem and panitumumab were administered as above and the EI-Tandem was active (TGI = 58.6%, p = 0.044) while panitumumab showed no activity (Fig. 3C).

The lack of efficacy for panitumumab is expected in RH41, as these tumors do not respond to EGFR inhibition. An IGF-IR antibody, MAB391 (R&D Systems®) or the IGF-IR-Adnectin alone exhibited similar activity to the EI-Tandem (%TGI = 58% and 61%, respectively) in RH41 tumor xenograft studies (data not shown) but were not included in the study shown in Figure 3C.

The EI-Tandem inhibited phosphorylation of EGFR in the H292 treated tumors shown in Figure 3B and also reduced total levels of EGFR, by the end of the study (Fig. 3D). Levels of phosphorylated ErbB2 were also lower in H292 treated tumors.

Blockade of EGFR and IGF-IR pathways in vitro and in vivo. To understand the dynamics of EGFR/IGF-IR signaling and its inhibition by the EI-Tandem, BxPC3 cells were serum-starved, exposed to 1 μM PEGylated EGFR-Adnectin, IGF-IR Adnectin, EI-Tandem Adnectin, or vehicle control for 1 h, then stimulated with either EGF, IGF-I or EGF + IGF-I for 10 min (Fig. 4A). The basal levels of phosphorylated EGFR, IGF-IR and AKT were nearly undetectable after serum deprivation. Stimulation with EGF induced EGFR phosphorylation, but did not trans-activate IGF-IR. EGFR phosphorylation was blocked by the
EGFR-Adnectin and the EI-Tandem, but not the IGF-IR-Adnectin. Similarly, stimulation with IGF-I induced strong phosphorylation of IGF-IR that was partially blocked by the IGF-IR-Adnectin and the EGFR-Adnectin. Interestingly, complete blockade of IGF-I-stimulated IGF-IR phosphorylation could only be obtained by the EI-Tandem and not by the IGF-IR or EGFR-specific monoadnectins alone, indicating crosstalk between the two pathways. EGF stimulation did not activate AKT signaling in this cell line, but IGF-I and EGF+IGF-I strongly induced phosphorylation of AKT that was partially suppressed by the IGF-IR-Adnectin and reduced to basal levels by the EI-Tandem. The EGFR-Adnectin partially reduced pAKT induced by the combination of EGF and IGF-I while the IGF-IR-Adnectin showed a greater degree of suppression of pAKT. However, only the EI-Tandem completely reduced IGF-I or EGF+IGF-I-stimulated pAKT, suggesting that the EGFR-pathway activates AKT signaling following IGF-I stimulation. Levels of total EGFR and IGFIR protein did not change from these treatments due to the short time cells were exposed to compound (1 h). Levels of GAPDH protein were also unchanged. These results illustrate the complex cross-talk between the EGFR and IGF-IR pathways and feed-back mechanisms.

In mice bearing BxPC3 xenografts, the EI-Tandem inhibited tumor growth more (TGI = 78%) than the EGFR-mononectin (TGI = 61.2%), the IGF-IR mononectin (TGI = 14.3%) and cetuximab (TGI = 62.6%; p = 0.027 for EI-Tandem vs. cetuximab, Fig. 4B). The EI-Tandem was also significantly more potent than a mixture of the individual EGFR and IGFIR monospecific Adnectins (TGI = 68.8%, p = 0.04), suggesting that there may be functional benefit to having both activities in a single molecule.

Identification of EI-Tandem binding sites on EGFR. Biacore competition binding assays were used to test whether the Adnectin binding site on EGFR overlaps with those of clinically approved EGFR antibodies (Fig. 5A). The binding of EGFR-Adnectin to EGFR adsorbed on the Biacore chip did not competitively inhibit the binding response of cetuximab, panitumumab or nimotuzumab to EGFR, indicating that the EGFR-Adnectin binds to a site distinct from those bound by the antibodies. Furthermore, an X-ray crystal structure of the EGFR-Adnectin in complex with EGFR supported the binding studies, showing that the EGFR-Adnectin bound to domain I of EGFR (unpublished results). The Adnectin binding site overlaps with the EGF and TGFα binding sites on EGFR, but not with the cetuximab or nimotuzumab binding sites, which are in domain III.54,55 Biacore experiments using sequential injections of the EGFR, bispecific Adnectin and IGF-IR demonstrated that bispecific Adnectin is capable of binding to EGFR and IGF-IR at the same time (Fig. 5B).

Discussion

Adnectins that specifically inhibited either EGFR or IGF-IR were selected for and optimized using a previously published mRNA display and selection technique.3 Monospecific Adnectins against EGFR or IGF-IR were linked to form bispecific EI-Tandem proteins. The EI-Tandems retained the individual receptor-specific antagonism of the parent molecules and inhibited the phosphorylation of EGFR and IGF-IR at nanomolar concentrations. These bispecific Adnectins had favorable drug-like physical characteristics in that they had a high $T_m$ and existed as homogeneous, stable monomers. An unoptimized expression system in
panitumumab and nimotuzumab, although both the EGFR-Adnectin and the EI-Tandem were competitive with EGF binding to EGFR. Our results demonstrate a novel approach to engineering bispecific biologics, illustrated here by a bispecific Adnectin that binds and blocks EGFR and IGF-IR pathways with high potency.

The modularity of Adnectins allows greater ease and versatility in the construction of multispecific molecules than afforded by antibodies. While research on bispecific antibodies has progressed steadily since the early innovations of “knobs-into-holes,” a strategy adopted to engineer Cβ3 domains for heterodimerization,36,37 development of bispecific antibody drugs remains challenging. These difficulties include the production of soluble single chain antibodies in E. coli, requirement for disulfide linkage for stabilization, low level of expression in mammalian cells, or rapid clearance of antibody fragments from the circulation.6,38-41 Adnectin tandems, in contrast, can be assembled readily from individual functional elements using simple linker designs. Further, antibodies requiring glycosylation and other post-translational modifications for full activity are generally produced in Chinese hamster ovary cells.42,43 In contrast, Adnectins do not require post-translational modification for activity and have excellent thermal stability. Furthermore, Adnectins can be rapidly developed in comparison to the extended periods required for development of antibody constructs.

PEGylation of the EI-Tandem Adnectin increases its solubility in aqueous solutions and will increase circulating serum half-life. It may also result in decreased immunogenicity and stabilize the protein against proteolysis. In mice, antibodies like cetuximab can have a half-life of several weeks while in humans the half-life at therapeutic doses ranges from 66–97 h.44 This is probably due to the fact that cetuximab does not bind mouse EGFR and thus is not cleared as quickly in mice by receptor mediated mechanisms as it is in humans. Because the EI-Tandem does bind mouse EGFR the mouse data may more readily be scaled to humans. While it is not

Figure 3. Pharmacokinetic profile and in vivo activity of EI-Tandem. (A) Plasma levels of EI-Tandem after dosing i.p. with 100 mg/kg (○) and 10 mg/kg (□) of EI-Tandem. (B) H292 xenografts were either untreated (○); dosed three times a week with 100 mg/kg PEGylated EI-Tandem formulated in PBS (□) or dosed every three days i.p. with panitumumab (○). Dosing schedule of EI-Tandem (I) and panitumumab (x) are indicated on the x-axis. (C) Efficacy of PEGylated EI-Tandem against RH41 Ewing sarcoma xenografts. Treatments and schedule are as described for the H292 model. The EI-tandem dosed in the RH41 model was the I-GS10-E4-PEG construct (Table 1). Inhibition of growth was significantly different for panitumumab and EI-Tandem vs. control in H292 and EI-Tandem vs. control in RH41 by a two-tailed paired t-test as described in Experimental Procedures. (D) The effects of EI-Tandem (open bars) and panitumumab (hatched bars) on pEGFR, total EGFR and pErb-B2 in H292 tumors as determined by Meso Scale analysis. “C” signifies control levels (black bars) measured in untreated H292 tumors sampled on day 20 from the experiment shown in (B). Time points indicate hours after the last dose of treatment in the study presented in (B). Error bars indicate SD.

shake flasks provided 230 mg/L of purified, PEGylated material showing the potential for scalable manufacturing of bispecific Adnectins. The high yield of purified product obtained at the research scale suggests the likelihood that efficient manufacturing and long-term product stability are readily achievable. The EI-Tandem inhibited tumor growth of H292 xenografts in vivo comparably to panitumumab and was active against RH41 xenografts where panitumumab was not active. Competitive binding studies indicated that the EGFR-Adnectin bound to a site on EGFR distinct from that of marketed antibodies cetuximab,
known what the half-life of the PEGylated EI-Tandem Adnectin will be in humans, by comparison to another PEGylated protein of similar size, it can be expected to exhibit a half-life on the order of 50–80 h, making once a week dosing possible. PEGasys\textsuperscript{\textregistered} is recombinant interferon α-2a (IFNα2a) a protein of 20 kDa (comparable in size to the EI-Tandem Adnectin) conjugated to a 40 kDa branched PEG molecule. Without the PEG, IFNα2a has a plasma half-life of 3–8 h while the PEGylated form has a half-life of 50–80 h.\textsuperscript{45}

EGFR and IGF-IR share downstream signaling pathways such as PI3K/AKT and RAF/MEK/ERK, facilitating cell proliferation and survival. The EI-Tandem inhibited phosphorylation of pERK and pEGFR at similar concentrations in A431 and H292 cells. Treatment of BxPC3 cells with the EI-Tandem demonstrated the crosstalk that operates between the EGFR and IGF-IR pathways and the ability of EGFR to transactivate IGF-IR and induce AKT signaling through IGF-IR. Only simultaneous blockade of EGFR and IGF-IR completely inhibited IGF-I-stimulated increases in pEGFR, pIGF-IR and pAKT, demonstrating transactivation of IGF-IR through EGFR. Indeed, IGF-I has been shown to transactivate the EGFR through the IGF-IR.\textsuperscript{48} Furthermore, the bispecific inhibition provided by the EI-Tandem resulted in enhanced activity for inhibition of BxPC3 tumor growth in mice compared with monospecific EGFR or IGF-IR Adnectins alone.

The EI-Tandem inhibited EGFR or IGF-IR phosphorylation in several cell lines (H292, A431 and BxPC3), as measured by multiple techniques including In-Cell westerns, ELISA and immunoblots. These results suggest a potential to inhibit tumor growth broadly.

In vivo, the EI-Tandem downregulated pEGFR, total EGFR, as well as pERB2 (HER-2) in tumors. Since the EGFR family of receptors form homo- and heterodimers with each other and contribute to the regulation of cell proliferation, decreased phosphorylation of HER-2 could play a role in the antitumor effects of the EI-Tandem. It has been recently reported that treatment with cetuximab also decreased phosphorylation of HER-2 in several cancer cell lines.\textsuperscript{45}

A key feature of anti-EGFR antibodies is that, upon binding to EGFR, the receptors undergo internalization and degradation,\textsuperscript{46,47} suggesting that this activity may be part of the antiproliferative and antitumor effects of these antibody drugs. Similar to the antibody drugs, the EI-Tandem also induced EGFR downregulation, potentially due to receptor degradation. While the E\textsuperscript{1}-GS10-I-PEG EI-Tandem clone did not induce significant downregulation of IGF-IR in vitro, the L-GS10-E\textsuperscript{4}-PEG clone was a potent inducer of IGF-IR degradation in vitro and this may offer therapeutic advantages.\textsuperscript{35}

Biologic therapeutics are considered to be the new frontier for the pharmaceutical industry\textsuperscript{49} and there is increasing interest in combining multiple biologics to treat cancer. However, combinations of biologics can be prohibitively expensive and are limited by the number of available biologics in the market. Building multiple activities into one molecule should be more cost-effective, allowing more efficient product development. Adnectins offer many advantages including speed of development and ease of engineering multi-target specificity.

In summary, our results demonstrate a novel, straightforward technology for designing multi-specific biologic agents. Here we developed a bispecific Adnectin that blocked two growth factor receptor pathways operative in tumor tissues and demonstrated its antitumor activity. Targeting two specific receptors of critical importance in tumor growth by this novel technology holds promise for cancer therapy. As new receptor targets are identified by microarray and proteomics technologies, the Adnectin platform could be used to rapidly build potent multi-specific therapeutics against these new targets.

Figure 4. Effect of EI-Tandem on EGFR and IGF-IR crosstalk in vitro and in vivo. (A) BxPC3 cells were serum starved before stimulation with either EGF, IGF-I or a combination of EGF+IGF-I. All treatments were separated by SDS-polyacrylamide gel electrophoresis, transferred to membrane and probed with various antibodies, followed by GAPDH as an internal loading control. (B) Efficacy of PEGylated EI-Tandem against BxPC3 xenografts. Animals were untreated (○); dosed three times a week with 100 mg/kg EI-Tandem (□), dosed three times a week with 50 mg/kg EGFR-Adnectin (solid red line), IGF-Adnectin (solid blue line) or a combination of 50 mg/kg EGFR-Adnectin + 50 mg/kg IGF-Adnectin (solid green line). For comparison, an EGFR antibody was administered in one group with the optimal dose of 40 mg/kg cetuximab every three days (○). All treatments were by the i.p. route. The data represent the means ± SE of tumor sizes from eight animals in each group. Dosing schedule of EI-Tandem or monospecific Adnectins (I) and cetuximab (x) are indicated on the x-axis. BxPC3 immunoblot and xenograft were carried out with the L-GS10-E\textsuperscript{4}-PEG construct (Table 1).
mRNA-peptide display technology were described previously.\textsuperscript{8-11} Primary selection was based on the binding of Adnectins in the library to EGFR or IGF-IR extracellular domain. After four rounds of selection and enrichment, populations of binders were sequenced and several hundred Adnectins were expressed and assayed for binding affinity and cell-based functional activity against the targets. Based on this data, a parental clone for each target was chosen and optimized by additional rounds of PROfusion. Dozens of optimized clones were tested for binding affinity and in cell-based functional assays (see methods below) prior to selection of clones for the construction of EI-Tandems.

Construction and characterization of EI-Tandems. EI binders were produced by covalently linking an EGFR-binding Adnectin to an IGF-IR-binding Adnectin using a glycine-serine linker. Four different EGFR (E) clones (E#1, E#2, E#3, E#4) and one IGF-IR (I) clone (I-lead) were used in the construction of bispecific EI-Tandems in two possible orientations; either EI or IE. Adnectins or Adnectin-tandems were cloned into the pET9d or pET29 vectors as His\textsubscript{6} tag fusions. DNA was transformed into \textit{E. coli} HMS174(DE3) or BL21(DE3) (EMD Biosciences) and cells were inoculated in LB medium containing 50 \(\mu\)g/mL kanamycin. Expression cultures were grown in Studier ONE auto-induction medium (Novagen/EMD Biosciences\textsuperscript{®}). The clarified lysate was purified using a Ni-NTA Superflow matrix column (Qiagen\textsuperscript{®}) with standard washing and elution methods.\textsuperscript{23} To allow for PEGylation, the protein was modified near the C-terminus by a serine to cysteine mutation. PEGylation of the protein was accomplished by incubating maleimide-derivatized PEG reagent with the protein solution.\textsuperscript{24} Progress and confirmation of the PEGylation reaction was confirmed by SDS-PAGE and SEC. Separation of reaction products from precursors was accomplished by ion exchange chromatography. Lipo-polysaccharides were depleted during this process and further removed with SartoBind Q charged membranes (Sartorius AG). Endotoxin levels were determined using the EndoSafe PTS LAL assay (Charles River Laboratories\textsuperscript{®}). The average yield of this purified, PEGylated EI-Tandem from three different preparations was 230 mg per liter of \textit{E. coli}.

Competitive ligand-binding assays. To measure inhibition of EGF binding, A431 cells were plated at 15,000 cells/well in 96-well plates and incubated for 48 h. Cells were washed and incubated with Adnectins diluted in starvation media (DMEM + 0.1% BSA) for 30 min at 37°C. Europium (Eu)-labeled EGF (PerkinElmer\textsuperscript{®}) was added at 10 nM and plates were incubated for 3 h at 4°C in the dark. After washing, Enhancement solution (PerkinElmer\textsuperscript{®}) was added to the plates and incubated for 1 h at 37°C, and the plates were read on the Flexstation II (Molecular Devices\textsuperscript{®}). IC\textsubscript{50} values were calculated with Softmax plus software. To measure inhibition of IGF-I binding, MCF-7 cells were plated at 50,000 cells/well in 24-well plates in RPMI 1640 with 10% FBS. After 24 h, cells were washed with RPMI 1640 containing 0.1% BSA and pre-incubated for 30 min on ice with 200 \(\mu\)L binding buffer and IGF-IR Adnectin. [\textsuperscript{125}I]-IGF-I, (40 pM ~ 60,000 cpm) from Perkin Elmer, was added and incubated for 3 h on ice. Cells were then washed with PBS containing 0.1% BSA and lysed with 500 \(\mu\)L of 0.1% SDS + 0.5 N NaOH.

**Experimental Procedures**

Cell lines. The following human cell lines were used: H292 (non-small cell lung carcinoma), A431 epidermoid carcinoma, MCF7 (breast carcinoma), BxPC3 (pancreatic carcinoma) and DiFi (colorectal carcinoma). DiFi cells were obtained from Dr. Zhen Fan (MD Anderson Cancer Center, Houston, TX) and all other cell lines were purchased from American Type Culture Collection. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 10 mM HEPES except A431, which were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate and 1.0 mM sodium pyruvate.

Initial identification of EGFR and IGF-IR-binding adnectins. The generation of libraries of variants based on the tenth human fibronectin type III domain scaffold and PROfusion

![Figure 5. The EGFR-Adnectin does not compete for binding of EGFR antibodies to EGFR. (A) Initial injection of the EGFR-Adnectin shows binding to EGFR on the surface of the chip. A second injection of EGFR-Adnectin mixed with an equal amount of cetuximab, panitumumab, or nimotuzumab showed no competition for binding of antibodies to EGFR by the Adnectin. (B) The EI-Tandem Adnectin can bind EGFR and IGF-IR simultaneously. Initial injection of EI-Tandem Adnectin showed binding to EGFR immobilized on the chip surface. A second injection of EI-Tandem plus soluble IGF-IR showed a second binding event for sIGF-IR to other end of the immobilized EI-Tandem Adnectin.](image-url)
Radioactivity of the lysates was measured using a Wallac 1470 Gamma Counter (Perkin Elmer®) and the data analyzed using SigmaPlot® (Systat®).

In-cell western assay. A431 cells were seeded into poly-D-lysine coated 96-well microtiter plates (Becton Dickinson®). Inhibition of EGF-induced phosphorylation of EGFR and ERK1/2 by Adnectins was determined using appropriate antibodies™ using an Odyssey® Infrared Imaging System (LI-COR®). Each clone was assayed in duplicate or triplicate and values were normalized to β-actin or total ERK1/2 levels. IC<sub>50</sub> values were calculated from linear regression analysis of percent inhibition of maximum signal minus background.

Size exclusion chromatography and LC-MS. SEC was performed on the purified proteins using a Superdex 200, 5/150 column with UV detection at 214 nm and 280 nm and with fluorescence detection (excitation = 280 nm, emission = 350 nm). Adnectins were further analyzed by LC-MS liquid chromatography HPLC system coupled with Waters® Q-ToF API mass spectrometer. Samples were diluted to approximately 0.5 mg/mL with HPLC-grade water and analyzed using a Jupiter C18 column (Phenomenex®).

Determination of binding affinity. Surface plasmon resonance (Biacore®) analysis was performed to determine off-rates or binding affinities of Adnectins to EGFR-Fc or IGF-IR-Fc. 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 expression vector containing the hinge and constant regions of human IGF-IR (aa 1–932) was purchased as a fusion protein, IGF-IR-Fc, which was purified by Protein A chromatography. Human EGF-R-Fc (aa 1–645) was purchased from R&D Systems®. Typically, anti-human IgG (25 µg/mL in sodium acetate pH 5.0) was immobilized on flow cells 1 and 2 of a CM5 chip and protein A (50 µg/mL in sodium acetate pH 4.5) was immobilized on flow cells 3 and 4 following the manufacturer’s recommendations (GE Healthcare). EGF-R-Fc (7 µg/mL) and IGF-IR-Fc (9 µg/mL) were captured on flow cell 2 and 4, respectively, using two minute injections at 5 µL/min. Binding of Adnectin analytes (1–600 nM) was examined in 10 mM Hepes, 150 mM NaCl, 3 mM EDTA and 0.05% Tween-20, pH 7.4 using a 240 sec contact time and 600 sec dissociation time at a flow rate of 30 µL/min. The anti-human IgG surface was regenerated between cycles using two 30 sec injections of 3 M MgCl₂ (30 µL/min) while the protein A surface was regenerated using two 60-sec injections of 10 mM glycine pH 1.5 (10 µL/min). Sensorgrams were evaluated using Biacore® T100 Evaluation Software, Version 1.1.1 (GE Healthcare/Biacore®) to determine the rate constants k<sub>a</sub> (k<sub>aoff</sub>) and k<sub>s</sub> (k<sub>aon</sub>) and the affinity (K<sub>d</sub>) was calculated from the ratio of rate constants k<sub>a</sub>/k<sub>s</sub>. Adnectins were evaluated for specificity in a similar format using anti-human IgG to capture HER2-Fc, HER3-Fc and HER4-Fc (R&D Systems®).

For Biacore® competition experiments, EGF-R-Fc (3 µg/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. EGF-Fc antibodies were obtained as marketed drug and competition between Adnectins and antibodies for binding to EGFR-Fc was assessed by binding 450 nM EGFR clone E#1 (30 µL/min, 200 s contact time), immediately followed by 450 nM E#1 alone, or a mixture of 450 nM E#1 plus 450 nM cetuximab, panitumumab or nimotuzumab (30 µL/min, 200 sec contact time). The surface was regenerated between cycles using two 10 sec pulses of 50 mM NaOH at a flow rate of 30 µL/min.

Thermal scanning fluorimetry. All samples were diluted with PBS to 0.2 mg/mL. Sypro Orange (Invitrogen™) was added to the sample in a 96-well plate and mineral oil was overlaid on top of the sample to prevent evaporation. The plate was covered and centrifuged for 30 sec to remove air bubbles and the samples scanned from 25 to 95°C on a Biorad® CFX-96 (Bio-Rad®) at a scan rate of 120°C/h. Data analysis was performed for the inflection point with the CFX software.

Anti-proliferative activity in H292 and RH41 cells. Briefly, 2,000 cells/well were plated into 96-well microplates in RPMI-1640 medium and allowed to adhere for 24 h. Seeding density was selected to maintain exponential growth without the control cells reaching confluence during the assay. Serial dilutions of Adnectins were added 24 h after plating and cells were incubated for 72 h. Cells were treated with CyQUANT® NF reagent (Invitrogen™) for 1 h at 37°C and total DNA was quantified by reading fluorescence with 485 nm excitation and 530 nm emission on a CytoFluor® 4000 instrument (Applied Biosystems®). Linear regression analysis of the percent of inhibition by test compound was used to determine IC<sub>50</sub> values.

Immunoblot analysis. Cells were treated with Adnectins for the indicated time periods and analyzed by immunoblotting as described in reference 27 except membranes were incubated with the appropriate secondary antibodies (LI-COR® Biosciences) and protein visualization was performed on an Odyssey® Infrared Imaging System. The total IGF-IR antibody was from Santa Cruz Biotechnology® and GAPDH antibody was from Cell Signaling Technology®.

Inhibition of IGF-IR/EGFR phosphorylation in H292 cells. H292 cells (65,000 cells/well) were plated in 96-well plates and incubated overnight. After 24 h, cells were washed once and then incubated for 24 h in serum-free media. Serial dilutions of Adnectins were added, and cells were incubated for 3 h. Cells were stimulated with 100 ng/mL of IGF-1 or EGF for 10 min at 37°C. Media was removed and cells lysed in 100 µL of cell lysis buffer as described for immunoblotting. Cells were incubated at room temperature for 15 min, lystate was transferred to an enzyme-linked immunosorbent assay (ELISA) measuring phospho-IGF-IR (tyrosine 1131) or phospho-EGFR (tyrosine 1068) (Cell Signaling Technology®), and the assay was completed according to the manufacturer’s procedure.

Antitumor testing. Female athymic (nude) mice 5–6 weeks of age were obtained from Harlan Laboratories. Mice were quarantined for three weeks prior to their use in experiments. Animals were provided food and water ad libitum and cared for according to Association for Assessment and Accreditation of Laboratory Animal Care International and Bristol-Myers Squibb guidelines. Tumors were propagated by subcutaneous implantation in nude mice. Tumors were implanted subcutaneously with 1 mm³ fragments in the hind flank and allowed to establish to a size of 50–150 mg prior to initiation of treatment, which occurred...
Pharmacokinetics of the EI-Tandem 40 kDa PEG conjugate was studied in mice at 10 and 100 mg/kg. Animals (n = 3 per group) were injected i.p. with Adnectin diluted in PBS and serial blood samples (~0.05 mL) were obtained by nicking the lateral tail vein. Blood samples were diluted into a citrate phosphate dextrose solution in a 1:1 ratio and plasma was prepared. A quantitative electrochemiluminescence (ECL) assay was developed to detect the EI-Tandem Adnectin in plasma samples. In this assay, IGF-IR-Fc protein was adsorbed to a Meso Scale Discovery® plate overnight at 4°C. Plasma was added to the plate and incubated at 22°C for 1 h to capture the Adnectin. Immobilized EI-Tandem was detected by a rabbit polyclonal antibody specific to the PEG attached to the Adnectin mixed with a goat anti-rabbit antibody linked with a SULFO-TAG™. Unbound SULFO-TAG™ reagent was washed away and ECL detection was used to quantify EI-Tandem based on comparison to a 4-parameter fit of a standard curve of the EI-Tandem Adnectin.

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Conflict of Interest

Authors are employees of Adnexus or Bristol-Myers Squibb.

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