Anti-tumor effect of CT-322 as an adnectin inhibitor of vascular endothelial growth factor receptor-2

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Abbreviations: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; TKI, tyrosine kinase inhibitor; 10Fn3, tenth human fibronectin type III domain; RGD, arginine-glycine-aspartate sequence; FG, the peptide loop between the F and G strands of the 10Fn3; SGE, serine-glycine-glutamic acid sequence; HUVEC, human umbilical vein endothelial cells; EBM, endothelial basal medium; FGF-2, fibroblast growth factor-2; RIPA, radio-immunoprecipitation; SDS, sodium dodecyl sulfate; KDR, kinase insert domain receptor; ip, intraperitoneally; SPR, surface plasmon resonance

CT-322 is a new anti-angiogenic therapeutic agent based on an engineered variant of the tenth type III domain of human fibronectin, i.e., an Adnectin™, designed to inhibit vascular endothelial growth factor receptor (VEGFR)-2. This PEGylated Adnectin was developed using an mRNA display technology. CT-322 bound human VEGFR-2 with high affinity (KD, 11 nM), but did not bind VEGFR-1 or VEGFR-3 at concentrations up to 100 nM, as determined by surface plasmon resonance studies. Western blot analysis showed that CT-322 blocked VEGF-induced phosphorylation of VEGFR-2 and mitogen-activated protein kinase in human umbilical vascular endothelial cells. CT-322 significantly inhibited the growth of human tumor xenograft models of colon carcinoma and glioblastoma at doses of 15–60 mg/kg administered 3 times/week. Anti-tumor effects of CT-322 were comparable to those of sorafenib or sunitinib, which inhibit multiple kinases, in a colon carcinoma xenograft model, although CT-322 caused less overt adverse effects than the kinase inhibitors. CT-322 also enhanced the anti-tumor activity of the chemotherapeutic agent temsirolimus in the colon carcinoma model. The high affinity and specificity of CT-322 binding to VEGFR-2 and its anti-tumor activities establish CT-322 as a promising anti-angiogenic therapeutic agent. Our results further suggest that Adnectins are an important new class of targeted biologics that can be developed as potential treatments for a wide variety of diseases.

Introduction

Angiogenesis, the growth of new microvessels, is a key contributor to tumor growth.1 Vascular endothelial growth factor (VEGF)-A and its isoforms are pro-angiogenic factors that bind and activate the receptor VEGFR-2, which is expressed on the surface of vascular endothelial cells. VEGF-A binding to VEGFR-2 leads to autophosphorylation of tyrosine residues at the carboxy-terminal of the receptor, which initiates cell signaling, vasculogenesis and angiogenesis. Two other homologous VEGF receptors, VEGFR-1 and VEGFR-3, have overlapping and regulatory functions, including feedback regulation for VEGFR-1 and formation of lymphatic vasculature for VEGFR-3.2,3 Three members of the human VEGF family, VEGF-A, VEGF-C and VEGF-D, activate VEGFR-2. While VEGF-A stimulates the growth of new blood vessels, VEGF-C and VEGF-D are primarily known as stimulators of lymphangiogenesis by activating VEGFR-3, although they may also stimulate blood vessel growth since they activate VEGFR-2.2,4

The development of drugs that inhibit angiogenesis has been a breakthrough in cancer treatment. Currently marketed anti-angiogenic agents include a monoclonal antibody (bevacizumab) that specifically binds VEGF-A, thereby preventing its binding to VEGFR-1 and VEGFR-2, and small molecule tyrosine kinase inhibitors (sorafenib, sunitinib and pazopanib) that inhibit multiple receptor tyrosine kinases. However, the treatment of metastatic cancer and other indications requires new agents that are selective for specific targets or activate a small subset of kinases of the family. The high affinity and specificity of CT-322 for VEGFR-2 and its anti-tumor activities establish CT-322 as a promising anti-angiogenic therapeutic agent. Our results further suggest that Adnectins are a new class of targeted biologics that can be developed as potential treatments for a wide variety of diseases.
inhibitors (TKIs) (sorafenib and sunitinib) that bind to ATP binding sites in the intracellular domains of VEGR-1, VEGFR-2, or VEGFR-3, as well as several other tyrosine kinases. The different mechanisms of action of these agents result in different therapeutic indices of tumor suppression and toxicity, and suggest advantages of specificity in VEGFR-2 blockade. For example, the lack of specificity of TKIs may result in adverse effects, as well as the desired blockade of VEGF-A-mediated activities. In addition, an antibody specific for VEGF-A may not have maximal efficacy if VEGF-C and -D are overexpressed in tumor specimens since VEGF-C and VEGF-D are ligands for VEGFR-2 and VEGFR-3.

CT-322 is a PEGylated Adnectin, designed to bind potently and specifically to VEGFR-2. A recent report illustrated the anti-tumor effects of CT-322 in a single orthotopic mouse model of pancreatic cancer. We now present the full preclinical in vitro and in vivo characterization of CT-322, from its early discovery to biological activity in vivo, including anti-tumor effects in xenograft models.

**Results**

In vitro characterization of CT-322. The amino acid sequences of the binding loops of C7 and (thus CT-322) are devoid of homology to wild type or SGE control (Table 1). PEGylation of C7 with a 40-kDa branched PEG created CT-322, which retained a relatively high affinity for hVEGFR-2 (K_D, 11 nM) and reduced but substantial affinity (K_D, 250 nM) for mVEGFR-2.

Migration of CT-322 on SEC column indicated that CT-322 was a single species with an apparent MW >450 kDa (Fig. 1A Suppl. Material). Apparent molecular weight of PEGylated proteins are generally reported to be 10 to 15-fold higher than the expected molecular weight. To resolve the molecular mass of CT-322, a recent report illustrated the anti-tumor effects of CT-322 in a single orthotopic mouse model of pancreatic cancer. We now present the full preclinical in vitro and in vivo characterization of CT-322, from its early discovery to biological activity in vivo, including anti-tumor effects in xenograft models.

CT-322 bound specifically to hVEGFR-2 with a K_D of 11 nM, as measured by surface plasmon resonance (SPR; Fig. 1A, Table 1). Binding to hVEGF-R1 or hVEGF-R3 was not detected at CT-322 concentrations up to 100 nM. As shown in Figure 1B, CT-322 competitively inhibited cellular proliferation induced by the activation of VEGFR-2 by VEGF-A, VEGF-C, and VEGF-D in a murine pre-B cell line transfected with KDR from hVEGF-R2. The IC_50 values were in the range of 10–80 nM. CT-322 and C7 competitively inhibited the proliferation of HUVEC in response to VEGF-A with similar potency (Fig. 2, Suppl. Material).

VEGF-A-induced HUVEC proliferation was completely blocked by CT-322 at 55 nM, p = 0.004 (Fig. 1C). This effect was specific for the VEGF-2 binding of CT-322 as the control Adnectin, SGE, had no effect on the VEGF-A-induced endothelial cell proliferation (p = 0.5). Furthermore, the inhibition of proliferation was specific for VEGF-A-mediated effects, since 600 nM CT-322 had no effect on FGF-induced proliferation. Thus, CT-322 inhibited VEGF-A-induced endothelial cell functions, which defined its anti-angiogenic mechanism in vitro.

Western blot analysis (Fig. 1D) qualitatively demonstrated that CT-322 inhibited VEGF-A-induced intracellular signaling in HUVEC. Addition of VEGF (20 ng/mL) produced a dramatic increase in the level of phosphorylated MAP kinase (p = 0.5). Furthermore, the inhibition of phosphorylation was specific for VEGF-A-mediated effects, since 600 nM CT-322 had no effect on FGF-induced phosphorylation. As an independent dimerization assay also showed inhibition by CT-322 (see Fig. 3 Suppl. Materials).

Pharmacokinetics of CT-322 in cynomolgus monkeys. The plasma time-concentration curves following dosing with C7, C7-20PEG and CT-322 are shown in Supplemental Figure 4. C7 was cleared rapidly from the blood with a terminal T1/2 of 4 ± 2 hours, while a 20 kDa and 40 kDa PEG increased the T1/2 to 17 ± 2 hours and 50 ± 20 hours, respectively (Suppl. Table 1). These results indicated that the clearance rate of an Adnectin could be modified with the size of the PEG moiety.

Anti-tumor effects of CT-322. CT-322 significantly inhibited growth of established U87 human glioblastoma xenografts

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**Table 1.** In vitro binding characteristics of C7 and CT-322

| Adnectin | BC | DE | FG | Human | Murine | Human | Murine | Human | Murine | Human | Murine |
|----------|----|----|----|-------|--------|-------|--------|-------|--------|-------|--------|
| WT       | DAPAVTV | PGSKS | GRGDSPASSK |       |        |       |        |       |        |       |        |
| SGE      | DAPAVTV | PGSKS | GSGESPASSK |       |        |       |        |       |        |       |        |
| C7       | RHPHFPT | PLQPP | DGRNRLSLSI | 0.31  | 4.5    | 3.1 x 10^3 | 3.6 x 10^3 | 9.4 x 10^3 | 1.7 x 10^3 | 1.2   | 16    |
| CT-322   | RHPHFPT | PLQPP | DGRNRLSLSI | 11    | 250    | 7.7 x 10^3 | 7.5 x 10^3 | 8.1 x 10^3 | 1.9 x 10^3 | 16    | 240   |

| KD (nM) | kon (M^-1s^-1) | koff (s^-1) | IC_50 (nM) |
|---------|----------------|-------------|-------------|
|          |                |             |             |

WT, wild type; BC, DE, FG refer to specific amino acid loops of the Adnectin molecule. The binding affinities for VEGF receptors were determined by surface Plasmon resonance as described in the text.
in nude mice by 45% at 3 mg/kg dose (p < 0.05, Fig. 2A). The growth inhibition with the 30 mg/kg dose was similar to that seen with the monoclonal anti-VEGFR-2 antibody DC101 administered at 40 mg/kg twice weekly (BIW), the optimal dose and schedule in mouse models.19 As can be seen from (Fig. 2B) microvessel density in the tumors was reduced to a similar degree after 20 days of CT-322 or DC101. Comparison of CT-322 treatment group vs. control group (ANOVA) indicated significant differences (p < 0.05), whereas no significant difference was found between CT-322 vs. DC101 (p > 0.05).

CT-322 effectively reduced lung metastases in an orthotopic breast carcinoma adjuvant therapy model (Fig. 3A). Of 19 vehicle-treated mice, 16 (84%) developed visible lung tumors, with a median of 9 metastases per animal. Six of 20 (30%) mice treated with CT-322 developed lung tumors, with a median of 1 metastasis per animal. These results indicate that CT-322 reduced both the total number of metastases and the number of animals with metastases, with the mean reduction of metastasis significantly lower (p = 0.001). Treatment with anti-VEGF-A bevacizumab as a positive control resulted in 14 of 19 (74%) mice developing a lung metastasis, with a median of three tumors per animal, with the mean number of metastases also significantly lower than control (p = 0.004).

Prior xenograft results indicated that 30 mg/kg BIW was a highly active dose of CT-322,15 thus, this dose was used to compare anti-tumor effects to marketed TKIs. CT-322 administered at 30 mg/kg twice weekly suppressed Colo-205 xenograft tumor growth with an efficacy comparable to that of sunitinib and sorafenib, administered daily at their maximally active doses (Fig. 3B).6,20 Differences between CT-322 and control group (ANOVA) demonstrated statistical significance (p < 0.05) whereas the differences between the effects of CT-322, sorafenib
tumor volume of 150 mm³, none of the mice in the 60 or 120 mg/kg arms of the study had tumors that reached 1,000 mm³, which was the cut-off in this Kaplan-Meier analysis. CT-322-mediated tumor growth inhibition was evident even at 1 or 5 mg/kg three times weekly (TIW) doses, and there was a substantial increase in the number of animals without 1,000 mm³ tumors at the 30 mg/kg dose. In the groups administered 60 and 120 mg/kg, tumor growth was reduced by 93% after 5 weeks of treatment. Mice treated for a period of 64 days had no observable drug-related adverse effects except for broken teeth or malocclusions in animals treated with 30, 60 and 120 mg/kg doses, during the latter part of the experiment. These adverse effects were likely due to an effect of VEGFR-2 blockade that is specific to rodents because the animals have continually growing teeth.21,22

CT-322 induced a dose- and time-dependent increase in mVEGF-A as determined from experiments on non-tumor bearing mice (Fig. 4B). For any given dose, VEGF-A increased to an approximate sustained peak by 24 hours after initiation of drug administration. The dose required for maximum VEGF-A increase was 60 mg/kg TIW, analogous to the dose required for maximal tumor suppression of Colo-205 xenografts in the same strain of mice. VEGF-A was also moderately elevated by 3–7 days at the 1 and 5 mg/kg doses that had exerted moderate anti-tumor effects in the Colo-205 model. Blockade of VEGFR-2 by antibodies also caused an increase in VEGF-A.19

A single administration of 50 mg/kg CT-322 significantly increased mean blood pressure by about 10 mm Hg in rats within three days of administration (see Fig. 5 Suppl. Material), indicating that an increase in blood pressure may be another biological marker of VEGFR-2 blockade. Blood pressure returned to near baseline levels over the course of the following week, presumably due to elimination of CT-322. Other blockers of the VEGF-A pathway also increase blood pressure.23

CT-322 combined effectively with a small molecule anti-tumor agent to reduce tumor growth. CT-322 at 60 mg/kg three times weekly inhibited Colo-205 tumor growth at a rate comparable to that of temsirolimus, and the combination of the two drugs was superior to either agent alone (Fig. 5). The improved efficacy of the combination treatment over monotherapies began to emerge on Day 21 when all treatments were stopped. Differences in the treatment groups were maintained over time and there were statistically significant differences (p < 0.01) in tumor volumes between single agent treatment groups and the combination group on Days 34 and 40. The tumor burden was substantially lower in the combination group, despite the lack of treatment for 40 days.
Figure 3. (A) The effect of CT-322 and bevacizumab on orthotopic breast carcinoma adjuvant therapy model. Each group included 19 or 20 athymic mice. The vertical bars represent the range of observation in each group and the box and horizontal bar represent the SD and mean of the observations in each group. (B) Comparative anti-tumor effects of CT-322 and sorafenib and sunitinib on Colo-205 tumor model. Colo-205 tumor bearing mice (n = 15 mice per group) were treated with vehicle, CT-322 (30 mg/kg, ip twice per week), sunitinib, or sorafenib for 34 days. Sunitinib (80 mg/kg) and sorafenib (60 mg/kg) treatments were administered daily by oral gavage.

Figure 4. (A) Anti-tumor effect of CT-322 expressed as time needed to reach a tumor volume of 1,000 mm$^3$ in mice implanted with Colo-205 cells. Treatment with CT-322 BIW was started when tumor volume was about 150 mm$^3$ (n = 9 mice/group). Differences between control and all treatment groups were statistically significant (p = 0.001; Mann Whitney test). (B) The effect of CT-322 on serum VEGF levels; n = 3 for each group. Mean ± SD.

Figure 5. Anti-tumor effects of the combination of CT-322 and temsirolimus. CT-322 was administered at 60 mg kg, TIW and temsirolimus at 20 mg/kg, BIW in mice bearing Colo-205 tumor cells. Treatments were stopped on Day 21 and tumor volumes were measured TIW for an additional 40 days. Statistical significance of differences between combination group and single agent treatment groups was determined using data on Days 21, 34 and 40 (p < 0.01).
CT-322, a VEGFR-2 antagonist, exemplifies a new class of targeted biologics called Adnectins that are genetically engineered variants of the tenth type III domain of human fibronectin. CT-322 specifically and potently bound VEGFR-2 without detectable binding to VEGFR-1 or VEGFR-3, and blocked the activation of VEGFR-2 by its three ligands, VEGF-A, -C and -D. The affinity of CT-322 for mouse VEGFR-2 was sufficient to demonstrate anti-angiogenic and anti-tumor effects in vivo in preclinical models while having higher affinity for human VEGFR-2. The peptide moiety of CT-322 (with MW of approximately 10 kDa) is modified with a single 40 kDa branched PEG to demonstrate anti-angiogenic and anti-tumor effects in vivo.

PEGylation of C7 decreased the affinity of the protein moiety for VEGFR-2 to approximately 1/30th of the original value. This reduction was primarily the result of a decrease in the association rate constant of CT-322 compared to C7. This type of reduction of association rate constant and potency occurs for PEGylated proteins and may be the result of steric interactions and/or the large hydrodynamic radius of the PEGylated protein (apparent molecular weight on SEC >450 kDa compared to actual MW of approximately 50 kDa). Despite the reduction of binding affinity in vitro, the activity in vivo is substantially improved due to the dramatic increase in half-life and exposure of CT-322 compared to C7.

In vitro studies on CT-322 provided insight into its anti-angiogenic mechanism of action. VEGF-A homodimer mediates signaling by inducing dimerization of VEGFR-2 and subsequent auto-phosphorylation of the intracellular domains. CT-322 blocked VEGF-induced receptor dimerization, the resultant receptor auto-phosphorylation, and intracellular signaling through MAP kinase. Because of this dimerization mechanism of receptor activation, anti-VEGFR-2 antibodies have the potential to at least partially activate VEGFR-2, despite blocking VEGF binding. Because CT-322 is a monomeric and monovalent species, there may be an advantage to not inducing any receptor activation. The blockade of VEGFR-2 signaling also inhibited HUVEC proliferation. The inhibitory effect of CT-322 on HUVECs was specific for VEGFR-2 because CT-322 did not block PGE2-induced proliferation. Additional evidence of specificity in vivo was provided by the fact that CT-322 inhibited VEGF-A mediated vascular leak in mice used in a modified Mile’s assay, but did not inhibit the PBS-induced vascular leak (see Fig. 6 Suppl. Materials).

CT-322 inhibited U87 tumor growth in a human xenograft model in mice. The tumor suppression was associated with a potent anti-angiogenic effect on tumor vasculature, characterized by a reduction in CD31 staining of tumor specimens. These anti-tumor and anti-vasculature effects in this study were similar to that of DC101, an anti-VEGFR-2 antibody.

Preclinical evidence of the potency of CT-322 as a tumor growth inhibitor and the potential safety advantages of CT-322 were gained from comparisons of CT-322 to the tyrosine kinase inhibitors sorafenib and sunitinib. CT-322 at 30 mg/Kg TIW was as effective as the maximal doses of sorafenib and sunitinib in suppressing the growth of Colo-205 xenografts. In addition, sorafenib and sunitinib had overt toxicity, whereas CT-322 and vehicle treatment were comparably tolerated.

CT-322 reduced total macroscopic lung metastases and the number of mice with detectable metastasis in the orthotopic xenograft model. Importantly, treatment was started post resection of the primary tumor to mimic the clinical set up of adjuvant therapy. Our results suggest that blockade of VEGFR-2 is an attractive mode of action for adjuvant therapy. The low activity of bevacizumab compared to CT-322 in this study may be due to a substantive role of stromal-derived (mouse) VEGF-A versus tumor-derived human VEGF-A in the metastatic growth of MDA-MB-231 cells because bevacizumab is selective for human VEGF-A and does not neutralize mouse VEGF-A; however, while not known for MDA-MB-231, some tumor cell lines, such as A673 are not substantively dependent on stromal-derived VEGF-A. Therefore, it is possible that the mechanistic difference between the two agents might have a role in the observed efficacy difference. Alternatively, specific VEGF-R2 signaling blockade might have superior effects to the specific blockade of VEGF-A. The relevance of these differences to human disease is so far unknown.

Specificity of inhibition of VEGFR-2 signaling by CT-322 may be advantageous compared to anti-VEGF-A antibody because VEGF-A signals through both VEGFR-1 and VEGFR-2 and blockade of VEGFR-1 may lead to adverse effects. Similarly, there may also be an advantage of specific VEGFR-2 blockade compared to inhibition of multiple tyrosine kinases (e.g., by sorafenib and sunitinib). Blockade of VEGFR-2 may provide sufficient efficacy because signaling through VEGFR-2 is considered the primary stimulus for angiogenesis. Specific VEGFR-2 blockade may even have efficacy advantages over specific VEGF-A blockade in some populations due to blockade of potential VEGF-C and VEGF-D signaling through VEGFR-2 since these growth factors also signal through VEGFR-2 and are upregulated in many tumors. However, VEGF-C and -D are primarily considered activators of lymphangiogenesis and their contributions to angiogenesis in tumors in humans are not clearly defined.

Because bevacizumab is currently approved for use in combination therapies only, it was important to determine whether the CT-322 had activity in combination with other chemotherapeutic agents. We used temsirolimus, an approved mTOR inhibitor for renal cell cancer treatment, for the combination studies. The CT-322/temsirolimus combination exerted substantially higher efficacy compared to single agents on Colo-205 xenografts. Given that anti-VEGF blockade is used clinically for treatment of renal cell cancers (sorafenib and sunitinib are approved for renal cell cancers), the combination of CT-322 with temsirolimus may provide benefit beyond either therapy alone in humans. Furthermore, recent studies indicate that the mechanism of action of an mTOR inhibitor in blocking angiogenesis is distinct from that of VEGFR signaling pathways, revealing potential pathways for synergy in these two classes of therapeutic agents. In our study, the efficacy of combination of CT-322 with temsirolimus was remarkable, since the anti-tumor effect was maintained over 40 days after stopping the treatment.
Biomarkers of pharmacological activity are an important part of Phase 1 and Phase 2 clinical studies. For example, bevacizumab was well tolerated in its first Phase 1 study. Although clinical tumor responses were not confirmed, biological markers, such as increased blood pressure and increased plasma levels of VEGF-A, indicated that the drug was active. Increases of plasma VEGF-A and blood pressure are also observed during sorafenib and sunitinib treatment. DC101, an anti-mouse VEGFR-2 antibody that blocks mouse VEGFR-2 signaling, also led to elevated VEGF-A levels and dose-dependent tumor suppression. Therefore, the effect of CT-322 on blood pressure and VEGF-A levels in animals were assessed. The dose response of increase of plasma VEGF-A was similar to that of growth inhibition of Colo-205 xenografts with maximal effects at 60 mg/kg TIW. This result suggests that VEGF-A might be a useful biological marker for dose selection in the clinic. CT-322 also increased blood pressure of telemetered rats after a single administration at a dose similar to that yielding tumor growth inhibition in mouse tumor xenograft models. Thus, blood pressure may be another biological marker for selecting active doses of CT-322 in clinical studies.

A recent report indicated that CT-322 is effective in controlling pancreatic tumor growth and metastasis in xenograft models. Initial results from the first Phase 1 clinical trial were presented in 2008 at the American Society of Clinical Oncology meeting. CT-322 was found to be well tolerated at 2 mg/kg/week. Thirty-seven patients with solid tumors or non-Hodgkin lymphoma were treated with CT-322, and 49% of patients experienced stabilization of the disease. The encouraging results from this Phase 1 study currently are being pursued in Phase 2 trials on the treatment of glioblastoma multiforme.

In terms of clinical development, Adnectins are the most advanced of a number of formats of protein engineering for targeted therapies. Adnectins bind target molecules with $K_D$ values comparable to those reported for the interaction of antigens with therapeutic antibodies. Affinities comparable to antibodies are accomplished despite the fact that Adnectins, which are approximately 10 kDa, are much smaller than monoclonal antibodies (e.g., approximately 149 kDa). Because Adnectins are small proteins that are not glycosylated and have no disulfide bonds, they can be easily manufactured from E. coli. The ease of engineering and selection of Adnectins with desired binding and pharmaceutical properties establish them as attractive options for development as therapeutics for the treatment of a wide variety of diseases.

In summary, CT-322 was developed as the first of the Adnectin family of targeted biologics. Being a potent and specific VEGFR-2 blocker with activity against rodent and human receptors, CT-322 suppressed angiogenesis and tumor growth in a wide range of preclinical models. Combination of CT-322 with temsirolimus demonstrated promising anti-tumor effects. Our results also suggest potential safety benefits of CT-322 compared to single compound inhibition of multiple tyrosine kinases. Biological markers associated with anti-tumor effects have been identified in the preclinical models. These studies will assist in the clinical development of CT-322 and spur research on this anti-angiogenic agent and more generally advance the study of Adnectins in multiple therapeutic areas.

Materials and Methods

Generation and selection of adnectins. The methods used to generate and select Adnectins with selective binding affinity to VEGFR-2 have been previously described in detail. Preliminary experiments identified clone VR28 as a lead candidate. VR28 was subjected to multiple rounds of site-directed mutagenesis and selection to increase the binding affinity to human VEGFR-2. The resulting clone was used as the starting point for development of an Adnectin with affinity to both human and murine VEGFR-2. After 3 rounds of selection using mVEGFR-2 as the target and one round using hVEGFR-2 as the target, a candidate with high affinity binding to both targets was identified. Sequence activity relationship guided incorporation of a single point mutation to this clone to create the final molecule, C7+. CT-322 was generated by PEGylation of C7+ with a single 40 kDa branched PEG via an engineered cysteine (see below). The amino acid sequence of CT-322 varied from that of VR28 only in the FG region and the variant sequence was DGRNRLLLS1 (Table 1).

Control adnectin. The wild-type 10Fn3 contains an alpha5, beta1 integrin-binding motif, RGD (arginine-glycine-aspartate), within the FG (fibronectin G-strand) loop. To generate an Adnectin control most analogous to the wild type sequence, but without known binding activity (e.g., no integrin binding), the RGD sequence was mutated to SGE (serine-glycine-glutamic acid). The variant sequence of the FG region was GSGESPASSK (Table 1).

Protein expression and purification. Clones were expressed in E. coli BL21(DE3) pLysS (Invitrogen, Carlsbad, CA) in a construct that encoded residues 1–101 of each Adnectin using standard methods. Selected Adnectins were tagged at the C-terminus by a His-tag. Pellets of E. coli were lysed by sonication, centrifuged, and the supernatant was filtered and loaded onto a HisTrap column (Amersham Biosciences, Piscataway, NJ). Adnectins were eluted with 50 mM HEPES, 500 mM NaCl and 500 mM imidazole, dialyzed against 50 mM sodium acetate (NaAcOH; pH 4.5), 100 mM NaCl, and concentrated.

Adnectins without His-tags were refolded and purified from E. coli inclusion bodies. E. coli cell pellets were re-suspended in 50 mM HEPES 500 mM NaCl, 5 mM EDTA, and lysed with a M-110EH microfluidizer (Microfluidics, Newton, MA). Inclusion bodies were isolated, washed, and solubilized with 6 M guanidine-HCl, 50 mM Tris (pH 8), 5 mM EDTA, and 2 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP). Adnectins were refolded by dialysis against 50 mM NaAcOH (pH 4.5) and 0.1 mM TCEP. Adnectins were purified by a SP-Sepharose column (Amersham Biosciences) with a linear elution gradient of 0–1 M NaCl and 50 mM NaAcOH (pH 4.5). Adnectins were dialyzed against 50 mM NaAcOH (pH 4.5) and 100 mM NaCl, then concentrated.

PEGylation of C7+. Clone C7+ was modified by introduction of a single cysteine at position 100 instead of serine that was used to conjugate a single PEG molecule. One mg/mL C7-
CT-322 was performed using a SuperdexTM 200 10/300 GL column (GE Healthcare). A buffer of 100 mM sodium sulfate, 100 mM sodium phosphate, 150 mM sodium chloride, pH 6.8 at a flow rate of 0.6 mL/min was employed. CT-322 (20 µg) was injected at a concentration of 10 mg/mL.

SEC was coupled with light scattering for SEC MALD. SEC-exclusion chromatography (SEC) of CT-322 was performed using a Superdex™200 10/300 GL column (GE Healthcare). A buffer of 100 mM sodium sulfate, 100 mM sodium phosphate, 150 mM sodium chloride, pH 6.8 at a flow rate of 0.6 mL/min was employed. CT-322 (20 µg) was injected at a concentration of 10 mg/mL.

Size exclusion chromatography and SEC multi-angle light scattering (MALS). Size-exclusion chromatography (SEC) of CT-322 was performed using a Superdex™200 10/300 GL column (GE Healthcare). A buffer of 100 mM sodium sulfate, 100 mM sodium phosphate, 150 mM sodium chloride, pH 6.8 at a flow rate of 0.6 mL/min was employed. CT-322 (20 µg) was injected at a concentration of 10 mg/mL.

Surface plasmon resonance. The binding affinity of Adnectins for VEGF receptors was determined by surface plasmon resonance. Target proteins (R&D Systems) were immobilized on a CM5 chip (Biacore International AB, Switzerland). Binding was analyzed in 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20, and Adnectin concentrations ranging from 1 nM to 10 µM, at 25°C on a BIAcore 2000 or 3000 (Biacore) instrument. Association was measured using the kinetic function with a 10 min dissociation time at a 30 µL/min flow rate. After each run, the chip was regenerated. Tests were run in duplicate and responses from an empty flow cell and from buffer injections were subtracted from recorded values. Binding rate constants were determined using BIAeval software (Biacore) using global fitting to a 1:1 Langmuir binding model or mass transfer limited model.

VEGF-response assay of transfected pre-B cell line. The construction of a Ba/F3 cell line that would proliferate in response to VEGF has been previously described in detail. To determine VEGF-induced growth response, cells were seeded on 96-well plates (2–5 x 10⁴ cells/well) in 95 µL of growth medium. Test protein (CT-322 or SGE) was added as a 5 µL solution in PBS/20% minimal Ba/F3 medium. After incubation for 72 hours at 37°C, proliferation was measured by the addition of 20 µL of CellTiter 96 AQueous One solution (Promega) to each well, followed by measurement of absorbance at 490 nm using a microplate reader (Molecular Dynamics).

Endothelial cell proliferation assay. Primary human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex Bioproducts (East Rutherford, NJ) and were maintained according to the supplier’s directions. HUVEC between passages 3 to 7 were seeded into 96-well dishes at 2,000 cells/200 µL/well in EBM (endothelial basal medium) containing 2% FBS. After 24 hours, hVEGF165 (15 ng/mL), fibroblast growth factor-2 (FGF-2, 5 ng/mL), and CT-322 or SGE at various concentrations were added to the wells. The cells were incubated for 72 hours and ³H-thymidine (1 mCi/mL) was added for the final 10 to 12 hours. The cells were harvested and ³H-thymidine incorporation into DNA was determined using a MicroBeta counter (Packard, Meriden, CT).

Western blot analysis. Cells were grown in 6 well dishes to 80% confluence and starved for 5 hours in EBM-2. Cells were pre-incubated for 30 min with CT-322 or control and treated with VEGF₁₆₅ (20 ng/mL) for 5 to 8 min, washed with ice-cold PBS, and lysed with RIPA (radio-immunoprecipitation assay) buffer supplemented with phosphatase inhibitor cocktail (Sigma, St. Louis, MO) and complete-Mini protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN). SDS-sample buffer was added to samples that were separated on 6% polyacrylamide gel, transferred to nitrocellulose membranes (Invitrogen) and immunoblotted with anti-phosphoVEGFR-2 antibody (PC460, Calbiochem, La Jolla, CA). The blot was stripped and the membrane was re-probed with an antibody against total kinase insert domain receptor (KDR; A3, Santa Cruz Biotechnology, CA). Samples were also separated on 10% SDS/PAGE and immunoblotted with phospho-p44/42 MAP kinase (Thr202/Tyr204; 9101 Cell Signaling, Beverly, MA). The blot was stripped and probed with anti-ERK-1 antibody (Santa Cruz Biotechnology).

Pharmacokinetics of CT-322. The pharmacokinetics of C7’, C7’ conjugated to 20PEG, and CT-322 (C7’ conjugated to 40PEG) were determined in cynomolgus monkeys as described in the Supplemental Information for this article.

In vivo tumor models. The human Colo-205 colorectal carcinoma, U87 glioblastoma, and MDA-MB-231 breast carcinoma cell lines, routinely used in preclinical studies, were obtained from American Type Culture Collection (ATCC; Manassas, VA). Cells were grown and maintained as recommended by ATCC. Six to eight-week-old female athymic NCRNU-M-F nude mice (Taconic, Hudson, NY) were housed in a standard, pathogen-free, animal-care facility on a 12-hour light/dark cycle, with food and water ad libitum, as recommended by Guide for the Care and Use of Laboratory Animals. For subcutaneous implantation of tumor cells, sub-confluent cultures were harvested. Colo-205 or U87 cells (5 x 10⁶ cells per mouse) were injected subcutaneously into the backs of 6-week-old female athymic nude mice. Primary tumors were allowed to reach 50–100 mm³ before randomization into groups of 9–10 mice. Adnectins were administered intraperitoneally (ip) at doses and schedules as indicated in the Results section. Sunitinib (80 mg/kg) or sorafenib (60 mg/kg) were administered daily by oral gavage. Mouse VEGFR-2 monoclonal antibody DC101 (ATCC) was administered ip at 40 mg/kg twice weekly at its previously determined optimal dose. CT-322 was administered at the indicated doses and schedule. Tumors were measured three times weekly using a caliper and tumor volume calculated. For studies on the combination of CT-322 with temsirolimus, animals bearing Colo-205 tumors...
were treated with CT-322, temsirolimus, or the combination for 21 days. Subsequently, all treatments were terminated and the animals were monitored through 60 days from the start of treatment. Tumors were measured three times weekly using a caliper and tumor volumes were calculated. Control mice were euthanized on Day 41.

Orthotopic breast carcinoma adjuvant therapy model. A total of 1 x 10^6 MDA-MB-231 (LT subclone) cells were implanted into the right mammary fat pad of athymic mice. Tumors grew until the average tumor size reached about 400 mm^3 (Day 24 post-implantation), and visible tumor was dissected from the surrounding tissues en bloc. Mice were randomly assigned to treatment groups post-resection to ensure similar mean primary tumor burden. Treatment with CT-322 (30 mg/kg), bevacizumab (5 mg/kg), or control (PBS) was initiated on Day 28 post-implantation, and visible tumor was dissected from the surrounding tissues. Treatments were administered as 100 μL i.p injections twice weekly. Mice were sacrificed on Day 118 post-implantation, and lungs were resected and placed in Bouins solution to visualize macroscopic lung metastases. Surface lung metastases were counted by a single observer blinded to treatment group assignment. Local tumor re-growth was measured with calipers and resected at the time of sacrifice.

Measurement of VEGF-A levels. NCRNu female mice were administered CT-322 ip at 1, 5, 60 and 120 mg/kg three times weekly for 12 days. Blood samples were obtained at the indicated times by cardiac puncture under anesthesia, and plasma murine (m)VEGF-A was assessed using an ELISA (R&D systems) following the manufacturer’s specifications with the exception of the plasma dilution, which was diluted 1:2.

Blood pressure analysis in CT-322 treated rats. The effect of CT-322 on blood pressure was determined in male Sprague-Dawley rats as described in the Supplemental Information for this article.

Microvessel density. U87 tumors from control and CT-322-treated animals were examined by immunohistochemistry using a CD31 antibody staining which is a common measure of microvessel density. CD31 is a glycoprotein constitutively expressed on the surface of endothelial cells, and concentrated at the junction between them. Microvessel density was determined by counting the number of cell junctions per mm^2.

Statistical data analysis. Statistical comparisons between 3 or more groups were performed using a Kruskal-Wallis test followed by a Student-Newman-Keuls test or one-way ANOVA followed by Dunnett’s test. Statistical significance was defined by p < 0.05. Comparison between 2 groups was performed by a Mann-Whitney test. Data analysis was performed using SigmaStat version 3.11 (Systat Software, Inc., Point Richmond, CA).

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Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/MamlukMABS2-2-Sup.pdf

References

1. Kerbel RS. Tumor angiogenesis. N Engl J Med 2008; 358:2093-9.
2. Padera TP, Jain RK. VEGFR3: a new target for antiangiogenesis therapy? Dev Cell 2008; 15:178-9.
3. Jain RK. Tumor angiogenesis and accessibility: role of vascular endothelial growth factor. Semin Oncol 2002; 29:3-9.
4. Neufeld G, Cohen T, Gengrinovich S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J 1999; 13:9-22.
5. Ferrara N, Hillan KJ, Novotny W, Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. Biochem Biophys Res Commun 2005; 333:328-35.
6. Wilhelm SM, Adnane L, Newell P, Villanueva A, Llover JM, Lynch M. Preclinical overview of sorafenib, a multi-kinase inhibitor that targets both Raf and VEGF and PDGF receptor tyrosine kinase signaling. Mol Cancer Ther 2006; 7:3129-40.
7. Le TC, Raymond E, Faivre S. Sunitinib: a novel multitargeted tyrosine kinase inhibitor. A brief review of its therapeutic potential in the treatment of renal carcinoma and gastrointestinal stromal tumors (GIST). Ther Clin Risk Manag 2007; 3:341-9.
8. Piao WG, Li JW, Feng R, Beveridge M, Yue E, Lu AG, et al. Vascular endothelial growth factors C and D represent novel prognostic markers in colorectal cancer using quantitative image analysis. Eur Surg Res 2007; 39:229-38.
9. Achen MG, Jehlisch M, Kukl E, Makinen T, Vitali A, Wilks AF, et al. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci USA 1998; 95:548-53.
10. Hamada K, Okie Y, Takekura N, Ito Y, Jussila L, Dumont DJ, et al. VEGF-C signaling pathways through VEGFR-2 and VEGFR-3 in vascularangiogenesis and hematopoiesis. Blood 2000; 96:3793-800.
11. Koide A, Bailey CW, Huang X, Koide S. The fibronectin type III domain as a scaffold for novel binding proteins. J Mol Biol 1998; 284:1141-51.
12. Xu L, Aha P, Gu K, Kuimelis RG, Kurz M, Lam T, et al. Directed evolution of high-affinity antibody mimics using mRNA display. Chem Biol 2002; 9:933-42.
13. Parker MH, Chen Y, Danchev F, Dufa K, Ekstrom J, Germaine E, et al. Antibody mimics based on human fibronectin type three domain engineered for thermostability and high-affinity binding to vascular endothelial growth factor receptor two. Protein Eng Des Sel 2005; 18:435-44.
14. Germanova EV, Chen Y, Bloom L, Gokemeijer J, Shamah S, Warikoo V, et al. Antagonists to human and bovine serum albumin in aqueous solution with added salt. J Chromatogr A 2000; 867:105-12.
15. Bocci G, Man S, Green SK, Francia G, Ehs JM, du Manoir JM, et al. Increased plasma vascular endothelial growth factor (VEGF) as a surrogate marker for optimal therapeutic dosing of VEGF receptor-2 monoclonal antibodies. Cancer Res 2004; 64:6616-25.
16. Abrams TJ, Murray LJ, Pesenni E, Holway YW, Colombo T, Lee LB, et al. Preclinical evaluation of the tyrosine kinase inhibitor SU11248 as a single agent and in combination with “standard of care” therapeutic agents for the treatment of breast cancer. Mol Cancer Ther 2003; 2:1011-21.
17. Dai J, Rabin AB, VEGF: an essential mediator of both angiogenesis and endochondral ossification. J Dent Res 2007; 86:937-50.
18. Kohno S, Kaku M, Kawata T, Fujita T, Tsutsui K, Ohnita J, et al. Neutralizing effects of an anti-vascular endothelial growth factor antibody on tooth movement. Angle Orthod 2005; 75:797-804.
19. Rodhart JM, Langenberg MH, Witterwee E, Voest EE. The molecular basis of class side effects due to antiangiogenic and endochondral ossification. J Dent Res 2007; 86:937-50.
24. Fishburn CS. The pharmacology of PEGylation: balancing PD with PK to generate novel therapeutics. J Pharm Sci 2008; 97:4167-83.
25. Dhalluin C, Ross A, Huber W, Gerber P, Brugger D, Gsell B, et al. Structural, kinetic, and thermodynamic analysis of the binding of the 40 kDa PEG-interferon-alpha2a and its individual positional isomers to the extracellular domain of the receptor IFNAR2. Bioconjug Chem 2005; 16:518-27.
26. Liang WC, Wu X, Peale FV, Lee CV, Meng YG, Gutierrez J, et al. Cross-species vascular endothelial growth factor (VEGF)-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF. J Biol Chem 2006; 281:951-61.
27. Gerber HP, Kowalski J, Sherman D, Eberhard DA, Ferrara N. Complete inhibition of rhabdomyosarcoma xenograft growth and neovascularization requires blockade of both tumor and host vascular endothelial growth factor. Cancer Res 2000; 60:6253-8.
28. Duff SE, Jezierska M, Rosa DD, Kumar S, Haboubi N, Sherlock D, et al. Vascular endothelial growth factors and receptors in colorectal cancer: implications for anti-angiogenic therapy. Eur J Cancer 2006; 42:112-7.
29. Enholm B, Jussila L, Karkkainen M, Altitalo K. Vascular endothelial growth factor-C: a growth factor for lymphatic and blood vascular endothelial cells. Trends Cardiovasc Med 1998; 8:292-7.
30. Lane HA, Wood JM, McSheehy PM, Allogreni PR, Boulad A, Brueggen J, et al. mTOR inhibitor RAD001 (Everolimus) has antiangiogenic/vascular properties distinct from a VEGFR tyrosine kinase inhibitor. Clin Cancer Res 2009; 15:1612-22.
31. Gordon MS, Margolin K, Talpaz M, Sledge GW Jr, Holmgren E, Benjamin R, et al. Phase I safety and pharmacokinetic study of recombinant human anti-vascular endothelial growth factor in patients with advanced cancer. J Clin Oncol 2001; 19:843-50.
32. Lainakis G, Bamias A. Targeting angiogenesis in renal cell carcinoma. Curr Cancer Drug Targets 2008; 8:349-58.
33. Sweeney CJ, Chriorean EG, Mita MM, Papadopoulos KP, Silver B, Freed M, et al. Phase I study of CT-322, first adnectin protein therapeutic and potent inhibitor of VEGFR-2, in patients (pts) with advanced solid tumors (ST). J Clin Oncol 2008; 26:158.
34. Molckovsky A, Siu LL. First-in-class, first-in-human phase I results of targeted agents: Highlights of the 2008 American Society of Clinical Oncology meeting. J Hematol Oncol 2008; 1:20.
35. Gebauer M, Skerra A. Engineered protein scaffolds as next-generation antibody therapeutics. Curr Opin Chem Biol 2009; 13:245-55.
36. Koide A, Koide S. Monobodies: antibody mimics based on the scaffold of the fibronectin type III domain. Methods Mol Biol 2007; 352:95-109.