A dual-targeting PDGFRβ/VEGF-A molecule assembled from stable antibody fragments demonstrates anti-angiogenic activity in vitro and in vivo

Robert Mabry,1,* Debra G. Gilbertson,1 Amanda Frank,1 Tuyen Vu,1 Dan Ardourel,1 Craig Ostrander,2 Brenda Stevens,2 Susan Julien,2 Selcic Franke,1 Brent Meengs,2 Jennifer Brody,4 Scott Presnell,4 Nels B. Hamacher,2 Megan Lantry,2 Anita Wolf,2 Tom Bukowski,2 Robert Rosler,2 Cindy Yen,2 Monica Anderson-Haley,2 Kenneth Brasel,2 Qi Pan,1 Hank Franklin,1 Penny Thompson,1 Mike Dodds,9 Sara Underwood,3 Scott Peterson,1 Pallavur V. Sivakumaran2,5 and Mark Snavely1,2

1Antibody Discovery and Assay Technology; 2Protein Biochemistry; 3Oncology; 4Scientific Computing; and 5Preclinical Development; ZymoGenetics, Inc.; Seattle, WA USA

*Correspondence to: Robert Mabry; Email: mabryr@zgi.com
Submitted: 10/06/09; Accepted: 11/02/09
Previously published online: www.landesbioscience.com/journals/mabs/article/10498

Key words: bispecific, antibody, PDGFRβ, VEGF-A, stability, angiogenesis

Introduction

Angiogenesis, the formation of sprouts from pre-existent blood vessels and their invasion into surrounding tissue, has been implicated in the development of cancer and age-related macular degeneration (AMD). Targeting the vascular endothelial growth factor (VEGF) pathway has provided significant benefit to patients with both cancer and AMD.1 However, efficacy is often moderate at best, and there is substantial room for improvement. Some patients show an intrinsic refractoriness to anti-VEGF therapy, while others show transient benefit followed by tumor progression.4,6 One mechanism of adaptive resistance to anti-VEGF-therapy in preclinical models involves pericytes that express platelet-derived growth factor receptor (PDGFR) β.7,9 Several lines of evidence support co-targeting the PDGF and VEGF pathways to enhance the efficacy of anti-angiogenic treatment. Co-inhibition of signaling mediated by PDGF/PDGFRβ and VEGF-A/VEGFR more effectively prevents the growth of new blood vessels and is superior to anti-VEGF therapy alone in inhibiting tumor growth in preclinical cancer models and in a mouse model of ocular neovascularization.10-14 A number of small molecule tyrosine kinase inhibitors (TKIs) have recently been approved for the treatment of cancer.15 These TKIs target a wide range of intracellular kinases, including VEGFR and PDGFR.16 However, treatment with these inhibitors is limited by toxicity, especially when combined with chemotherapy.17,18 An attractive approach to inhibition of angiogenesis in tumors is the use of a single protein therapeutic that interrupts both VEGF and PDGF
pathways, and has the potential for superior anti-angiogenic activity over targeting VEGF alone. In addition, this approach is likely to result in lower toxicity than “broad spectrum” TKIs.

Bispecific antibodies (bsAbs) represent an intriguing approach to antibody engineering by combining two different binding specificities into one antibody-like molecule. The rationale for this approach was first described over two decades ago, and potential applications of bsAbs range from immunohistochemistry and diagnostics to human therapeutics. The promise of dual-targeting therapeutics has prompted the design of various formats of antibody-like proteins that can be tailored to specific needs. However, the manufacturability of these complex proteins at large scale continues to be a significant hurdle to the development of bsAbs. To date, no dual targeting proteins have been approved by the US Food and Drug Administration (FDA) for any indication.

Single-chain variable fragments (scFvs) are extremely useful in the construction of molecules with the complexity required for a dual-targeting approach. ScFvs contain both light (V_L) and heavy (V_H) variable domains in a single polypeptide, and can be fused to other proteins in modular form. The proximity of the V_H and V_L domains obviates the pairing of independent polypeptides, greatly facilitating production. In addition, the specificity, small size, short half-life in vivo, and potential for production from prokaryotic cells make scFvs attractive molecules for certain biomedical applications.

Despite the advantages of scFvs, the development of these molecules has been limited by the prevalence of stability issues. Since the early reports on construction of scFvs, numerous groups have struggled with poor expression, insufficient affinity, aggregation, limited shelf-life and poor serum stability. Some of these issues have been addressed recently by applying techniques designed to enhance stability through protein engineering, resulting in a number of new drug candidates that demonstrate efficacy in clinical settings. However, the lack of FDA approval of a therapeutic molecule containing a scFv highlights the limitations of these proteins. As a result, attention continues to be focused on the folding and stability of scFvs.

The observation that scFvs isolated from libraries of antibody fragments or assembled from IgGs frequently require structural modification to improve potency, stability, solubility or production has led to extensive examination of the factors that contribute to their inferior biophysical properties. In many cases, maturation techniques have been implemented to obtain the desired activity by mutation followed with rigorous functional screening. However, the theoretical diversity generated by combinatorial libraries of random or semi-random mutations can be very large. In order to sample a large population of variants, a display platform is typically used that maintains the link between a protein and the nucleic acid sequence of the encoding gene. This strategy of screening large numbers of candidates for the desired function, in combination with the evolution of molecular properties through mutation, is an alternative to rational or computational design, and circumvents the difficulty of predicting both the impact of mutations on structure and function of proteins and the greater diversity of options that can be explored.

Previously, we described a combinatorial method for selecting stable antibody fragments from a human phage display library and used the resulting scFvs to construct stable bispecific molecules against two soluble cytokines. In this report, we performed stress-guided selections for the isolation of stable antibody fragments targeting PDGFRβ and VEGF-A. The scFvs demonstrate high affinity binding to each target, high potency in cell-based assays, and remarkable stability in the absence of a traditional maturation campaign. The scFvs were fused to both ends of the Fc region of human IgG1 to construct a tetravalent bsAb. The final bispecific molecule binds both targets simultaneously, possesses the desired activity in cell-based assays, and is stable in vivo. Using a combination of analytical tools, we show that the stability of the final candidate is superior to that of extensively engineered antibody fragments. Furthermore, the dual-targeting molecule demonstrates the potential for production at large scale.

The process we describe overcomes significant challenges in the development of this complex class of proteins, and generates a highly efficacious anti-angiogenic molecule with considerable therapeutic potential.

**Results**

Identification and characterization of stable molecules targeting VEGF-A and PDGFRβ. To identify antibody fragments with superior stability, scFvs were selected against soluble VEGF-A and PDGFRβ-Fc under conditions that incorporate incubation at elevated temperature (see Materials and Methods). We previously reported a strategy that converts antigen-binding fragments (Fabs) to scFvs in batch mode and incorporates a step that shuffles the variable regions to increase diversity. The conversion process was followed by additional rounds of panning that integrated thermal stress to select for stable scFvs that bind either VEGF-A or PDGFRβ and function as inhibitors of the respective pathways. The binding characteristics were determined for unique scFvs, and two fragments, one directed against VEGF-A and one directed against PDGFRβ, were selected for construction of a dual-targeting molecule. The biophysical properties of the component scFvs and the bispecific, IgG-like protein were characterized using several analytical tools: differential scanning calorimetry (DSC), size-exclusion chromatography combined with multi-angle light scattering (SEC-MALS), and dynamic light scattering (DLS). Collectively, these three techniques provide a thorough assessment of protein integrity.

DSC measures phase transitions in solution. Upon thermal treatment, changes in heat capacity are measured that correlate with protein unfolding or precipitation. The observed melting temperatures (Tm)s represent the midpoint of protein unfolding where 50% of the molecules are folded and 50% unfolded. The two scFvs chosen for construction of the bispecific molecule demonstrated extraordinary stability with Tm of 74°C (anti-VEGFα scFv) and 78°C (anti-PDGFRβ scFv) (Fig. 1A). These are the highest reported values for scFvs of which we are aware, including those that have been subjected to maturation campaigns. The bispecific scFv-Fc-scFv constructed using these stable scFvs exhibits a primary Tm of 72°C, indicating that it too is highly...
Both antibody fragments belong to the same V<sub>H</sub> family due to the design of the Dyax library. V<sub>H</sub>3-3-23 was selected as the heavy chain framework because it possesses key amino acid residues in the framework region that have been reported to contribute to stability. One of the key issues with bispecific IgG-like molecules has been production. The DNA encoding the scFv-Fc-scFv was cloned into an expression vector for evaluation of production in CHO cells (as described in Materials and Methods). The bispecific molecule was produced at approximately 120 mg/L from an unoptimized pool under standard conditions, a level of production we typically observe with a native IgG antibody under similar conditions.

The affinities of all three proteins were measured using surface plasmon resonance (SPR). The anti-PDGFR<sub>β</sub> scFv displayed impressive affinity for the PDGFR<sub>β</sub> receptor with a calculated K<sub>D</sub> of 90 pM. The anti-VEGFA scFv also displayed subnanomolar affinity for the target ligand (K<sub>D</sub> of 600 pM). When

The extraordinary stabilities observed for the two scFvs are likely the result of the rescue of particular amino acid sequences following selection under thermal stress. The variable region sequences were aligned with that of the germline families that most resemble the amino acid sequence of each scFv (Fig. 4).

Both antibody fragments belong to the same V<sub>Hi</sub> family due to the design of the Dyax library. V<sub>Hi</sub>3-3-23 was selected as the heavy chain framework because it possesses key amino acid residues in the framework region that have been reported to contribute to stability.

One of the key issues with bispecific IgG-like molecules has been production. The DNA encoding the scFv-Fc-scFv was cloned into an expression vector for evaluation of production in CHO cells (as described in Materials and Methods). The bispecific molecule was produced at approximately 120 mg/L from an unoptimized pool under standard conditions, a level of production we typically observe with a native IgG antibody under similar conditions.

The affinities of all three proteins were measured using surface plasmon resonance (SPR). The anti-PDGFR<sub>β</sub> scFv displayed impressive affinity for the PDGFR<sub>β</sub> receptor with a calculated K<sub>D</sub> of 90 pM. The anti-VEGFA scFv also displayed subnanomolar affinity for the target ligand (K<sub>D</sub> of 600 pM). When
Figure 2. For figure legend, see page 24.
In a long-term assay (48 hours), the anti-VEGF-A scFv and the bsAb inhibited VEGF-A induced proliferation of human umbilical vein endothelial cells (HUVECs) with IC₅₀ values in the picomolar range (Fig. 6A). The level of inhibition was comparable to that seen with bevacizumab (Fig. 6A).

To test for neutralization of the PDGFRβ pathway, the ability to block the activity of PDGF-BB on human brain vascular pericytes (HBVPs) was analyzed. In a short-term assay (15 minutes), the anti-PDGFRβ scFv and the bsAb exhibited sub-nanomolar affinities to each target (Table 1). However, there was an observed decrease in the dissociation (off rate) of the bsAb to PDGFRβ, with no observed alteration in dissociation (off rate). The scFv-Fc-scFv construct was also evaluated for simultaneous binding to both targets. Figure 5 depicts the binding of the bsAb to immobilized VEGFA and the subsequent binding of PDGFRβ-Fc added in the final mobile phase. The association and dissociation curves generated for these interactions are consistent with binding curves generated against each target separately, suggesting that the bispecific molecule is capable of binding to both targets simultaneously without significant changes in affinity.

**Inhibition of biological activity mediated by VEGF and PDGF in vitro.** The anti-PDGFRβ scFv, the anti-VEGF-A scFv and the anti-PDGFRβ/VEGF-A scFv-Fc-scFv were evaluated for potency in cell-based assays that measure neutralization of the activity mediated through human VEGF-A and human PDGFRβ. In a short-term assay (15 minutes), the anti-VEGF-A scFv and the bispecific scFv-Fc-scFv neutralized VEGF-A-induced phosphorylation of VEGFR2 in HEK293 cells transfected with the receptor (IC₅₀: 0.04 and 0.07 nM respectively, Suppl. Table 1). In a long-term assay (48 hours), the anti-VEGF-A scFv and the bsAb inhibited VEGF-A induced proliferation of human umbilical vein endothelial cells (HUVECs) with IC₅₀ values in the picomolar range (Fig. 6A). The level of inhibition was comparable to that seen with bevacizumab (Fig. 6A). To test for neutralization of the PDGFRβ pathway, the ability to block the activity of PDGF-BB on human brain vascular pericytes (HBVPs) was analyzed. In a short-term assay (15 minutes), the anti-PDGFRβ scFv and the scFv-Fc-scFv blocked PDGF-BB-induced phosphorylation of PDGFRβ on HBVPs (IC₅₀: 0.3 and 0.1 nM respectively, Suppl. Table 1). In a long-term assay (18 hours), the anti-PDGFRβ scFv and the scFv-Fc-scFv neutralized PDGF-BB-induced proliferation of HBVPs with picomolar IC₅₀ values (Fig. 6B). These data suggest that the scFvs and the bispecific molecule are very potent inhibitors of activity mediated by both targets. The decrease in observed on-rate of the scFv-Fc-scFv binding to PDGFRβ, relative to the anti-PDGFRβ scFv, had no impact on potency in the cell-based assay. In addition, anti-PDGFRβ/VEGF-A scFv-Fc-scFv did not inhibit the activity of mouse VEGF-A or activity mediated through mouse PDGFRβ (Suppl. Table 1).
that differentiate from hMSCs. Endothelial sprouting is dependent on the presence of VEGF-A in the media, whereas pericyte differentiation and coverage of endothelial cells is dependent on PDGF-β (produced by endothelial cells) and PDGFRβ on the surface of the hMSCs and pericytes. Blocking the activity of VEGF-A at the start of the assay led to complete inhibition of endothelial sprouting, whereas blockade of activity mediated by PDGFRβ resulted in inhibition of pericyte differentiation and coverage of endothelial sprouts (data not shown). To mimic therapeutic inhibition of angiogenesis, antibodies or anti-PDGFRβ/VEGFR inhibitors were used. To mimic this in vitro, a co-culture system using HUVECs and human mesenchymal stem cells (hMSCs) was developed to test inhibition of proliferation and sprouting of endothelial cells. It was reported previously that, when cultured together, endothelial cells induce differentiation of murine mesenchymal stem cells into pericytes. In the co-culture sprouting assay, hMSC and cytodex beads coated with HUVECs were embedded in fibrin gel to form endothelial sprouts, which are covered with pericytes that differentiate from hMSCs. Endothelial sprouting is dependent on the presence of VEGF-A in the media, whereas pericyte differentiation and coverage of endothelial cells is dependent on PDGF-β (produced by endothelial cells) and PDGFRβ on the surface of the hMSCs and pericytes. Blocking the activity of VEGF-A at the start of the assay led to complete inhibition of endothelial sprouting, whereas blockade of activity mediated by PDGFRβ resulted in inhibition of pericyte differentiation and coverage of endothelial sprouts (data not shown). To mimic therapeutic inhibition of angiogenesis, antibodies or anti-PDGFRβ/
VEGF-A scFv-Fc-scFv were added after sprouts and pericyte coverage had formed. Addition of the anti-VEGF-A mAb bevacizumab inhibited the formation of new sprouts, but had little effect on preformed sprouts (Fig. 7). Addition of anti-PDGFRβ mAb E9899 induced pericyte dissociation from endothelial cells, but had no effect on endothelial sprouting (data not shown). In contrast, addition of the scFv-Fc-scFv alone or of both anti-VEGF-A and anti-PDGFRβ antibodies together resulted in significant reduction of endothelial sprouting in this model and induced pericyte dissociation from endothelial cells (Fig. 7A and B). The effect on endothelial sprouting was dose dependent (Fig. 7C).

Pharmacokinetics of the scFv-Fc-scFv. The pharmacokinetic properties of the anti-PDGFRβ/VEGF-A scFv-Fc-scFv were examined with a single intravenous injection in C.B-17 SCID mice. SCID mice were used for this experiment to understand better the behavior of the molecule in the strain used for tumor models. As shown in Table 2, a single injection of the scFv-Fc-Fv resulted in antibody-like clearance with a $t_{1/2}$ of about 460 hours. Furthermore, sera isolated from these mice neutralized the activity of VEGF-A and PDGFRβ in the short-term activity assays (Suppl. Table 2). These data suggest that the dual-targeting molecule is not only stable in vivo, but also retains its functional properties in mouse serum for up to a week.

Activity of the scFv-Fc-scFv in vivo. The potency of the anti-PDGFRβ/VEGF-A scFv-Fc-scFv was tested in vivo in a tumor model. Both scFvs in this engineered molecule bind specifically to the human protein and do not cross-react with mouse VEGF-A or mouse PDGFRβ. For this reason, the potency of both arms of the molecule could not be tested in a mouse model. However, a large number of human tumors secrete VEGF-A and growth of these tumors in mice has been shown to be dependent on production of the growth factor by the tumor. The A673 tumor model has been used extensively to study the potency of anti-human VEGF-A antibodies, including bevacizumab. This model was used to test the potency of the bsAb relative to bevacizumab. Prophylactic and therapeutic treatment with the scFv-Fc-scFv inhibited significantly the growth of A673 tumors in mice with efficacy similar to that seen with bevacizumab (Fig. 8A and B). Furthermore, treatment with the dual-targeting molecule or bevacizumab
significantly decreased micro-vessel density within the tumors (Suppl. Fig. 1).

**Internalization of the scFv-Fc-scFv.** Classical antibodies to a number of cell surface receptors induce internalization of the receptor (and the antibody). The anti-PDGFRβ/anti-VEGF-A scFv-Fc-scFv was tested for internalization over time by incubating with HBVPs expressing endogenous human PDGFRβ. As shown in Figure 9, the scFv-Fc-scFv bound to the cell membrane at T0 (on ice) as evidenced by the plasma membrane staining. As early as thirty minutes after warming at 37°C, internalization was apparent as cytoplasmic punctuate staining. Internalization was monitored over time, and punctuate staining increased and

| Parameter                  | scFv-Fc-scFv |
|----------------------------|-------------|
| C₀ (μg/mL)                 | 113         |
| AUC₀₉₀ (h*μg/mL)           | 13200       |
| AUCᵣτ/D (h*μg/mL)/(μg)    | 243         |
| t½₂₅x (h)                  | 460         |
| Vᵣ (mL)                   | 2.65        |
| Cl (mL/h)                  | 0.0041      |

**Figure 7.** Anti-PDGFRβ/VEGF-A scFv-Fc-scFv inhibits endothelial sprouting and pericyte/endothelial association in a coculture sprouting assay. Bevacizumab, anti-PDGFRβ mAb E9899 or the anti-PDGFR/VEGF-A scFv-Fc-scFv were used as antagonists in a endothelial/pericyte sprouting coculture assay as described in Methods. (A) Representative figures from each of the four groups (25 nM concentration). (B) Quantitative data using metamorph software for the four groups (25 nM concentration). (C) Dose response curve for bevacizumab alone or anti-PDGFRβ/VEGF-A scFv-Fc-scFv. The anti-PDGFRβ mAb E9899 did not have any effect on endothelia sprouting in the assay (data not shown).
then decreased, indicating the PDGFRβ/VEGF-A scFv-Fc-scFv was efficiently bound, and internalized by the HBVP. After two hours, almost no plasma membrane staining was visible, although in some cells hazy staining could be observed, possibly a result of constitutive receptor expression. Similar results were obtained with a mouse-anti-human PDGFRβ antibody E9899 (data not shown). Furthermore, VEGF-A bound to the VEGF-A-specific scFv of the dual-targeting entity did not inhibit internalization (data not shown).

Discussion

Antibodies possess a modular structure that suggests numerous possibilities for the development of bispecific molecules mediating biological activity specific to each target. However, with the use of antibody fragments to construct IgG-like proteins capable of dual-targeting, stability issues tend to increase as the complexity of the molecules increases. The data in this report suggest that one effective strategy for the successful generation of bsAbs is to select stable components early in the development process. One of the V region pairs identified by stress-guided selection would not have been predicted to be exceptionally stable. The combination of V<sub>H3</sub> and V<sub>I</sub> has been reported to be one of the more stable V region pairs. However, the V<sub>H3</sub>/V<sub>IV</sub> pairing was not noted to be particularly stable. One of the advantages of stress-guided selection is the identification of atypical V region pairings with the desired properties through functional analysis.

ScFvs can serve as versatile building blocks for the construction of bsAbs, but they typically require engineering or thorough characterization during the selection process due to stability issues with this class of proteins. Here, we incorporated stress-guided selection during panning of the library and chain shuffling during the conversion of the Fab output to scFvs in order to avoid the need for extensive maturation of individual antibody fragments. Our previous study indicated that shuffling of variable regions using a combinatorial strategy may enhance isolation of scFvs with high affinity for the target, and permits the construction of bsAbs with stable monomeric units. Thermal stress has been applied previously at both selection and screening steps of the process. Phage display is one of the few display systems in which thermal stress can be applied during selection. Thermal treatment prior to phage panning prevents the enrichment of unstable antibody fragments, while rescuing more stable fragments that either have the capacity to withstand unfolding or may refold upon cooling prior to selection against the target. In addition, thermal stress has been suggested to be superior to other stress-guided methods for maturation of individual fragments. However, stress during the selection process is directed toward proteins that are fused to the p3 coat protein of the phage, which may influence the stability of the displayed protein. The incorporation of thermal treatments in both the selection and screening processes would interrogate stability of fragments both as fusions and as soluble, independent proteins.

Another key element of the process is the incorporation of a combination of biophysical analyses to verify stability. While one analytical tool can provide insight into the biophysical behavior of the antibody fragments, a comprehensive approach that uses multiple methods will provide a more complete picture of the stability of the bsAbs. Our approach of incorporating stress-guided selection and chain shuffling during the construction of bsAbs is a promising strategy for the development of therapeutics that target multiple receptors.

Figure 8. Prophylactic and therapeutic treatment with anti-PDGFRβ/VEGF-A scFv-Fc-scFv inhibits growth of A673 rhabdomyosarcoma tumors. (A) Prophylactic treatment—female SCID were injected with A673 tumor cells on Day 0. Starting Day 1, groups of mice were injected intraperitoneally (i.p.) with the indicated drugs (at specified concentrations) twice a week for a total of eight doses. (B) Therapeutic treatment—female SCID were injected with A673 tumor cells on Day 0. Starting at a tumor volume of 150–200 mm<sup>3</sup> groups of mice (n = 10/gp) were injected i.p. with the indicated drugs (at specified concentrations) twice a week for a total of five doses. Mean tumor volume change over time is shown in both figures.
of a protein, the combination of multiple techniques provides comprehensive characterization during the selection of antibody fragments for assembly of complex, bispecific molecules. The anti-PDGFRβ/VEGF-A bsAb described here displays both potency and stability equivalent to typical therapeutic mAbs. The high MW species observed in preparations of the candidate scFvs are minimized when the single chain fragments are fused to an Fc domain. This effect is especially evident at higher concentrations, as determined by DLS and SEC-MALS. Results of analysis by DSC indicate that the thermal stability of less stable scFv is not altered by fusion to the Fc domain. The reduction in aggregation that is apparent upon evaluation using SEC-MALS and DLS may result from physical separation of aggregation-prone scFvs by anchoring them to the Fc domain. It is possible that the use of thermal stress during the selection of scFvs fused to p3 identifies antibody fragments that are stable when fused to Fc. Since the stability of the final molecule appears to be influenced significantly by the stabilities of the individual antibody fragments, as measured by DSC, our data suggest that the stability of a dual-targeting molecule in this format depends on the stabilities of the individual antibody units.

Stability of protein therapeutics has gained considerable attention due to the complexity of developing molecules with binding sites for different targets. However, confronting issues involving degradation, denaturation and aggregation downstream in the development process may not be the best approach. Engineering processes applied to “evolve” proteins by rational design late in the molecular development process are time consuming and labor intensive. On the other hand, the use of directed evolution to enhance stability is adaptable to high-throughput platforms, and can significantly reduce the time between discovery and lead development.

Targeting angiogenesis has been shown to be effective in the treatment of solid tumors and wet AMD. However, while inhibitors specific for VEGF-A have demonstrated efficacy, there is considerable room for improvement. In preclinical models, co-targeting the VEGF and PDGF pathways is more efficacious than targeting VEGF-A alone. VEGF-resistance in these preclinical models has been shown to be mediated by emergence of pericytes expressing PDGFRβ within tumors. Also, upregulation of PDGF-C has been demonstrated to play a role in resistance to anti-VEGF treatment both in preclinical and clinical settings. Thus, there is justification for the development of treatments that target both the VEGF and PDGF pathways. One successful approach is the use of multi-targeted TKIs that inhibit the intracellular kinase domains of both of these receptors. Although these molecules have been approved and successful in the clinic, their use is limited by toxicity, either as monotherapy or in combination with chemotherapy. As an alternative approach to the treatment of such tumors, a bispecific molecule that targets both the VEGF and PDGF pathways specifically offers the potential for effective inhibition of angiogenesis with reduced toxicity, relative to small molecule inhibitors, and may facilitate combination therapies with other drugs, including standard chemotherapy. This approach could provide more effective treatment for a variety of solid tumors.

The bsAb described in this manuscript is a high-affinity, dual-targeting protein specific for both PDGFRβ and VEGF-A. This scFv-Fc-scFv is effective in inhibiting VEGF-A and PDGFRβ activity in vitro (PDGF-BB, Fig. 6; PDGF-AB, CC, DD, Suppl. Table 1) and shows anti-tumor activity in vivo. The observed decrease in affinity of the bsAb to PDGFRβ, measured by SPR, did not appear to influence biological activity. We previously observed the changes in affinity from scFv to bispecific assembly that did not correlate with biological activity. Whether the changes in affinity are true or a result of steric hindrance related to Biacore formatting remains to be determined. Furthermore, the bsAb shows enhanced efficacy in an endothelial:pericyte co-culture assay, dependent on VEGF-A and PDGFRβ activity. These data support the use of this bsAb to block both pathways.

Figure 9. Internalization of anti-PDGFRβ/VEGF-A scFv-Fc-scFv after binding to HBVps. Chamber slides containing HBVps were incubated with 1 μg/mL of anti-PDGFRβ/VEGF-A scFv-Fc-scFv for 1 hour on ice to allow binding. Following a PBS rinse, cells were cultured at 37°C for the indicated times, then fixed and permeabilized prior to staining with Alexafluor-488 labeled goat anti-human IgG antibody (green) and DAPI nuclear stain (blue). The t = 0 time point was from cells cultured with scFv-Fc-scFv for 1 hour on ice.
and suggest that the molecule will provide improved efficacy in both oncology and wet AMD. Recent data with ranibizumab and a PDGF aptamer have shown encouraging anti-angiogenic effects in patients with wet AMD. However, this procedure involves two concurrent intravitreal injections at each dose. The engineered bsAb described here is expected to show enhanced efficacy in wet AMD with a single molecule. Furthermore, we anticipate reduced toxicity, either as a monotherapy or in combination with chemotherapeutics, relative to the less specific TKIs.

The method we have applied to engineering a dual-targeting molecule focuses on early identification of stable antibody fragments. As the next wave of protein-based therapeutics advance to lead development, the result of the successful application of engineering platforms, the value of applying engineering strategies and rigorous characterization upstream in the drug development process will become apparent.

Materials and Methods

Mice, cells and reagents. Female C.B-17 SCID mice (CB17/ Icr-Pkdcsild(+/−/IcrIcoCrl, Charles River Laboratories, Wilmington MA) were used for pharmacokinetic and tumor experiments. Bevacizumab (Genentech, San Francisco, CA) was obtained from the local Pharmacy. The rat anti-human PDGFRβ antibody (E9899) was generated by immunizing rats with soluble human PDGFRβ and selecting for neutralizing antibodies from hybridomas. Human umbilical vein endothelial cells (HUVEC) and primary human mesenchymal stem cells (HMSC) were obtained from Lonza (Walkersville, MD) and primary human brain vascular pericytes (HBVP) were purchased from ScienCell Research (San Diego, CA). Recombinant human VEGF-Aβ was cloned expressed and purified from E. coli at ZymoGenetics. Recombinant human PDGFR-BB was generated in S. cerevisiae at Novo Nordisk (Copenhagen, Denmark) and provided to ZymoGenetics. A673 (CRL-1598) rhabdomyosarcoma was obtained from American Type Culture Collection (Manassas, VA). Human PDGFRβ-Fc, human VEGFR2-Fc, human VEGFA, human PDGF-BB and mouse anti-human PDGFRβ antibody were produced at ZymoGenetics. Monomeric PDGFRβ was prepared by a Lys-C digest of PDGFRβ-Fc, followed by affinity purification (anti-PDGFRβ sepharose). Biotin labeling of ligands was performed at ZymoGenetics.

Phage selections and screening. Antibodies generated against both VEGF-A and PDGFRβ were derived from the Dyax libraries. The selections were performed as previously described with modifications. Anti-PDGFRβ antibodies were identified by selecting on biontilated target (in-house) captured on magnetic beads (Dynabeads M-280 Streptavidin, #112-06D, Invitrogen Dynal AS, Oslo, Norway). Anti-VEGF-A antibodies were identified by selecting on immunotubes (NUNC, Denmark) coated with antigen (VEGF-A in-house) at various concentrations. Following three rounds of selections, the Fabs in the enriched pool were converted to scFvs with shuffling of V regions through a combinatorial method. Additional rounds of panning were performed with the integration of thermal treatment (50–80°C, 1 hr) prior to incubation with target molecule. After 1–2 rounds of panning, scFvs were screened for activity using soluble scFv produced in E. coli as described previously. Anti-PDGFRβ clones were screened for antigenism using a blocking ELISA. Costar (#9018) 96-well plates were coated with an anti-human IgG antibody specific for Fcγ (109-005-098, Jackson Immunology) in 0.1 M NaHCO3, pH 9.6 overnight at 4°C. The next day, plates were washed three times with 0.1% Tween-20/PBS (PBST) and blocked with 5% milk (#170-6404, Bio-Rad)/PBST for one hour at room temperature (RT). Next PDGFRβ was added at 0.25 μg/mL in 2% BSA (#160069 MB Biomedicals)/PBST and incubated for one hour at RT. Plates were washed and blocked again with 5% milk/PBST for one hour at RT. After another wash with PBS, a (1:1) mixture of supernatant containing either Fab or scFv and biontilated PDGF-BB at 0.0112 μg/mL in 2% BSA/PBST was added for one hour at room temperature. Plates were washed with PBST followed by the addition of a 1:3,000 dilution of Streptavidin-HRP (#21124, Pierce) in 2% BSA/PBST for one hour at room temperature. Plates were then washed with PBST and 50 μL of TMB (TMWB-1000-01, BioFX Laboratories) added. The color was allowed to develop for 20–30 min, followed by the addition of 50 μL of stop buffer (STPR-1000-01, BioFX Laboratories) to quench the reaction. Plates were then read at 450 nm on a plate reader.

Antibodies selected against VEGF-A were also screened for blocking the interaction between receptor and ligand. Costar (#9018) 96-well plates were coated with anti-human IgG Fcg-specific antibody (109-005-098, Jackson Immunology) at 1 μg/mL in 0.1 M NaHCO3, pH 9.6 overnight at 4°C. The next day, plates were washed with 0.1% Tween-20/PBS (PBST) and blocked with 1% BSA (#A3059-100G, SIGMA)/PBST for one hour at room temperature (RT). Following a wash with PBST, VEGFR2-Fc at 0.2 μg/mL in 1% BSA/PBST was added and incubated for one hour at room temperature. Concurrently, in a separate 96 well plate (Costar 3357) a 1:1 dilution of supernatant, containing either Fab or scFv and biontilated VEGF-A (ZymoGenetics) in 1% BSA/PBST at 20 ng/mL was made and incubated for 1 hr at RT. Blocked assay plates were washed with PBST followed by the addition of the supernatant/biontilated VEGF-A complex for 1 hr at RT. After washing, a 1:4,000 dilution of Streptavidin-HRP (#21124, Pierce) in 1% BSA/PBST was added for one hour at RT. Plates were then washed and TMB (TMWB-1000-01, BioFX Laboratories) added to develop for 20 min, followed by the addition of 100 μL of stop buffer (STPR-1000-01, BioFX Laboratories) to quench the reaction. Plates were then read at 450 nm on a plate reader.

Antibody expression and purification. Selected scFvs were expressed using a proprietary vector with a pha promoter in either BL21 (#69449-4, Novagen, Madison, WI) or TG1 cells (#200123, Stratagene, La Jolla, CA). Once transformed, colonies were selected for inoculation into 2 ml LB medium supplemented with antifoam at 100 μg/L (#A8311, Sigma-Aldrich, St. Louis, MO) and kanamycin (#60615, Sigma-Aldrich, St. Louis, MO) at 25 μg/mL. Cultures were grown overnight at 37°C, shaking at 250 rpm. The overnight cultures were then diluted to a 0.2% inoculum into 0.5 L of phosphate-limiting media and
supplemented with antifoam and antibiotic as above. Cultures were grown in 2 L baffled flasks for 24 hrs at 30°C. Soluble scFv samples were recovered from wet cell pellets by treatment with periplasting buffer containing Ready-Lyse lysozyme (#R1802, Epicentre Technologies, Madison, WI) as per manufacturer’s instructions in the Protocol for the Preparation of Periplasmic and Spheroplastic Proteins from >1 mL Bacterial Culture (Epicentre Biotechnologies).

For the Immobilized Metal Affinity Chromatography (IMAC) enrichment of scFvs, the periplasmic fraction was passed through a 0.22 µm filter and purified by affinity capture with a HisTrap HP column (GE Healthcare, Piscataway, NJ) on a liquid chromatography instrument (Akta Explorer System, GE Healthcare, Piscataway, NJ). The bound protein was eluted using 400 mM imidazole in 50 mM NaPO₄, 500 mM NaCl pH 7.4 and assayed for protein content by absorbance at 280 nm, and for quality by analytical size exclusion chromatography and SDS-PAGE. For enrichment of Fabs, affinity purification was performed using Protein A (#17-1279-04, GE Healthcare, Piscataway, NJ) in batch mode. The Protein A resin was added to the periplasmic extract for overnight incubation at 4°C with mixing. The following day, the protein was eluted by a low pH step elution using 50 mM NaH₂PO₄, pH 2.5, 150 mM NaCl, immediately neutralized with 1 M HEPES pH 7.2 and evaluated as described above. Protein was dialyzed to a final buffer composition of 25 mM histidine, 125 mM NaCl, pH 6.8.

The scFv-Fc-scFv construct was assembled by a combination of PCR and homologous recombination in yeast, as an alternative to ligation, using a plasmid which was constructed from pZMP21 (patent pub. No. US 2003/0232414 A1) (deposited at the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209, designated as ATCC# PTA-5266) with DHFR as the selectable marker. The expression construct was electroporated into CHO DXB11 cells, adapted to grow in suspension in protein-free medium and subjected to nutrient selection in -HT medium followed by selection in methotrexate. Pools of selected cells were scaled up in spinner flask culture for purification and analysis of bispecific molecules. Conditioned media were harvested, passed through a 0.2 µm filter and adjusted to pH 7.4. The protein was purified from the filtered media using a combination of POROS® A50 Protein A Affinity Chromatography (Applied Biosciences, Foster City, CA) and Superdex 200 Size Exclusion Chromatography (GE Healthcare, Piscataway, NJ). Column fractions were analyzed by SDS-PAGE to determine the appropriate pools. Enriched protein was quantified by UV at A280 nm and an analytical SEC method was used to characterize the purified protein. Final buffer composition for the bsAbs was equivalent to the individual scFvs (25 mM histidine, 125 mM NaCl, pH 6.8).

Biophysical characterization. Differential scanning calorimetry (DSC) measurements were performed using a single cell VP-DSC instrument (MicroCal, Northampton, MA, USA) at a heating rate of 1°C/min for bispecific molecules and 1.5°C/min for single chain Fv. Working concentrations of these molecules were 0.35 mg/mL. Samples were buffer-exchanged over a Superdex 200 column, and a single peak of the target protein was collected, concentrated and filtered. The diluent was collected prior to injection of a bispecific molecule and was also used as reference blank for DSC analysis. All samples were stored frozen at -80°C until time of assay. Samples were thawed at room temperature, diluted to 0.35 mg/mL according to calculated extinction coefficient, and degassed prior to analysis.

Size Exclusion Chromatography Multi-Angle Light Scattering (SEC-MALS) analysis was used to provide an absolute measurement of MW and an assessment of oligomer formation from static light scattering in-line with SEC. Samples were analyzed with the three detectors connected in series with UV first (Agilent 1100 HPLC with diode array detector), followed by the LS (Wyatt Technology DAWN EOS Multi-angle laser light scattering detector) and RI (Wyatt Technology Optilab REX Differential Refractometer) detectors. For sample analysis, 150 µg of each sample was injected to the SEC-MALS system (GE Healthcare Superdex 200 SEC column, 10 x 300 mm) in a mobile phase consisting of 25 mM Histidine, 150 mM NaCl at pH 6.8 at a flow rate of 0.5 mL/minute run at ambient temperature. Data were analyzed with Wyatt Technology ASTRA software, version 5.3, according to the two-detector method, in the case of the individual scFvs, or the three-detector method for the scFv-Fc-scFv. The propensity of the antibody fragments and bispecific antibodies to aggregate was evaluated using dynamic light scattering (DLS), a technique that measures the hydrodynamic size of molecules in solution as a function of diffusion. Due to the extreme sensitivity to very large species, DLS can be used to reliably quantify large aggregate down to at least 0.01% by weight in a sample containing a range of species. Each of the bsAbs was analyzed in a generic buffer solution (25 mM histidine, 125 mM NaCl, pH 6.8). No attempt was made to improve stability through formulation. Samples were stored at -80°C prior to thawing and underwent one freeze-thaw cycle. Each sample was thawed at room temperature and scanned after dilution to 1 mg/mL in the standard buffer or after spin-concentrating to 25 mg/mL. All samples were centrifuged briefly at 16,000 xg and immediately prior to DLS analysis (DynaPro Plus Plate Reader, Wyatt Technology).

Affinity measurements. All binding kinetics and affinity studies were performed on a Biacore T-100™ system, (GE Healthcare). Affinity analyses were performed by capturing the purified anti-VEGFA and anti-PDGFRβ scFvs on anti-His/Myc immobilized sensor chip. Anti-His (GTX18184, Gentex, Irvine, CA) and anti-Myc (GTX20032, Genetex, Irvine, CA) antibodies were mixed in 1:1 molar ratio and covalently immobilized to CM5 chip using amine coupling chemistry (EDC:NHS) to a density of approximately 7500RU. The anti-VEGFA-A or the anti-PDGFRβ scFvs were then diluted to 10 nM and injected onto separate flow cells of the sensor chip at a flow rate of 10 uL/min for 1 minute. Serial 1:3 dilutions of VEGFA-A or PDGFRβ-Fc from 33.3 nM–0.14 nM were injected over this surface and allowed to specifically bind to captured antagonists on the sensor chip. Injections of each target concentration were performed in duplicate at a flow rate of 30 uL/min with 5 minutes of association and 10 minutes of dissociation time. These experiments were performed at 25°C in a buffer of 10 mM HEPES, 500 mM NaCl,
Affinity measurements of the tetravalent bsAb with PDGFRβ were performed with an Fc capture format. Goat anti-human IgG Fc-gamma (Jackson ImmunoResearch, West Grove, PA) was covalently immobilized on a CM4 chip (GE Healthcare/Biacore, #BR-1005-34) to a density of approximately 3500 RU using amine coupling as described above. The bsAb was captured onto a single flow cell of the CM4 chip at a density of 100 RU. Serial 1:3 dilutions of monomeric PDGFRβ (prepared from a Lys-C digest of PDGFRβ-Fc) were prepared from 100 nM–0.015 nM and injected over the surface at a flow rate of 30 μL/min. Duplicate injections of each antigen concentration were performed, with an association time of 9 minutes and dissociation time of 15 minutes. Between cycles, the flow cells were regenerated with a 30 μL injection of 20 mM HCl. The PDGFRβ binding curves were fit to a 1:1 binding model to calculate the association and dissociation rate constants.

Affinity measurements of the bsAb with VEGF-A were performed with the target antigen covalently immobilized to the sensor chip. VEGF-A was immobilized on a CM4 chip to a density of approximately 15 RU using amine coupling as described above. Serial 1:3 dilutions of the bsAb were prepared from 100 nM–0.015 nM and injected over the surface at a flow rate of 30 μL/min. Duplicate injections of each bsAb concentration were performed, with an association time of 9 minutes and dissociation time of 15 minutes. Between cycles, the flow cells were regenerated with a 30 μL injection of 10 mM glycine, pH 1.5. The VEGF-A binding curves were fit to a bivalent analyte model, and the monovalent component of the interaction (kₐ, kᵋ) was reported.

For co-binding analysis of the bsAb, a high density (1000 RU) of VEGF-A was covalently immobilized onto a CM4 sensor chip using amine coupling as described above. The bsAb was diluted to 100 nM and injected over the immobilized VEGF-A surface at a flow rate of 10 μL/min for 5 minutes. The capture level of the bsAb was approximately 700 RU. PDGFRβ-Fc (500 nM) was then flowed over the surface for 10 minutes at a flow rate of 30 μL/min. Between cycles, the flow cells were regenerated with 3 x 30 μL injections of 10 mM glycine, pH 1.5. The VEGF-A binding curves were fit to a bivalent analyte model, and the monovalent component of the interaction (kₐ, kᵋ) was reported.

All data were evaluated using Biacore Evaluation software to define either the affinity or the kinetics of the interactions. Baseline stability was assessed to ensure that the regeneration step provided a consistent binding surface throughout the sequence of injections. Binding curves were normalized by double-referencing and the resulting binding curves were globally-fit using the Biacore Evaluation Software v1.1.1.

**Endothelial: pericyte co-culture sprouting assay.** Cytodex-3 beads (Sigma-Aldrich, St. Louis, MO) were coated with HUVECs (Lonza) overnight, and then embedded (200 beads/well) with human mesenchymal stem cells (Lonza, Walkersville, MD, 40 K cells/well) in fibrin gel in 12-well tissue culture plates. A 1:1 mixture of fresh EGM-2 complete media (Lonza) and D551 fibroblast conditioned EGM-2 media were added on top of the fibrin gel along with 2 μg/mL of recombinant human HGF. Medium was replaced every two days till the end of the experiment. Bevacizumab, anti-PDGFRβ E9899 or anti-PDGFRβ/VEGF-A scFv-Fc-scFv were added to the culture medium at the indicated concentrations starting from day 8 (after EC sprouts and pericyte covering were formed). Seven days after addition of antagonists, cells were fixed in 4% PFA overnight at 4°C. Endothelial cells were stained with anti-PECAM (R&D systems, BAF806), followed by fluorescein-conjugated secondary antibody, and pericytes were labeled with anti-αSMA-Cy3 (Sigma, C6198). Cells were then viewed by an inverted fluorescence microscope and 6 x images were captured. A representative set of ten beads/well for each condition were chosen randomly. The total length of all the sprouts around a bead was measured in MetaMorph (version 7.1.6.0) by utilizing the angiogenesis tube formation application.

**Cell proliferation assays.** HUVECs were plated in 96-well flat-bottom plates (Falcon, Colorado Springs, CO) at a density of 1,000 cells per well. The HUVEC cells were plated for two days in complete EGM-2 MV media (Lonza, Walkersville, MD) at 37°C, 5% CO₂. The cells were starved of serum using serum free media (SFM:DMEM-F12 (1:1) with 1x insulin-transferrin-selenium, Invitrogen, Carlsbad, CA) for 24 h and then stimulated for 24 h with 2.6 nM human VEGF-A₁₆₅ with or without the serially diluted bevacizumab, anti-VEGF-A scFv, or the scFv-Fc-scFv at concentrations from 0.0005 nM to 500 nM. Cells were then pulsed with 1 μCi per well of ³H-thymidine for an additional 24 hrs at 37°C, 5% CO₂. Human brain vascular pericytes (HBVPs) were seeded in 96 well flat-bottom plates (Falcon, Colorado Springs, CO) at a density of 500 cells/well in complete media (SciGen Pericyte Media (PM) plus SciGen supplements Fetal Bovine Serum, Pericyte Growth Supplement, and Penicillin-Streptomycin) at 37°C in 5% CO₂ for 24–48 hours. The cells were starved of serum using SFM for 24 h and then stimulated with 0.4 nM human PDGF-BB in the presence or absence of a control rat anti-human PDGFRβ antibody E9899, anti-PDGFRβ scFv or the anti-PDGFRβ/VEGF-A scFv-Fc-scFv at concentrations from 2,000 nM to 0.02 nM. After 18–24 hours, 1 μCi of ³H-thymidine (Amersham, Piscataway, NJ) was added to each well and cells were incubated for 3–6 hours. Cells were harvested onto filter plates and incorporation of ³H-thymidine was determined using a Packard Topcount machine. Data analysis was done using GraphPad Prism software (LaJolla, CA).

**Pharmacokinetic analysis.** Unanesthetized female C.B-17 SCID mice were injected intravenously (i.v.) with 100 μg of anti-PDGFRβ/VEGF-A scFv-Fc-scFv in a 100 μL volume. Whole blood was collected at various times by cardiac puncture. Serum was generated and stored at -80°C. An enzyme linked immunosorbent assay (ELISA) was used to analyze test samples. The ELISA used recombinant human VEGF-A to capture scFv-Fc-scFv or appropriate standards. A biotinylated goat anti-human Fc antibody was used to bind captured Fc-containing protein, followed by incubation with streptavidin-HRP and the substrate tetramethylbenzidine. The colorimetric read-out was
analyzed. The resulting concentration versus time profile was subjected to noncompartmental PK analysis using WinNonlin 5.0.1 (Pharsight Inc., Mountain View, CA). Values for the area under concentration versus time curves extrapolated to infinity (AUC_{inf}) were calculated using the linear trapezoidal method with uniform weighting.

In vivo tumor experiments. Groups of C.B-17 SCID mice (8–12 weeks of age) were injected subcutaneously with 2 x 10^6 A673 cells in the mammary fat pad. For prophylactic treatment, cohorts of mice (n = 10/gp) were injected intraperitoneally twice a week with varying concentrations of bevacizumab or anti-PDGFRβ/VEGF-A scFv-Fc-scFv in a 100 μL volume starting one day after tumor inoculation. Mice received a total of eight treatments. For the therapeutic treatment, cohorts of mice (n = 10/gp) were injected intraperitoneally twice a week with varying concentrations of bevacizumab or the scFv-Fc-scFv in a 100 μL volume, starting with a tumor volume of 150–200 mm^3. Mice received a total of five doses before termination of the experiment. Weight of the mice and tumor volume were assessed three times under concentration versus time curves extrapolated to infinity (AUC_{inf}) were calculated using the linear trapezoidal method with uniform weighting.

The binding of α PDGFRβ/anti-VEGF-A scFv-Fc-scFv and control mouse α human PDGFRβ antibody were diluted to 1 μg/mL in binding buffer consisting of DMEM + 3% BSA and Hepes buffer.

References

1. Carmeliet P. Angiogenesis in health and disease. Nat Med 2003; 9:653-60.
2. Folkman J. Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Disc 2007; 6:273-86.
3. Pardoll DM. Monoclonal antibodies as immunotherapeutic agents for cancer. Nat Rev Cancer 2005; 5:320-31.
4. Bentink S. Antiangiogenic bispecific antibodies for cancer therapy. World J Gastroenterol 2009; 15:3801-3809.
5. Hynes RO, Strong L. Integrins: bidirectional signaling mechanisms for biology and medicine. Cell 2009; 138:825-37.
6. Norwell D, Hunt JA, Lewis CG. The β1 integrin ligand fibronectin. Biochim Biophys Acta 2003; 1582:19-33.
7. Norwell D, Hunt JA, Lewis CG. The β1 integrin ligand fibronectin. Biochim Biophys Acta 2003; 1582:19-33.
31. Honegger A, Malebranche AD, Rothlisberger D, Pluckthun A. The influence of the framework core residues on the biophysical properties of immunoglobulin heavy chain variable domains. Protein Eng Des Sel 2009; 22:121-34.
32. Honegger A. Engineering antibodies for stability and efficient folding. Handb Exp Pharmacol 2008; 47:68.
33. Maynard JA, Maassen CB, Leplla SH, Braxy K, Patterson JL, Ivenson BL, et al. Protection against anthrax toxin by recombinant antibody fragments correlates with antigen affinity. Nat Biotechnol 2002; 20:597-601.
34. Zhou Y, Drummond DC, Zou H, Hayes ME, Adams GP, Kiprotin DB, et al. Impact of single-chain Fv antibody fragment affinity on nanoparticle targeting of epidermal growth factor receptor-expressing tumor cells. J Mol Biol 2007; 371:934-47.
35. Zahnd C, Spinelli S, Lagunihud B, Amstruth P, Cambillau C, Pluckthun A. Directed in vitro evolution and crystalllographic analysis of a peptide-binding single chain antibody fragment (scFv) with low picomolar affinity. J Biol Chem 2004; 279:18870-7.
36. Mahy R, Lewis KE, Moore M, McKernan P, Bukowski T, Bontadelli K, et al. Engineering of Stable Bispecific Antibodies Targeting IL-17A and IL-23. Protein Eng Des Sel 2009; In press.
37. Michelson JS, Demarest SJ, Miller B, Amatucci A, Snyder WB, Wu X, et al. Anti-tumor activity of stability-engineered IgG-like bispecific antibodies targeting TRAIL-R2 and LTRβ. mAbs 2009; 1:128-41.
38. Hoet RM, Cohen EH, Kent RB, Rookey K, Schoonhooijs S, Hogan S, et al. Generation of high-affinity human antibodies by combining donor-derived and synthetic complementarity-determining-region diversity. Nat Biotechnol 2005; 23:344-8.
39. Hirschi KK, Rohovsky SA, Beck LH, Smith SR, D’Amore PA. Endothelial cells modulate the proliferation of mural cell precursors via platelet-derived growth factor-BB and heterotypic cell contact. Circ Res 1999; 84:298-305.
40. Jung S, Honegger A, Pluckthun A. Selection for improved protein stability by phage display. J Mol Biol 1999; 294:163-80.
41. Senino B, Kuhnert F, Tabruyn SP, Mancuso MR, Hu-Lowe DD, Kuo CJ, et al. Cellular source and amount of vascular endothelial growth factor and platelet-derived growth factor in tumors determine response to angiogenesis inhibitors. Cancer Res 2009; 69:4527-36.
42. Crawford Y, Kasman I, Yu L, Zhong C, Wu X, Modrusan Z, et al. PDGF-C mediates the angiogenic and tumorigenic properties of fibroblasts associated with tumors refractory to anti-VEGF treatment. Cancer Cell 2009; 15:21-34.
43. di Tomaso E, Landon N, Fuja D, Logie J, Tyrrell JA, Kansoun W, et al. PDGF-C induces maturation of blood vessels in a model of glioblastoma and attenuates the response to anti-VEGF treatment. PloS One 2009; 4:5123.
44. Boyer DS. Combined Inhibition of Platelet Derived (PDGF) and Vascular Endothelial (VEGF) Growth factors for the treatment of Neovascular Age-related Macular Degeneration (NV-AMD)—Results of a Phase 1 study. ARVO 2009 Annual Meeting 2009; Poster 1260.
45. Simmons LC, Reilly D, Klimowski L, Raju TS, Meng G, Sims P, et al. Expression of full-length immunoglobulins in Escherichia coli: rapid and efficient production of aglycosylated antibodies. J Immunol Methods 2002; 263:133-47.
46. Wen J, Arakawa T, Phleo JS. Size-exclusion chromatography with on-line light-scattering, absorbance and refractive index detectors for studying proteins and their interactions. Anal Biochem 1996; 240:155-66.