Characterization of a Bicyclic Peptide Neuropilin-1 (NP-1) Antagonist (EG3287) Reveals Importance of Vascular Endothelial Growth Factor Exon 8 for NP-1 Binding and Role of NP-1 in KDR Signaling

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Neuropilin-1 (NP-1) is a receptor for vascular endothelial growth factor-A165 (VEGF-A165) in endothelial cells. To define the role of NP-1 in the biological functions of VEGF, we developed a specific peptide antagonist of VEGF binding to NP-1 based on the NP-1 binding site located in the exon 7- and 8-encoded VEGF-A165 domain. The bicyclic peptide, EG3287, potently (IC50 1.2 μM) and effectively (>95% inhibition at 100 μM) inhibited VEGF-A165 binding to porcine aortic endothelial cells expressing NP-1 (PAE/NP-1) and breast carcinoma cells expressing only NP-1 receptors for VEGF-A, but had no effect on binding to PAE/KDR or PAE/Flt-1. Molecular dynamics calculations, a nuclear magnetic resonance structure of EG3287, and determination of stability in media, indicated that it constitutes a stable subdomain very similar to the corresponding region of native VEGF-A165. The C terminus encoded by exon 8 and the three-dimensional structure were both critical for EG3287 inhibition of NP-1 binding, whereas modifications at the N terminus had little effect. Although EG3287 had no direct effect on VEGF-A165 binding to KDR receptors, it inhibited cross-linking of VEGF-A165 to KDR in human umbilical vein endothelial cells co-expressing NP-1, and inhibited stimulation of KDR and PLC-γ tyrosine phosphorylation, activation of ERKs1/2 and prostaglandin production. These findings characterize the first specific antagonist of VEGF-A165 binding to NP-1 and demonstrate that NP-1 is essential for optimum KDR activation and intracellular signaling. The results also identify a key role for the C-terminal exon 8 domain in VEGF-A165 binding to NP-1.

Vascular endothelial growth factor A (VEGF-A) 2 is an essential mediator of vasculogenesis and angiogenesis during embryonic development and plays a central role in pathophysiological neovascularization in human disease (1, 2). Five isoforms of VEGF-A have been identified, VEGF-A121, VEGF-A145, VEGF-A165, VEGF-A189, and VEGF-A206, generated by alternative mRNA splicing of a single VEGF gene transcript containing 8 exons (3–5), of which VEGF-A165 is the most abundant and biologically active.

VEGF-A165 exerts its biological effects through high affinity binding to two tyrosine kinase receptors, Flt-1 (VEGFR1) and KDR (VEGFR2), which are expressed in most vascular endothelial cells (6). KDR binds VEGF-A165 with lower affinity than Flt-1, and is also recognized by VEGF-C, VEGF-D, and VEGF-E (7–11), whereas Flt-1 is also a receptor for PlGF and VEGF-B (12, 13). After binding and activation of KDR, VEGF-A165 stimulates ERK activation and an array of other early signaling events followed by short and long term cellular biological effects including production of prostacyclin (PGI2) and nitric oxide, increased cell survival, cell migration, proliferation, and angiogenesis (14–22). The function of Flt-1 in the endothelium is unclear, but it is thought to regulate the activity of VEGF-A partly by acting as a decoy receptor, and in part through direct regulatory effects on KDR (23).

Neuropilin-1 (NP-1) has recently been identified as a non-tyrosine kinase receptor for VEGF-A165, the heparin-binding PlGF-2 isoform, VEGF-B, and VEGF-E (24–26). NP-1 was first identified as a receptor for semaphorin 3A (Sema3A), a member of a family of proteins involved in axonal guidance (27, 28), and is expressed in endothelial cells, several tumor cell types, and in certain classes of neuron including cells of the dorsal root ganglion (DRG), olfactory, and optic nerves (24, 29). NP-2 has a similar domain structure to NP-1 with 44% amino acid identity, and exhibits a distinct expression pattern in the developing nervous system (28, 30). Sema3A induces neuronal growth cone collapse specifically through NP-1, whereas sema 3B, 3C, 3E, and 3F recognize both neuropilins, acting as NP-1 antagonists and NP-2 agonists (30–33). A growing body of evidence also indicates a role for neuropilins in angiogenesis. Overexpression of NP-1 in mice results in increased capillary formation, vasodilatation, and malformation of the heart (34), whereas mice deficient in NP-1 exhibit defects in embryonic axonal patterning and an array of vascular abnormalities including defective development of large vessels and impaired neural and yolk sac vascularization (35). Inactivation of both NP-1 and NP-2 causes a more severe failure of embryonic vascularization resulting in death at E8.5 (36). Despite the strong evidence that NP-1 is essential for normal vascular development, the
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underlying mechanisms remain obscure, and the role of NP-1 in the biological functions of VEGF-A are not fully understood.

We recently reported that a specific bicyclic peptide based on the C-terminal NP binding domain of VEGF-A165 (37) is an antagonist of VEGF binding to NP-1 and inhibits the anti-chemorepulsive effect of VEGF-A165 in DRG neuronal explants (38). Here, we have identified the key features of EG3287 responsible for its antagonistic properties through a detailed structure-function analysis, and investigated its biological effects in vascular endothelial cells. Our findings show that the C-terminal six amino acid domain encoded by exon 8 plays a crucial role in VEGF-A165 binding to NP-1. Evaluation of EG3287 in endothelial cells demonstrates that VEGF-A165 binding to NP-1 is required for stable binding to KDR, full activation of KDR and downstream signaling and biological responses. This antagonist should be a valuable tool for probing the biological role of NP-1 in diverse cell types, and will also be useful for designing improved neuropilin antagonists.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—Amino acids were purchased from Calbiochem Novabiochem, Alexis (both Nottingham, UK) or Bachem AG (Bubendorf, Switzerland). Linear, monocyclic, and bicyclic peptides based on residues 111–165 in VEGF-A165 were synthesized by Fmoc solid-phase synthesis using either Wang or Rink amide linkers. Side chain protections were: Arg(Pbf), Asn(Trt), Asp(OtBu), Cys(Trt), Cys(Acm), Gln(Trt), Glu(OtBu), Lys(Boc), Ser(tBu), Thr(tBu), and Tyr(tBu); additional side chain protections were: Arg(Pbf), Asn(Trt), Asp(OtBu), Cys(Trt), Cys(Acm), Gln-Trt, Glu(OtBu), Lys(Boc), Ser(tBu), Thr(tBu), and Tyr(tBu); additional linear peptides (peptides 12–19) were synthesized by Novabiochem, Alexis (both Nottingham, UK) Ltd. Additional bicyclic peptides (peptides 3–8) were synthesized by Bachem AG (Bubenrord, Switzerland). Linear, monocyclic, and bicyclic peptides based on residues 111–165 in VEGF-A165 were synthesized by Fmoc solid-phase synthesis using either Wang or Rink amide linkers. Side chain protections were: Arg(Pbf), Asn(Trt), Asp(OtBu), Cys(Trt), Cys(Acm), Gln-Trt, Glu(OtBu), Lys(Boc), Ser(tBu), Thr(tBu), and Tyr(tBu); additional linear peptides (peptides 12–19) were synthesized by Pepceuticals Ltd; additional bicyclic peptides (peptides 3–8) were synthesized by Bachem (UK) Ltd.

Linear peptides were synthesized by an automated multiple solid phase approach using the Fmoc-Arg(Pbf)-p-alkoxybenzyl alcohol resin (0.59 mmol/g loading) or Fmoc-Rink Amide MBHA resin (0.59 mmol/g or 0.68 mmol/g loading). Amino acids were attached by Fmoc strategy (0.59 mmol/g loading) or Fmoc-Rink Amide MBHA resin (0.59 mmol/g loading). Amino acids were attached by Fmoc strategy (0.59 mmol/g loading) or Fmoc-Rink Amide MBHA resin (0.59 mmol/g loading). Amino acids were attached by Fmoc strategy (0.59 mmol/g loading) or Fmoc-Rink Amide MBHA resin (0.59 mmol/g loading). Amino acids were attached by Fmoc strategy (0.59 mmol/g loading) or Fmoc-Rink Amide MBHA resin (0.59 mmol/g loading).

The coupling reagent, Pybop, NMM, and all amino acid derivatives were washed once with trifluoroacetic acid, filtered, and combined with the filtrates. The remaining resin was filtered, washed once with trifluoroacetic acid, and precipitated in ice-cold diethyl ether. The remaining resin was washed once with trifluoroacetic acid, filtered, and combined with the fractions. The precipitates were stored at 4 °C overnight and were collected by filtration, washed with ice-cold diethyl ether, and allowed to dry at room temperature. For non-Cys-containing peptides the cleavage mixture was filtered and precipitated in ice-cold diethyl ether. The remaining resin was washed once with trifluoroacetic acid, filtered, and combined with the fractions. The precipitates were stored at 4 °C overnight and were collected by filtration, washed with ice-cold diethyl ether, and allowed to dry at room temperature. For non-Cys-containing peptides the cleavage mixture was filtered and precipitated in ice-cold diethyl ether. The remaining resin was washed once with trifluoroacetic acid, filtered, and combined with the fractions. The precipitates were stored at 4 °C overnight and were collected by filtration, washed with ice-cold diethyl ether, and allowed to dry at room temperature. For non-Cys-containing peptides the cleavage mixture was filtered and precipitated in ice-cold diethyl ether. The remaining resin was washed once with trifluoroacetic acid, filtered, and combined with the fractions. The precipitates were stored at 4 °C overnight and were collected by filtration, washed with ice-cold diethyl ether, and allowed to dry at room temperature. For non-Cys-containing peptides the cleavage mixture was filtered and precipitated in ice-cold diethyl ether. The remaining resin was washed once with trifluoroacetic acid, filtered, and combined with the fractions. The precipitates were stored at 4 °C overnight and were collected by filtration, washed with ice-cold diethyl ether, and allowed to dry at room temperature. For non-Cys-containing peptides the cleavage mixture was filtered and precipitated in ice-cold diethyl ether. The remaining resin was washed once with trifluoroacetic acid, filtered, and combined with the fractions. The precipitates were stored at 4 °C overnight and were collected by filtration, washed with ice-cold diethyl ether, and allowed to dry at room temperature.
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ANSIG for openGL v1.0.3 (41). H, 13C, and 15N chemical shifts were referenced indirectly to DSS, using absolute frequency ratios (42). Interproton distance restraints were derived from two-dimensional 1H NOESY spectra with a mixing time of 250 ms. Cross-peaks were grouped into four categories according to their relative peak intensities: strong, medium, weak, and very weak, and were designated with interproton distance restraint bounds of 1.8–2.5 Å, 1.8–3.0 Å, 1.8–3.5 Å, and 1.8–4.5 Å, respectively. 0.5 Å was added for distances that involved methyl groups. The structure calculations were carried out using the PARALL-HDGv5.1 parameter, with the PROLSQ non-bonded energy function (43) within the CNS program (44), modified to allow fast stereochemistry, and to include active swapping, of prochiral centers (45). 229 interproton distance restraints (138 intraresidue, 56 sequential, 9 short range, 17 long range, and 9 ambiguous) were applied in restrained molecular dynamics simulated-annealing calculations.

Measurement of EG3287 Stability—EG3287 was recovered from tissue culture medium by a 1:1 addition of 20% trifluoroacetic acid and centrifugation of the precipitate. This method resulted in ~90% recovery. Quantitation of EG3287 was performed by Inpharmatica Ltd (Cambridge, UK) using a liquid chromatography mass spectrometric (LC-MS/MS) assay on a Micromass Quattro Micro.

Computational Chemistry—Molecules were analyzed using SYBYL® 7.0 (Tripos Inc., St. Louis, MO). The Biopolymer tools in Sybyl were used to build some of the peptides, check structures for errors, correct atom types, and add/remove hydrogens as necessary. For energy minimizations or molecular dynamics simulations we used starting conformations as described by Fairbrother et al. (46) (VEGF-A55, Protein Data Bank code: 2ygh, Ala111–Arg155, VEGF-A165) or EG3287 VEGF-A55 (Protein Data Bank code: 1KMX, Ser138–Arg153 VEGF-A165). The terminal amino acids and polar side chains were charged, and the peptides were solvated in a box of water molecules (Tripos explicit box solvation algorithm). Electrostatic charges were calculated with the method of Gasteiger and Marsili (47). Structures were minimized using the Tripos force field with a steepest descent gradient of 100 iterations followed by a conjugate gradient of 0.01 kcal/mol or a maximum of 10,000 iterations as termination criteria. During minimizations, a 12-Å non-bonded cut-off was applied, and when solvent was not present and ionic strength was set to 4.00.

For molecular dynamics simulations, all starting structures were solvated in a cubic box of explicit water molecules (edge 40 Å) using the XFIT solvation algorithm. This led to approximately 1700 water molecules depending on the solute starting conformation. All simulations were performed using periodic boundary conditions and the SHAKE algorithm. Constant temperature simulations (NTV, 300K) were run at a time step of 0.1 ps for a total period of 2000 ps. The Lennard-Jones interactions were evaluated with an 8.0-Å cutoff value. The non-bonded pair list was updated every 100 steps. Generated structures were stored in trajectory files every 5 ps, providing 400 conformers for each run. The collected structural data were analyzed with the graphic tools of the SYBYL® 7.0. All calculations were performed on the dual Hewlett-Packard work station XW60000, 2 × 2.8 GHz CPUs running REDHAT Enterprise Linux WS5.

Cell Culture—Human umbilical vein endothelial cells (HUVECs) were obtained from TCS CellWorks (Buckingham, UK) and cultured in endothelial cell basal medium (EBM) supplemented with 10% fetal bovine serum (FBS), 10 ng/ml human epidermal growth factor, 12 µg/ml bovine brain extract, 50 µg/ml gentamicin sulfate, and 50 ng/ml amphotericin-B. Porcine aortic endothelial cells expressing NP-1 (PAE/NP-1) (16) were provided by Dr. Shay Soker and grown in Ham’s F12 medium containing 10% FBS and 25 µg/ml hygromycin B. PAE cells expressing either KDR (PAE/KDR) (36) or Flt-1 (PAE/Flt-1) were provided by Professor Lena Claesson-Welsh (Uppsala University, Sweden) and grown in Ham’s F12 medium containing 10% FBS and 250 µg/ml Gentamicin G4H8. MDA-MB-231 breast carcinoma cells (gift of Professor Mike O’Hare) were cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS.

Radiolabeled Ligand Binding—Confluent cells in 24-well plates were washed twice with phosphate-buffered saline. At 4 °C various concentrations of peptides diluted in binding medium (Dulbecco’s modified Eagle’s medium, 25 mM HEPES pH 7.3 containing 0.1% bovine serum albumin) were added, followed by addition of the indicated concentration of 125I-VEGF-A165 (1200–1800 Ci/mmol, GE Healthcare Plc) or 125I-EGF. After 2 h of incubation at 4 °C (or the indicated time at 37 °C), the medium was aspirated, and washed four times with cold phosphate-buffered saline. The cells were lysed with 0.25 M NaOH, 0.5% SDS solution, and the bound radioactivity of the lysates was measured. Nonspecific binding was determined in 100-fold excess unlabeled VEGF-A165 or EGF (R & D Systems). Equilibrium dissociation constants (Kd) for peptides were calculated from IC50 values and the Kd of VEGF-A165 for NP-1 (0.3 nM) from Soker et al. (24), using the formula Kd = IC50/[1+(125I-VEGF-A165/Kd)].

Cross-linking—Confluent cells were bound with 125I-VEGF-A165 at 4 °C for 2 h as described above and then washed three times with phosphate-buffered saline. The bound 125I-VEGF-A165 was cross-linked to the cells by incubation with 1.5 M disuccinimidyl suberate (DSS) for 20 min at room temperature. After three washes with phosphate-buffered saline at 4 °C, the cells were solubilized in lysis buffer (64 mM Tris-HCl, pH 6.8, 0.2 mM Na3VO4, 2% SDS, 10% glycerol, 0.1 mM AEBSF, 5 µg/ml leupeptin) and scraped off the plates. After centrifugation at 16,000 × g for 20 min at 4 °C, cross-linked 125I-VEGF-A165-receptor complexes were subjected to 7.5% SDS-PAGE. Gels were dried and exposed to x-ray film.

Immunoblotting—Cells were pretreated with peptides for 15 min followed by treatment with growth factors for 10 min, and cells were immediately extracted by lysis buffer (64 mM Tris-HCl, pH 6.8, 0.2 mM Na3VO4, 2% SDS, 10% glycerol, 0.1 mM AEBSF, 5 µg/ml leupeptin). Activation of KDR, epidermal growth factor receptor (EGFR), PLC-γ, Akt, and ERKs1/2 was determined by immunoblotting cell extracts with antibodies that recognize KDR phosphorylated at tyrosines 1054 and 1059 (Oncogene Ltd), or 1175 (gift of Professor Shibuya), EGFR phosphorylated at tyrosines 845 and 1068, PLC-γ phosphorylated at tyrosine 783, Akt phosphorylated at serine 473 and ERK1 and 2 phosphorylated at threonine 202 and tyrosine 204 (all Cell Signaling Technology Inc, Beverly, MA). The same samples were immunoblotted with antibodies to the corresponding total proteins. Immunoreactive bands were visualized by chemiluminescence using horseradish peroxidase-conjugated anti-rabbit IgG and ECL reagent.

PGI2 and PGE2 Assay—After treatments, prostaglandin E2 (PGE2) and the stable PGI2 metabolite, 6-keto-prostaglandin F1α (PGF1α), were measured in cell culture supernatants using specific enzyme immunoassays kits (GE Healthcare Plc).

Apoptosis—Subconfluent HUVECs in 6-well plates were washed twice with serum-free M199 medium (Invitrogen), and incubated with the indicated additions for 24 h. The cells were then trypsinized, collected by centrifugation, and stained with fluorescein-conjugated annexin V and propidium iodide (Roche Applied Science). After staining, the cells were analyzed by flow cytometry using a FACScan (Becton Dickinson). Annexin V-positive staining cells were counted as apoptotic cells. Propidium iodide-positive and annexin V-negative cells were not counted as apoptotic cells.

Cell Proliferation—HUVECs were seeded at a density of 1 × 104 cells per well in 24-well plates. 24 h after plating, the medium was replaced...
with fresh EBM containing 0.5% FBS and 25 ng/ml VEGF-A165 in the absence or presence of EG3287. After 3 days, the cell numbers were determined using a Sysmex CDA-500 cell counter.

Data Analysis—Data were analyzed using Prism (version 3.0) statistical packages. Differences in prostanoid production among four treatment groups were evaluated using the one-way analysis of variance with Bonferroni’s multiple comparison tests. Differences in apoptotic frequencies between two groups were assessed by the chi-squared test. A value of \( p < 0.05 \) was taken as statistically significant.

RESULTS

Design and Synthesis of Bicyclic Peptides Mimicking the VEGF-A111–165 Region—The NMR-derived three-dimensional NMR solution structure of the VEGF-A111–165 region comprises two distinct protein subdomains, VEGF-A111–137 and VEGF-A138–165, each constrained and stabilized by two disulfide bonds and a network of hydrogen bonds (46). The C-terminal subdomain (Ser138-Arg165) features a typical \( \beta \)-hairpin turn combined with a short (2-step) \( \alpha \)-helix. Four positively charged amino acids are present in the helical region and three in the C-terminal exon 8 peptide. To elucidate the contribution of each subdomain to the binding and biological activity of VEGF-A165 mediated through neuropilin, we evaluated the biological effects of peptides corresponding to the subdomains in endothelial cells.

The formation of one or two specific disulfide bonds in peptides corresponding to each subdomain was achieved by pairwise selective deprotection of cysteine residues. The first disulfide bridge was formed by oxidation using either Me2SO (48), or potassium ferricyanide (III) (49), the second by a simultaneous Acm deprotection/oxidation step mediated by iodine, under acidic conditions (Fig. 1). The route was optimized such that only a single purification step, using reverse-phase HPLC-MS, was required to isolate the final compound.

Structure and Stability of EG3287—The structure of each subdomain in solution was determined by a series of molecular dynamic simulations. The root mean-squared values of the C\( \alpha \) chain for the two subdomains, between the average molecular dynamics structure and the starting conformation, were 3.5 and 0.45 Å for the N- and C-terminal subdomains, respectively. These values suggested that the C-terminal domain was more stable than the N-terminal domain (Fig. 2, A and B).

The resolved solution NMR structure for the C-terminal subdomain, described as EG3287, is shown in Fig. 2, C and D. The structure showed strong similarity both to the solution NMR structure of a recombinant protein comprising the C-terminal 55 amino acid residues of VEGF-A165 (46, 50), and to the molecular dynamics simulations for this region derived from the NMR structure (Fig. 2B). A 2.0-Å root mean-squared deviation value between the backbone carbons of EG3287 and the corresponding C-terminal domain of VEGF-A111–165 indicates the close similarity of the three-dimensional polypeptide fold in the excised subdomain and correlates well with the molecular dynamics simulations.

A liquid chromatography mass spectrometric (LC-MS/MS) assay demonstrated no significant degradation when the peptide was incubated at 37 °C for up to 24 h in water, EBM, or EBM supplemented with 10% fetal bovine serum, 10 ng/ml human epidermal growth factor, 12 \( \mu \)g/ml bovine brain extract, 50 \( \mu \)g/ml gentamicin sulfate, and 50 ng/ml

FIGURE 1. Synthesis of EG3287. The peptide chain was synthesized on Wang resin using Fmoc chemistry. Connectivity of the disulfides was achieved using an unambiguous cyclization protocol. The first cyclization was achieved using Fe(CN)6. Simultaneous deprotection of the Acm-protected Cys residues and cyclization using iodine provided, after HPLC purification, the bicyclic peptide EG3287.

FIGURE 2. Molecular dynamics and NMR structure of EG3287. A, N-terminal, and B, C-terminal subdomains excised from the 1KMX protein structure and subjected to molecular dynamics. The C-terminal domain was the more stable with a root mean-squared value of 0.45 Å compared with 3.5 Å; C, representation of the bundle of the 20 lowest energy conformers of NMR restraint-derived EG3287. The disulfide bonds are highlighted in yellow; N and C termini are indicated. D, ribbon drawing of one of the lowest energy conformations of EG3287. Note the close similarity between the structures of EG3287 and the corresponding region in native VEGF-A165 in B.
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Effects of peptides derived from VEGF-A_{165} exons 7 and 8 on VEGF-A_{165} binding to neuropilin

TABLE 1

Effects of peptides derived from VEGF-A_{165} exons 7 and 8 on VEGF-A_{165} binding to neuropilin

Sequences of exon7/8-derived peptides and positions of disulfide bonds are shown with IC\(_{50}\) values \(\pm\) S.E.M. and \(K\_\text{i}\) values \(\pm\) S.E.M. (\(\mu\)M) for inhibition of \(^{125}\text{I}-\text{VEGF-A}_{165}\) binding to PAE/NP-1 cells and (\% inhibition at 100 \(\mu\)M peptide). The structure of the VEGF-A_{165} C-terminal domain is shown above. X denotes aminobutyric acid (isosteric non-cyclizing substitution for cysteine); Z denotes aminoisobutyric acid; residues highlighted in red are mutations (alanine substitutions); Ac denotes amino-terminal acetylation.

| Peptide | VEGF-A_{165} residues | PAE/NP-1 IC\(_{50}\) (\(\mu\)M) | (\% inhibition at 100 \(\mu\)M) | Peptide structure |
|---------|-----------------------|------------------|-----------------|-----------------|
| 1       | 111-138               | No inhibition    |                 | H-ARGEPGCSRRKHLVFGDQPTCKGSCSKNTDSRCKARQLENNRTCCDKPRR-OH |
| 2       | 138-165               | 2.8\pm0.8/1.2\pm0.10 | (97)            | H-SCKNTDSRCKARQLENNRTCCDKPRR-OH |
| 3       | 138-165               | (72)             |                 |                 |
| 4       | 138-165               | 4.3\pm0.2/3.8\pm0.4 | (94)            | H-SCKNTDSRCKARQLENNRTCCDKPRR-OH |
| 5       | 138-165               | 11.5\pm0.3/6.4\pm0.3 | (89)            |                 |
| 6       | 138-165               | 3.0\pm0.7/2.5\pm0.9 | (91)            |                 |
| 7       | 138-165               | 4.3\pm0.3/2.9\pm0.6 | (85)            |                 |
| 8       | 138-165               | 12.0\pm0.8/6.8\pm0.4 | (82)           |                 |
| 9       | 138-165               | 0.0\pm0.9/6.2\pm0.13 | (86)           |                 |
| 10      | 138-165               | 6.9\pm0.0/6.2\pm0.13 | (86)           |                 |
| 11      | 138-165               | 3.8\pm0.2/2.6\pm0.24 | (79)          |                 |

amphotericin-8. Measurement of EG3287 after incubation with HUVECs at 37 °C indicated that whereas EG3287 was also largely unaffected with a half-life of \(~39\) h.

Structure-Function Analysis of the Requirements for VEGF-A_{165} Binding to Neuropilin—Initially the two bicyclic peptides corresponding to the subdomains VEGF-A_{111-137} (peptide 1) and VEGF-A_{138-165} (EG3287) were examined for binding to PAE/NP-1 cells. Only EG3287 exhibited significant activity (Table 1), localizing the main binding motif to the C-terminal subdomain. EG3287 selectively inhibited \(^{125}\text{I}-\text{VEGF-A}_{165}\) binding to PAE/NP-1 cells with an IC\(_{50}\) of 2.8 \(\mu\)M, and had little effect on binding to PAE/KDR or PAE/Flt-1, with a very similar IC\(_{50}\) of 3 \(\mu\)M (Fig. 3A). EG3287 also inhibited \(^{125}\text{I}-\text{VEGF-A}_{165}\) binding to MDA-MB-231 breast carcinoma cells, naturally expressing NP-1 but not KDR or Flt-1, with a very similar IC\(_{50}\) of 3 \(\mu\)M and complete inhibition of specific binding at 30 \(\mu\)M (Fig. 3A). In HUVECs, naturally expressing KDR, Flt-1 and NP-1 receptors, EG3287 inhibited \(^{125}\text{I}-\text{VEGF-A}_{165}\) binding with an IC\(_{50}\) of 20 \(\mu\)M, and caused a maximum 72% inhibition at 100 \(\mu\)M. When radiolabeled ligand binding experiments were performed at 37 °C for different times, EG3287 also effectively inhibited \(^{125}\text{I}-\text{VEGF-A}_{165}\) binding to PAE/NP-1 cells and HUVECs, but had no effect on binding to PAE/KDR cells (Fig. 3B). EG3287 did not affect binding of \(^{125}\text{I}-\text{EGF}\) to high affinity binding sites in MDA-MB-231 breast carcinoma cells expressing both NP-1 and EGF, and EGF had little effect on \(^{125}\text{I}-\text{VEGF-A}_{165}\) binding to PAE/NP-1 at concentrations up to 1 \(\mu\)g/ml (Fig. 3C), indicating that EGF does not compete with VEGF-A_{165} for binding to NP-1.

Prevention of cyclization by substituting all four cysteines with the non-cyclizing substitution for cysteine; Z denotes aminoisobutyric acid; residues highlighted in red are mutations (alanine substitutions); Ac denotes amino-terminal acetylation.
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Role of the VEGF Exon 8-encoded Domain in Neuropilin Binding—Whereas acetylation of the N terminus (peptide 9) had little effect on binding, transformation of the C terminus into the carboxamide (peptide 10) drastically reduced binding, indicating a key interaction with neuropilin at this point (Table 1). Surprisingly, an 8-residue linear peptide containing the 6 residues encoded by exon eight (peptide 11) demonstrated 73% inhibition of VEGF-A165 binding at 100 μM (Table 1). The contribution of the residues located in the region encoded by exon 8 was determined by means of an alanine scan of a 7-residue exon 8 peptide (peptide 12) in which the cysteine was replaced by aminobutyric acid to prevent problems arising from peptide dimerization (Table 2). Compared with peptide 12, mutation of either Lys162 (peptide 15), Pro163 (peptide 16), or Arg164 (peptide 17) had little effect, whereas mutation of Arg159 (peptide 13) or Asp161 (peptide 14) resulted in complete loss of activity, and substitution of Arg165 (peptide 18) increased the activity (Table 2). We also examined the effect of a peptide corresponding to the C terminus encoded by an alternative exon 8 found in VEGF-A165b (peptide 19), a recently identified isoform that is unable to stimulate endothelial cell proliferation, angiogenesis, or other biological activities (53). The peptide corresponding to the VEGF-A165b C terminus, Abu-RSLTRKD, had no effect on VEGF binding to PAE/NP-1 cells (Table 2).

EG3287 Inhibits Cross-linking of VEGF with KDR and NP-1 in HUVECs—Though EG3287 had no effect on VEGF-A165 binding to KDR in PAE/KDR cells in the absence of NP-1, we examined whether EG3287-mediated inhibition of VEGF binding to NP-1 in HUVECs could affect the ability of VEGF to bind to KDR. This question was addressed by investigating the effect of EG3287 on covalent cross-

![Image 3](https://via.placeholder.com/150)

**FIGURE 3.** Specific inhibition of 125I-VEGF-A165 binding to endothelial and breast carcinoma cells by EG3287. A, confluent PAE/NP-1, MDA-MB-231 breast carcinoma cells, HUVECs, PAE/Flt-1, and PAE/KDR cells were incubated for 2 h at 4 °C with 0.1 nM 125I-VEGF-A165 in the presence of the indicated concentrations of EG3287. Values represent mean percentages ± S.E. of specific 125I-VEGF-A165 binding calculated from the results of two or three independent experiments each performed in triplicate. B, confluent PAE/NP-1, PAE/KDR, and HUVECs were incubated for the indicated times (in hours) with 0.1 nM 125I-VEGF-A165 at 4 °C (hatched bars) or at 37 °C (black bars) in the presence of 100 μM EG3287. Bars represent mean percentages ± S.E. of specific 125I-VEGF-A165 binding calculated from the results of two or three independent experiments each performed in triplicate. The specific 125I-VEGF-A165 binding in the absence of any peptide is 100%. All peptides were readily soluble in water. C, left, MDA-MB-231 cells were incubated for 2 h with 0.1 nM 125I-EGF at 4 °C in the presence of the indicated EGF or EG3287 concentrations. Right, PAE/NP-1 was incubated for 2 h with 0.1 nM 125I-VEGF-A165 at 4 °C in the presence of 100 μM EG3287, 1 μg/ml VEGF-A165, or 1 μg/ml EGF. EG3287 has no effect on high affinity EGF binding, and EGF does not significantly compete with 125I-VEGF-A165 binding to PAE/NP-1.

![Image 4](https://via.placeholder.com/150)

**FIGURE 4.** Structures of disulfide-linked isomers of EG3287. A, single NMR conformer of EG327 with disulfides at Cys139-Cys158, Cys146-Cys160. B, molecular dynamics simulations of isomeric structure showing disulfides at Cys139-Cys146 and Cys158-Cys160 (peptide 3). C, isomeric structure with disulfides at Cys139-Cys158 and Cys146-Cys160 (peptide 4). B shows the least distortion from the EG3287 structure and has the highest biological activity.

**TABLE 2**

| Peptide | VEGF-A165 residues | PAE/NP-1 IC50/K | (%) inhibition at 100 μM | Sequence |
|---------|--------------------|-----------------|--------------------------|----------|
| 12      | 159-165            | (77)            | 100%                     | Ac-IBMCKRRR-OH |
| 13      | 159-165            | (12)            | 100%                     | Ac-IBMCKRRR-OH |
| 14      | 159-165            | (20)            | 100%                     | Ac-IBMCKRRR-OH |
| 15      | 159-165            | No inhibition   | 100%                     | Ac-IBMCKRRR-OH |
| 16      | 159-165            | No inhibition   | 100%                     | Ac-IBMCKRRR-OH |
| 17      | 159-165            | 10.5±0.1/9.8±0.12 | 71%                     | Ac-IBMCKRRR-OH |
| 18      | 159-165            | No inhibition   | 100%                     | Ac-IBMCKRRR-OH |
| 19      | (2)                |                 | 100%                     | Ac-XSSSlRKD-OH |
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FIGURE 5. EG3287 inhibits 125I-VEGF-A165 cross-linking to NP-1 and KDR in HUVECs. Confluent cells as indicated were incubated with 0.1 nM 125I-VEGF-A165 for 2 h at 4°C in the presence of EG3287 at the indicated concentrations, or in the presence of a 100-fold excess of unlabeled VEGF (NS, non-specific cross-linking). Cells were cross-linked to the bound 125I-VEGF-A165 as described under "Experimental Procedures." Cells were then solubilized and cross-linked proteins separated by SDS-PAGE and detected by autoradiography. The positions of KDR and NP-1 are indicated by arrowheads.

linking of 125I-VEGF-A165 to its receptors in HUVECs. As shown in Fig. 5, EG3287 strongly inhibited specific cross-linking of 125I-VEGF-A165 to two major bands of 250 kDa and 160 kDa corresponding to VEGF/KDR and VEGF/NP-1 complexes, respectively. In parallel experiments, EG3287 had no effect on 125I-VEGF-A165 cross-linking to KDR in PAE/KDR cells, but abolished cross-linking to NP-1 in PAE/NP-1 cells.

EG3287 Inhibits KDR Signaling—It was next determined whether inhibition of VEGF-A165 binding to NP-1 by EG3287 had any effect on the ability of VEGF-A165 to activate KDR. KDR phosphorylation at tyrosines 1054 and 1059 has been shown to be essential for kinase activity, whereas phosphorylation at Tyr1175 is required for tyrosine phosphorylation of PLC-γ and ERK activation (20, 54). EG3287 markedly inhibited phosphorylation of KDR at Tyr1175 in a concentration-dependent manner with a significant effect at 10 μM and almost complete inhibition at 100 μM (Fig. 6A). EG3287 also reduced VEGF-A165-induced Tyr1175 phosphorylation with a detectable effect at 30 μM and maximum but not complete inhibition at 100 μM.

A key early event following KDR activation is PLC-γ tyrosine phosphorylation and association of PLC-γ with KDR. EG3287 attenuated the increase in PLC-γ phosphorylation at tyrosine 783 induced by treatment with VEGF-A165 for 10 min, but did not cause a complete inhibition (Fig. 6A). Because VEGF-A165 induces activation of ERKs1/2 via PLC-γ and subsequent PKC activation, we next examined the effects of EG3287 on ERK activity. EG3287 inhibited VEGF-A165-induced activation of ERK1/2 in a concentration-dependent fashion, with a detectable decrease at 10 μM, and a maximum effect at 30–100 μM, similar to the concentration dependence of the inhibition of high affinity VEGF-A165 binding to NP-1 (Fig. 6A). In contrast to the inhibitory effects of the NP antagonist on VEGF-A165 signaling, EG3287 had no effect on EGF-induced EGFR phosphorylation at tyrosines 845 or 1068 and EGF-induced ERK1/2 activation in MDA-MB-231 breast carcinoma cells, or on FGF-2-induced activation of ERKs 1 and 2 in HUVECs (Fig. 6B).

The PKC-dependent ERK activation pathway is essential for VEGF-A165 stimulation of cPLA2 activity leading to increased PGI2 production (17, 21), a biological response that has been implicated in mediating VEGF-A-induced vascular permeability and vasculoprotective effects if VEGF-A in vivo (6). EG3287 caused a concentration-dependent decrease in VEGF-induced PGI2 generation measured either 30 min or 2 h after addition of VEGF-A165. Inhibition was detectable at 10 μM EG3287, with maximum inhibition at 100 μM (Fig. 7). EG3287 also caused a similar concentration-dependent inhibition of VEGF-A165 stimulation of PGE2 production (Fig. 7).

We also examined the ability of EG3287 to inhibit activation of Akt. EG3287 caused a partial inhibition of VEGF-A165-induced Akt activation, and, similar to its effects on ERK activity, also attenuated basal Akt phosphorylation (Fig. 8A). However, treatment with EG3287 caused no increase in cell death and did not decrease the anti-apoptotic effect of VEGF-A165 after 24 h, as determined by annexin V staining (Fig. 8B). EG3287 also had no significant effect on VEGF stimulation of endothelial cell proliferation (results not shown).
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FIGURE 7. Effect of EG3287 on VEGF-A165-induced prostanoid generation. Confluent cultures of HUVECs were incubated in serum-free medium for 30 min or 2 h with 25 ng/ml VEGF-A165 in the presence of the indicated concentration of EG3287; control cultures (C) were incubated in parallel for 30 min (hatched bars) or 2 h (black bars) with no additions. Supernatants were used to determine release of either the stable metabolite of PGI2, 6-keto-PGF1α (upper) or PGE2 (lower). Values for production of 6-keto-PGF1α, and PGE2 represent the means ± S.E. of results from three independent experiments. *, p < 0.05 versus VEGF treatment only; **, p < 0.01 versus VEGF treatment only; ***, p < 0.001 versus VEGF treatment only.

DISCUSSION

The conclusion that EG3287 is a specific inhibitor of binding to NP-1 is supported by the ability of the peptide to specifically compete with radiolabeled VEGF-A165 for binding to PAE/NP-1 cells, and to MDA-MB-231 breast carcinoma cells endogenously expressing NP-1 but not KDR or Flt-1, with very similar potency, whereas VEGF-A165 binding to PAE cells expressing either KDR or Flt-1 was unaffected. Furthermore, whereas the peptide completely abolished VEGF-A165 binding to cells expressing only NP-1 receptors, it caused only a partial inhibition of binding to HUVECs expressing all three receptors, and inhibited binding to HUVECs with significantly reduced potency, consistent with a selective effect on binding to the NP-1 receptor population. In addition, the present study aimed to elucidate the features of EG3287 required for inhibition of VEGF binding to NP-1 and to characterize the effects of EG3287 on VEGF-A165 receptor activation, intracellular signaling and downstream biological responses in endothelial cells.

The C-terminal 23 residues encoded by exon 7 in VEGF-A165 were previously identified as the core NP-1 binding domain (37). EG3287 overlaps with this domain, except that it contains the C-terminal six residues of VEGF-A165 encoded by exon 8 and lacks the first cysteine residue corresponding to position 22 of exon 7, previously identified as critical for the inhibitory activity of a glutathione S-transferase/exon 7 fusion protein (55). In addition, the structure of EG3287 is constrained by the introduction of two disulfide bonds, which were essential for optimal inhibition.

The strong similarity of the NMR-derived solution structures for EG3287 and the corresponding region of native VEGF-A165 obtained from the recombinant C-terminal 55 amino acids of VEGF-A165 (46), indicates that the constrained, disulfide-bonded structure of EG3287 closely resembles the native structure of the VEGF-A165 NP-1-binding domain. The EG3287 structure indicates several defined structural motifs, of which the most striking are a short α-helical region extending from residues 143–150, and a projecting region comprising the C-terminal six residues, encoded by exon 8. Similar stable small protein motifs have been observed in the Zn2+ binding domain of carbonic anhydrase, the scorpion toxin scyllatoxin and a charybdo toxin analogue (56–58), but EG3287 is unusual in constituting a stable protein domain with only 2 disulfide bridges between the α-helix and one of the β-sheet domains.

A surprising discovery was that peptides lacking the exon 7-encoded N-terminal residues and comprising largely the exon 8-derived sequence retained a significant degree of inhibitory activity, though with reduced potency compared with EG3287. Substitutions in this region indicated that the lysine, proline, and C-terminal arginine were all essential for inhibition of binding. The fact that amidation at the C-terminus almost completely prevented inhibition of VEGF-A165 binding to NP-1, further underscores the importance of the exon 8-encoded domain for NP-1 binding. Several substitutions designed to disrupt the predicted α-helical region reduced EG3287 activity, but did not abolish it, while alterations of the positions of the intramolecular disulfide bonds had significant though limited effects on the ability of EG3287 to inhibit NP-1 binding. Taken together, these results indicate an especially critical role for the C-terminal exon 8-derived region in VEGF-A165 recognition of NP-1. This conclusion is further supported by the recent finding that the naturally occuring immunostimulatory peptide, Tuftsin (sequence TKPR), and its higher affinity antagonist (TKPPR), are both similar in sequence to the exon 8 region of VEGF-A, and block VEGF-A165 binding to NP-1 (59).

The lack of a clearly defined role for the NP-1 intracellular domain suggests that this receptor is unlikely to play an independent role in VEGF-A165 signal transduction, and NP-1 has been proposed to act principally as a docking molecule for VEGF-A165 in endothelial cells. Given that EG3287 had no effect on VEGF-A165 binding to KDR in
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PAE/KDR cells, it was surprising that EG3287 markedly inhibited KDR phosphorylation at Tyr\(^{1054}\) and Tyr\(^{1059}\) induced by a maximally active VEGF-A\(_{165}\) concentration. Tyr\(^{1054}\) and Tyr\(^{1059}\) are in the activation region of the tyrosine kinase domain and phosphorylation at these sites has been shown to be essential for maximal activation of the KDR kinase, mutation of both these residues reducing VEGF-A-stimulated receptor activation to \(\sim 10\%\) of that for the native receptor (54). One explanation for the inhibitory effect of EG3287 on KDR activation is that NP-1 mediates a more stable physical association between VEGF-A\(_{165}\) and KDR, consistent with the reduction of radiolabeled VEGF-A\(_{165}\) cross-linking to KDR in HUVECs by EG3287, even though VEGF-A\(_{165}\) cross-linking to KDR in PAE/KDR cells lacking NP-1 was unaffected. These findings are in agreement with a previous report showing that formation of complexes between KDR and NP-1 enhance VEGF-A\(_{165}\) receptor binding (60), and consistent with a previous report showing that formation of complexes

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