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Enhancing Peptide Ligand Binding to Vascular Endothelial Growth Factor by Covalent Bond Formation

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Abstract

Formation of a stable covalent bond between a synthetic probe molecule and a specific site on a target protein has many potential applications in biomedical science. For example, the properties of probes used as receptor-imaging ligands may be improved by increasing their residence time on the targeted receptor. Among the more interesting cases are peptide ligands, the strongest of which typically bind to receptors with micromolar dissociation constants, and which may depend on processes other than simple binding to provide images. The side chains of cysteine, histidine, or lysine are attractive for chemical attachment to improve binding to a receptor protein, and a system based on acryloyl probes attaching to engineered cysteine provides excellent positron emission tomographic images in animal models (Wei et al. (2008) *J. Nucl. Med.* 49, 1828-1835). In nature, lysine is a more common but less reactive residue than cysteine, making it an interesting challenge to modify. To seek practically useful cross-linking yields with naturally occurring lysine side chains, we have explored not only acryloyl but also other reactive linkers with different chemical properties. We employed a peptide-VEGF model system to discover that a 19mer peptide ligand, which carried a lysine-tagged dinitrofluorobenzene group, became attached stably and with good yield to a unique lysine residue on human vascular endothelial growth factor (VEGF), even in the presence of 70% fetal bovine serum. The same peptide carrying acryloyl and related Michael acceptors gave low yields of attachment to VEGF, as did the chloroacetyl peptide.

Keywords

infinite affinity; aryl fluoride; lysine; mutation; mass spectrometry; VEGF

INTRODUCTION

Specific binding of one molecule to another is a widespread phenomenon in biology and is the basis of numerous practical applications directed at analysis, imaging, or therapy. In most familiar cases, the binding is reversible and may be characterized by an equilibrium

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Supporting Information Available: Experimental results for L19K-FDNB reaction with mutant VEGF K48A, and mass spectra of peptide-electrophiles. This material is available free of charge via the Internet at <http://pubs.acs.org>.

constant K_D ; a common rule of thumb in medicinal chemistry is that a drug should have a K_D value on the order of 10^{-9} M for its specific target, and negligible binding to other sites. At equilibrium, half the target sites will be occupied when the concentration of free drug equals K_D ; so for nanomolar K_D , nanomolar free drug will achieve half-saturation. In a living body, clearance of the drug from the tissues and other effects of molecular transport and chemical modification complicate this simple picture. Receptor ligands used as target-imaging probes may have properties similar to drugs, but often have a stronger constraint to remain on target for periods of hours or even days. The development of a drug, or ligand, that binds with at least nanomolar affinity without loss of other desirable properties is usually not a simple matter, even though sophisticated technologies have been developed for this purpose. Among the more challenging examples is the improvement of peptide ligands, the best of which typically bind in the micromolar instead of nanomolar range. This has attracted the attention of several labs that have chosen to explore the use of covalent attachment to deal with the shortcomings of low peptide affinity. A notable recent instance is the work of Holm et al., who used 58-residue affibody peptides carrying acryloyl groups to cross-link cysteine, histidine, or lysine side chains on protein targets.¹

Previously, we had developed antibodies with infinite affinity, which employed imaging probes carrying acryloyl groups to covalently label engineered cysteine side chains in the binding sites of anti-probe antibodies.²⁻⁴ Cysteine not involved in disulfide bonds is uncommon in extracellular proteins, usually making it necessary to use protein engineering to introduce a cysteine side chain at a desired site *in vitro*.⁵ Unlike cysteine and histidine, lysine residues are abundantly present on practically all proteins; if the covalent methodology were developed further to target naturally occurring lysine residues, it could lead to a new and interesting class of infinite-affinity ligands for biomedical use. Similar to the report by Holm et al.,¹ our efforts to employ ligands bearing acryloyl groups to cross-link specific lysine residues have achieved limited success, resulting in specific reactions but low yields. Here we explore some alternative reactive groups, finding one in particular that gives better yields in a relevant example. The system we use for illustration is a small 19-residue peptide ligand with modest $K_D \sim 10^{-6}$ M affinity for the 165-residue human signal protein Vascular Endothelial Growth Factor (VEGF).⁶

EXPERIMENTAL PROCEDURES

Chemicals

Maleic anhydride and chloroacetyl chloride were purchased from Sigma-Aldrich. Biotin-PEG₄-NHS, 1,5-difluoro-2,4-dinitrobenzene, 1-step TMB-Blotting substrate, and 1-step TMB-ELISA substrate were purchased from Thermo Fisher Scientific Pierce products (Rockford, IL). SYPRO Ruby® total protein stain was purchased from Bio-Rad (Hercules, CA).

Proteins

Recombinant human VEGF (VEGF₁₆₅ or VEGF-A) produced in *E. coli* was first purchased from Prospec-Tany (Rehovot, Israel). To obtain larger amounts, we turned to expression of VEGF in *E. coli*. Prof. Samuel Gellman and Holly Haase (University of Wisconsin-Madison) kindly provided plasmid pET-3d-VEGF165 transformed in *E. coli* BL21(DE3). Expression of VEGF was carried out as described.^{7, 8} Recombinant human VEGFR1-Fc chimera (rhFlt-1-Fc) was purchased from R&D Systems (Minneapolis, MN). Polyclonal rabbit anti-VEGF was purchased from Santa Cruz Biotech (Santa Cruz, CA). Streptavidin-AP and -HRP conjugates were purchased from Southern Biotech (Birmingham, AL). Goat anti-rabbit-HRP (1 mg/mL) was purchased from Abcam (Cambridge, MA). Rabbit anti-human Fc was purchased from Jackson ImmunoResearch (West Grove, PA). Trypsin and

chymotrypsin were purchased from Promega (Madison, WI). Bovine serum albumin fraction V was purchased from Fisher Scientific (Pittsburg, PA).

Miscellaneous

For control experiments, mutant VEGF K48A was cloned using QuikChange®XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers were purchased from Invitrogen (Carlsbad, CA), and expressed following the same procedures as VEGF. Competent *E. coli* BL21(DE3) was purchased from New England Biolabs (Ipswich, MA). The DNA mini-prep kit was purchased from Qiagen (Valencia, CA). Monomeric avidin resin was purchased from Thermo Fisher Scientific Pierce products (Rockford, IL).

Mass spectrometry

High-resolution mass spectra were acquired on an LTQ Orbitrap XL mass spectrometer equipped with an electrospray ionization source (ThermoFisher, San Jose, CA), operating in the positive ion mode. Samples were introduced into the source via direct infusion at a rate of 10 μ L/min. Collision-induced dissociation (CID) was used for MSⁿ experiments; the precursor ions were isolated with an isolation width of 3 Da, the ions were activated with a 25–35% normalized collision energy for 100 ms, and the q_z value was maintained at 0.250. The ions were subjected to CID in the ion trap portion of the instrument, and transferred to the Orbitrap for detection for high-resolution MSⁿ measurements. Spectra were acquired using Xcalibur, version 2.0.7 SP1 (ThermoFinnigan), and are an average of 16–53 scans. The spectra were externally calibrated using the manufacturer's standard calibration mixture, and then further calibrated internally with the lock mass tool.

Additional high-resolution mass spectra were acquired using Liquid Chromatography Tandem Mass spectrometry (LC-MS/MS). Peptides were separated and analyzed using a Michrom Paradigm MG4 HPLC system, (Michrom Bioresources, Auburn, CA) coupled with a Thermo Scientific LTQ-FT Ultra hybrid linear ion trap Fourier transform ion cyclotron resonance mass spectrometer (Thermo-Scientific, San Jose, CA) through a Michrom Bioresources Captivespray source (Michrom Bioresources, Auburn, CA). Peptides were loaded onto a C18 trap column (Zorbax 300SB-C18, 5 μ m, 0.3 mm $\times$ 5 mm: Agilent) and desalted online. Peptides are then eluted from the trap and separated with a reverse-phase Michrom Magic C18AQ (200 μ m $\times$ 150 mm) capillary column at a flow rate of 2 μ L/min. Peptides are eluted using a 45 min gradient of 2% B to 35% B over 30 min, 35% B to 80% B for 7.5 min, held at 80% B for 1 min, 80% B to 2% B in 0.5 min, and re-equilibrated for 11 min at 2% B (solvent A = aqueous 0.1% HCOOH; solvent B = 100% CH₃CN). The mass spectrometer was operated using a standard top 10 method where one high resolution survey scan (FT, 50,000 resolution) was followed by 10 low energy CID MS/MS LTQ ion-trap scans of the most intense ions eluting from the column. Dynamic exclusion was enabled.

Protein and Peptide identification were performed using Peaks Studio 5.3 (Bioinformatics Solutions Inc). Raw mass spectrometry files were loaded directly into Peaks Studio and searched against the VEGF protein sequence appended to the Common Repository of Adventitious Proteins database (www.thegpm.org/crap), which contains 112 sequences of common laboratory protein contaminants. The data were searched using a parent ion mass of 25 ppm and fragment ion mass of 0.5 Da, with trypsin and chymotrypsin enzyme specificity. Carboxamidomethylation of cysteine was searched as a fixed modification and the following masses were searched as variable modifications to lysine (K(amide) + linker = 309.11, ERK(amide) + linker = 594.25, EC(CAM)FERK(amide) + linker = 1030.39). In addition, oxidation of methionine and deamidation of asparagine and glutamine were searched as

variable modifications. A false discovery rate was calculated using an appended decoy database.

Low-resolution mass spectra were acquired in the positive ion mode with an AB Sciex Qtrap 2000 (Foster City, CA). Source parameters were 5kV spray voltage, with a curtain plate temperature of 275 °C and sheath gas setting of 15. Samples were analyzed via flow injection analysis by injecting 20 μ L samples into a stream of 50% CH₃CN:50% 0.1% HCOOH flowing at 200 μ L/min. Protein Prospector (www.prospector.ucsf.edu) was used to calculate theoretical m/z values.

Molecular modeling and peptide synthesis

To determine candidate sites for cross-linking on VEGF and peptide v107, the NMR structure of the complex (PDB 1KAT) was visualized using PyMOL Molecular Viewer. We explored possible modifications of peptide v107 to introduce a reactive side chain for cross-linker attachment. Distances between reactive residues on VEGF and potential v107 reactive side chains were modeled, resulting in the hypothesis that mutating Leu-19 to Lys would give the closest proximity to VEGF Lys-48 with no apparent steric hindrance from neighboring residues.

Solid phase peptide synthesis of v107 L19K, consisting of the sequence GGNECDARMWEWECFERK-NH₂ with either free or acetylated N-terminus, was performed by Biomatik (Wilmington, DE). Note that residues A7 and I8 were transposed from the original sequence.⁶ The two cysteine residues were bridged by a disulfide bond. The peptide was characterized via HPLC and electrospray ionization mass spectrometry (ESI-MS).

N-terminal biotinylation of peptide v107 L19K to form “L19K”

This protocol was modified from Sélo and co-workers.⁹ The v107 L19K peptide (1.4 μ mol, 1.8 mM) was prepared in 150 μ L DMF and 650 μ L of aqueous 0.1 M Et₃N•HOAc (pH 6.5). Peptide concentration was determined by measuring absorbance at 280 nm using $\epsilon_{280} = 11,500 \text{ M}^{-1}\text{cm}^{-1}$.⁸ Biotin-PEG₄-NHS (2 mg, 3.4 μ mol) was dissolved in 100 μ L of 0.1 M Et₃N•HOAc (pH 6.5) and immediately added to the peptide solution to a final concentration of 4 mM biotin-PEG₄-NHS and 1.5 mM peptide. The reaction was incubated at 4 °C overnight with shaking. The crude product was purified by RP-C18 HPLC using a semi-prep column (Phenomenex Luna 5 μ C18 300Å 250 $\times$ 10 mm). Mobile phases were A: aqueous 50 mM Et₃N•HOAc (pH 7), and B: CH₃CN with 0.1% CF₃COOH; gradient 25-50% B (0-50 min); flow rate = 3 mL/min. Yield = 90% for the product conjugated only at the N terminus (retention time = 18.1 min), determined by HPLC peak areas. Free peptide eluted at 15.4 min with a yield of 4%, biotinylation only at the lysine side chain eluted at 19.5 min with a yield of 2%, and doubly biotinylated peptide (both the N-terminus and the lysine side chain) eluted at 21.4 min with a yield of 4%. Identification of biotinylated species was performed by tandem mass spectrometry (see Supporting Information).

Synthesis of L19K-chloroacetamide (L19K-Cl)

Neat chloroacetyl chloride (0.5 μ L) was added to 100 μ L of 1 mM L19K dissolved in 1:1 CH₃CN:aqueous 0.1 M Et₃N•HOAc (pH 9). The reaction mixture was readjusted to pH 9 with neat Et₃N and incubated at RT with shaking for 15 min. The reaction was monitored by fluorescamine spot test for free amines until negative.^{10, 11} Hydrolysis of the acid chloride formed chloroacetic acid, significantly acidifying the reaction mixture to $\approx$ pH 2. The mixture was extracted with EtOAc several times to remove the chloroacetic acid. A modified 4-(4'-nitrobenzyl)pyridine (NBP) spot test for alkylating agents was used to monitor the progress of extraction.^{12, 13} EtOAc extracts were spotted on a silica plate. NBP

(5% w/v in acetone) was sprayed on the plate and heated at 100 °C for 5 min. A drop of neat Et₃N was applied to each spot, resulting in a violet color if chloroacetyl chloride or chloroacetic acid was present in the extract. Mass spec confirmed the chloroacetyl adduct (see Supporting Information).

Synthesis of L19K-Maleyl (L19K-M)

Maleic anhydride (10 μL of 100 mM) in CH₃CN was added to 100 μL of 1 mM L19K dissolved in 1:1 CH₃CN: aqueous 0.1 M Et₃N•HOAc (pH 8) and incubated at RT with shaking for 3 h. Completion of reaction was monitored by fluorescamine spot test. Purification was performed by several EtOAc extractions. Baeyer's test was used to confirm the efficiency of extraction for maleic anhydride, wherein a drop of 1% aqueous KMnO₄ was added to the ethyl acetate extracts to check for alkenes.¹⁴ HR-MS confirmed the maleyl adduct (in the absence of formic acid due to the instability of the adduct under acidic conditions, see Supporting Information).

Synthesis of L19K-5-fluoro-2,4-dinitrobenzene (L19K-FDNB)

As shown in Scheme 1, one μmol (10 μL of 100 mM) 1,5-difluoro-2,4-dinitrobenzene (DFDNB) was added to 0.1 μmol (100 μL of 1 mM) L19K dissolved in 1:1 CH₃CN:aqueous 0.1 M Et₃N•HOAc (pH 9) and incubated at RT for 2 h. Completion of the reaction was monitored by fluorescamine spot test. Purification was performed with several EtOAc extractions. A simple UV quench test was used to determine the efficiency of DFDNB extraction, with 254 nm radiation from a hand-held lamp applied to the EtOAc extracts spotted on a fluorescent silica TLC plate. Mass spec confirmed the FDNB adduct (see Supporting Information).

All the L19K-electrophiles were evaporated under reduced pressure and re-suspended in DMF as 1 mM solutions.

Reaction of L19K-electrophiles with VEGF

For all reactions, concentrations of 0.5 μM VEGF (1 μM binding sites) and 1 μM peptides (-M, -FDNB, and -Cl derivatives) were studied in PBS (pH 7.2), unless stated otherwise. All reactions were performed at 37 °C with shaking. VEGF concentrations are stated in terms of binding sites hereafter. For *comparing electrophilic linker reactivity*, 4 μL of 10 μM (40 pmol) peptide analog prepared in PBS was added to 10 μL of 4 μM VEGF sites (40 pmol binding sites) plus PBS to a total volume of 40 μL. The reactions were stopped after 24 h by adding 5× SDS-PAGE sample application buffer containing 20% v/v β-mercaptoethanol (5× SABR20) for subsequent western blot analysis.

For *pH-dependent* reactions, in addition to pH 7.2, additional samples were prepared using PBS adjusted to pH 8 and 9. These reactions were stopped after 1 h.

For *competitive binding* with unlabeled L19K, a tenfold dilution series (5 mM, 500 μM, 50 μM, and 5 μM) of N-terminal acetylated v107 L19K (competitor) was first prepared in PBS. A 2.5 μL aliquot of each concentration (containing 12,500; 1,250; 125; and 12.5 pmol respectively) was added to 4.3 μL of 5.8 μM VEGF binding sites (25 pmol), 2.5 μL of 10 μM L19K-FDNB (25 pmol), and PBS to a total volume of 25 μL in this order. These reactions were stopped after 1 h.

For *time-dependent* covalent attachment, 7.5 μM L19K-FDNB was reacted with 6 μM VEGF binding sites. A 10 μL aliquot from the reaction was collected after each time point (0.1, 0.2, 0.4, 0.8, 1.5, 3, 6, 9, and 24 h) and quickly frozen in liquid N₂ until electrophoretic analysis.

For *off-target reactions* of L19K-electrophile with other VEGF residues, L19K-FDNB and -Cl were each added to mutant VEGF K48A (1 μ M binding site final concentration), in which lysine-48 has been replaced by alanine. Each L19K-electrophile was also reacted with parental VEGF as a positive control.

For *protein selectivity* of L19K-FDNB for VEGF in the presence of serum proteins, fetal bovine serum was added to a final concentration of 2, 20, or 70% v/v. These reactions were stopped after 1 h. Control reactions include no added FBS, no VEGF, no peptide, or no electrophile.

Western blot to characterize L19K-electrophiles covalently bound to VEGF

L19K-electrophile-VEGF reaction mixtures were diluted with 5 $\times$ sample application buffer containing 20% v/v β -mercaptoethanol (5 $\times$ SABC20). Each 10 μ L sample was loaded on 10-20% Novex Tris-glycine gels for SDS-PAGE at 200 V for 50 min. Protein was electroblotted onto a PVDF membrane and blocked with 50 mL of 3% w/v bovine serum albumin (BSA) in Tris-buffered saline (TBS), for 1 h at 37 $^{\circ}$ C. For *anti-VEGF* western blots, the membrane was probed with 25 mL rabbit polyclonal anti-VEGF (1:500) in binding buffer (TBS containing 0.05% tween-20 and 3% BSA) for 1 h at RT. The membrane was washed 3 $\times$ with TBST. Secondary antibody conjugate goat anti-rabbit-HRP (1:500 dilution from 10 μ g/mL) in binding buffer was incubated for 1 h at RT. The membrane was washed 5 $\times$ with TBST. Turbo TMB-Blotting substrate (10 mL) was added and incubated at 37 $^{\circ}$ C until blue color developed.

Stripping western blots for re-probing

After anti-VEGF analysis, the blot was stripped of its antibody probes in 40 mL stripping buffer (62.5 mM Tris-HCl pH 7, 2% SDS, and 100 mM β -mercaptoethanol) and incubated at 50 $^{\circ}$ C with shaking at 70 rpm for 30 min. The blot was washed several times with H₂O and re-blocked with BSA for streptavidin-AP probing.

For *streptavidin-AP* western blots, the membrane was probed with 25 mL of streptavidin-AP (1:10,000) in binding buffer at RT for 1 h. The membrane was washed 4 $\times$ with TBST and 1 $\times$ with AP developing buffer (100 mM Tris-HCl, 100 mM NaCl, and 5 mM MgCl₂, pH 9.5). Protein bands were stained with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT, 0.4 mM) substrates in AP developing buffer until color developed.

Site-directed mutagenesis of VEGF K48A

The VEGF K48A mutant gene was constructed using a QuikChange[®] II XL site-directed mutagenesis kit according to the supplier's instructions. Plasmid pET-3d-VEGF₁₆₅ was used as the DNA template, in which the VEGF gene is located between NcoI and BamHI restriction sites and under control of a phage T7 promoter. Sequences of primers were as follows, with the mutation underlined in bold letters: Forward 5'-

GATCGAGTACATCTTC**GCG**CCATCCTGTGTGCCCC; Reverse 5'-

GGGGCACACAGGATGG**GCG**GAAGATGTACTCGATC. DNA template (50 ng) was mixed with 100 ng of each primer along with 1 $\times$ reaction buffer, dNTPs, Quick solution, DNA polymerase (2.5 μ L) and DNase-free water to a total volume of 50 μ L. The reaction was thermally cycled with an initial cycle at 95 $^{\circ}$ C for 1 min followed by 18 cycles (95 $^{\circ}$ C for 50 s, 68 $^{\circ}$ C for 50 s, 68 $^{\circ}$ C for 15 min), and a final cycle at 68 $^{\circ}$ C for 7 min. Note that the annealing temperature was optimal at 68 $^{\circ}$ C and is the same temperature as the elongation cycle. Dpn I was added and incubated at 37 $^{\circ}$ C for 3 h. The mutant plasmid was transformed into ultracompetent XL2-Blue *E. coli* following standard transformation protocol. Each reaction was plated onto LB (+100 μ g/mL carbenicillin) plates. Selected colonies were purified using a Qiagen mini-prep kit, characterized using a 1.2 % agarose gel, and

submitted for DNA sequencing (Davis Sequencing, Davis, CA). The resulting sequence was analyzed using Bioedit (Supporting Information). The selected plasmid was transformed into competent *E. coli* BL21(DE3) for protein expression and isolation as described for parental VEGF by Peterson and co-workers.⁸

Characterization of binding activity of VEGF mutant K48A by competitive ELISA

The activity of mutant VEGF K48A was characterized by competitive ELISA with biotinylated parental VEGF conjugate (biotin-VEGF) against immobilized recombinant human VEGFR1-Fc chimera (rhFlt-1-Fc). Biotin-VEGF was prepared by conjugating 1 mM biotin-PEG₄-NHS with 200 µg/mL parental VEGF in 50 µL PBS at 4 °C overnight, and desalted with gel filtration spin columns. For ELISA, 50 µL of 5 µg/mL rhFlt-1-Fc in PBS was immobilized in a 96-well microtiter plate at 4 °C overnight. The wells were washed with PBS and blocked with 300 µL of 1% BSA in PBS at RT for 1 h. Four-fold serial dilutions (50 µL) of VEGF mutant K48A starting from 200 nM in binding buffer (1% BSA in PBS) was added to rhFlt-1-Fc and incubated at RT for 1 h in triplicate wells. The wells were washed 2× with PBST. Biotin-VEGF (50 µL of 6 nM sites) in binding buffer was added and incubated at RT for 1 h. The wells were washed 3× with PBST. Streptavidin-HRP (1:4000) in binding buffer was added and incubated at RT for 1 h. The wells were washed 5× with PBST. 1-Step Turbo-TMB ELISA (100 µL) was added and incubated at 37 °C until blue color developed. Final yellow color was stabilized with 100 µL of 1 M H₂SO₄. Absorbance was read at 450 nm. As a positive control, rhFlt-1-Fc was detected using rabbit anti-human Fc (1:5000), followed by goat anti-rabbit-HRP conjugate (1:500 dilution from 10 µg/mL). Negative controls included omission of Flt-1-Fc, biotin-VEGF, or streptavidin-HRP.

Affinity purification of L19K-FDNB-VEGF and enzymatic digestion for mass spectrometric analysis

Using the biotin tag on the peptide, cross-linked L19K-FDNB-VEGF (100 µg, 900 µL) was purified from unreacted VEGF using 1 mL of monomeric avidin resin (Pierce), following the manufacturer's protocol. The conjugate was eluted with 5 mM biotin in PBS containing 0.02% NaN₃, in 1-mL fractions. A Bradford colorimetric assay was used to determine which fractions contained the conjugate. The conjugate was precipitated using trichloroacetic acid with sodium deoxycholate, followed by acetone precipitation, as described by Pierce protocols (Tech Tip #8).

The pellet was resuspended in 50 µL of 50 mM NH₄HCO₃ (pH 8.5) and up to 10% of CH₃CN by volume. Trypsin digestion was performed following standard procedures. Briefly, the conjugate was reduced and denatured first by the addition of TCEP (1 µL of 500 mM; final 10 mM) and heated at 95 °C for 10 min. The conjugate was alkylated with 15 mM iodoacetamide at RT in the dark for 1 h, and quenched with 5 mM DTT for 10 min. Trypsin (1.5 µg) was added and the mixture incubated at 37 °C overnight. The reaction was stopped by heating to 95 °C for 5 min. Chymotrypsin (1.6 µg) was added and the mixture incubated at 37 °C overnight. The reaction was stopped by heating to 95 °C for 5 min.¹⁵ The peptides were desalted using a Sep Pak C18 cartridge, and the solvent was evaporated using a speed-vac. The peptide fragments were resuspended in aqueous 10% CH₃CN. LC-MS/MS was performed by UC Davis Proteomics Core facility.

RESULTS

N-terminal biotinylation of L19K

The NMR structure¹⁶ indicated that labeling with a tag at the N-terminus of the peptide would not interfere with binding to VEGF (Figure 1). We chose biotin-PEG₄-as the tag

because it is convenient for subsequent western blotting with streptavidin conjugates. In addition, the PEG₄ spacer between biotin and the peptide should allow room for streptavidin to capture biotin without steric hindrance from the L19K-VEGF cross-linked product. Analytical results demonstrating isolation of the pure N-terminal conjugate are provided in Supporting Information.

Conjugation of peptides to VEGF: yields with L19K-FDNB, -M, and -CI

As an example, the L19K-FDNB reaction chemistry is shown in Scheme 1. Reducing, denaturing conditions were employed for anti-VEGF western blot analysis to confirm irreversible covalent reactions and to obtain distinguishable bands between unreacted monomer VEGF (~20 kDa) and the cross-linked VEGF-peptide (~23 kDa). Next, the blot was stripped of its anti-VEGF probes and re-probed with streptavidin-AP to detect the biotinylated peptide (~3 kDa) and its 23-kDa VEGF conjugate.

After 24 h incubation of 1 μ M L19K-electrophiles with 1 μ M VEGF (sites) at pH 7.2 and 37 °C, biotin-tagged peptide in conjugates was detected at ~23 kDa using the sensitive streptavidin western blot (Figure 2A). Control reactions with no electrophile or no peptide did not show bands at ~23 kDa. Free biotinylated peptide was observed to migrate below 10 kDa.

Using the less sensitive anti-VEGF western blot, 1 μ M L19K-FDNB gave a modest conversion of 1 μ M VEGF to the conjugate migrating at ~23 kDa (Figure 2B), while the ~23 kDa conjugates of VEGF with L19K-M and -CI were not detected. Free VEGF was observed at ~20 kDa.

The reactivity of L19K-FDNB was further investigated by varying the pH of the reaction. As shown in Figure 2C, after 1 h the bands corresponding to L19K-FDNB-VEGF (~23 kDa) had higher intensities at pH 8 and 9 compared to pH 7.2. SYPRO total protein stain confirmed the presence of unconjugated VEGF at ~20 kDa, but the conjugated product was not detectable during the first hour (Figure 2D).

Competitive binding between excess peptide L19K and 1 μ M L19K-FDNB for VEGF (1 μ M sites) was briefly explored. After 1 h, the highest concentration of the competitor (500 μ M) completely prevented the permanent attachment of L19K-FDNB to VEGF (Figure 2E).

A time course study of a small excess of L19K-FDNB (7.5 μ M) binding to 6 μ M VEGF sites was performed at pH 7.2 from 5 min to 24 h of incubation at 37 °C (Figure 3, lanes 0.1–24 h). The yield of covalent product increased over 24 h, approaching saturation of VEGF (~23 kDa) as shown on a total protein-stained gel under reducing conditions. A control lane under *non-reducing* conditions (lane 24*) shows that after 24 h almost no monomer VEGF is visible at ~20 kDa, indicating that the VEGF dimer is stable under these incubation conditions.

To confirm that L19K-FDNB reacts specifically with VEGF residue Lys-48, we performed trypsin and chymotrypsin double digests of the L19K-FDNB-VEGF conjugate and analyzed the peptide fragments by LC-MS/MS as described in Experimental Procedures. A possible solution to the VEGF peptide fragment containing Lys-48 was identified as IFK(309.11)PSC(CAM)VPL (Figures 4 and 5). Mass modification of 309.11 on VEGF Lys-48 corresponds to the L19K-FDNB fragment, 2,4-dinitrobenzyl-5-lysine. CAM represents a carboxamidomethyl modification on the cysteine.

Mutating VEGF Lys-48 to Ala eliminates reaction between 1 μ M L19K-FDNB and 1 μ M VEGF mutant. Though the reported affinity of v107 for VEGF K48A ($K_D > 10 \mu$ M) is

weaker than for parental VEGF ($K_D \approx 1 \mu\text{M}$),¹⁶ we can still gain valuable information regarding the background reactivity of L19K-electrophiles with the other 10 lysine residues on the protein. L19K-FDNB and -Cl were each incubated with equimolar $1 \mu\text{M}$ mutant VEGF K48A sites at 37°C for 24 h, comparing parental VEGF as positive control. A streptavidin western blot showed that the analogs did not yield covalent products with the mutant, while they did yield covalent products with parental VEGF (see Supporting Information). This supports the expectation that L19K-FDNB and -Cl do not react with the other lysines on parental VEGF or mutant K48A under these conditions. To assure that VEGF mutant K48A was folded properly, we measured its ability to compete with biotinylated parental VEGF for binding to an immobilized VEGF receptor (rhFlt-1-Fc). Concentrations of VEGF K48A $>10 \text{ nM}$ reduce the binding of 6 nM parental VEGF to Flt-1-Fc in a dose-dependent manner (see Supporting Information), implying that the mutant is functional and binds to the same site on the receptor as parental VEGF.

In the presence of 2, 20, or 70% v/v fetal bovine serum, L19K-FDNB ($1 \mu\text{M}$) reacted with VEGF ($1 \mu\text{M}$ sites), but showed minimal attachment to serum proteins after 1 h (Figure 6). Streptavidin western blots showed that the band for L19K-FDNB-VEGF conjugate at $\sim 23 \text{ kDa}$ is significantly darker than the smeared background in the lane due to serum. The bloom from the large concentration of serum albumin loaded on the gel (see SYPRO, Figure 6 inset) gives an idea of the selectivity of the reaction.

DISCUSSION

Previous work by our colleagues successfully used a DOTA-acrylamide ligand to tag an engineered cysteine adjacent to the binding pocket of an antibody for imaging *in vivo*.^{3, 4} We wished to extend this approach to specific attachment of a probe to a naturally occurring lysine residue on a protein target. Our initial results were similar to Holm and co-workers, who used an affibody-acrylamide ligand to target lysine, cysteine, and histidine near the binding pocket of its protein target.¹ For our initial studies we prepared derivatives of peptide L19K not only with acryloyl, but also with closely related crotonoyl, fumaryl, and maleyl tags as potential aza-Michael acceptors to explore whether adding substituents would enhance the conjugation yield with the VEGF site (structures are provided in Scheme 1). As a typical representative of this class of tagged peptides, the maleyl derivative **4** is included in the experiments below.

All the aza-Michael acceptors above gave low conjugation yields with VEGF under physiological conditions. While streptavidin western blots detected the biotin-tagged peptide in conjugation products, yields were so low that anti-VEGF western blots and also SYPRO total protein stain showed that practically all the VEGF remained unmodified. These low yields could be due to multiple factors such as weak affinity of L19K-acrylamide for VEGF, steric constraints on reaction between the bound acryloyl and the Lys-48 nucleophile, and a small population of the unprotonated free amine available to undergo an aza-Michael addition with the acryloyl moiety during its non-covalent bound lifetime. Other systems of affinity-induced covalent capture by Michael addition rely on stronger non-covalent association and a sulfur nucleophile.^{3, 4, 17} The acryloyl-affibody ligand model system of Holm and co-workers, which reacts covalently with an engineered lysine near the protein binding pocket, gave a significantly lower yield than its engineered cysteine counterpart.¹

To seek improved cross-linking yields with lysine, we explored other electrophilic linkers with different chemistry. While they react with nitrogen nucleophiles, chloroacetamide groups are typically more reactive with sulfur, and can attach fluorescent tags with affinity to a molecular probe via alkylation of a nearby cysteine side chain.¹⁸ We examined the

reaction of L19K–Cl **5** with VEGF Lys-48; similar to the acrylamide analogs and perhaps for similar reasons, the yield was poor.

Next we examined the classic reagent DFDNB, which has been used to cross-link molecules via primary amines in a variety of circumstances such as attaching peptides to protein binding sites,^{19, 20} amino acids to each other,²¹ or phospholipids to cell-surface proteins.²² Due to the electron withdrawing and resonance properties of –NO₂ groups in the 2- and 4-positions of the benzene ring of 1,5-difluoro-2,4-dinitrobenzene (DFDNB), in addition to the activating effect of the 5-fluorine on the 1-fluorine,²³⁻²⁵ DFDNB appears to be a very reactive cross-linker. The symmetric nature of DFDNB may give the impression that the reactivity of both fluorine leaving groups is equal. However, after displacement of the first fluorine by an amine nucleophile to form **6** (Scheme 1), the reactivity of the remaining fluorine is significantly lower owing to the amine's inductive effect. This makes L19K-FDNB **6** intrinsically less reactive than the similar-looking small molecule 1-fluoro-2,4-dinitrobenzene (FDNB or DNFB), which Sanger famously used in the sequencing of insulin.²⁶ The markedly low reactivity of L19K-FDNB with serum proteins (Figure 6) is likely to depend on steric hindrance by the L19K peptide in addition to the electronic properties of the fluorodinitroaryl group. The latter group is not hydrophilic and so might interact favorably with the peptide surface, while the rather restricted geometry for nucleophilic attack by a protein-bound lysine could further disfavor reactions with all but specifically bound proteins. Such effects may be important, since any applications beyond these *in vitro* experiments would depend on the reagent maintaining its specificity in biological environments similar to serum.

Another topic to consider is the classical experiments of Landsteiner and Jacobs, who prepared guinea-pig immunogens from 1-halo-2,4-dinitrobenzenes.^{27, 28} Landsteiner used intracutaneous administration techniques designed to efficiently produce sensitization in order to study the immunological properties of these electrophilic small molecules, which are much less shielded than **6** in Scheme 1. Nevertheless, the possibility that *in vivo* applications of molecules related to L19K-FDNB would stimulate an immune response requires further investigation.

The reaction of L19K-FDNB **6** with VEGF gave yields superior to L19K–Cl **5** or L19K–M **4** in PBS (pH 7.2) at 37 °C under equimolar reaction conditions and at low micromolar concentration (Fig. 2A, B). The size and shape of the cross-linker, nature of the reaction, and microenvironment of the peptide-VEGF binding pocket could all affect the yield of the covalent product. In this case, the “reach” of all three cross-linkers is comparable (lysine – (CH₂)₄NH-plus~2-3 Å), but their shapes are distinct and the relative bulk of the –FDNB is evident. The local environment of the peptide-VEGF binding pocket contains aromatic residues such as VEGF Phe-17 and Tyr-21, which may interact favorably with the aromatic ring of –FDNB and aid in prolonging the residence lifetime of the peptide on VEGF prior to covalent labeling. Our time-course study (Figure 3) shows that covalent labeling can be observed as early as 5 min and increases to almost quantitative after several hours. This yield is comparable to systems with engineered cysteines near the protein binding pocket and acryloyl-based ligands.^{1, 29}

The mass spectrometric analysis of the VEGF conjugate tryptic fragments (Figures 4 and 5) agrees with our expectation that VEGF Lys-48 was the site of covalent labeling with L19K–FDNB. Other products were not detected by mass spectrometry. Study of the VEGF mutant K48A indicates that L19K–FDNB does not react detectably with any of the other 10 lysine residues or the N-terminus of mutant K48A. Both mass spectrometry and western blotting with mutant K48A imply that the peptide's affinity for parental VEGF drives L19K–FDNB to selectively tag Lys-48. Previous studies with intramolecular cross-linking of insulin

chains and also a peptide with affinity for thrombin using DFDNB, are consistent with these findings of site-selective cross-linking to a nearby lysine.^{19, 20}

CONCLUSIONS

The covalent attachment of ligand to target is interesting as an alternative strategy to the extensive use of combinatorial library technology to seek synthetic ligands that bind usefully to naturally occurring proteins. In order for covalent attachment to be useful, the chemistry involved must be so mild (or so shielded) that reactions with off-target molecules are negligible, but a significant portion of the target protein should be affected. This need not be a high percentage in all cases, since applications such as imaging do not require it. When designing covalent ligands for natural biological targets, engineering highly nucleophilic residues such as cysteine near the ligand-binding site on the protein target is usually not an option. Since lysine side chains occur in high abundance, finding a suitable lysine cross-linker is an important challenge. The results described here suggest that electrophiles inspired by aryl halides may provide a way forward.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DFDNB	1,5-difluoro-2,4-dinitrobenzene
Et₃N•HOAc	Triethylammonium acetate
FBS	fetal bovine serum
L19K	Biotin-peptide v107 L19K
L19K-Cl	Biotin-peptide v107 L19K-Chloroacetamide
L19K-M	Biotin-peptide v107 L19K-Maleyl
L19K-FDNB	Biotin-peptide v107 L19K-5-Fluoro-2,4-dinitrobenzene
NBP	4-(4'-nitrobenzyl)pyridine
PBST	PBS containing 0.05% tween-20
streptavidin-AP	conjugate of streptavidin with alkaline phosphatase
RT	room temperature
TCEP	<i>tris</i> (2-carboxyethyl)phosphine
DTT	dithiothreitol
VEGF	human vascular endothelial growth factor (Human VEGF ₁₆₅ or VEGF-A)

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Figure 1.

Covalent labeling of VEGF Lys-48 with biotinylated L19K-electrophiles. **A)** Molecular modeling of NMR structure PDB 1KAT, showing ribbon structures of peptide in red and VEGF in teal. Peptide N-terminus does not participate in binding to VEGF. Lysine side chains are shown in yellow (CPK coloring). Boxed area was zoomed to show v107 L19K side chain (orange) in close proximity to VEGF Lys-48. **B)** Schematic representation of reversible interaction between peptide-electrophile and VEGF, followed by covalent labeling to VEGF Lys-48 with electrophiles maleyl (**M**), 5-fluoro-2,4-dinitrobenzene (**FDNB**), or chloroacetamide (**Cl**).

Figure 2.

Western blots and SYPRO total protein stain characterization of VEGF-peptide conjugates under reducing conditions. Red arrows indicate the position of bands representing the conjugate on the blot or gel at ~23 kDa. Unreacted peptide (~3 kDa) is shown below 10 kDa. Protein standards (L) are labeled in kDa units. **A**) The maleyl analog was included as a representative aza-Michael acceptor for comparison with electrophiles -Cl and -FDNB. After 24 h incubation of 1 μ M L19K-electrophiles with 1 μ M VEGF (sites) at pH 7.2 and 37 $^{\circ}$ C, comparison of reactivity with VEGF by detecting peptide with Streptavidin. Bands corresponding to unreacted peptide are at the bottom of the blot. Control reactions: no electrophile (lane 4) and no peptide (lane 5). **B**) The same blot as **A** probed with the less sensitive anti-VEGF shows that only L19K-FDNB gave a detectable product (lane 2). The bottom of the blot is not shown because no bands were visible. **C**) Reaction of L19K-FDNB and VEGF after 1 h at pH 7.2, 8, or 9 (lanes 1-3, respectively) probed with Streptavidin, showing increasing product band intensities with pH 8 and 9. Control reactions: no electrophile (lanes 4-6) and no peptide (lane 7). **D**) SYPRO stain of **C** confirmed the same amount of VEGF loaded on each lane, but is not sensitive enough to detect VEGF conjugate after 1 h. **E**) Competition between 1 μ M L19K-FDNB and 0.5, 5, 50, and 500 μ M unlabeled L19K (lanes 2-5 respectively) for 1 μ M VEGF sites shows that during 1 h incubation, 500 μ M competitor prevents L19K-FDNB reaction with VEGF (lane 5). Control reactions: no competitor (lane 1) and VEGF alone (lane 6). **F**) SYPRO stain of **E** confirmed the same amount of VEGF. Covalent product was not yet detectable after 1 h. Unreacted peptide competitor is visible at higher concentrations (lanes 4 and 5).

Figure 3. SYPRO stain from time course study of 7.5 μ M L19K-FDNB and 6 μ M VEGF sites from 5 min (*lane 0.1*) up to 24 h (*lane 24*) at pH 7.2, 37 °C, showing almost quantitative conversion of VEGF to the conjugate after $\approx$ 9 h of incubation. Red arrow indicates the position of VEGF conjugate migration on the gel. Control: non-reducing aliquot from 24 h reaction, showing practically no monomer VEGF at $\sim$ 20 kDa.

Figure 4. FT-ICR-MS1 of tryptic and chymotrypsin fragment of VEGF conjugate, IFK(309.11)PSC(CAM)VPL. Doubly charged $[M+2H]^{2+}$ m/z calcd 685.3516, observed 685.3548. A modification of mass 309.11 corresponds to the L19K-FDNB fragment.

Figure 5. MS2 of IFK(309.11)PSC(CAM)VPL. Theoretical values were calculated using Protein Prospector. Peaks Studio 5.3 software was used to identify the modified peptide. A modification of 309.11 corresponding to the L19K-FDNB fragment is indicated on VEGF Lys-48, as K(+309.11). The spectrum and table highlight the observed y ions in red and b ions in blue. The table lists the peptide sequence along with m/z values corresponding to theoretical b and y ions. Errors for the observed masses are plotted on the graph and are within 0.5 Da.

Figure 6.

Streptavidin western blot of 1 μ M L19K–FDNB and 1 μ M VEGF sites in the presence of 2, 20, and 70% fetal bovine serum (*lanes 3-5*) showing selectivity of L19K–FDNB for VEGF after 1 h. Inset: SYPRO stain shows an overwhelming amount of proteins, particularly albumin, in these reactions. On the left side, L denotes protein standards. Controls: peptide only (*lane 1*), no serum (*lane 2*), no electrophile (*lane 6*), and no peptide (*lane 7*).

Scheme 1.

Electrophiles attached to peptide L19K (denoted R): **1** acryloyl, **2** crotonoyl, **3** fumaryl, **4** maleyl, **5** chloroacetyl, **6** FDNB. Example reactions: tagging the peptide's unique lysine with DFDNB, and cross-linking of **6** (peptide L19K-FDNB) to VEGF Lys-48 in the peptide•VEGF complex. The structure of unmodified peptide was taken from PDB 1KAT; the fluorodinitroaryl group may interact with the peptide surface in ways not suggested by the drawing.