Discovering Small Molecule Ligands of Vascular Endothelial Growth Factor That Block VEGF–KDR Binding Using Label-Free Microarray-Based Assays

James P. Landry,[1] Yiyan Fei,[1] Xiangdong Zhu,[1] Yaohuang Ke,[2] Guoliang Yu,[2] and Pierre Lee[2]

[1]Department of Physics, University of California at Davis, Davis, California.
[2]Epitomics, Inc., Burlingame, California.

ABSTRACT

We present here a label-free microarray-based assay platform that we used to identify inhibitors of vascular endothelial growth factor (VEGF)–kinase-insertion domain receptor (KDR) binding. Supported by a combination of special ellipsometry-based optical detection and small molecule microarrays (SMM), this platform consists of three assays: (1) the first assay detects binding of a target protein with SMM and identifies ligands to the protein as inhibitor candidates; (2) the second assay detects binding of a receptor protein with identical SMM and subsequent binding of the target protein (a sandwich assay) to identify the ligands to the receptor protein that do not interfere with the target-receptor binding; (3) the third assay detects binding of the target protein to the receptor protein in the presence of the ligands of the target protein identified from the first assay, with the receptor protein immobilized to a solid surface through the ligands identified in the second assay, to yield dose–response curves. Using this platform, we screened 7,961 compounds from the National Cancer Institute and found 12 inhibitors to VEGF–KDR (VEGFR2) interactions with IC_{50} ranging from 0.3 to 60 μM. The inhibitory potency of these inhibitors found in the microarray-based assay was confirmed by their inhibition of VEGF-induced VEGFR2 phosphorylation in a cell-based assay.

INTRODUCTION

The vascular endothelial growth factor (VEGF) is a homodimeric member of the cystine knot family of growth factor proteins.[1] It has a high specificity for vascular endothelial cells and functions as a potent mitogen in angiogenesis through binding to cell-surface receptors of the tyrosine kinase family such as the kinase domain receptor (KDR) and the fms-like tyrosine kinase (Flt-1). VEGF in a dimeric form binds to extracellular domains (ECDs; primarily domains 2–3)[2] of the KDR and cause the latter to form dimers and, in turn, autophosphorylate the intracellular domains. This event activates a host of downstream signaling pathways, including angiogenesis. Excessive expression of VEGF is one of the several means that cancerous cells employ to survive and grow. As a result, suppression of VEGF–KDR binding activity is one of the cancer intervention strategies in drug development.[2–13] So far, small molecule compounds have been explored almost exclusively for KDR ligands that bind to the intracellular tyrosine-kinase domain of KDR and, in turn, block the kinase activity of the membrane protein.[3–5,12,13] Most anti-VEGF agents in research and drug development have been neutralizing proteins such as monoclonal antibodies (*e.g.*, Bevacizumab or Avastin from Genentech),[6,7] peptides (*e.g.*, Cyclo-VEGI from Merck),[9] aptamers (*e.g.*, Macugen from Eyetech Pharmaceuticals and Pfizer),[10] and soluble decoy receptors (*e.g.*, VEGF-Trap from Regeneron Pharmaceutics).[11] Anti-VEGF monoclonal antibodies and other large neutralizing proteins have the advantage of being highly specific and, thus, of low toxicity in general, and yet suffer from high cost of manufacturing and the requirement of parenteral administration. Only a handful of peptides, such as cyclic vascular endothelial growth inhibitor (Cyclo-VEGI) have been explored as small molecule ligands of VEGF for their blocking effect on VEGF–KDR binding. Given the advantage of low cost of manufacturing and the ease of administration and the fact that small molecule compounds other than peptides have not been extensively studied as novel VEGF ligands against VEGF–KDR binding, our present study focused on the discovery of VEGF ligands that interrupt the VEGF–KDR binding.

In this article, we report the application of a label-free microarray-based assay platform[14–24] to screen 7,961 compounds from the National Cancer Institute Developmental Therapeutics Program (NCI/DTP) for ligands of VEGF and VEGF receptor, Type-2 (VEGFR-2; also known as KDR), with the goal to identify compounds that inhibit

ABBREVIATIONS: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; ECD, extracellular domain; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; Fc-KDR, KDR fused to the immunoglobulin G Fc domain; Fc-VEGF, VEGF fused to the immunoglobulin G Fc domain; HEK, human embryonic kidney; KDR, kinase-insertion domain receptor; NCI, National Cancer Institute; NCI/DTP, NCI Developmental Therapeutics Program; NSC, Cancer Chemotherapy National Service Center; OI-RD, oblique-incidence reflectivity difference; SMM, small molecule microarray; VEGF, vascular endothelial growth factor; VEGF-A, VEGF, Type-A; VEGFR-2, VEGF receptor, Type 2 (also known as KDR).

DOI: 10.1089/adt.2012.485

VEGF–KDR binding. Using this assay platform, we identified 12 compounds that bind to VEGF with high affinity and interrupt VEGF–KDR binding with half-maximal inhibitory concentrations (IC_{50}s) ranging from 0.3 to 60 μM (IC_{50} here is defined as the ligand concentration at which the amount of VEGF captured by the immobilized KDR is reduced by half from the maximum level). The inhibitory effects of these 12 compounds were confirmed in a cell-based VEGFR2 phosphorylation inhibition assay.

MATERIALS AND METHODS

The method, as illustrated in *Figure 1*, consists of three microarray-based, label-free binding assays: (1) the first assay (Assay #1) identifies high-affinity VEGF ligands by incubating VEGF with small molecule compounds immobilized on isocyanate-functionalized glass slides; (2) the second assay (Assay #2, a sandwich assay) identifies high-affinity KDR ligands by reacting KDR with identical compound microarrays and by subsequently incubating VEGF with the reacted microarrays further singles out those ligands that do not interfere with VEGF–KDR binding; (3) the third assay (Assay #3, an inhibition assay) identifies inhibitors to VEGF–KDR binding by incubating mixtures of VEGF and its ligands (discovered from Assay #1) with KDR immobilized to isocyanate-functionalized glass surface through the KDR ligands (discovered from Assay #2).

Small Molecule Microarrays of Compounds from the Open Repository of the NCI/DTP for Assay #1 and Assay #2

We used plated sets from NCI/DTP with a total of 7,961 compounds: (1) a Challenge set with 57 compounds (10 mM); (2) a Natural Products set with 235 compounds (10 mM); (3) a Structural Diversity set with 1,990 compounds (10 mM); (4) a Mechanistic Diversity set with 879 compounds (1 mM); and (5) an Open set with 4,800 compounds (1 mM). We printed these compounds (all dissolved in dimethyl sulfoxide) into two microarrays on two separate isocyanate-functionalized glass slides.[17] One half of the compounds, along with a set of control compounds, were printed in duplicate on one slide over an area of 2 cm × 4 cm, with a total of ~10,700 printed spots. The other half of the compounds with the same set of control compounds were printed (also in duplicate) on the second slide. A printed slide is assembled with a fluid chamber so that the microarray can be processed and scanned simultaneously with an ellipsometry-based optical scanner as briefly described below.[21–24]

KDR Ligand Microarray for Assay #3

We printed two of the KDR ligands that were identified from Assay #2, National Service Center (NSC)-20596 and NSC-78033, in 12 replicate spots each to a small microarray on an isocyanate-functionalized glass slide. The KDR ligand microarray is first incubated in a solution of KDR fused to the immunoglobulin G Fc domain (Fc-KDR) at 100 nM and washed with 1× PBS so that a full layer of KDR was captured by the immobilized NSC 20596 and NSC 78033. We then incubated the KDR microarray in the mixture of VEGF (60 nM) and one of the VEGF ligands (from Assay #1) at a concentration between 1 nM and 100 μM in 1× PBS and recorded binding curves of the VEGF to the immobilized KDR. The net change in oblique-incidence reflectivity difference (OI-RD) signal, proportional to the surface mass density of the captured VEGF molecules, varies with the ligand concentration.

Ellipsometry-Based Optical Scanner for Label-Free Small Molecule Microarray Detection

We read out VEGF- and KDR-binding processes on NCI/DTP compound microarrays with a scanning ellipsometry microscope based on measurements of OI-RD signals, as described previously.[22,24] The OI-RD signals are defined as the difference in the fractional reflectivity change between *p*-polarized (transverse magnetic) and *s*-polarized (transverse electric) components of a monochromatic light from a solid surface due to the addition of a biomolecular layer, $(r_p - r_{p0})/r_{p0} - (r_s - r_{s0})/r_{s0} \equiv \Delta_p - \Delta_s$. r_{p0} and r_{s0} are reflectivities of the bare surface and r_p and r_s are reflectivities of the surface when it is covered with the molecular layer. $\Delta_p - \Delta_s$ is proportional to the surface mass density Γ (g/cm^2) of the molecular layer. Using a pixel step size of 20 μm, our OI-RD scanning microscope enables us to acquire a $\Delta_p - \Delta_s$ (*i.e.*, surface mass density) image of a 10,000-spot compound microarray in 20 min.

Phospho-VEGFR2 Capture Enzyme-Linked Immunosorbent Assay

The 293/KDR cell line that stably expresses the human VEGFR-2 (KDR) was purchased from SibTech, Inc. The cells were maintained in culture with the Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. For enzyme-linked immunosorbent assay (ELISA), the recombinant human VEGF, Type-A (VEGF-A) 165 (#293-VE) and the monoclonal anti-human VEGFR-2/KDR mouse IgG (#MAB3573) for plate coating were purchased from R&D Systems. The biotinylated anti-phospho-

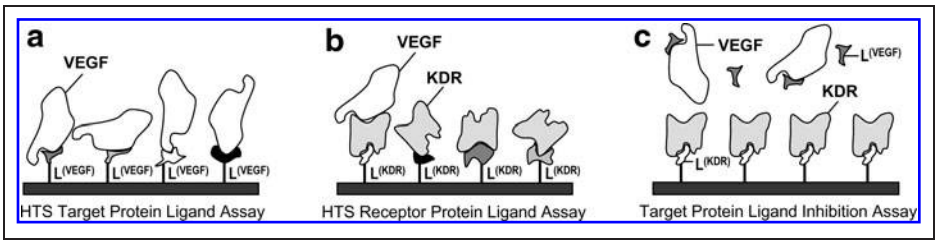


Fig. 1. Microarray-based tri-assay platform for discovery of inhibitors of the vascular endothelial growth factor (VEGF)–kinase-insertion domain receptor (KDR) binding from a large library of small molecules. **(a)** Assay #1: binding of VEGF with small molecule microarrays (SMM) yields VEGF ligands as inhibitor candidates. **(b)** Assay #2: binding of KDR with SMM, followed by incubation with VEGF reveals KDR ligands that capture KDR and yet retain its reactivity with subsequent VEGF. **(c)** Assay #3: binding of mixtures of VEGF and VEGF ligands with KDR immobilized using the KDR ligands identified in Assay #2 yields the IC_{50} of the ligands and, in turn, inhibits the VEGF–KDR interaction.

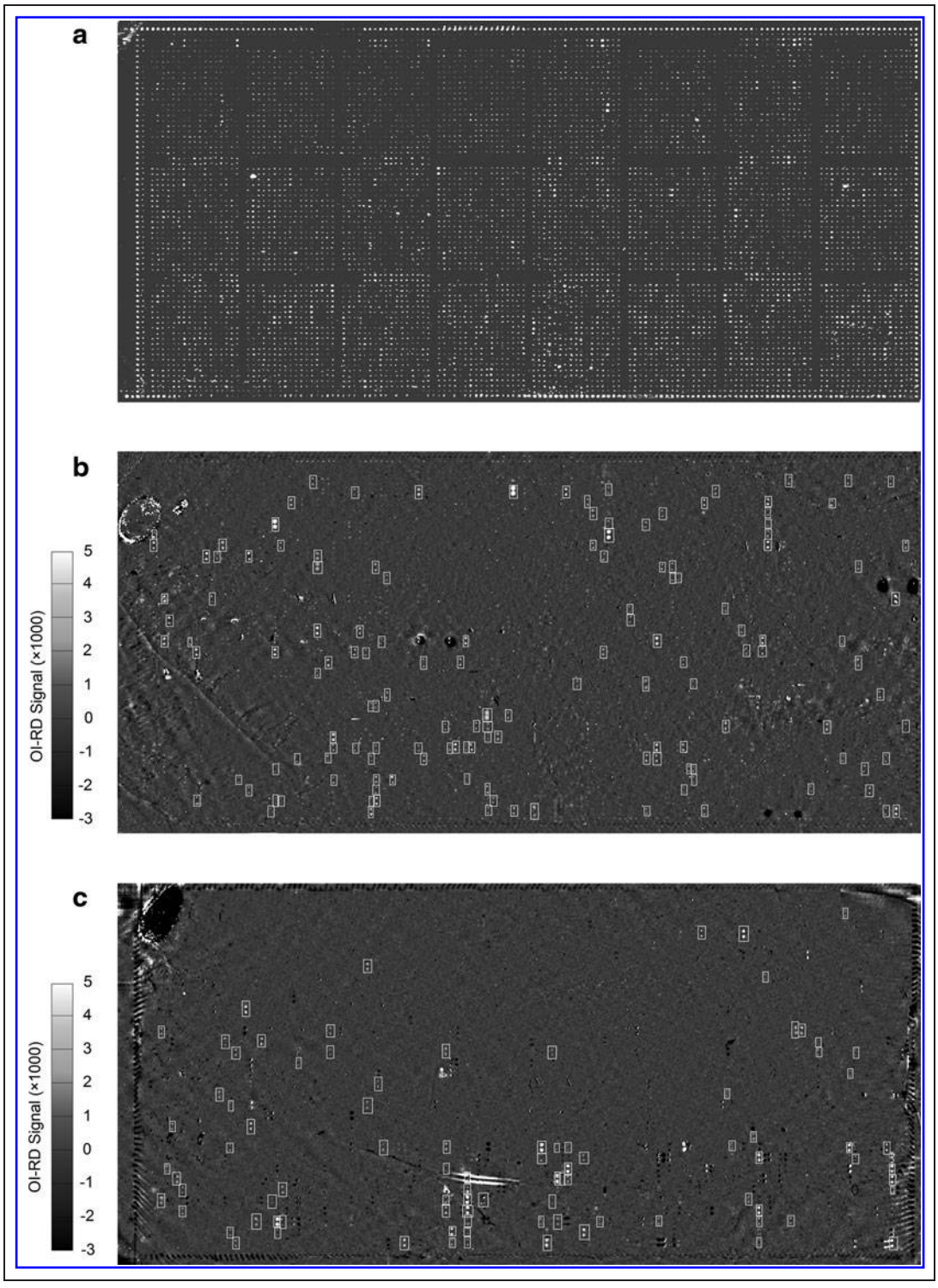


Fig. 2. **(a)** Oblique-incidence reflectivity difference (OI-RD) image of a 10,800 compound microarray with 4,080 National Cancer Institute (NCI) compounds printed in vertical spot doublets along with a set of control compounds on an isocyanate-functionalized glass slide over an area of 2 cm × 4 cm. The image was acquired with a pixel step size of 20 μm before further processing. **(b)** Change in OI-RD image of the compound microarray that contains 4,080 NCI compounds after incubation with VEGF fused to the immunoglobulin G Fc domain (Fc-VEGF). **(c)** Change in OI-RD image of another compound microarray that contains the remaining 3,881 NCI compounds after incubation with Fc-VEGF. The compounds that bound with Fc-VEGF appear as vertical spot doublets in the images, which we enclosed with rectangular boxes.

tyrosine mouse monoclonal antibody (P-Tyr-100) was purchased from Cell Signaling Technology (#9417). Peroxidase-conjugated streptavidin was purchased from Jackson ImmunoResearch Laboratories (#016-030-084). 293/VEGFR-2/KDR cells were seeded onto 96-well tissue culture plates (50,000 cells per well) and incubated overnight. Cells were washed twice in 1× PBS and starved in DMEM containing 0.2% FBS for at least 4 h. Individual NCI/DTP compounds at various concentrations in a solvent buffer were premixed with human VEGF-A165 in DMEM with 0.2% FBS for 1 h at 37°C in a CO_2 incubator before being added to the serum-starved cells for 5 min at 37°C. The final concentration of human VEGF-A165 was 25 ng/mL and the assay volume was 100 µL per well. After stimulation with the compound-VEGF-A165 mixture, cells were washed with ice-cold 1× PBS once and lysed with a cold lysis buffer (100 µL/well) on ice for 10 min, and then frozen at −80°C. The lysis buffer contained 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor cocktail (#5871; Cell Signaling Technology).

The phosphorylation of VEGFR-2/KDR on tyrosine residues is quantified by the following capture ELISA assay: Well bottoms of ELISA microplates (Microlite2 flat bottom white strips, #7410; Thermo Scientific) were coated with 50 µL of monoclonal anti-human VEGFR-2/KDR mouse IgG at 4 µg/mL in 1× PBS overnight at 4°C. The plates were washed twice with a wash buffer (1× PBS containing 0.05% Tween 20), and then blocked with a blocking buffer (1% bovine serum albumin [BSA] in 1× PBS) at room temperature for 1 h. The plates were washed twice with the wash buffer before lysate samples were added. The frozen cell lysates were thawed before being transferred to the coated microplates (80 µL/well) and allowed to incubate at 37°C for 2 h. Following incubation, the plates were washed five times in the wash buffer. The phosphorylation status of the captured VEGFR-2 was determined by incubating the plates in 0.5 µg/mL of biotinylated anti-phospho-tyrosine mouse monoclonal antibody for 1 h at room temperature (followed by five washes in the wash buffer), and then incubated in a solution of peroxidase-conjugated streptavidin (at 1:1,000 dilution from the stock) for 30 min at room

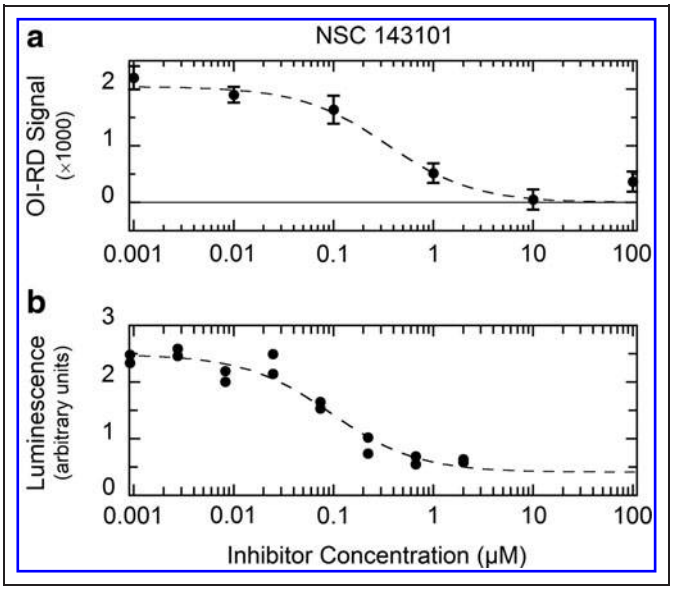


Fig. 3. **(a)** The equilibrium OI-RD signal, proportional to surface mass density of VEGF captured by the immobilized KDR, following incubation of the KDR microarray with a range of concentrations of the inhibitor NSC 143101 together with VEGF (60 nM). The error bar at each concentration represents the standard error of measurements made at 12 KDR microarray spots. The microarray-based IC_{50} value is found to be 0.33 ± 0.05 µM with the standard error computed from nonlinear curve fitting to the data. **(b)** Chemiluminescence signal, proportional to the amount of autophosphorylated intracellular KDR in the lysate of 293/KDR cells after their being treated with mixtures of a range of concentrations of NSC 143101 and a fixed concentration of VEGF (25 ng/mL). The cell-based IC_{50} value is found to be 0.09 ± 0.03 µM with the standard error computed from nonlinear curve fitting to the chemiluminescence data.

Table 1. NSC ID, Molecular Weight, and IC_{50} of 12 VEGF Ligands That Exhibit an Inhibitory Effect on VEGF–KDR Binding

NSC ID number (NCI/DTP)	M.W. (Da)	IC_{50} (µM) (*microarray-based*)	IC_{50} (µM) (*cell-based*)
143101	610	0.33 ± 0.05	0.09 ± 0.03
35676	230	1.1 ± 0.2	0.3 ± 0.1
236657	572	9 ± 2	0.6 ± 0.3
275425	315	17 ± 6	3 ± 1
17008	414	3.6 ± 0.8	5 ± 3
72284	316	5 ± 1	9 ± 2
128884	300	22 ± 5	10 ± 6
21607	124	11 ± 2	10 ± 7
26989	280	12 ± 3	13 ± 9
134159	294	24 ± 7	15 ± 10
293161	635	10 ± 3	17 ± 8
774	321	14 ± 4	40 ± 23

At each ligand concentration, 12 endpoints of the VEGF-KDR binding reaction in the presence of the ligand were measured. The error limits in IC_{50} were obtained from a nonlinear curve fitting of the dose–response data. The last column lists IC_{50} of the same ligands against VEGF-KDR binding, but obtained with a cell-based phosphor-KDR enzyme-linked immunosorbent assay.

VEGF–KDR, vascular endothelial growth factor–kinase-insertion domain receptor; NCI/DTP, National Cancer Institute Developmental Therapeutics Program.

temperature. After five washes, the microplates were processed for enhanced chemiluminescence (ECL) substrate development (Femtoglow horseradish peroxidase (HRP) substrate, #SHRPE23007, Michigan Diagnostics). The chemiluminesence signals were recorded by Modulus II microplate multimode reader (Promega) and the data were analyzed by Graphpad Prism (GraphPad Software).

For comparison, mixtures of Avastin at various concentrations with human VEGF-A (25 ng/mL) were also used to treat 293/KDR cells and the phospho-KDR proteins in the cell lysates were detected with the same sandwich ELISA.

RESULTS AND DISCUSSION

Figure 2a shows the OI-RD image of one NCI/DTP compound microarray before it was further processed. The spots along the perimeter are BSA; immediately inside the BSA spots are biotin-conjugated BSA. Further inside are 4,080 NCI compounds printed as vertical doublets.

Identifying High-Affinity VEGF Ligands from 7,961 NCI/DTP Compounds (Assay #1)

We obtained the human VEGF and KDR by expressing fusion proteins of the human VEGF165 and KDR ECD with the Fc fragment of rabbit IgG in HEK293 cells, and then purifying the fusion proteins with Protein A column chromatography.[8] We incubated the NCI compound microarrays in VEGF fused to the immunoglobulin G Fc domain (Fc-VEGF) at 290 nM (13.8 μg/mL) in 1× PBS for 60 min and washed the microarrays with 1× PBS *in situ*. *Figure 2b* and *c* shows the change in the OI-RD image of the microarrays by subtracting the OI-RD images taken before incubation from the images taken after. The distinguishable vertical doublets (enclosed by rectangular boxes) reveal compounds that captured Fc-VEGF. In addition to compounds of interest for subsequent inhibition assays, the boxed hits include (1) those that bind to the Fc fragment of rabbit IgG instead of VEGF; (2) those that bind to VEGF in such a way that the captured VEGF still binds to KDR and, thus, are not of interest in our search for VEGF ligands that block VEGF–KDR binding; and (3) those that are not reproducible when the VEGF binding assays are repeated. We eliminated those compounds from the hit list by (1) repeating the VEGF binding assays, (2) performing control assays that incubated fresh NCI compound microarrays in the rabbit IgG Fc fragment solution, and (3) performing sandwich assays that incubated VEGF-reacted microarrays in the KDR solution. Finally, from the two NCI compound microarrays with a total of 7,961 compounds, we found 107 hits that bind to human VEGF with the potential to block VEGF–KDR binding. We subsequently screened these compounds for inhibitors against VEGF–KDR binding in Assay #3.

Identifying KDR Ligands from 7,961 NCI Compounds That Do Not Interfere with KDR-VEGF Binding (Assay #2)

Using the same procedure as Assay #1, we incubated two fresh NCI compound microarrays in Fc-KDR at 64 nM in 1× PBS for 60 min and washed the microarrays *in situ* with 1× PBS. Out of 7,961 compounds, we found 27 hits that bound to KDR. Immediately after the reaction with KDR, we incubated the microarrays in Fc-VEGF at 290 nM. From this sandwich assay, we identified four hits that captured KDR in the primary reaction in such a way that the captured KDR was still capable of binding to VEGF in the secondary reaction. We selected two of them, NSC 20596 (*5-hydrazinyltetralin-2-amine*) and NSC 78033 ($C_{15}H_{15}N_2.1/2Cl_6Sn$), for immobilization of the KDR to an isocyanate-functionalized glass surface in Assay #3 (see below). The structures of these two compounds are shown in *Supplementary Figure S1* (*Supplementary Data* are available online at www.liebertpub.com/adt).

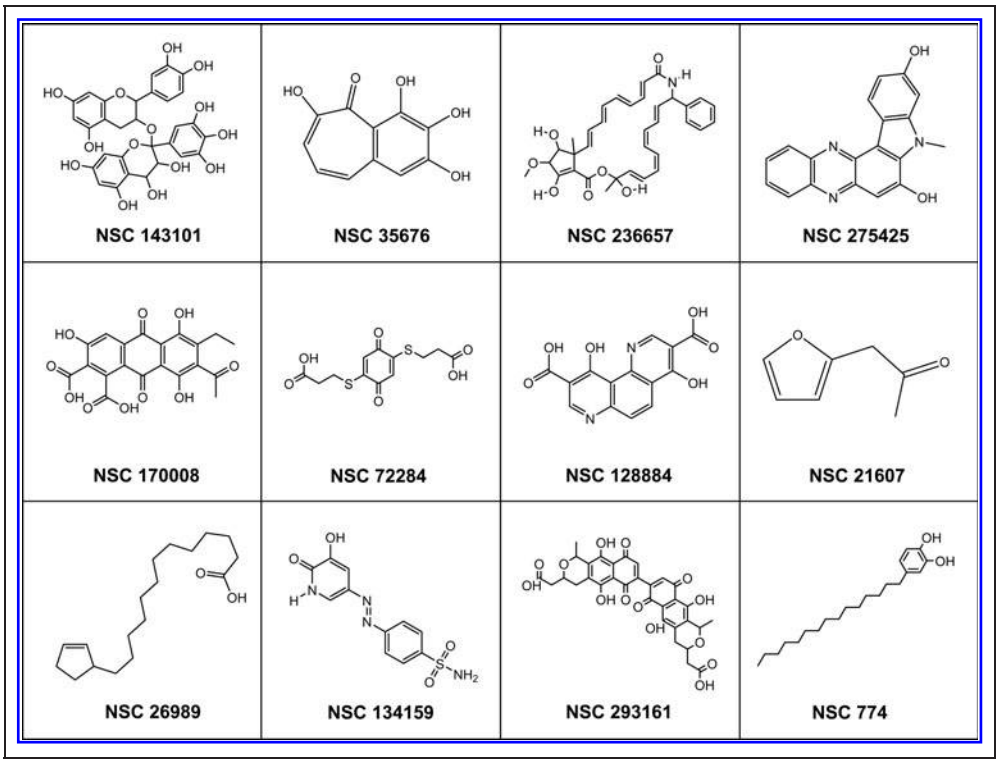


Fig. 4. Molecular structures of 12 small molecule ligands of VEGF that block VEGF–KDR binding. The structural data are obtained from the National Cancer Institute Developmental Therapeutics Program.[25]

Table 2. Tri-Assay Protocol Table for Inhibitory Ligand Discovery

Step	Parameters	Value	Description
Assay #1: VEGF ligand discovery from library compounds			
1-1	Library compounds	10 μL	7,961 NCI/DTP-plated compounds in 384-well plates
1-2	Controls	10 μL	Bovine serum albumin, peptides, in 384-well plate
1-3	Compound microarray	1 nL	4,000 compounds in pairs on isocyanate-functionalized slide
1-4	Microarray washing	30 min	1× PBS
1-5	Microarray blocking	10 min	10 μM BSA in 1× PBS
1-6	Microarray readout	633 nm	Prereaction image with *in-situ* OI-RD scanner
1-7	VEGF reaction	1 mL	290 nM Fc-VEGF in 1× PBS
1-8	Incubation time	60 min	Room temperature
1-9	Microarray readout	633 nm	Postreaction image with *in-situ* OI-RD scanner
Assay #2: KDR ligand discovery from library compounds Steps 2-1 to 2-5, see Steps 1-1 to 1-5.			
2-6	Microarray readout	633 nm	Prereaction image with *in-situ* OI-RD scanner
2-7	KDR reaction	1 mL	64 nM Fc-KDR in 1× PBS
2-8	Incubation time	60 min	Room temperature
2-9	Microarray readout	633 nm	Postreaction image with *in-situ* OI-RD scanner
2-10	VEGF reaction	1 mL	290 nM Fc-VEGF in 1× PBS
2-11	Incubation time	60 min	Room temperature
2-12	Microarray readout	633 nm	Postreaction image with *in-situ* OI-RD scanner
Assay #3: dose–response curve of VEGF ligands against VEGF–KDR binding reaction			
3-1	KDR ligand array	1 nL	Two special ligands printed on isocyanate-functionalized slide
3-2	Microarray readout	633 nm	Prereaction image with *in-situ* OI-RD scanner
3-3	KDR Reaction	300 μL	100 nM Fc-KDR in 1× PBS
3-4	Incubation time	30 min	Room temperature
3-5	Microarray readout	633 nm	Binding curve acquisition with *in-situ* OI-RD scanner
3-5	Microarray readout	633 nm	Postreaction image with *in-situ* OI-RD scanner
3-6	(VEGF + ligand) reaction	300 μL	Ligand (100 μM to 1 nM) mixed with 125 nm VEGF
3-7	Incubation time	30 min	Room temperature
3-8	Microarray readout	633 nm	Binding curve acquisition with *in-situ* OI-RD scanner
3-7	Microarray readout	633 nm	Postreaction image with *in-situ* OI-RD scanner

OI-RD, oblique-incidence reflectivity difference; BSA, bovine serum albumin; Fc-KDR, kinase-insertion domain receptor fused to the immunoglobulin G Fc domain; Fc-VEGF, vascular endothelial growth factor fused to the immunoglobulin G Fc domain.

Screening VEGF Ligands That Were Discovered in Assay #1 for Inhibitors to VEGF–KDR Binding (Assay #3)

Many (if not most) ligands of VEGF are expected to bind to the protein at sites that have neither direct nor indirect modulation effects on the VEGF–KDR interaction. As a result, an inhibition assay is required to further screen the VEGF ligands for those that inhibit the VEGF–KDR interaction. To do so in a microarray format, we need to immobilize KDR on a solid surface in such a way that the KDR retains its affinity to VEGF. The KDR ligands discovered in Assay #2 serve just such a need. From the OI-RD change versus the ligand concentration (*i.e.*, the dose–response curves), we identified 12 compounds that blocked the reaction of VEGF with KDR that demonstrated IC_{50}s ranging between 0.3 and 60 μM. *Figure 3a* shows the microarray-based dose–response curve for the most potent ligand, NSC 143101 (M.W. = 610 Da), with $IC_{50} = 0.33 \pm 0.05$ μM. *Table 1* lists all 12 inhibitory ligands and the corresponding IC_{50} values. The structures of these ligands are shown in *Figure 4*.[25] The microarray-based dose–response curves for all 12 inhibitors are listed in *Supplementary Figure S2*. We summarize the details of the tri-assay inhibitor discovery protocol in *Table 2*.

Validation of Microarray-Discovered Small-Molecule Inhibitors with a Cell-Based Downstream Pathway Inhibition Assay

Microarray-based inhibition assays, as presented here, need to be confirmed with accepted cell-based assays in which the putative inhibitors encounter VEGF and KDR in their native cellular environment. For this purpose, we examined the inhibitory effect of the identified compounds on VEGF binding to KDR (VEGFR-2) on the membrane of cells. The binding of VEGF to KDR induces autophosphorylation in the intracellular domain of KDR, and thus, an inhibition effect is exhibited by a decrease in the level of phospho-KDR in KDR-expressing cells after treatment with inhibitors (at a concentration between 1 nM and 30 μM) in the presence of human VEGF-A (25 ng/mL). We found that all 12 inhibitors identified in the microarray-based assay inhibited VEGF-induced KDR autophosphorylation, indicating that these compounds indeed disrupt VEGF binding to KDR (VEGFR-2) in their native cellular environment. The IC_{50} values obtained by the cell-based assay, in essence, agree with the values deduced from the microarray-based assay. In *Figure 3b*, we show the cell-based dose–response curve for NSC 143101 with

the $IC_{50} = 0.09 \pm 0.03$ μM. The same cell-based IC_{50} for Avastin was found to be 0.033 ± 0.006 μM.[7] *Table 1* lists the cell-based IC_{50} values for all 12 ligands. The cell-based dose–response curves for all 12 inhibitors are listed in *Supplementary Figure S2*.

CONCLUSION

We have presented a microarray-based tri-assay platform for finding VEGF ligands that modify VEGF–KDR binding, starting from a library of 7,961 small molecule compounds. The platform is enabled by the combination of small molecule microarrays with a high-throughput label-free optical scanner. With further carefully designed validation based on other established methods, it can become generally useful for discovery of ligands of other proteins that modify binding of the proteins with its targets. We note that the third assay (dose–response determination) of this tri-assay platform is a one-on-one assay that involves incubation of immobilized KDR with one mixture of VEGF and a ligand at a time. If one combines the printed microarray with the microplate format by immobilizing the KDR on the surface of a glass-bottomed microplate in the form of 2×2 or other small square microarrays, one should be able to simultaneously measure binding assays involving 384 or even 1,536 different protein–ligand mixtures for dose–response determination. In this case, the ellipsometry scanner will be employed to detect 384–1,536 binding processes simultaneously using previously demonstrated techniques.[24] If 12 concentrations of a probe protein are used to obtain one dose–response curve for one ligand, one will be able to measure dose–response curves of 32–128 ligands in a single experiment. The development of such a microarray/microplate format for the third assay is under way.

ACKNOWLEDGMENT

This work was supported by NIH under NIH-R01-HG003827.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

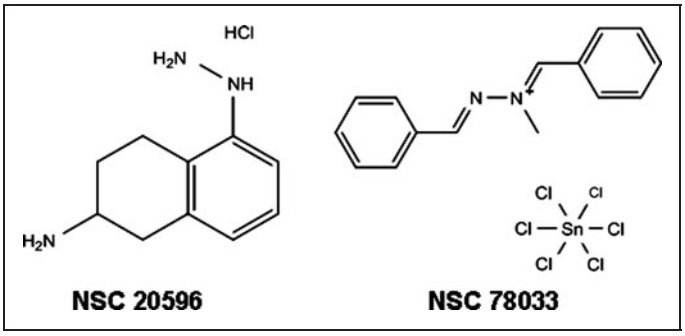
REFERENCES

1. Muller YA, Li B, Christinger HW, Wells JA, Cunningham BC, de Vos AM: Vascular endothelial growth factor: crystal structure and functional mapping of the kinase domain receptor binding site. *Proc Natl Acad Sci USA* 1997;94:7192–7197.
2. Fuh G, Li B, Crowley C, Cunningham B, Wells JA: Requirements for binding and signal of the kinase domain receptor for vascular endothelial growth factor. *J Biol Chem* 1998;273:11197–11204.
3. Kiselyov A, Balakin KV, Tkachenko SE: VEGF/VEGFR signalling as a target for inhibiting angiogenesis. *Expert Opin Investig Drugs* 2007;16:83–107.
4. Shinkaruk S, Bayle M, Lain G, Deleris G: Vascular endothelial cell growth factor (VEGF), an emerging target for cancer chemotherapy. *Curr Med Chem Anticancer Agents* 2003;3:95–117.
5. Moreira IS, Fernandes PA, Ramos MJ: Vascular endothelial growth factor (VEGF) inhibition—a cirtical review. *Anti-Cancer Agents Med Chem* 2007;7:223–245.
6. Ferrara N, Hillan KJ, Gerber HP, *et al.*: Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat Rev Drug Discov* 2004;3:391–400.
7. Ranieri G, Patruno R, Ruggieri E, Montemurro S, Valerio P, Ribatti D: Vascular endothelial growth factor (VEGF) as a target of bevacizumab in cancer: from the biology to the clinic. *Curr Med Chem* 2006;13:1845–1857.
8. Yu Y, Lee P, Ke Y, *et al.*: A humanized anti-VEGF rabbit monoclonal antibody inhibits angiogenesis and blocks tumor growth in xenograft models. *PLoS One* 2010;5:e9072.
9. Bikfalvi A: Recent developments in the inhibition of angiogenesis: examples from studies on platelet factor-4 and the VEGF/VEGFR system. *Biochem Pharmacol* 2004;68:1017–1021.
10. Ng EW, Shima DT, Calias P, *et al.*: Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. *Nat Rev Drug Discov* 2006;5:123–132.
11. Holash J, Davis S, Papadopoulos N, *et al.*: VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc Natl Acad Sci USA* 2002;99:11393–11398.
12. Schenone S, Bondavalli F, Botta M: Antiangiogenic agents: an update on small molecule VEGFR inhibitors. *Curr Med Chem* 2007;14:2495–2516.
13. Underiner TL, Ruggeri B, Gingrich DE: Development of vascular endothelial growth factor receptor (VEGFR) kinase inhibitors as anti-angiogenic agents in cancer therapy. *Curr Med Chem* 2004;11:731–745.
14. MacBeath G, Schreiber SL: Printing proteins as microarrays for high-throughput function determination. *Science* 2000;289:1760–1763.
15. Schena M: *Microarray Analysis*. Wiley, New York, 2003.
16. Zhu H, Bilgin M, Bangham R, *et al.*: Global analysis of protein activities using proteome chips. *Science* 2001;293:2101–2105.
17. Bradner JE, McPherson OM, Koehler AN: A method for the covalent capture and screening of diverse small molecules in a microarray format. *Nat Protoc* 2006;1:2344–2352.
18. MacBeath G, Koehler AN, Schreiber SL: Printing small molecules as microarrays and detecting protein-ligand interactions en masse. *J Am Chem Soc* 1999;121:7967–7968.
19. Vegas AJ, Fuller JH, Koehler AN: Small-molecule microarrays as tools in ligand discovery. *Chem Soc Rev* 2008;37:1385–1394.
20. Stanton BZ, Peng LF, Maloof N, *et al.*: A small molecule that binds Hedgehog and blocks its signaling in human cells. *Nat Chem Biol* 2009;5:154–156.
21. Fei YY, Landry JP, Sun YS, *et al.*: A novel high-throughput scanning microscope for label-free detection of protein and small-molecule chemical microarrays. *Rev Sci Instrum* 2008;79:013708.
22. Fei YY, Landry JP, Sun YS, *et al.*: Screening small-molecule compound microarrays for protein ligands without fluorescence labeling with a high-throughput scanning microscope. *J Biomed Opt* 2010;15:016018.
23. Landry JP, Fei YY, Zhu XD: High throughput, label-free screening small molecule compound libraries for protein-ligands using combination of small molecule microarrays and a special ellipsometry-based optical scanner. *Int Drug Discov* 2012;6:8–13.
24. Landry JP, Fei Y, Zhu XD: Simultaneous measurement of 10,000 protein-ligand affinity constants using microarray-based kinetic constant assays. *Assay Drug Dev Technol* 2012;10:250–259.
25. NCI/NIH Developmental Therapeutics Program: Basic chemical data. Available at: http://dtp.nci.nih.gov/dtpstandard/ChemData/index.jsp (last accessed May 15, 2013).

Address correspondence to:
Xiangdong Zhu, PhD
Department of Physics
University of California at Davis
Davis, CA 95616

E-mail: xdzhu@physics.ucdavis.edu

SUPPLEMENTARY DATA



Supplementary Fig. S1. Structures of two molecules that capture kinase-insertion domain receptor (KDR) from a solution, while allowing the captured KDR to bind with the solution-phase vascular endothelial growth factor (VEGF).