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VEGFR1 domain 2 covalent labeling with horseradish peroxidase: Development of a displacement assay on VEGF

Marie Reille-Seroussi^{a,1}, Jean-François Gaucher^{b,*}, Laure-Anne Cussac^a,
Isabelle Broutin^b, Michel Vidal^{a,c}, Sylvain Broussy^{a,**}

^a UMR 8638 CNRS Université Paris Descartes, Faculté de Pharmacie de Paris, Sorbonne Paris Cité, 4 Avenue de l'Observatoire, Paris 75006, France

^b UMR 8015 CNRS Université Paris Descartes, Faculté de Pharmacie de Paris, Sorbonne Paris Cité, 4 Avenue de l'Observatoire, Paris 75006, France

^c UF Pharmacocinétique et Pharmacochimie, Hôpital Cochin, AP-HP, 27 rue du Faubourg Saint Jacques, Paris 75014, France

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ABSTRACT

The VEGFR1 has been shown to play a role in the regulation of angiogenesis, and has therefore been associated to several pathologies. In order to extend our toolbox of screening methods for the identification of compounds disrupting the VEGF receptor 1/VEGF interaction, we developed a fast and accurate displacement assay, in which VEGF receptor 1 domain 2 is directly labeled with an enzyme, bypassing the classical streptavidin-biotin interaction system. A description of this straightforward strategy is provided here, including its advantages and disadvantages. Optimization of the reagents preparation, purification and conservation, and displacement assay with known molecular entities are presented.

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Introduction

The deregulation of angiogenesis is involved in cancer and inflammatory diseases like atherosclerosis, rheumatoid arthritis, and age-related macular degeneration (AMD) [1,2]. The development of molecules able to control angiogenesis is therefore the subject of intense research [3]. Vascular endothelial growth factor A (VEGF) is the main pro-angiogenic factor, which interacts with the extracellular domain (ECD) of two cellular receptors (VEGFR1 and VEGFR2) [4]. Among the strategies developed to stop abnormal angiogenesis, disrupting the interaction of the VEGF with its receptors has led to the clinical use of the VEGF₁₋₁₆₅ targeting antibodies bevacizumab (Avastin) and ranibizumab (Lucentis) for the treatment of cancer and AMD, respectively [5]. Therefore, the identification of peptides, peptidomimetics and small molecules able to block these protein/protein interactions, either through binding to the receptor or to the VEGF, has been actively pursued [6–10]. For this purpose,

several biochemical assays have been employed for an *in vitro* evaluation of molecules. A classical assay format was based on radioactive labeled VEGF [6,11,12]. Another homogeneous assay used fluorescent peptides competing for the VEGF₁₋₁₆₅ at the receptor binding site and detection by fluorescence polarization [13]. Displacement assays with chemiluminescence-based detection systems for the screening of compounds targeting the VEGFR1/VEGF interaction have been widely employed in our group using both the seven Ig-like structural domains constituting the ECD (d1-d7) or the d1-d3 domains [7,9,14–20]. Genentech, Inc. has also described two assays for the specific evaluation of the second domain of the VEGFR1_{ECD} (VEGFR1_{d2}), which is the main VEGF binding site. The first one used biotinylated VEGF (*bt*VEGF₈₋₁₀₉) and a coated anti-VEGF monoclonal antibody that competes with VEGFR1_{d2} binding [11]. The second one used the biotinylated domain d2 of the VEGFR1 (*bt*VEGFR1_{d2}) and coated VEGF₈₋₁₀₉, but few details on the experimental procedures have been disclosed [21]. This assay holds some interesting features. First, it allows for the identification of compounds that targets the VEGF/VEGFR1_{d2} binding site, either to the VEGF₈₋₁₀₉ or to the VEGFR1_{d2} domain. It is therefore complementary to the previously described fluorescence polarization assay [13], which only detects compounds binding to the VEGF₁₋₁₆₅. Moreover, it is complementary to the VEGFR1_{d1-d3} or

* Corresponding author.

** Corresponding author.

E-mail addresses: jean-francois.gaucher@parisdescartes.fr (J.-F. Gaucher), sylvain.broussy@parisdescartes.fr (S. Broussy).

¹ Both authors contributed equally to this work.

VEGFR1_{ECD}/*bt*VEGF₁₋₁₆₅ assays [7,9,14–20], which may detect compounds binding outside the targeted interface, on the whole d1-d3 or ECD surface. In order to develop a similar method, we tried to bind the VEGFR1_{d2} onto a 96-well plate and to incubate with *bt*VEGF₁₋₁₆₅. Unfortunately, random and weak signal was obtained instead of the expected saturation binding curve. We hypothesized that the VEGFR1_{d2} interacted with the high binding surface of the plates through its hydrophobic VEGF binding area, therefore preventing further *bt*VEGF₁₋₁₆₅ fixation.

Interestingly, inspection of the VEGFR1_{d2}/VEGF₈₋₁₀₉ crystal structure [11] showed that all six lysine residues of the VEGFR1_{d2} were located outside the binding interface. This encouraged us to try a direct chemical labeling of VEGFR1_{d2} with the horseradish peroxidase (HRP). The conjugate, named VEGFR1_{d2}-HRP, was used as ligand of the VEGF and as enzyme-reporter. Therefore we developed a VEGF binding assay, in which the VEGF₉₅ (residues 13–107) was coated onto plates. After an incubation step with the VEGFR1_{d2}-HRP followed by a washing step, the remaining VEGF-bound VEGFR1_{d2}-HRP, was quantified by a chemiluminescence reaction. The assay may be used for competitive binding in two different ways: the competitors may be ligands of the VEGF or of the VEGFR1_{d2} (Fig. 1). We provide here a detailed description of the procedures required to perform the assay, and of its usefulness and limitations.

Material and methods

Protein expression, purification and refolding

The human VEGFR1_{d2} protein (residues 132–226, calculated molecular mass = 11006 g mol⁻¹ and $\epsilon_{275} = 11150$ L mol⁻¹ cm⁻¹) was obtained as previously described [18] and stored at –20 °C as 1 mg dry aliquots (protein content based on UV absorption at 280 nm) lyophilized from a 5 mM HEPES/NaOH pH 6.8 solution.

VEGF expression and purification were adapted from Ref. [22]. The synthetic gene of the human VEGF₉₅ homodimer (residues 13–107, calculated molecular mass = 23056 g mol⁻¹ and $\epsilon_{280} = 12920$ L mol⁻¹ cm⁻¹) was cloned into pet22b (+) expression vector (Novagen, Merck, Darmstadt, Germany) and transformed in *E. coli* Rosetta 2 bacterial strain (Novagen, Darmstadt, Germany). Cultures were grown at 37 °C in LB supplemented with 150 µg/mL ampicillin, and protein expression was induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside. VEGF₉₅ was expressed as an insoluble protein. Cells were lysed by ultrasonic disruption in Tris/Hepes buffer (50 mM/50 mM pH 8.1 containing 0.1% (w/v) Triton X-100). Inclusion bodies were isolated by centrifugation at 9500 g for 20 min. The pellet was further purified by three successive steps of sonication, washing (Tris/Hepes 50 mM/50 mM pH 8.0, NaCl 1 M) and centrifugations at 40000 g for 20 min. Typically, 160 mg of protein were solubilized in 125 mL of buffer containing urea 8 M, DTT 4 mM, Tris/HCl 12.5 mM pH 8.1, NaCl 250 mM, imidazole 5 mM and dialyzed overnight in 20 vol of Na₃ citrate 50 mM, cysteine 1 mM at 4 °C. Then it was dialyzed successively in 20 vol Tris/HCl 20 mM pH 8.1, CuSO₄ 1 mM and 20 vol Tris/HCl 20 mM pH 8.1 NaCl 250 mM. Misfolded protein was discarded by centrifugation at 40000 g during 20 min, and the supernatant was dialyzed two times against Tris/HCl 20 mM pH 8.1. Refolded protein was purified by anion-exchange chromatography on a Source 15Q column (Amersham-GE Healthcare, Little Chalfont, UK) in Tris/HCl 20 mM pH 8.1 and eluted with a 1 M NaCl gradient in the same buffer. The main elution peak corresponded to refolded VEGF₉₅. Purity and homogeneity of disulfide bonding were checked by SDS-PAGE, with and without thiol reduction. The protein was eluted as a unique peak of 20.3 kDa apparent mass on analytical size exclusion

chromatography (SEC) (Superdex™ 75 HR 10/30 Amersham-GE Healthcare, Little Chalfont, UK). It was dialyzed three times in pure water, concentrated by ultrafiltration, lyophilized and stored at –80 °C.

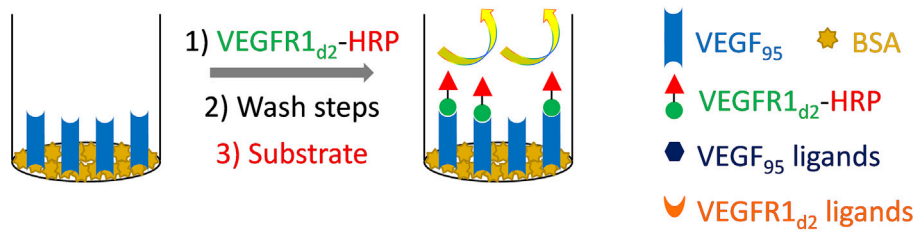
VEGFR1_{d2}-HRP conjugation and purification

The coupling procedure was adapted from the recommended method provided by the HRP maleimide activated supplier (Sigma Aldrich), using SATA (S-acetylthioglycolic acid *N*-hydroxysuccinimide ester) to introduce an additional thiol functional group [23]. One VEGFR1_{d2} aliquot (91 nmol, 1 mg) was dissolved in 100 µL water and further diluted to 1 mL with KH₂PO₄/Na₂HPO₄ 50 mM pH 7.5 with 1 mM EDTA. To the resulting 90 µM VEGFR1_{d2} solution was added a 100 mM solution of SATA in DMF (typically 1.8 µL for 2 equivalents SATA). Some precipitate was observed and its quantity increased for higher SATA to VEGFR1_{d2} ratios. After 10 min at room temperature, the protein was separated from small molecules by SEC into the reaction buffer on an Econo-Pac® 10DG column (Bio-Rad). Fractions containing the protein were identified by UV, pooled and concentrated to 1 mL by ultrafiltration (MWCO 3000, Millipore). The thiol groups were deprotected by addition of 100 µL of a hydroxylamine solution (prepared from 50 mmol of H₂NOH·HCl and 2.5 mmol of EDTA in 100 mL of water, with the pH adjusted to 7.5 with solid Na₂HPO₄) [23]. After 1 h at room temperature in the dark, thiol content was titrated by Ellman analysis using $\epsilon_{412} = 14150$ L mol⁻¹ cm⁻¹ [24,25] and total protein content was determined by UV at 280 nm (see Table S1 for an optimization study of the ratio SATA/VEGFR1_{d2}). Typically, about 30% of recovered protein functionalized with 1.0–1.2 thiol group per protein was obtained with 2 equivalents of SATA. Solid HRP maleimide activated (1 equivalent corresponding to the measured thiol content, 25 nmol, 1.0 mg) was added and the reaction mixture was kept in the dark at room temperature for 3 h. β-mercaptoethanol was then added to a final concentration of 1.5 mM, and after 15 min at room temperature, *N*-ethyl maleimide was added to a final concentration of 3.0 mM. After 15 additional minutes, the reaction was stopped by removing excess reagents on an Econo-Pac® 10DG column equilibrated with the reaction buffer.

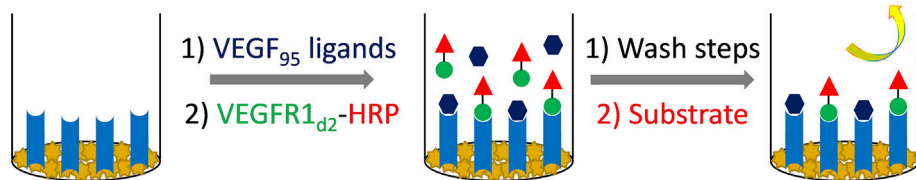
Because several lysine side-chains of the VEGFR1_{d2} may be functionalized, we obtained a mixture of VEGFR1_{d2} covalently linked to several HRP, herein named VEGFR1_{d2}-(HRP)_n (n: number of HRP molecules bound to one VEGFR1_{d2} molecule). The VEGFR1_{d2}-(HRP)_n conjugates were further purified by SEC on a Superdex™ 75 HR 10/30 (Amersham) with isocratic elution in NaCl 250 mM, NaH₂PO₄/NaOH 50 mM pH 7.2 buffer (4 °C). Fractions containing the desired product (as determined by the elution profile and SDS-PAGE analysis) were pooled and concentrated (fractions f to i in supporting Fig. S1). For long-term storage, an equal volume of glycerol was added and the VEGFR1_{d2}-HRP solution was stored at –20 °C.

The proportions of VEGFR1_{d2}-(HRP)₂, VEGFR1_{d2}-HRP (for n = 1) and HRP in pooled fractions were estimated by densitometry of SDS-PAGE (LAS 4000 imager and Multigauge 2000 software, Fuji-film) (supporting Fig. S2). The molar proportions of each species were VEGFR1_{d2}-(HRP)₂: 4 ± 1%, VEGFR1_{d2}-HRP: 59 ± 6% and HRP: 37 ± 7%, allowing to calculate a ([VEGFR1_{d2}-HRP] + [VEGFR1_{d2}-(HRP)₂])/[HRP]_{total} molar ratio of 0.61. This molar ratio corresponds to the effective concentration of conjugate and was used in the binding assay to correct the [HRP]_{total} concentration measured by UV at 403 nm (ϵ_{403} (HRP) = 100000 L mol⁻¹ cm⁻¹). In addition, the quantitative comparison of the UV/visible absorptions at 275 and 403 nm agreed with the proportions obtained using the densitometry method (supporting Fig. S3).

A- Binding assay of the VEGFR1_{d2}-HRP conjugate with coated VEGF₉₅



B- Displacement assay using VEGF₉₅ ligands



C- Displacement assay using VEGFR1_{d2} ligands

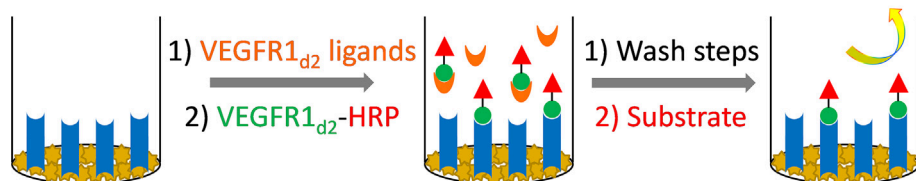


Fig. 1. Schematic drawing of the binding (A) and displacement assays using competing ligands (B and C).

Binding of VEGFR1_{d2}-HRP to coated VEGF₉₅, and displacement assays

Tween 20 and bovine serum albumin fraction IV (BSA) were obtained from Sigma-Aldrich.

Binding assay of the VEGFR1_{d2}-HRP conjugate with coated VEGF₉₅

A white high-binding 96-well plate (Costar) was coated overnight at 4 °C with VEGF₉₅ (10, 20 or 40 ng/well) in 100 μL PBS (10 mM, pH 7.4, 137 mM NaCl, and 2.7 mM KCl) per well. The plate was washed 3 times with PBS containing 0.1% Tween 20 (v/v, buffer A) and 200 μL of 3% BSA in PBS (w/v) were added. The plates were washed 3 times with buffer A after 1.5 h incubation at 37 °C. Increasing concentrations of the VEGFR1_{d2}-HRP conjugate were added to the wells (100 μL final volume). After 1 h incubation at 37 °C, the plate was washed 4 times with buffer A and 100 μL SuperSignal West Pico Substrate (Pierce) were added. The plate chemiluminescence (as relative lights units, RLU) was rapidly read on a Perkin Elmer Victor 2 spectrophotometer. Saturation curves corresponding to the specific signal (total signal – non-specific signal) of VEGFR1_{d2}-HRP bound to coated VEGF₉₅ were analyzed with the software Graphpad Prism. Non-specific signal was obtained without coated VEGF₉₅.

Displacement assay using VEGFR1_{d2} and VEGF₉₅ ligands

Transition metals and DMSO were obtained from Sigma-Aldrich. Peptides used as VEGF ligands were synthesized as previously described (see the supporting information) [9].

The displacement assay was carried out following the binding procedure above with 40 ng/well VEGF₉₅, but with addition of ligands at twice the desired final concentration in 50 μL of PBS with or without 2% DMSO. If desired, a pre-incubation period could be

inserted at this point. Then, a solution of VEGFR1_{d2}-HRP at twice the desired final concentration (typically 2 nM) in 50 μL PBS was added to each well and the resulting solutions were mixed with 2 micropipette actions.

The percentages of displacement of the VEGFR1_{d2} by the different ligands were calculated using the formula: % displacement = $100 \times [1 - (S - NS)/(MS - NS)]$, where S is the measured signal, NS and MS are non-specific (no VEGF₉₅ coated) and maximum (no ligand) signals, respectively. Data were analyzed with the software Graphpad Prism.

Results and discussion

VEGFR1_{d2}-HRP conjugation

The VEGFR1_{d2} was covalently linked to commercially available horseradish peroxidase (HRP). The chemical coupling procedure followed a known two-steps method for the preparation of conjugates for immunoassay [23]. It was mediated through the introduction of a protected thiol group onto one of the six lysine residues of the domain 2, by the *N*-hydroxysuccinimide ester of S-acetylthioglycolic acid (SATA). After removal of the thiol protecting group, the resulting functionalized protein was covalently conjugated to maleimide activated HRP. Interestingly, as observed by SDS-PAGE, the mono-conjugated adduct VEGFR1_{d2}-HRP was obtained as the major product at approximately 55 kDa. Other bands were attributed to excess HRP (44 kDa) and VEGFR1_{d2} (11 kDa), as well as some poly-conjugation products VEGFR1_{d2}-(HRP)_n (99 kDa for n = 2, supporting Fig. S1). To optimize the reaction conditions, several runs have been carried out with 1, 2 and 4 equivalents of SATA for 1 equivalent of VEGFR1_{d2}. The best compromise appeared to be 2 equivalents of SATA, giving a labeling of 1.1 thiol groups per

protein molecule and a low quantity of poly-conjugated products, while keeping the unreacted VEGFR1_{d2} and the total protein recovery to acceptable levels (see the supporting information for details). Although the crude mixture obtained at this point was suitable for binding and displacement assays, we purified further the conjugate by size exclusion chromatography, to remove the unreacted VEGFR1_{d2} and most of the poly-conjugated VEGFR1_{d2}-(HRP)_n. Finally, the VEGFR1_{d2}-HRP conjugate containing a small amount of VEGFR1_{d2}-(HRP)₂ (4 ± 1%) and some HRP (37 ± 7%) was obtained. A further purification of the conjugated product resulted in very low yield because the commercial HRP-maleimide is a mixture of isozymes with different physicochemical properties (isoelectric point, molecular weight and hydrophobicity). So we decided to build our assay on the semi-purified product.

Binding assay of the VEGFR1_{d2}-HRP conjugate with coated VEGF₉₅

In order to measure the affinity of the VEGFR1_{d2}-HRP conjugate for the VEGF₉₅, the following assay was carried out (Fig. 1-A). The VEGF₉₅ was coated onto white high binding 96-well plates following established procedures (at 10, 20 and 40 ng/well), and increasing concentrations of the conjugate were added to the wells. We obtained sigmoidal saturation curves in semi-log plot, for which luminescence was proportional to the remaining ligand (VEGFR1_{d2}-HRP) bound to the coated VEGF₉₅. [VEGFR1_{d2}-HRP] at half-saturation corresponded to an apparent K_d of 15 nM (95% confidence interval 13–20 nM, Fig. 2-A and supporting Fig. S4). Note that at 40 ng/well VEGF₉₅, its concentration was high compared to the K_d value. Therefore, the usual assumption that the free ligand concentration [VEGFR1_{d2}-HRP] was equal to the total ligand concentration was not true anymore, because a significant fraction was bound to the coated VEGF₉₅. The resulting ligand depletion was taken into account for the K_d calculations according to references [26] and [27] (supporting Fig. S5 and Table S2). The obtained K_d value showed the high affinity of the VEGFR1_{d2}-HRP conjugate for the VEGF₉₅ and was compatible with previously reported K_d and IC₅₀ values, in the low nanomolar range, for the binding of VEGF to monomeric VEGFR1_{d2}. K_d values were determined by surface plasmon resonance assays using VEGF₁₋₁₆₅ (K_d = 2.9 ± 0.2 nM [28,29], and K_d = 19.8 nM [30]), and IC₅₀ values were measured by two ligand binding assays: the first assay used biotinylated VEGF₈₋₁₀₉ and coated anti-VEGF monoclonal antibody that competes with VEGFR1_{d2} binding (IC₅₀ = 1.4 ± 0.4 nM [11]), and the second assay used a biotinylated VEGFR1_{d2} and coated VEGF₈₋₁₀₉ (IC₅₀ = 10 ± 1 nM [21]). As a control experiment of our displacement assay, we verified that free HRP at concentrations up to 100 nM did not interact with coated VEGF₉₅. Finally, the effect of incubation time on signal intensity was evaluated. It appeared that with 1 nM VEGFR1_{d2}-HRP and 40 ng per well of coated VEGF₉₅, at 37 °C, the maximum signal was achieved after 30 min and was stable for at least 1 h. A slight decrease was observed after 2 h. Therefore, an incubation time of 45 min was chosen for the subsequent displacement experiments, to ensure equilibrium conditions and maximum signal. Conveniently, we observed that the activity of the VEGFR1_{d2}-HRP conjugate was conserved during a period of one year, when stored at –20 °C in 50% glycerol and PBS buffer (v:v).

Displacement assay using VEGFR1_{d2} and VEGF₉₅ competing ligands

Displacement assays were carried out using a sub-saturating concentration of VEGFR1_{d2}-HRP (typically 1 nM) in the presence of competing ligands.

Validation of the competition assay with VEGF₉₅ ligands

We evaluated the assay efficiency using several VEGF ligands: VEGFR1_{d2} and a series of peptides able to bind the VEGF at the VEGFR1_{d2} interacting surface (Fig. 1-B, Fig. 2-B and Table 1). The representative member was the peptide v114* (VEPNCDIHV^hLWE-WECFERL-NH₂, with the two cysteine residues involved in a disulfide bond), identified by Genentech, Inc., of which we recently synthesized and assayed a variety of derivatives [9,31,32] (peptides numbering is given according to reference [9]).

The effects of incubation and pre-incubation time were investigated for the VEGFR1_{d2}-HRP/VEGF₉₅ interaction displacement by peptide (1) (supporting Figs. S6–A and Table S3). The correlation coefficient was poor at 15 min incubation, due to a steeper Hills slope (1.8 for the best fit with a variable slope analysis), probably because the equilibrium between VEGF₉₅/VEGFR1_{d2}-HRP had not been reached. The IC₅₀ values increased slightly from 15 min to 45 min, and remained constant after 45 min incubation. Moreover, we observed for peptide 1 that an additional pre-incubation time of 45 min in the absence of VEGFR1_{d2}-HRP had no effect on the IC₅₀ in the optimized conditions used (supporting Figs. S6–B).

An IC₅₀ of 58 nM was obtained for unlabeled VEGFR1_{d2}. This value is higher than expected. Indeed, in this competitive displacement assay, according to the Cheng - Prusoff equation [33], the IC₅₀ value should be close to the K_d value, because [VEGFR1_{d2}-HRP] = 1 nM and K_d = 15 nM. We hypothesize that the hydrophobic surface of VEGFR1_{d2} that constitutes the VEGF binding site adsorbs non-specifically onto the remaining space on the plate, which increases the IC₅₀ value. This can be correlated with our observation that the direct coating of the VEGFR1_{d2} domain prevents VEGF binding (see above).

However, the IC₅₀ obtained here for v114* and the modified peptide 1 were in good qualitative agreement with both the IC₅₀ values measured in the VEGFR1_{ECD}/btVEGF₁₋₁₆₅ displacement assay and the K_d values determined by ITC (Table 1). Importantly, for peptides 10, 11 and 12, a good relative correlation was obtained with K_d values determined by ITC, whereas the VEGFR1_{ECD}/btVEGF₁₋₁₆₅ displacement assay provided misleading results.

Validation of the displacement assay with VEGFR1_{d2} ligands

Ligands binding the VEGFR1_{d2}-HRP conjugate should also induce a displacement of the interaction (Fig. 1-C, Fig. 2-C and Table 1), which was confirmed with the VEGF₉₅ (IC₅₀ = 45 nM). Moreover, some peptides have been described to bind the VEGFR1_{d2} at the VEGF binding site. Unfortunately, for the cyclic peptide c [YYDEGLEE]-NH₂ [7,14], no activity was detected up to 100 μM. This synthetic peptide was also inactive in our hands when assayed with the VEGFR1_{ECD}/btVEGF₁₋₁₆₅ displacement assay [15]. We recently discovered that some divalent metal cations can bind the VEGFR1_{d2} and displace its interaction with the VEGF [34]. This observation combined with the variability of the displacement activity among batches of the same peptide prompted us to test metal complexes, which may be present as trace amounts and lead to false positive hits. Pd(OAc)₂ was a potent inhibitor with an IC₅₀ of 47 nM. CuSO₄ had also some activity, but did not reach 100% displacement, even above 30 μM. This may be due to the low solubility of copper hydroxide at pH 7.4. CoCl₂ and FeSO₄ were not able to displace the interaction. These results correlate fairly well with those obtained with the VEGFR1_{ECD}/btVEGF₁₋₁₆₅ assay [34]. Finally, we verified that the displacement observed was not due to interference of the metal cations with the HRP detection system at the low concentrations used (see the supporting information). A detailed structural investigation of the transition metal mediated inhibition of the VEGF/VEGFR1_{d2} interaction is reported elsewhere [34].

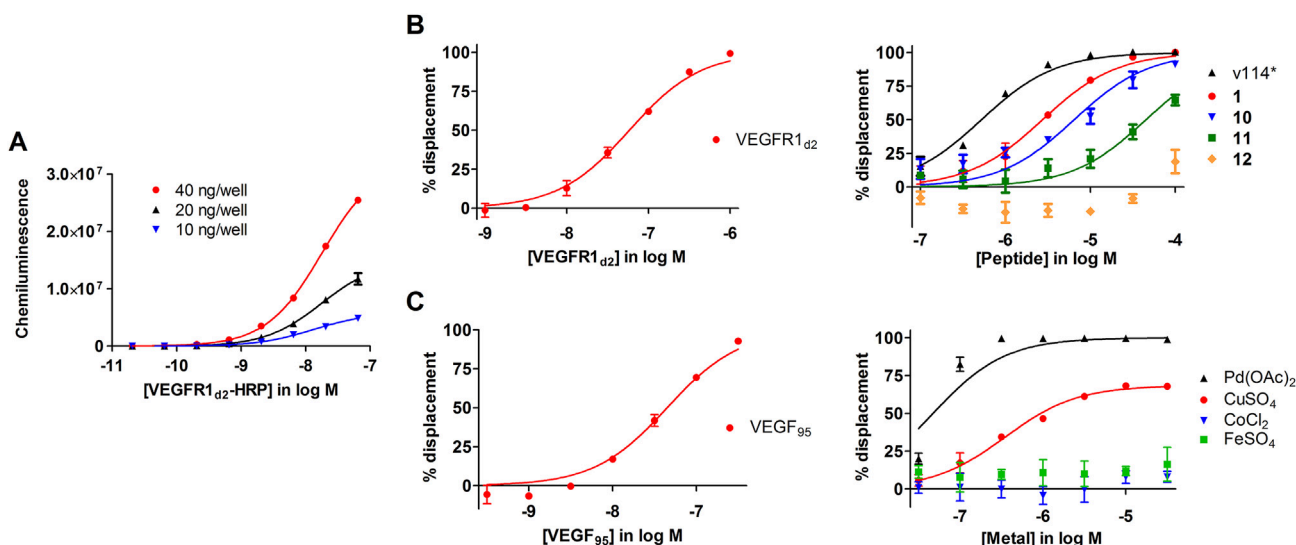


Fig. 2. A: Binding assay of the VEGFR1_{d2}-HRP conjugate with coated VEGF₉₅. The analysis was performed with the software GraphPad Prism using a “one site-accounting for ligand depletion” non-linear regression [26,27]. B and C: displacement curves obtained for the indicated competitors (VEGF₉₅ ligands in B and VEGFR1_{d2} ligands in C) in the herein developed assay employing VEGFR1_{d2}-HRP (1 nM) and coated VEGF₉₅ (40 ng/well). Data are presented as percentages of displacement of VEGFR1_{d2}-HRP. The analysis was performed with the software GraphPad Prism using a “log [inhibitor] vs. response” non-linear regression, with bottom and top values fixed at 0 and 100% respectively (excepted for CuSO₄ where the top value was not fixed). Mean numbers from at least triplicate wells are plotted. Error bars represent standard deviations. The assay was performed in the presence of 1% DMSO for the metals and the peptides.

Table 1
IC₅₀ and K_d values determined for ligands disrupting the VEGFR1_{d2}/VEGF₉₅ interaction.

	Competing ligands	IC ₅₀ VEGFR1 _{d2} (μM) ^a	IC ₅₀ ECD (μM) ^b	K _d (μM) ^c
VEGF ligands	VEGFR1 _{d2}	0.058 [0.053; 0.064]	0.70	0.020 [0.006; 0.044]
	v114*	0.52 [0.45; 0.61]	1.8 [1.1; 3.0]	0.028 [0.022; 0.035]
	1	2.7 [2.2; 3.3]	5.2 [3.6; 7.5]	0.15 [0.10; 0.19]
	10	6.5 [4.7; 8.9]	12 [10; 15]	0.29 [0.27; 0.33]
	11	45 [35; 58]	7.8 [5.9; 11]	2.3 [2.1; 2.4]
	12	>100	7.3 [5.4; 10]	20 [17; 24]
VEGFR1 _{d2} ligands	VEGF ₉₅	0.045 [0.037; 0.054]	0.012	0.020 [0.006; 0.044]
	Pd(OAc) ₂	0.047 [0.032; 0.070]	0.36 [0.27; 0.48]	Nd
	CuSO ₄	0.15 [0.091; 0.25]	0.97 [0.63; 1.5]	Nd
	CoCl ₂	>100	47 [35; 63]	Nd
	FeSO ₄	>100	>100	Nd

95% confidence intervals are given in brackets.

Nd: not determined.

^a “IC₅₀ VEGFR1_{d2}” refers to the IC₅₀ values measured in the herein described VEGF₉₅/VEGFR1_{d2}-HRP displacement assay.

^b “IC₅₀ ECD” refers to the IC₅₀ values measured in the VEGFR1_{ECD}/btVEGF₁₋₁₆₅ displacement assay [9].

^c K_d values for VEGF₉₅ determined by ITC at 293 K [9], and unpublished results.

Conclusions

The direct chemical modification of the VEGFR1_{d2} domain through one of its 6 lysine residues produces a conjugate which retains a high affinity for the VEGF. The conjugate can be used in a displacement assay, which bypasses the widely used antibodies or the streptavidin-biotin interaction system. It accelerates the process by avoiding some incubation steps. The optimized assay can be carried out in only 2.5 h after the VEGF coating step. The displacement assay can be used to identify ligands of either VEGFR1_{d2} or VEGF. Some potential disadvantages exist. In particular, the assay may lead to false positive for molecules that bind tightly to HRP and inhibits it in spite of the washing steps. However, such molecules have not been found during the course of the optimization study. In any case, adventitious HRP inhibition can be checked afterwards in separate wells with the substrate and plate reader required for the assay. Finally, the functional VEGFR1_{d2}-HRP conjugate described herein may also be used for the screening of

peptides and small molecules attached to various types of solid supports, for the identification of VEGFR1_{d2} ligands.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ab.2017.05.004>.

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