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Short title: Chemiluminescent screening assay for VEGF-R1 ligands

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List of abbreviations : VEGF, Vascular endothelial growth factor; PlGF, Placenta growth factor; Flt-1, Fms Like Tyrosine 1; KDR, Kinase insert domain receptor; ECD, Extra-cellular domain; VEGF-R, Vascular endothelial growth factor receptor; NP-1, Neuropilin-1; PBS, Phosphate buffered saline; DMSO, Dimethylsulfoxyde; BSA, Bovine serum albumin; Fmoc, 9-Fluorenylmethoxycarbonyle; RLU, Relative light units; bt$VEGF_{165}$, Biotinylated $VEGF_{165}$

Angiogenesis is the growth of new blood vessels from a pre-existing vasculature. The deregulation of this physiological process is involved in several pathologies including cancer and inflammatory diseases (atherosclerosis, rheumatoid arthritis or age-related macular degeneration). Consequently, the research for drugs able to disrupt abnormal angiogenesis constitutes an essential research field [1].

Angiogenic sprouting of blood vessels depends on the balance between pro- and anti-angiogenic molecules. Among them, the vascular endothelial growth factor (VEGF A) is the major contributor to tumor angiogenesis [2]. The predominant isoform of VEGF A in humans is named $VEGF_{165}$, according to its length in amino acids. $VEGF_{165}$ pro-angiogenic activity is mediated through binding to two high-affinity tyrosine kinase receptors: VEGF receptor 1 (VEGF-R1, Flt-1) and VEGF receptor 2 (VEGF-R2, KDR), located predominantly at the surface of endothelial cells but also on several tumour cell lines [3]. Neuropilin-1 (NP-1), a non-tyrosine kinase receptor of $VEGF_{165}$, acts as a co-receptor, enhancing the activity of VEGF-R2 [4]. The specific role of VEGF-R1 is still a matter of debate. It acts as a negative regulator of angiogenesis during development but an increasing number of papers tend to demonstrate its specific implication as a promoter of angiogenesis under pathological conditions [5].

Considering the central role displayed by the system $VEGF_{165}$/VEGF-R1 in disease-associated angiogenesis, it constitutes an attractive target for the development of anti-angiogenic therapies. Among the several strategies developed to disrupt this interaction, a promising approach is constituted by antagonist molecules, with a high affinity for the extracellular domain of the receptor, able to prevent $VEGF_{165}$ binding on VEGF-R1 [6].

The affinity of these molecules is commonly evaluated thanks to a radioactive competition test: the tested compound and radiolabeled [^{125}I]$VEGF_{165}$ are incubated with cells expressing VEGF receptors [7], membranes from endothelial cells [8] or directly with

recombinant receptors coated on microplates [9], and the remaining activity after washes is measured. Unfortunately, the increasing logistical and regulatory difficulties associated with the use of radioactive isotopes threaten to limit the usefulness of this method. Consequently, the discovery of antagonists is limited by the unavailability of a safe and low-cost high-throughput screening test, and therefore, few antagonists have been reported to date [6]. To help in the discovery of potential new antiangiogenic therapeutics, we have developed a robust non-radioactive assay system for screening of VEGF receptors ligands. Our test is based on competition between compounds and biotinylated $VEGF_{165}$ ($btVEGF_{165}$), which is detected by chemiluminescence (Fig. 1). This test is performed with the extra-cellular domain (ECD) of recombinant human VEGF-R1, allowing the discovery of receptor-specific molecules. Indeed, cell lines over-expressing one of the receptors proved to be not always a reliable system for testing ligand specificity due to the constitutive co-expression of other receptors, as attested by Perret et al who described for the first time a VEGF-R2 specific antagonist whose biological effect was in fact triggered by interaction with NP-1 [7, 10].

In a first step, we determined the affinity of $btVEGF_{165}$ for VEGF-R1 ECD. The surface of white high binding 96-well microplates (Corning Life Sciences, Netherlands) was coated with 100 µl of phosphate buffer saline (PBS; pH = 7.4) solution containing 200 ng/mL of VEGF-R1 ECD/Fc chimera (R&D Systems, UK). The plate was sealed and incubated overnight at 4°C. After 3 washes with 250 µl of PBS 0.5% (v/v) Tween 20 (Buffer A), the plate was blocked by adding 200 µl of PBS with 2% (w/v) bovine serum albumin fraction IV (BSA; Sigma-Aldrich, France) and agitated at 25°C for 2 hours. The plate was washed carefully three times with buffer A. The plates were then ready for sample addition. Alternatively, the plates could be dried under vacuum, sealed with a drying agent and stored at 4°C for 1 week.

Then, 100 µl of a solution of $btVEGF_{165}$ (obtained as part of a Fluorokine biotinylated VEGF kit from R&D Systems, UK) diluted at varying concentrations in PBS were added to each well. The plate was sealed again and incubated 2,5 hours at 25°C. Three washes were performed with buffer A and 100 µl of AMDEX™ streptavidin-horse radish peroxydase (Amersham Biosciences, UK) diluted at 1:8000 in PBS containing 0.5% (v/v) Tween 20 and 0.3% (w/v) BSA were added to each well. After 45 min incubation at 25°C under obscurity, the plate was washed 5 times with buffer A and 100 µl of SuperSignal west pico chemiluminescent substrate (Pierce, USA) were added. Luminescence was quantified with an EnVision™ 2101 Multilabel reader (Perkin Elmer, USA). Signal is given as relative light units (RLU). Non-specific binding is defined as the signal measured in the absence of receptor coated on the microplate. The specific binding was calculated as the difference between total binding and non-specific binding. Data were analyzed using the non-linear regression function in Prism® version 4.03 (GraphPad Software, USA).

In a second step, to verify the specificity of the interaction, we realized a competition assay with unlabeled recombinant human $VEGF_{165}$. The plate was coated with 20 ng/well of VEGF-R1 ECD, then blocked by BSA, and 50 µL of $VEGF_{165}$ (Abcys, France) at various concentrations were added to 50 µL of $btVEGF_{165}$ at 5 ng/mL (131 pM). After 2.5 h of incubation at 25°C, wells were washed three times with buffer A and the $btVEGF_{165}$ remaining was detected as previously described. The percentages of displacement were calculated by the following formula: Percentage of displacement = 100*[1-(S-NS)/(MS-NS)], where S refers to the signal measured, NS is the non specific binding signal and MS is the maximum binding signal obtained with $btVEGF_{165}$ without competitor.

The saturation curve is represented in figure 2 A. The binding capacity of $btVEGF_{165}$ reached its maximum when added at a concentration of 10 nM. Based on three independent experiments, the dissociation constant (K_d) was estimated at 750 pM, a value comparable to

the one found by Barleon et al. [11]. Considering that performing the assay with recombinant receptors does not allow to reproduce the favourable effect of membrane microenvironment existing in cell based assays, it is not surprising to obtain a value superior to the K_d previously described for $VEGF_{165}$ interaction with Cos cells transfected with VEGF-R1 (10-30 pM) [12]. In addition, our competition assay with unlabeled $VEGF_{165}$ (Fig. 2 B) demonstrates that 50% of biotinylated ligand binding (introduced at 131pM) was inhibited by 387 ± 60 pM of unlabeled $VEGF_{165}$. This result proves that the $btVEGF_{165}$ exhibits roughly the same affinity for VEGF-R1 ECD as unlabeled $VEGF_{165}$.

Another key point in developing a screening assay is the assessment of its robustness in the presence of dimethylsulfoxyde (DMSO). As a matter of fact, screening of chemical libraries is typically conducted in the presence of low concentrations of DMSO to avoid problems of solubility. We controlled that weak DMSO concentrations (0.63%, 1.25% and 2.5% v/v) had no significant effect on $btVEGF_{165}$ binding to VEGF-R1 ECD. The effect was more obvious at 5% and 10% DMSO, leading to respectively, 32% and 46% decrease of specific binding signals. Nevertheless, the reproducibility of the experiment performed on triplicate wells was not affected in all cases. Based on these data, a final concentration of 1% DMSO was chosen for development of the assay, allowing the screening of most of chemical compound libraries.

It follows then that this assay represents a strong asset in the search of small-molecule ligands of VEGF-R1. Thus, we verified that molecules exhibiting a weak affinity for the receptor could be readily identified using this screening test. Therefore, we evaluated the affinity of peptides previously described in the literature as ligands of VEGF receptors (Fig. 2 B). Peptides were synthesized by solid phase peptide synthesis on an Applied Biosystems 433A synthesizer (Applied Biosystems, USA) using Fmoc chemistry. They were purified to

at least 95% purity by semi-preparative reverse phase HPLC and their identity was confirmed by electron spray MS (LCQ Advantage, Thermo Electron Corporation, USA).

QK (Ac-KLTWQELYQLKYKGI-NH_2) is a VEGF mimicking peptide [8], exhibiting an agonist activity for VEGF receptors. Its affinity was determined by competition with [^{125}I]$VEGF_{165}$ for the binding sites of VEGF on cell membranes isolated from bovine aorta endothelial cells which express VEGF-R1, VEGF-R2 and NP-1 receptors. This assay leads to an IC_{50} estimated in the nanomolar range. Tested on our screening assay, its IC_{50} for VEGF-R1 was measured at 32 µM. Based on this result, it can be hypothesized that the high affinity of QK for endothelial cells may be triggered essentially by its binding to VEGF-R2/NP-1.

SP5.2 (NGYEIEWYSWVTHGMY-NH_2) has been described as a VEGF-R1 specific antagonist peptide, identified by phage display library screening [13]. To evaluate its affinity for the receptor, El-Mousawi et al. synthesized a fluorescein-labeled SP5.2 peptide and measured the fluorescence signal due to peptide binding to immobilized VEGF-R1/Fc chimera (1 µg/well) in a saturation assay. Based on this experiment, SP5.2 binding was dose-dependent, with a half-maximum value at 40 µM. This *in vitro* value on purified receptor is in the same order of magnitude than the IC_{50} obtained for SP5.2 in our chemiluminescent competition assay, measured at 28 ± 7 µM.

As a negative control, we chose the peptide A7R (ATWLPPR) initially described as a VEGF-R2 specific antagonist and which recently proved to be a NP-1 specific ligand [7, 10, 14]. As expected, no displacement of bt$VEGF_{165}$ was observed in the range of concentrations tested (3-100 µM).

Finally, we verified if the screening assay could allow the identification of larger inhibitors of $VEGF_{165}$. Thus, we tested a monoclonal antibody raised again VEGFR-1 ECD (anti-VEGFR-1, clone FLT-19, Sigma-Aldrich). This antibody proved to be able to displace bt$VEGF_{165}$ binding to VEGFR-1 with an IC_{50} of 2.81 ± 1.59 nM (supplementary data).

In summary, the current study describes a method for identifying ligands of VEGF receptor 1 based on chemiluminescent detection of biotinylated $VEGF_{165}$ binding to the recombinant receptor. This assay has been developed on an easy to handle 96-well format. Considering the small amounts of proteins used and the suppression of radioactivity issues, this assay represents an interesting progress for the evaluation of new VEGF-R1 ligands and should allow the screening of chemical libraries, even by academic institutions.

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Fig. 1. Schematic representation of the chemiluminescent screening assay.

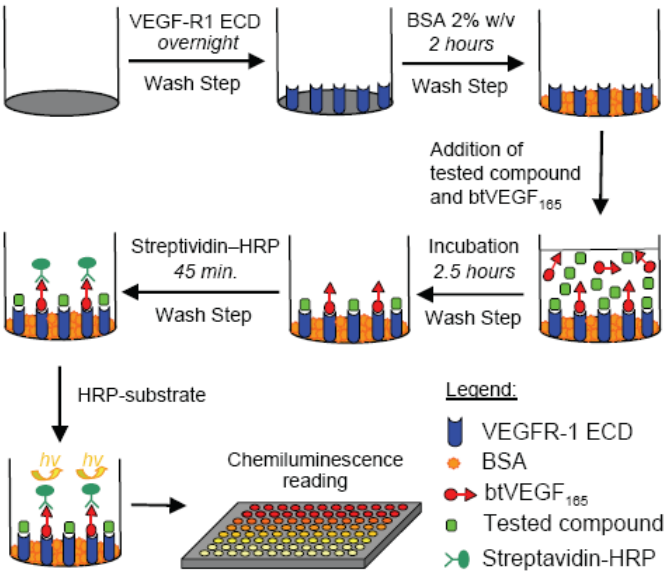

Fig. 2. (A) Solid-phase binding assay of biotinylated $VEGF_{165}$ to recombinant human VEGF-R1. Total and non-specific binding curves correspond to the signal observed, respectively in presence (20 ng/well) or in absence of VEGF-R1. The data show mean numbers from triplicate wells. Error bars represent for standard deviation. (B) Competition assay between human $VEGF_{165}$ and biotinylated $VEGF_{165}$ (5 ng/mL; 131 pM) for binding to VEGF-R1 ECD (20 ng/well). Data is presented as a percentage of displacement of biotinylated $VEGF_{165}$. (C)

Competition assay between peptides QK, SP5.2 and A7R and $btVEGF_{165}$ in presence of 1% DMSO.