Molecular modelling, synthesis and biological evaluation of peptide inhibitors as anti-angiogenic agent targeting neuropilin-1 for anticancer application

Ezatul E. Kamarulzamanab, Régis Vanderesseab, Amirah M. Gazzalib, Muriel Barberi-Heyoc, Cédric Boura, Céline Frochotd, Omar Shawkatay, André Aubryb and Habibah A. Wahaabf

aSchool of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia; bLCPM, UMR-CNRS 7375, Université de Lorraine, ENSIC, 1 Rue Grandville, F-54000 Nancy, France; cCRAN, UMR-CNRS 7039, Campus Science, BP 70239, F-54506 Vandœuvre-lès-Nancy, France; dLRGP, UMR-CNRS 7274, Université de Lorraine, ENSIC, 1 Rue Grandville, F-54000 Nancy, France; eChemical Sciences Programme, School of Distance Education, Universiti Sains Malaysia, 11800 Penang, Malaysia; fMalaysian Institute of Pharmaceuticals and Nutraceuticals, Ministry of Science, Technology and Innovation, Jalan Bukit Gambir, 11800 Penang, Malaysia

Communicated by Ramaswamy H. Sarma

(Received 11 September 2015; accepted 9 December 2015)

Vascular endothelial growth factor (VEGF) and its co-receptor neuropilin-1 (NRP-1) are important targets of many pro-angiogenic factors. In this study, nine peptides were synthesized and evaluated for their molecular interaction with NRP-1 and compared to our previous peptide ATWLPPR. Docking study showed that the investigated peptides shared the same binding region as shown by tuftsin known to bind selectively to NRP-1. Four pentapeptides (DKPPR, DKPRR, TKPPR and TKPRR) and a hexapeptide CDKPRR demonstrated good inhibitory activity against NRP-1. In contrast, peptides having arginine residue at sites other than the C-terminus exhibited low activity towards NRP-1 and this is confirmed by their inability to displace the VEGF165 binding to NRP-1. Docking study also revealed that replacement of carboxyl to amide group at the C-terminal arginine of the peptide did not affect significantly the binding interaction to NRP-1. However, the molecular affinity study showed that these peptides have marked reduction in the activity against NRP-1. Pentapeptides having C-terminal arginine showed strong interaction and good inhibitory activity with NRP thus may be a good template for anti-angiogenic targeting agent.

Keywords: neuropilin-1; VEGFR; angiogenesis; synthetic peptides; docking study; molecular modelling; ELISA; in vitro binding

Introduction

Despite recent advances in surgery, chemotherapy and radiation treatment, survival of patients with advanced malignancy remains suboptimal. Photodynamic therapy (PDT) is now regarded as a promising clinical treatment modality in cancer. PDT involves the combined action of a photosensitizer, visible light of an appropriate wavelength and molecular oxygen to produce reactive oxygen species, particularly singlet oxygen that plays the main role in mediating cellular death. The effectiveness of PDT could be enhanced by combining peptide inhibitors that target angiogenesis with the treatment regimen.

Angiogenesis, the formation of new blood vessels from the pre-existing microvasculature is important in the pathogenesis of malignant, infectious, fibro-proliferative and inflammatory diseases. In cancer, angiogenesis occurs in the tumour surrounding tissues to form a novel vascular tube-like structure to supply nutrients and oxygen to maintain the viability of tumour progression and also to facilitate metastatic spreading into other parts of the body.

Targeting angiogenesis would suppress or inhibit the formation of new blood vessels and consequently disrupt the tumour growth and metastases (Benouchan & Colombo, 2005; Denekamp, 1984; Folkman, 1971; Shimizu, Asai, & Oku, 2005).

A variety of pro-angiogenic factors have been identified (Folkman, 1995; Hanahan & Folkman, 1996). One of the most specific and important growth factors is vascular endothelial growth factor (VEGF) which is also known as vascular permeability factor or vasculotropin. VEGF is a key regulator involved in angiogenesis, vasculogenesis and vascular permeability (Dvorak et al., 1999; Ferrara & Davis-Smyth, 1997; Klagsbrun & Soker, 1993) which influences the progression of tumour growth. Five VEGF isoforms are commonly encountered, i.e. VEGF121, VEGF145, VEGF165, VEGF189 and VEGF206. All these isoforms are active in angiogenesis (Matthies, Low, Lingen, & DiPietro, 2002) via their interactions with the tyrosine kinase receptors; VEGFR-1, VEGFR-2 (also known as kinase domain receptor, KDR) and in
lymphangiogenesis with VEGFR-3. VEGFR-2 is expressed selectively on almost all endothelial cells, showing its crucial function in the tumour angiogenesis process. These VEGF isoforms also binds to a family of co-receptors called the neuropilins (NRP). NRP-1 especially has become a popular drug target due to its ability to bind and block VEGF binding and its function in inhibiting chemotaxis, phosphorylation, signalling and migration (Bernatchez, Rollin, Soker, & Sirois, 2002; Soker, Miao, Noimi, Takashima, & Klagsbrun, 2002; Soker, Takashima, Miao, Neufeld, & Klagsbrun, 1998).

Neuropilin-1 (NRP-1) has two (α1 and α2) complement-binding homology domains, two coagulation factors V and VII homology domains (b1 and b2) and a meprin domain (c) in their extracellular regions. Cumulative findings indicate that α and β domains are crucial for the ligand binding, including binding to semaphorin 3A (SEMA3A) (Gu et al., 2002; Ueyama et al., 2011) and vascular endothelial growth factor splice variant VEGF<sub>165</sub> (Hoeben et al., 2004; Krussel et al., 2001; Lange, Guttmann-Raviv, Baruch, Machluf, & Neufeld, 2003; Poltorak et al., 1997; Whittle, Gillespie, Harrison, Mathieson, & Harper, 1999; Woolard et al., 2004). The role of the loops at b1 domain of human NRP-1 as a targeted ligand-binding site has been determined by Vander Kooi and co-workers (Vander Kooi et al., 2007). They demonstrated that the interaction of tuftsin, an immunostimulatory tetrapeptide (TKPR) with the b1b2 domains was very similar to that of the VEGF<sub>165</sub> C-terminus (DKPRR).

Tuftsin is an immunostimulatory peptide that can be used to enhance the immunogenicity of proteins (Gao et al., 2015). It is a tetrapeptide (Thr-Lys-Pro-Arg) produced by enzymatic cleavage of the Fc-domain of the heavy chain of immunoglobulin G. It stimulates the phagocytic activity of polymorphonuclear leukocytes. At this time, as far as we know, no clinical trials are performed with tuftsin. In most recent research studies, tuftsin is used for the treatment of rheumatoid arthritis (Jain, Tran, & Amiji, 2015), for the modulation of murine lupus nephritis (Bashi et al., 2015) and a macrophage-targeting molecule (Horváti et al., 2014).

Tuftsin was shown to bind only at the b1 domain of NRP-1 in the conserved interstrand loops where it formed interactions with residues Y297 (of the first ligand binding loop, loop I), D320 (loop II), and S346, T349 and Y353 (loop III). These residues were also required for VEGF binding as shown by the overlapping binding competition study between tuftsin and mutational study of VEGF at the loop of residues S346, E348 and T349 (Vander Kooi et al., 2007).

Previously in our group, a peptide-conjugated photosensitizer (PS), TPC-Ahx-ATWLPPR targeting NRP-1 had showed an affinity for endothelial cells of tumour neovascularatures (Tirand et al., 2006). The molecule showed enhanced uptake and photodynamic properties compared to its nonconjugated counterpart. However, the peptide moiety of the conjugate degraded rapidly and was relatively unstable. Thus, the aim of the present study is to search for novel and stable peptide which could be conjugated to a photosensitizer and could act on NRP-1. A total of nine peptides of various sequence length (DKPRR, CDKPRR, TKPRR, TKPRR-NH<sub>2</sub>, APQPRPL, CPQPRPL, TKPPR, DKPPR, DKPPR-NH<sub>2</sub>) were synthesized and evaluated for their binding affinity towards recombinant NRP-1 through in vitro molecular affinity study.

Among some peptides known to modulate VEGF-dependent angiogenesis (Table 1), we chose some criteria: (1) the sequence (as short as possible); (2) the C-terminal residue generally involved in the recognition; (3) the hydrophilic/hydrophobic balance. Starting from tuftsin, we decided to change its C-terminal part by the PPR sequence of ATWLPPR. However, this PPR sequence being subject to turns mediated by cis/cis conformations, we also tested TKPRR (the choice of an additional arginine being related to potential additional hydrogen bonds). We also saw the option of a change at the C-terminus arginine either by amidification or coupling to another amino-acid. Concerning the N-terminus of the sequence, we chose an aspartate instead of threonine for hydrophilic and potential other hydrogen bonds.

Prior to that, the binding interaction of these peptides was investigated using molecular docking technique.

Table 1. Some specific peptides of VEGF receptors described in the literature.

| Peptide  | Target | Ref.          |
|----------|--------|---------------|
| TKPR     | NRP-1  | Vander Kooi et al. (2007) |
| ATWLPPR  | NRP-1, NRP-2 | Binetruy-Tournaire et al. (2000) |
| TKPPR    | NRP-1  | Von Wroński et al. (2006) |
| SCKNTDSRCARQLELNRTRCCDKPRR (EG3287) | KDR, Flt-1 | Jia et al. (2006) |
| CSDKNCRTCRC-NH<sub>2</sub> | NRP-1 | Soker et al. (1997) |
| Ac-KLTQWELQKYBGK-NH<sub>2</sub> | KDR/Flt-1 | D’Andrea, Del Gatto, Pedone, and Benedetti (2006) |
| SYGRKRRQRRRAPQAPQ | KDR, Flt-1 | Albini et al. (1996) |
| CPQPRPL  | Flt-1, NRP-1 | Giordano et al. (2001) |
| CPQPRPLC |         | Giordano et al. (2005) |
Five peptides (DKPRR, DKPPR, TKPRR, TKPPR and CDKPRR) showed better affinity than ATWLPPR (Thomas et al., 2010; Tirand et al., 2006). Of these five peptides, two are novel i.e. DKPPR and TKPRR and were then chosen to be conjugated photosynthesizer molecules (Kamarulzaman et al., 2015). It is hoped that
the information from this finding will provide deeper understanding towards the design of more active peptides as anti-angiogenic targeting agents.

**Materials and methods**

**Molecular docking**

The three-dimensional (3D) structure of tuftsin (TKPR) was taken from the crystal structure along with the NRP-1 receptor (PDB ID: 2ORZ) (Vander Kooi et al., 2007). For the peptides (Figure 1), the structures were built using HyperChem Pro 6.0 (Hypercube Inc., Gainesville, USA) based on the 3D structure of tuftsin and their geometry was optimized with HyperChem using steepest descents and conjugate gradient algorithms (termination conditions set to a maximum of 500 cycles or .1 kcal/Å mol rms gradient). For example, for TKPRR, an additional of linear peptide chain was added to the structure at the C-terminal while for DKPRR, the tyrosine residue was mutated to an aspartic acid residue and the final structure was optimized as described above.

Both the ligands and NRP-1 receptor were prepared for docking using AutoDockTools (ADT) (Sanner, 2005). The protonation for the ligands and NRP-1 were assigned using ADT where polar hydrogen atoms were added to all ligands and receptor. The protonation states of an amino acid side chains assigned depend on its environment in the protein. The flexibility of peptides which is contributed to the calculation of the torsional free energy was assigned with AutoTors. Since AutoDock can only accommodate 32 torsions in each docking run, the number of active bonds was reduced. This was carried out by first examining the crystal structure of tuftsin-bound to NRP-1. The side chain and the functional group of tuftsin that contributed in the binding with NRP-1 were identified in order to decide which bond to be rotated. In general, all the amide bonds were assigned non-rotatable while the side chains of the peptides are kept flexible. The example of selection of active torsion is provided in the supplementary information (Supplementary Figure S1).

Default Kollman charges and solvation parameters were assigned to the protein atoms (Weiner et al., 1984) and Gasteiger charges (Gasteiger & Marsili, 1980) were added to each ligand atom. A grid box with the size of 70 × 70 × 70 points spaced .375 Å apart and centred on the tuftsin’s centre of mass (−24.166, 7.136, −27.591) at the NRP-1 active site was used for all AutoDock 3.0.5 docking runs (Morris et al., 1998b). The parameters of the Lamarckian Genetic Algorithm (LGA) were as follows: population size of 50, 1,500,000 energy evaluations, 200 search runs. The ligand pose with the lowest predicted free energy of binding, chosen from the most populated cluster, was used in subsequent analysis.

**Synthesis and biological assay of peptides**

**Chemicals and reagents**

The Fmoc-amino acid-Wang resin and all other Fmoc-amino acid-OH were purchased from Senn Chemicals International (Gentilly, France). Reagents and solvents were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). NRP-1 and KDR recombinant chimeric proteins and its reagents were purchased from R&D Systems, Lille, France.

**Instruments**

The peptides were synthesized using an automated ResPepXL peptide synthesizer (Intavis AG, Bioanalytical Instruments) and operated with a Multiple-Parallel Peptide Synthesis Program. The peptides were purified using Shimadzu LC-10ATvp, column C18 reverse phase Delta Pak (5 um; 150 mm × 21.1 mm). Mass spectra were recorded on a Bruker Reflex IV time-of-flight mass spectrometer (Bruker-Daltonic, Bremen, Germany) equipped with the SCOUT 384 probe ion source and electrospary on a Platform Micromass apparatus. Nuclear magnetic resonance (NMR) spectra for 1H, TOCSY and COSY were recorded on Bruker Advance 300 (300 MHz) in DMSO-<i>d</i>6. The bioassays were measured using Microplate reader MCC/340 (Labsystems, Cergy-Pontoise, France).

**Synthesis of peptides for biological test**

The nine peptides (DKPRR, CDKPRR, TKPRR, TKPRR-NH2, APQPRPL, CPQPRPL, TKPPR, DKPPR, DKPPR-NH2) were synthesized through Fmoc chemistry with HBTU activation via solid phase peptide synthesis (SPPS) on our peptide synthesizer. The Fmoc-amino acid-Wang resin was used as the starting material and swelled in dichloromethane (DCM). The functional groups at the building block of the resin were removed by piperidine (20% in dimethylformamide (DMF). The next amino acid was then graftted with the activation step.

| Receptor (coating) (μg/mL) | Biotinylated VEGF (ng/mL) | VEGF competitor (μg/mL) |
|--------------------------|--------------------------|-------------------------|
| NRP-1                    | 2                        | 5                       | .5                       |
| KDR                      | 5                        | 20                      | 2                        |
where its carboxyl group was activated by adding a threefold excess of Fmoc-aminoacid-OH, 2-(1H-benzotriazol-1-yl)-1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzo-triazole (HOBt) and N,N-isopropylethyamine (DIEA) in DMF. The process of deprotection, activation and coupling were repeated until the desired sequences of amino acids were grafted on the resin. At the end of each step, all soluble reagents on the resin and the protecting group at the N-terminus of the peptide were removed by filtration and washing process without disturbing or damaging the resin. Cleavage of the peptide from the resin was done using trifluoroacetic acid, trisopropylsilane and water. The resin was then washed, purified using high performance liquid chromatography (HPLC) and further lyophilized and stored at −20°C.

Biological test – binding study of the peptides on recombinant NRP-1 and VEGFR-2 (KDR)

The affinity of the peptides towards NRP-1 was assessed through a competitive bioassay with biotinylated VEGF165. The surface of 96-well Maxisorp microplates (Dutscher) was coated with receptors NRP-1 and KDR recombinant chimeric proteins in phosphate-buffered saline (PBS) and left overnight at room temperature. After washing the wells with the wash buffer (PBS containing .05% Tween-20), the plates were then blocked with the blocking buffer containing PBS and .5% bovine serum albumin (BSA) for 1 h at 37°C to avoid nonspecific interactions. The biotinylated VEGF165 was then added to put in contact with the recombinant chimeric proteins in the wells with or without peptide, together with the blocking buffer with additional of 2 μg/mL heparin. After 2 h of incubation at room temperature, the wells were rinsed with the wash buffer and the amount of bound biotinylated VEGF165 was stained with streptavidin horseradish coupled to peroxide by adding this reagent to the wells for 20 min at room temperature. After rinsing, the colour reagent was added and the reaction was stopped after 30 min by the addition of the stop solution and the optical density was measured at 450 nm. The results were expressed as the relative absorbance from wells containing only biotinylated VEGF165. Three wells per condition were used in this test. The concentrations of receptors, biotinylated VEGF165 and VEGF165 competitor are summarized in Table 2.

Results and discussion

Validation of docking calculations

Docking methods are typically validated by ‘redocking’ experiments, where a series of known complexes are separated and then redocked, to ensure that the docking algorithm can reproduce the observed experimental binding mode (Cosconati et al., 2010). In this study, the validation was done by performing a redocking run of tuftsin, the co-crystalized ligand into the binding pocket of NRP-1. Tuftsin, an immunostimulatory tetrapeptide (TKPR) shares a sequence motif similar to the C-terminal of VEGF165, i.e. CDKPR, which has been tested to compete with VEGF165 for binding to NRP-1 (Von Wronski et al., 2006). The re-docked tuftsin was found to be similar to the crystallographic pose with an RMSD of 1.94 Å (Figure 2). Although the RMSD value is relatively high, however, considering this peptide is highly flexible and has a sizeable number of atoms, this value is deemed acceptable. An RMSD of less than 2.0 Å is

![Graphical representations of the NRP-1/Tuftsin complex. (a) superimposition of the crystallographic (blue) and docked (red) poses of tuftsin into the b1 domain of NRP-1 (b) Focused view of interaction of tuftsin at the binding site. The binding site is represented as stick and coloured as yellow for Y or Tyr, pink (E or Glu), green (T or Thr), light grey (S or Ser) and purple (D or Asp). Tuftsin is rendered as ball and stick and coloured based on their atom type: carbon (grey), oxygen (red), nitrogen (blue) and hydrogen (white). Hydrogen bond and π–π interactions between the tuftsin and the NRP-1 are shown as black and red lines, respectively.](image)
generally accepted in the reproduction of experimentally determined structure (Jones, Willett, & Glen, 1995; Wahab, Choong, Ibrahim, Sadikun, & Scior, 2009).

Furthermore, it was further verified by visual inspection that the docked ligand bound to the protein at the same binding site as that of the crystal structure, at the conserved interstrand loops of the NRP-1 b1 domain (Figure 2(b)). Both the crystal and docked conformations bound in a region close to residues Tyr297 (loop I), Asp320 (loop II), and Ser346, Glu348, Thr349 and Tyr353 (loop III) (Figure 2(b)). It is hence confirmed that the experimental conformation was successfully reproduced by the docking protocol developed and thus the same docking procedure was applied to dock the other peptides on NRP-1.

Molecular docking

In this study, the computational docking between the peptide ligands and NRP-1 was calculated using a semi-empirical free-energy force field method implemented in AutoDock. Although, it would be more preferable to calculate the free energy of binding using quantum chemical calculation, free energy perturbation method or full atomic molecular dynamic simulation, those methods are computationally demanding (Woo & Roux, 2005), (Huey, Morris, Olson, & Goodsell, 2007). In contrast, AutoDock provides rapid evaluation of the interaction energy between the ligand and its protein target using simpler force field but covering a wider region of conformational space (Morris et al., 1998a).

The free energy calculation in AutoDock utilizes a semi-empirical free-energy force field method which was parameterized using a large number of protein-inhibitor complexes for which both structure and inhibition constants, or $K_i$, are known. Figures 3 and 4 show the conformations of docked peptides with NRP-1. As expected, all the peptides bound to the same binding region as tuftsin at b1 domain of NRP-1 (Vander Kooi et al., 2007). The predicted binding free energy ($\Delta G_{\text{bind}}$) and the inhibition constant ($K_i$) of tuftsin and the peptides are given in Table 3. Tuftsin showed the highest activity with the estimated $\Delta G_{\text{bind}}$ of $-12.28$ kcal/mol followed by TKPRR and TKPPR ($-11.01$ and $-10.49$ kcal/mol, respectively). It is interesting to note that the longer the residue chain, the higher the binding energies as seen in the case of heptapeptides APQPRPL and CPQPRPL. In general, the peptides with arginine at the C-terminal showed higher activity than those with the other residues or with arginine in between the sequence. These findings demonstrated that the position of arginine and the length of the peptide sequence play an important role in their

Figure 3. The docked pose of all peptide ligands. The Tuftsin (TKPR) is rendered as CPK, while the other peptides are rendered as stick and coloured as green for (DKPRR), red (DKPPR), orange (DKPPR-NH2), black (TKPRR), cyan (TKPRR), yellow (TKPRR-NH2), blue (CDKPRR), pink (APQPRPL), grey (CPQPRPL) and purple (ATWLPPR).
Changing the carboxyl group with an amide did not have a consistent effect on the binding affinity towards NRP-1 as demonstrated by TKPRR-NH₂ and DKPPR-NH₂. In the case of TKPRR, this replacement significantly reduced the activity in TKPRR-NH₂ while in the case of DKPPR, the replacement slightly increased the binding affinity towards NRP-1.

Figure 4. The poses of docked peptides into the b1 domain of NRP-1. (a) ATWLPFR (b) TKPRR, (c) TKPRR (d) TKPRR-NH₂ (e) DKPRR, (f) DKPRR-NH₂ (g) CDKRPR (h) APQPRPL, (i) CPQPRPL and (j) DKPRR. NRP-1 is presented as ribbon and the binding site is represented as stick and colored as velvet for Y or Tyr, pink (G or Glu), green (T or Thr), light grey (S or Ser), purple (D or Asp), cyan (G or Gly) and W or Trp (light blue). Peptide ligands are rendered as ball and stick and colored based on their atom type: carbon (grey), oxygen (red), nitrogen (blue), hydrogen (white) and sulphur (yellow). Hydrogen bond and π-π interactions between the peptide ligands and the NRP-1 are shown as black and red lines, respectively.

Figure 4. (Continued).
Similar to tuftsin, it was observed that the docked peptides bound in close proximity to Tyr297 (loop I), Asp320 (loop II), Ser346, Glu348, Thr349 and Tyr353 (loop III). In general, the peptides stacked in between the two tyrosine residues, i.e. Tyr297 and Tyr353 where they formed hydrogen bonding with these residues. However, not all of the peptide formed hydrogen bond with Y297 (i.e. TKPRR-NH$_2$ and CPQPRPL) and Y353 (i.e. DKPPR-NH$_2$, CDKPRR, APQPRPL, CPQPRPL and ATWLPR). The arginine C-terminal of each peptide was also held by a number of $\pi-\pi$ interactions which mediated one, two or more interactions with NRP-1 through residues Y297, Y353 and W301 (see Figures 4 and 5). These interactions strengthen the binding of these peptides and increase their stability in the NRP-1 binding site. However, interestingly, DKPPR and TKPPR did not make any $\pi-\pi$ interaction with NRP-1. These peptides also formed a network of hydrogen bonding with D320, S346, E348 and T349. These findings are in agreement with other studies (Starzec et al., 2007; Vander Kooi et al., 2007).

The distances of arginine of each peptide with those important residues were also examined and summarized in the Table 4. It was observed that most peptides bind closer to these side chains; Y297 (loop I), D320 (loop II), S346, E348, T349 and Y353 (loop III) compared to ATWLPR, the peptide we previously demonstrated (Tirand et al., 2006) to inhibit NRP-1. This indicates that these peptides might have better interaction at the binding site of NRP-1 than ATWLPR. Therefore, from all these observations, the investigated peptides (TKPRR, TKPPR, DKPPR-NH$_2$, DKPRR, TKPPR-NH$_2$, CDKPRR, APQPRPL and CPQPRPL) were then synthesized in order to confirm their binding affinity from the molecular affinity study.

### Synthesis and characterization of the peptides

The peptides; DKPRR, DKPPR, DKPPR-NH$_2$, TKPRR, TKPPR, TKPRR-NH$_2$, CDKPRR, APQPRPL and CPQPRPL were synthesized through SPPS technique with a final purity obtained more than 95%, as assessed by HPLC. The identities of all peptides were confirmed by mass spectrometry and NMR (see supplementary material).
Table 3. The predicted binding free energy, $\Delta G$ and the inhibition constant, $K_i$ of tuftsin and the investigated peptides.

| Peptide       | Free energy of binding (kcal/mol) | Inhibition constant, $K_i$ (M) |
|---------------|-----------------------------------|--------------------------------|
| ATWLPPR       | $-5.26$                           | $1.38 \times 10^{-4}$          |
| Tuftsin (TKPR) | $-12.28$                          | $1.00 \times 10^{-9}$          |
| TKPR          | $-11.01$                          | $8.49 \times 10^{-9}$          |
| TKPRR         | $-10.49$                          | $2.04 \times 10^{-9}$          |
| TKPRR-NH$_2$  | $-6.94$                           | $3.85 \times 10^{-6}$          |
| DKPRR         | $-8.96$                           | $2.69 \times 10^{-7}$          |
| DKPRR-NH$_2$  | $-9.86$                           | $5.88 \times 10^{-8}$          |
| CDKPRR        | $-6.46$                           | $1.85 \times 10^{-5}$          |
| APQPRPL       | $-4.91$                           | $2.50 \times 10^{-4}$          |
| CPQPRPL       | $-4.55$                           | $4.60 \times 10^{-4}$          |
| DKPRR         | $-4.15$                           | $9.04 \times 10^{-8}$          |

**In vitro binding studies on NRP-1 (molecular affinity studies)**

The affinities of the different peptides against NRP-1 were evaluated by ELISA, a type of binding affinity test. It is well known that VEGF$_{165}$ binds specifically to VEGFR-1, VEGFR-2, NRP-1 and NRP-2, as well as nonspecifically to cell-surface and extracellular matrix proteoglycans (Von Wronska et al., 2006). Hence, if the peptides do have affinities to NRP-1, they will compete with VEGF$_{165}$ in order to bind to the said receptor.

The competitive binding experiments were carried out in the presence of heparin (2 μg/mL) because the binding of VEGF$_{165}$ into the receptors is heparin-dependent. This strategy was carried out due to the fact that heparin alone in the absence of VEGF$_{165}$ and reversely so, has not any significant binding capability (Von Wronska et al., 2006). Figure 6(a) shows the binding of biotinylated VEGF$_{165}$ to recombinant NRP-1 chimeric protein was displaced by five peptides i.e. DKPRR, DKPPR, TKPRR, TKPR and CDKPRR in a concentration-dependent manner (IC$_{50}$ = 11 μM for DKPRR and 1.0 μM for DKPPR, TKPRR, TKPR and CDKPRR, Figure 7). It is interesting to note that these peptides showed better affinity than the ATWLPPR from the previous studies (IC$_{50}$ = 19 μM (Thomas et al., 2010; Tirand et al., 2006)). Several studies have demonstrated that peptide could selectively block VEGF-NRP-1 interaction using fragments of exon 7 (sometimes combined with exon 8) from VEGF. The peptide that shared similar homology to tuftsin and TKPR could also significantly reduce VEGF signalling through VEGFR-2 (Jia et al., 2006; Von Wronska et al., 2006) which indicates the consistency of the role of NRP-1 as a VEGF co-receptor (Neufeld, Cohen, Gengrinovitch, & Poltorak, 1999; Soker, Fidder, Neufeld, & Klagsbrun, 1996; Soker, Gollamudi-Payne, Fidder, Charmahelli, & Klagsbrun, 1997; Soker et al., 1998, 2002; Von Wronska et al., 2006; Whitaker, Limberg, & Rosenbaum, 2001).

The replacement of carboxyl group by amide group at the C-terminal arginine of the peptides DKPRR and TKPRR showed lesser affinities for NRP-1 as compared to the acidic peptides. The APQPRPL and CPQPRPL (linear peptide) had no binding affinity towards NRP-1. Contrary to this finding, slightly different peptides that were synthesized by other group did show affinity towards NRP-1. Giordano and the team previously reported a sequence of PQPRPL (Giordano, Cardó-Vila, Lahdenranta, Pasqualini, & Arap, 2001) and CPQPRPLC in cyclic form CPQPRPLC (Giordano et al., 2001) which showed affinity towards NRP-1 receptor.

**Further discussion**

Protein–peptide interactions are involved in numerous cellular processes and therefore, it is not surprising that in the recent years, peptides have been attracting interests in drug discovery and development. Computational chemistry techniques have proven to successfully support the drug-discovery process; and computer simulations of peptide–protein binding, based on molecular docking and scoring, have been widely used in applications to computational structure prediction of ligand–protein complexes, virtual screening of large databases for active compounds and binding affinity prediction of inhibitor–protein complexes (Verkhivker et al., 2002). However, reliable prediction of protein–ligand docking structure remains a challenging problem. Peptide ligands present one of the more difficult cases because of their high flexibility, requiring extensive configurational sampling. Even for the rigid-protein model, it is demanding to handle flexible ligands with over 15–20 rotatable bonds, which is typical even for short peptides. This difficulty increases when the protein receptor is also flexible and a peptide ligand only binds to a small subset of the many possible conformations of the protein (Huang & Wong, 2009).

To date, there are a few web servers offer the computation of protein–peptide docking, such as FlexPrepDock (Raveh, London, & Schueler-Furman, 2010; Raveh, London, Zimmerman, & Schueler-Furman, 2011) which
Figure 5. The interactions between the peptide ligands with NRP-1 binding site as visualized using LigPlot (Wallace, Laskowski, & Thornton, 1995).
Figure 5. (Continued).
Figure 5. (Continued).
Figure 5. (Continued).
Figure 5. (Continued).
Table 4. The nearest distance (Å) of arginine C-terminal residue of each peptide to the six important residues (Y297, D320, S346, E348, T349 and Y353).

| Peptide*   | Y297 | D320 | S346 | E348 | T349 | Y353 |
|------------|------|------|------|------|------|------|
| ATWLPPR    | 5.05 | 2.47 | 14.46| 18.07| 14.22| 8.21 |
| Tuftsin    | 4.96 | 2.03 | 5.32 | 5.07 | 6.91 | 3.32 |
| TKPPR      | 8.22 | 5.94 | 10.10| 10.75| 7.17 | 6.38 |
| TKPRR      | 3.92 | 2.09 | 5.65 | 8.75 | 8.22 | 3.27 |
| TKPRR-NH₂  | 2.97 | 1.86 | 6.66 | 2.29 | 3.70 | 2.00 |
| DKPRR-NH₂  | 3.06 | 3.01 | 1.42 | 3.48 | 4.31 | 3.44 |
| DKPPR      | 3.28 | 5.77 | 15.14| 10.29| 15.22| 11.38|
| DKPPR      | 4.46 | 2.72 | 3.63 | 2.37 | 4.03 | 1.98 |
| CDKPRR     | 2.11 | 1.88 | 6.88 | 9.70 | 7.43 | 3.58 |
| APQPRPL    | 2.50 | 4.47 | 4.47 | 5.14 | 4.01 | 4.16 |
| CPQPRPL    | 2.72 | 2.98 | 6.76 | 7.75 | 6.73 | 2.60 |

*In cases where the C-terminal residue is not arginine, the arginine nearest to the C-end terminus will be selected instead.

Figure 6. Molecular affinity of the peptides to the recombinant NRP-1 (a) and KDR (b) recombinant chimeric protein. Binding of biotinylated VEGF (5 ng/mL) to NRP-1 and 20 ng/mL to KDR proteins in the presence of 2 μg/mL heparin was evaluated in the presence of VEGF as positive control (.5 μg/mL and 2 μg/mL of VEGF for NRP-1 and KDR, respectively) or in the presence of studied peptides (120 μM). Data points show the mean ± SD, n = 3. The horizontal grey line in (a) represents bound VEGF in presence of tuftsin (120 μM). Data points show the mean ± SD, n = 3.
up to seven amino acids (Arun Prasad & Gautham, 2008), peptide–antibody complexes (Chen, Simmonds, & Timkovich, 2013; Sotriffer et al., 2000), and even protein–protein interactions (Eyrisch & Helms, 2007). Based on these encouraging results, we are persuaded to apply AutoDock to predict the binding interaction of our protein–peptide complexes.

In our previous study, a peptide-conjugated photosynthesizer, TPC-Ahx-ATWLPPR targeting NRP-1 had demonstrated an affinity for endothelial cells of tumour neovascularature (Tirand et al., 2006). However, the peptide moiety (ATWLPPR) of the conjugate degraded rapidly and was relatively unstable. The peptide ATWLPPR identified from phage epitope library was first reported to bind at VEGFR-2 (KDR) (Binetruy-Tournaire et al., 2000) but has been demonstrated to bind specifically to NRP-1 (Perret et al., 2004) and then selectively inhibits VEGF165 binding to NRP-1 (Starzec et al., 2006). Besides ATWLPPR, PQPRPL (Giordano et al., 2001), CDKPRR (Jia et al., 2006) and TKPRR (Von Wronski et al., 2006) and two cyclic peptides CPQPRPLC (Giordano et al., 2005) and CPQPRPRL (Jia et al., 2006), have also been reported to inhibit NRP-1. The latter cyclic peptide was derived from exon 8 of VEGF (residues 138–165) (Jia et al., 2006). Thus, in this study, we evaluated the binding of the whole or modified sequence of these peptides onto NRP-1 and their affinity and molecular interactions were compared using molecular docking technique as well as in vitro molecular affinity study.

The docked peptides were shown to bind at the b1 domain of NRP-1 and they were found to form a network of interactions with residues of Y297 (loop I), D320 (loop II) and S346, E348 and T349 (loop III). They were also found stacking in between the two tyrosines, Y297 and Y353 which could strengthen the binding of ligand/receptor complex. These findings are similar to what observed previously in the crystal structure of NRP-1/tuftsins co-complex (Von Wronski et al., 2006) as well as in the molecular dynamic simulation of NRP-1/ATWLPPR system (Starzec et al., 2007).

In general, peptides with C-terminal arginine showed a more negative predicted binding energy than those without, which agreed well with the in vitro molecular affinity results (Figure 5). The APQPRPL and CPQPRPL, both which have a C-terminal leucine residue on the other hand failed to displace VEGF165 binding on NRP-1. The distance analysis between the C-terminal residues with the important residues of NRP-1 binding site also correlates with this finding. In particular, C-terminal arginine showed the strongest interaction with Asp320, which is consistent with a molecular dynamic study of NRP-1 with a model peptide, RPAR (Haspel et al., 2011). This finding also corresponded well with the previous alanine-scanning and amino acid-deletion analysis which showed that the C-terminal arginine played an important role in the inhibitory activity of ATWLPPR on VEGF165 binding to NRP-1 (Starzec et al., 2007). These findings are also consistent with the previous observation that the binding site for tuftsin (TKPR) in NRP-1 seems to optimally accommodate a C-terminal arginine (Vander Kooi et al., 2007; Von Wronski et al., 2006).

The results from molecular docking showed that changing the carboxyl group of the C-terminal arginine with a terminal amide group did not have consistent and significant effects on the binding capability of the peptide. From the in vitro binding, however, it was evidence that the peptides with a terminal amide group have much lower affinity to NRP-1 than the corresponding peptides with the carboxyl group. This result is in agreement with the previous studies which showed that the carboxyl group of the terminal arginine must be in its free acid form since inhibitory action drops dramatically for the peptide with a terminal amide group (Jia et al., 2006).

All the peptides used in this study had no affinity towards VEGFR-2 (KDR) which was evidence from their failure to displace the binding of biotinylated VEGF165 on the KDR protein. DKPRR, DKPPR, TKPRR, TKPPR and CDKPRR showed good inhibitory activity on NRP-1. However, among these peptides, only DKPPR and TKPRR were chosen for further study (Kamarulzaman et al., 2015). These two peptides not only showed favourable binding interaction with NRP-1 as demonstrated by molecular docking but also from the in vitro binding affinity test in which both of them showing the highest displacement of VEGF165 binding towards the recombinant NRP-1. DKPPR and TKPPR will hence be conjugated.

Figure 7. Binding affinity of the peptides to the recombinant NRP-1 recombinant chimeric protein as a function of concentration.
with a photosensitizer, and evaluated for their biological activity in vivo. This will be further discussed in our subsequent publication (Kamarulzaman et al., 2015).

Conclusion
The combination of computational docking study and biological evaluation in this study provide an insight into the design of peptide that might interact with NRP-1. Peptides having C-terminal arginine at position 4 and/or 5 i.e. DKPRR, DKPPR, TKPRR, TKPPR and CDKPRR made interaction with NRP-1 through hydrogen bond and π–π interaction. These peptides also demonstrated a good inhibition against NRP-1. On the contrary, peptides having seven amino acids and with arginine located at other than the C-terminal i.e. CPQPRLPL and APQPRPL failed to displace the VEGF₁₆₅ binding to NRP-1. A minor replacement of amide group instead of carboxyl group at C-terminal domain i.e. DKPPR-NH₂ and TKPRR-NH₂ showed lower activity against NPR-1. Among the evaluated peptides, two are novel i.e. DKPRR and TKPRR and were then chosen to be conjugated photosensitizer molecules (Kamarulzaman et al., 2015). It is hoped that the information from this finding will provide deeper understanding towards the design of more active peptides as anti-angiogenic targeting agent.

List of Abbreviations

- BSA: Bovine serum albumin
- DCM: Dichloromethane
- DIEA: N,N-isopropylethylamine
- DMF: Dimethylformamide
- ELISA: Enzyme-linked immunosorbent assay
- HOBt: Hydroxybenzotriazole
- HPLC: High performance liquid chromatography
- KDR: Kinase domain receptor
- NMR: Nuclear magnetic resonance
- NRP: Neuropilin
- PS: Photosensitizer
- PBS: Phosphate-buffered saline
- RMSD: Root-mean-square deviation
- SPPS: Solid phase peptide synthesizer
- TPC: [5-(4-carboxyphenyl)-10,15,20-triphenylchlorin]
- VEGF: Vascular endothelial growth factor
- VEGFR: Vascular endothelial growth factor receptor

Supplementary material
The supplementary material for this paper is available online at http://dx.doi.org/10.1080/07391102.2015.1131196.

Disclosure statement
No potential conflict of interest was reported by the authors.

References

- Albini, A., Soldi, R., Giuninucciolo, D., Girauo, E., Benelli, R., & Primo, L. (1996). The angiogenesis induced by HIV-1 Tat protein is mediated by the Flik-1/KDR receptor on vascular endothelial cells. Nature Medicine, 2, 1371–1375. doi:10.1038/nm1296-1371
- Antes, I. (2010). DynaDock: A new molecular dynamics-based algorithm for protein–peptide docking including receptor flexibility. Proteins: Structure, Function, and Bioinformatics, 78, 1084–1104. doi:10.1002/prot.22629
- Arun Prasad, P., & Gautham, N. (2008). A new peptide docking strategy using a mean field technique with mutually orthogonal Latin square sampling. Journal of Computer-Aided Molecular Design, 22, 815–829. doi:10.1007/s10822-008-9216-5
- Bashi, T., Blank, M., Ben-Ami Shor, D. B. A., Fridkin, M., Versini, M., & Gendelman, O. (2015). Successful modulation of murine lupus nephritis with tuftsin-phosphorylcholine. Journal of Autoimmunity, 59, 1–7. doi:10.1016/j.jaut.2015.03.001
- Benouchan, M., & Colombo, B. M. (2005). Anti-angiogenic strategies for cancer therapy (review). International Journal of Oncology, 27, 563–571. doi:10.3892/ijo.3827.3892.3563
- Bernatchez, P. N., Rollin, S., Soker, S., & Sirois, M. G. (2002). Relative effects of VEGF-A and VEGF-C on endothelial cell proliferation, migration, and PAF synthesis: Role of neuropilin-1. Journal of Cellular Biochemistry, 85, 629–639. doi:10.1002/jcb.10155
- Binetruy-Tournaire, R., Demangel, C., Malavaud, B., Vassy, R., Rouyre, S., & Kraemer, M. (2000). Identification of a peptide blocking vascular endothelial growth factor (VEGF)-mediated angiogenesis. The EMBO Journal, 19, 1525–1533. doi:10.1093/emboj/19.7.1525
- Chen, Y., Simmonds, R. S., & Timkovich, R. (2013). Proposed docking interface between peptidoglycan and the target recognition domain of zoscin A. Biophysical Journal, 105, 107–113. doi:10.1016/0006-3495(85)90497-X
- Cosconati, S., Forli, S., Perryman, A. L., Harris, R., Goodsell, D. S., & Olson, A. J. (2010). Virtual screening with AutoDock: Theory and practice. Expert Opinion on Drug Discovery, 5, 597–607. doi:10.1517/17460441.2010.484460
- D’Andrea, L. D., Del Gatto, A., Pedone, C., & Benedetti, E. (2006). Peptide-based molecules in angiogenesis. Chemical Biology & Drug Design, 67, 115–126. doi:10.1111/j.1747-0285.2006.00356.x
- Denekamp, J. (1984). Vascular endothelium as the vulnerable element in tumours. Acta Radiologica Oncology, 23, 217–225. doi:10.3109/02841868409136015
- Dvorak, H. F., Nagy, J. A., Feng, D., Brown, L. F., Dvorak, A. M., & Yoshiji, H. (1999). Vascular permeability factor/vascular endothelial growth factor and the significance of microvascular hyperpermeability in angiogenesis. Current Topics in Microbiology and Immunology, 237, 97–132. doi:10.1007/10669_2001_0001
- Ewing, T. J., Makino, S., Skillman, A. G., & Kuntz, I. D. (2001). DOCK 4.0: Search strategies for automated molecular docking of flexible molecule databases. Journal of Computer-Aided Molecular Design, 15, 411–428. doi:10.1023/A:101115820450
Molecular modelling, synthesis and biological evaluation of peptide inhibitors

Eyrisch, S., & Helms, V. (2007). Transient pockets on protein surfaces involved in protein–protein interaction. Journal of Medicinal Chemistry, 50, 3457–3465. doi:10.1021/jm070095g

Ferrara, N., & Davis-Smyth, T. (1997). The biology of vascular endothelial growth factor. Endocrine Reviews, 18, 4–25. doi:10.1210/edrv.18.1.0287

Folkman, J. (1971). Tumor angiogenesis: Therapeutic implications. New England Journal of Medicine, 285, 1182–1186. doi:10.1056/NEJM197111182582108

Folkman, J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. Nature Medicine, 1, 27–30. doi:10.1038/nm0195-7

Gao, Y., Su, Q. D., Yi, Y., Jia, Z. Y., Wang, H., & Lu, X. X. ... (2015). Enhanced mucosal immune responses induced by a combined candidate mucosal vaccine based on Hepatitis A virus and Hepatitis E virus structural proteins linked to tuftsin. PLoS One, 10, e0123400. doi:10.1371/journal.pone.0123400

Gasteiger, J., & Marsili, M. (1980). Iterative partial equalization of orbital electronegativity – A rapid access to atomic charges. Tetrahedron, 36, 3219–3228. doi:10.1016/0040-4020(80)80168-2

Giordano, R. J., Anobom, C. D., Cardó-Vila, M., Kalil, J., Valente, A. P., & Pasqualini, R. (2005). Structural basis for the interaction of a vascular endothelial growth factor mimic peptide motif and its corresponding receptors. Chemistry and Biology, 12, 1075–1083. doi:10.1016/j.chembiol.2005.07.008

Giordano, R. J., Cardó-Vila, M., Lahdenranta, J., Pasqualini, R., & Arap, W. (2001). Biopanning and rapid analysis of selective interactive ligands. Nature Medicine, 7, 1249–1253. doi:10.1038/nm1011-1249

Gu, C., Limberg, B. J., Brian Whitaker, G., Perman, B., Leahy, D. J., & Rosenbaum, J. S. (2002). Characterization of neuropilin-1 structural features that confer binding to semaphorin 3A and vascular endothelial growth factor 165. Journal of Biological Chemistry, 277, 18069–18076. doi:10.1074/jbc.M201681200

Hanahan, D., & Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell, 86, 353–364. doi:10.1016/S0092-8674(00)81018-7

Haspel, N., Zanuy, D., Nussinov, R., Teesalu, T., Rusoliatli, E., & Aleman, C. (2011). Binding of a C-end rule peptide to the neuropilin-1 receptor: A molecular modeling approach. Biochemistry, 50, 1755–1762. doi:10.1021/bi101662j

Hetenyi, C., & van der Spoel, D. (2002). Efficient docking of peptides to proteins without prior knowledge of the binding site. Protein Science, 11, 1729–1737. doi:10.1101/ps.020302

Hoeben, A., Landuyt, B., Highley, M. S., Wilhiers, H., Van Oosterom, A. T., & De Bruijn, E. A. (2004). Vascular endothelial growth factor and angiogenesis. Pharmacological Reviews, 56, 549–580. doi:10.1124/pr.56.4.3

Horváti, K., Bacsa, B., Kiss, Éva, Gyulai, G., Fodor, K., & Balka, G. (2014). Nanoparticle encapsulated lipopeptide conjugate of antitubercular drug isoniazid. In vitro intracellular activity and in vivo efficacy in a guinea pig model of tuberculosis. Bioconjugate Chemistry, 25, 2200–2208. doi:10.1021/bc500476x

Huang, Z., & Wong, C. F. (2009). Docking flexible peptide to flexible protein by molecular dynamics using two implicit-solvent models: An evaluation in protein kinase and phosphatase systems. The Journal of Physical Chemistry B, 113, 14343–14354. doi:10.1021/jp907375b

Huey, R., Morris, G. M., Olson, A. J., & Goodsell, D. S. (2007). A semiparametic free energy force field with charge-based desolvation. Journal of Computational Chemistry, 28, 1145–1152. doi:10.1002/jcc.20690

Jain, S., Tran, T. H., & Amiji, M. (2015). Macrophage repolarization with targeted alginate nanoparticles containing IL-10 plasmid DNA for the treatment of experimental arthritis. Biomaterials, 61, 162–177. doi:10.1016/j.biomaterials.2015.05.028

Jia, H., Bagherzadeh, A., Hartzoulakis, B., Jarvis, A., Lohr, M., & Shaikh, S. (2006). Characterization of a bicyclic peptide neuropilin-1 (NP-1) antagonist (EG3287) reveals importance of vascular endothelial growth factor exon 8 for NP-1 binding and role of NP-1 in KDR signaling. Journal of Biological Chemistry, 281, 13493–13502. doi:10.1074/jbc.M512121200

Jones, G., Willett, P., & Glen, R. C. (1995). Molecular recognition of receptor sites using a genetic algorithm with a description of desolvation. Journal of Molecular Biology, 245, 43–53. doi:10.1006/jmbi.1996.0073

Kamarulzaman, E. E., Amirah Mohd Gazzali, A. M., Acherar, S., Frochot, C., Barberi Heyob, M., & Boura, C. (2015). New peptide-conjugated chlorin-type photosensitizer targeting neuropilin-1 for anti-vascular targeted photodynamic therapy. International Journal of Molecular Sciences, 16, 24059–24080. doi:10.3390/ijms161024059

Klagsbrun, M., & Soker, S. (1993). VEGF/VPF: The angiogenesis factor found?. Current Biology, 3, 699–702. doi:10.1016/0960-9225(93)90077-W

Knusel, J. S., Behr, B., Milki, A. A., Hirchenhain, J., Sen, Y., & Bielfeld, P. (2001). Vascular endothelial growth factor (VEGF) mRNA splice variants are differentially expressed in human blastocysts. Molecular Human Reproduction, 7, 57–63. doi:10.1093/molehr/7.1.57

Kurcinski, M., Jamroz, M., Blaszczyz, M., Koliński, A., & Kmiecik, S. (2015). CABS-dock web server for the flexible docking of peptides to proteins without prior knowledge of the binding site. Nucleic Acids Research, 43, W419–W424. doi:10.1093/nar/gkv456

Lange, T., Guttmann-Raviv, N., Baruch, L., Machluф, M., & Neufeld, G. (2003). VEGF162, A new heparin-binding vascular endothelial growth factor splice form that is expressed in transformed human cells. Journal of Biological Chemistry, 278, 17164–17169. doi:10.1074/jbc.M212224200

London, N., Raveh, B., Cohen, E., Fathi, G., & Schueler-Furman, O. (2011). Rosetta FlexPepDock web server – High resolution modeling of peptide–protein interactions. Nucleic Acids Research, 39(suppl), W249–W253. doi:10.1093/nar/gkr431

Matthies, A. M., Low, Q. E. H., Lingen, M. W., & DiPietro, L. A. (2002). Neuropilin-1 participates in wound angiogenesis. The American Journal of Pathology, 160, 289–296. doi:10.1016/S0002-8440(10)64372-6

Morris, G. M., Goodsell, D. S., Halliday, R. S., Huey, R., Hart, W. E., & Belew, R. K. (1998a). Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. Journal of Computational Chemistry, 19, 1639–1662. doi:10.1002/(ISSN)1096-987X

Morris, G. M., Goodsell, D. S., Halliday, R. S., Huey, R., Hart, W. E., & Belew, R. K. (1998b). Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. Journal of Computational Chemistry, 19, 1639–1662. doi:10.1002/(ISSN)1096-987X
Whittle, C., Gillespie, K., Harrison, R., Mathieson, P. W., & Harper, S. J. (1999). Heterogeneous vascular endothelial growth factor (VEGF) isoform mRNA and receptor mRNA expression in human glomeruli, and the identification of VEGF<sub>148</sub> mRNA, a novel truncated splice variant. Clinical Science, 97, 303–312. doi:10.1042/cs0970303

Woo, H. J., & Roux, B. (2005). Calculation of absolute protein–ligand binding free energy from computer simulations. Proceedings of the National Academy of Sciences, 102, 6825–6830. doi:10.1073/pnas.0409005102

Woolard, J., Wang, W. Y., Bevan, H. S., Qiu, Y., Morbidelli, L., & Pritchard-Jones, R. O. (2004). VEGF<sub>165b</sub>, an inhibitory vascular endothelial growth factor splice variant: Mechanism of action, in vivo effect on angiogenesis and endogenous protein expression. Cancer Research, 64, 7822–7835. doi:10.1158/0008-5472.CAN-04-0934