A novel bioengineered fragment peptide of Vasostatin-1 exerts smooth muscle pharmacological activities and anti-angiogenic effects via blocking VEGFR signalling pathway

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ABSTRACT

Chromogranin A (CgA) is a hydrophilic glycoprotein released by post-ganglionic sympathetic neurons. CgA consists of a single peptide chain containing numerous paired basic residues, which are typical cleavage sites in prohormones to generate bioactive peptides. It is recognized as a diagnostic and prognostic serum marker for neuroendocrine tumours. Vasostatin-1 is one of the most conserved regions of CgA and has diverse inhibitory biological activities. In this study, a novel peptide fragment that contains three typical functional structures of Vasostatin-1 was synthesized. This unique bioengineered Vasostatin-1 Derived Peptide (named V1DP) includes a highly conserved domain between vertebrate species in its N-terminal region, comprising a disulphide bridge formed by two cysteine residues at amino acid positions 17 and 38, respectively. Besides, V1DP contains two significant tripeptide recognition sequences: the amino acid triplets, RGD and KGD. Our data demonstrated that V1DP could induce a dose-dependent relaxation of rat arterial smooth muscle and also increase the contraction activity of rat uterus smooth muscle. More importantly, we found that V1DP inhibits cancer cell proliferation, modulate the HUVEC cell migration, and exhibit anti-angiogenesis effect both in vitro and in vivo. We further investigated the actual mechanism of V1DP, and our results confirmed that V1DP involves inhibiting the vascular endothelial growth factor receptor (VEGFR) signalling. We docked V1DP to the apo structures of VEGFR2 and examined the stability of the peptide in the protein pockets. Our simulation and free energy calculations results indicated that V1DP can bind to the catalytic domain and regulatory domain pockets, depending on whether the conformational state of the protein is JM-in or JM-out. Taken together, our data suggested that V1DP plays a role as the regulator of endothelial cell function and smooth muscle pharmacological homeostasis. V1DP is a water-soluble and biologically stable peptide and could further develop as an anti-angiogenic drug for cancer treatment.

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1. Introduction

Chromogranin A (CgA) is a hydrophilic glycoprotein released by post-ganglionic sympathetic neurones and was first discovered in catecholamine-containing adrenal chromaffin cells [1]. It is co-stored and co-released in the secretory granules of endocrine, neuroendocrine, and immune cells [2–4]. CgA consists of a single
peptide chain containing numerous paired basic residues, which are typical cleavage sites in prohormones to generate bioactive peptides [5]. CgA-related polypeptides are detected in the blood of patients with neuroendocrine tumors, renal failure, heart failure, rheumatoid arthritis, hypertension and inflammatory bowel disease [6]. In human Full-length CgA and CgA-derived peptides are variously expressed with pathological conditions and exert distinctive paracrine, autocrine and endocrine functions [7]. For example, in human CgA derived peptide, Vasostatin-1 (CgA1–76), Pancrestatin (CgA250–301), Catestatin (CgA352–372) are the well-recognized CgA authentic products [3,5,6]. Catestatin is diminished in genetic hypertension, whereas the full length of CgA is overexpressed [6]. And in rodents, Catestatin could sensitize the insulin function and decrease hypertension, obesity, cardiac contractility, atherosclerosis and inflammation, while the Pancrestatin exerts an opposite function of Catestatin [6,8,9]. Interestingly, the N-terminal fragment Vasostatin-1 is a water-soluble and biologically stable peptide and has revealed the potential of antiangiogenic and modulate fibroblast and endothelial cell adhesion. While CgA fragments lacking the C-terminal region showed proangiogenic activity and induce tumor growth [6,7,9]. The capability of CgA in regulating cell adhesion may be crucial in the metastasis of neuroendocrine tumours as its abnormal circulating level in many neuroendocrine tumour tissues [10]. Overall, the CgA system, consisting of full-length CgA and its fragments, is emerging as an important and complex player in cardiovascular, immunometabolic, and cancer regulation.

The determination of these peptides/proteins primary structures, revealing their high degree of structural conservation across species, suggests that they may perform an important and fundamental biological role. Vasostatin-1 is one of the most conserved regions of CgA and has diverse inhibitory biological activities. In the amino acid sequence of Vasostatin-1, the presence of two significant tripeptide recognition sequences has been demonstrated: the amino acid triplets, Arg-Gly-Asp (RGD), residues (43–45) and Lys-Gly-Asp (KGD), residues (9–11). These motifs are known to play a major inhibitory role in cell adhesion and possibly angiogenesis. Some research related to the RGD motif peptides has demonstrated some typical inhibitory characteristics on platelet aggregation, angiogenesis and tumour growth [1,11]. The tripeptide RGD is also found in the sequences of a number of other proteins, where it has been shown to play a role in cell adhesion [12].

In this study, a novel peptide fragment that contains three typical functional structures of Vasostatin-1 was synthesized. This fragment is a selected fragment of Vasostatin-1, and also as sequences as the N-terminal 8–47 fragment of CgA. This unique bioengineered Vasostatin-1 Derived Peptide (named V1DP) includes the three typical functional structures within Vasostatin-1. It has been shown that V1DP contains a highly conserved domain between vertebrate species in its N-terminal region, comprising a disulphide bridge formed by two cysteine residues at amino acid positions 17 and 38. In addition, the sequence of V1DP contains two significant tripeptide recognition sequences: the amino acid triplets, RGD and KGD. This novel Vasostatin-1 derived has been characterized through various functional screening such as the MTT cell viability assay, haemolysis assay and smooth muscle bioassay. It was observed that V1DP induced a dose-dependent relaxation of rat arterial smooth muscle and also induced an increase in the contraction activity of rat uterus smooth muscle preparations. We also found its potential role in inhibiting cancer cell proliferation, modulating the HUVEC cell migration, exhibiting anti-angiogenesis effect both in vitro and in vivo. We further investigated the potential mechanism of the anti-angiogenesis effect of V1DP through western blot. The results indicated the anti-angiogenesis effect of V1DP might involve the inhibition of VEGFR signaling. Such data would be important clues to explain the mechanism of the biological function of Vasostatin-1 through these structure/activity studies.

2. Method

2.1. Peptide synthesis and purification

Peptides were synthesized using solid-phase Fmoc chemistry by means of a PS4 automated solid-phase peptide synthesizer (Protein Technologies, Inc., AZ, USA). The purification of the synthetic peptides was analyzed by both reverse-phase HPLC and MALDI-TOF mass spectrometry. The HPLC column C18 (Phenomenex C-18, 25 cm × 0.45 cm) was washed by Buffer B (0.05%/29.95/70.00 (TFA/water/Acetonitrile) (v/v/v)) for approximately 30–60 min and then it was equilibrated in Buffer A ( trifluoroacetic acid (TFA)/water (0.05%/99.95, v/v) for 1 h. Synthetic peptides were dissolved in Buffer A at a concentration of 1 mg/ml then injected onto the HPLC system and peptides were eluted using a gradient formed from 0.05%/99.95 (v/v) TFA/water to 0.05%/19.95/80.0 (v/v/v) TFA/water/acetonitrile in 80 min at a flow rate of 1 mL/min. For MALDI-TOF mass spectrometry, matrix solution (α-cyano-4-hydroxycinnamic acid (CHCA)) was prepared as a 10 mg/ml solution of CHCA in acetonitrile/TFA/Water (50/0.05/49.95, v/v/v).

The primary structures of the major products (>90%) in each synthesis mixture, were finally confirmed by LCQ-Fleet™ electro-spray ion-trap mass spectrometer. In the ESI source, nitrogen sheath and auxiliary gas flows were maintained at 30 and 10 arbitrary units as set by the software, respectively. The resulting fragment ion profile was then searched using the SEQUEST algorithm in the Thermo Scientific Proteome Discoverer 1.0 software Sequest algorithm (ThermoFinnigan, San Jose, California, USA) against a customized FASTA database of related sequences.

2.2. Rat smooth muscle bioassay

Female Wistar rats (200–300 g) were applied and sacrificed followed the principle of institutional animal experimentation ethics and UK animal research guidelines. All of the procedures involved in this study was approved by IACUC of Queen’s University Belfast. The tail artery and uterus were generated as described previously [13,14]. The peptide was diluted in Kreb’s solution to a range of concentrations from 10−11 to 10−8 M for the construction of dose–response curves. These peptides were added to the smooth muscle preparations in increasing concentrations followed by 5 min washing and 5 min equilibration periods between each dose. Changes in tension of the smooth muscle preparations were recorded and amplified through force transducers connected to a PowerLab System (AD Instruments Pty Ltd.).

2.3. Haemolysis assays

Forty percent of (packed cell volume) fresh horse blood (TCS Biosciences Ltd. Buc kinham, UK) was used [15]. 4% of horse erythrocytes were prepared and incubated with a range of concentration of peptide for 120 min. The incubation of erythrocytes with 1% (v/v) Triton X-100 and with PBS were used as positive and negative control, respectively. The supernatants were measured by Optical density (OD) at 550 nm using a Synergy HT plate reader.

2.4. Cell culture

HCT116, DLD-1 and CCD-18CO were purchased from American Type Culture Collection (ATCC). DKS8 and HKE3 were gifts from Prof. Senji Shirasawa’s Laboratory [16]. Human umbilical vein
endothelial cells (HUVEC) were purchased from Thermo Fisher Scientific. HCT116, DLD-1, HKE3, DKS8 and CCD-18CO were maintained in DMEM/F-12 (Gibco; Cat. no. 1320033) with 10% foetal bovine serum and 1% penicillin/streptomycin, HUVEC cell line was grown in Medium 200 supplemented with low serum growth supplement (LSGS) (Thermo Fisher Scientific, Waltham, MA). All cell lines were incubated at 37 °C, 5% CO2.

2.5. MTT cell viability assays

The synthetic peptides were analyzed by the MTT-based in vitro toxicity test [14,15]. The cells were seeded to the required numbers (5 x 10^3 cells per well) in a 96-well plate before further testing. After 24 h incubation, the medium in each well was removed with serum-free medium for 24 h. Afterwards, 10 μl with different peptide concentrations range from 10^{-5} to 10^{-12} M with serum-free medium for 24 h. Afterwards, 10 μl (5 mM) MTT solution (Sigma, UK) were added to each well, and the plate was incubated for 4 h at 37 °C, 5% CO2. The medium of each well gently removed, and 100 μl of DMSO was later added to each well and mixed thoroughly until all the formazan crystals were dissolved. The plates were then placed in an ELx808 Absorbance Microplate Reader (BioTek, USA) and the absorbance measured at λ = 550 nm.

2.6. Wound healing cell migration assay

HUVEC were seeded in a 96-well Microplate (Image Lock plates, Essen Bioscience) at the density of 1 x 10^4 cells/well and then incubated overnight, then changed the medium with and without peptide added for 6 h before wounding by WoundMaker™ (Essen BioScience). The cell plate was placed into the IncuCyte ZOOM (Essen BioScience) for 24 h and scanned every 2 h. The relative wound density was calculated by IncuCyte ZOOM Software [16].

2.7. Endothelial cell tube formation assay

HUVEC cells were treated with and without peptide added medium for 6 h. Then the cell lines were seeded into a 15-well m-Slide angiogenesis chamber (ibidi, Planegg, Germany) coated with 10 μl Matrigel (BD Biosciences) and calculated with 1.5 x 10^4/well. After incubation for 18 h, morphological changes of HUVECs and tube formation were visualized and photographed using the EVOS Cell Imaging System.

2.8. Western blots assay

The detailed protocol referred to a previous publication [16]. The cell line samples were lysed with ice-cold RIPA lysis buffer with protease inhibitor (Complete EDTA-free, #10634200, Roche) and phosphatase inhibitor (PhosSTOP, #04906837001, Roche) and centrifuged at 20,000 x g for 30 min at 4 °C. Supernatants were collected and quantified using Pierce BCA Protein Assay Kit (#23225). All samples were dissolved in LDS sample buffer and reducing agent (Life Technologies) and heated for 5 min at 95 °C. An equal concentration of proteins was electrophorized and separated with TGX Stain-FreeTM FastCast™ Acrylamide Kit, 12% and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad; Hercules, CA). Blocking was carried out with 5% non-fat milk, 0.1% Tween for 1 h in room temperature. Then the blots were incubated overnight with primary antibody at 4 °C. After washing with PBST for 10 mins three times, the membranes were incubated with secondary antibody for 1-hour prior. Finally, the bands were visualized by ChemiDoc MP Imaging System (Bio-Rad), with adding the chemiluminescent HRP substrate reagent (1:1) (Immobilon Western, WBKLS0500, Millipore). Primary antibodies used were phospho-ERK1/2 (Thr202/Tyr204) (1:1000, #9101, CST), total ERK1/2 (1:1000, #9102, CST), Phospho-VEGF Receptor 2 (Tyr1175) (1:1000, #2478S, CST), VEGF Receptor 2 (1:1000, #2479, CST), GAPDH (1:1000, #2118, CST).

2.9. Zebrafish angiogenesis assay

The Tg(fli1a:EGFP) transgenic zebrafish were used in this study, and all the procedures were approved by the Animal Research Ethics Committee of the University of Macau. The detailed protocol was described in our previous study [17]. The fish were maintained in the ZebTEC multilinking rack system (Tecniplast: Bugguggiate, Italy) at 28 °C on 14 h light:10 h dark photoperiod. Zebrafish embryos at 2 hpf (hours after fertilization) were placed in a dish with 30 mL system water containing 0.1% 1-phenyl 2-thiourea (PTU) to suppress pigment cell development. At 24 hpf, the eggshells (chorion) were gently removed with forceps, and the embryos were transferred to a 24-well plate at a density of 15 embryos/1 mL system water per well. Peptides were prepared with different concentrations and injected into the embryos at 9.2 nl/embryo, and the intersegmental vessels (ISVs) and subintestinal vessels (SIVs) of the embryos were observed at 48 (24-h treatment) and 72 hpf respectively.

2.10. Molecular model construction

The three-dimensional structure of V1DP was predicted using the PEPFOLD3 server [18]. The best model was selected based on the sOPEP energy and was submitted to MD to obtain an equilibrated structure. For the protein receptor, we chose two different ligand-bound crystal structures of the human VEGFR2 kinase domain to investigate how the peptide binds. The two structures are different in the way that the juxtamembrane (JM) domain is oriented. For PDB 4AGD, the JM domain is inserted into the regulatory domain pocket (RDP) and packed close to the DFG domain (the JM-in state). On the contrary, for PDB 4ASD, the JM domain is rearranged out of the RDP (the JM-out state), thereby increasing the RDP cavity volume. We followed these steps to prepare the protein models for peptide docking: In each case, the co-crystalized ligand was first removed, and the missing N-terminal residues were modeled using the Swiss-Model homology modeling server [19]. As the kinase insert domain (57 residues) is missing in the crystal structure, we followed a previous study [20] to construct five alanine to join residues Tyr938 to Tyr996. Finally, the remodelled protein was subjected to 200 ns MD simulation and then clustering analysis to select receptor conformations for peptide docking.

2.11. Molecular docking of V1DP with VEGFR2

BIOVIA Discovery Studio 2017 R2 (Biovia, San Diego, CA, USA) was used to carry out the molecular docking simulation. ZDOCK of Discovery Studio with ZRANK (ranks docked poses) was used by the following parameters: Angular step size as 15; RMSD cutoff as 6.0; interface cutoff as 9.0; the maximum number of clusters as 60, then RDOCK procedure was used to refine the docked poses by using the Chemistry at Harvard Macromolecular Mechanics (CHARMM) force field [14,21]. In addition to Discovery Studio, other docking methods, HPEPDOCK, AutoDock Vina, and ZDOCK were tried, but the peptide was docked with either unfolded conformation or docked with too low-affinity binding poses.

2.12. Molecular dynamics simulation of protein-peptide complex

Selected protein-peptide complexes from the results of molecular docking were subjected to MD simulations. All MD simulations
were performed using GROMACS 2019 and the CHARMM36m force field. The complex was placed in the centre of the TIP3P water box with a padding distance of 1.2 nm to the box boundary and was neutralized with counter ions Na\(^+\)Cl\(^-\). The system was energy minimized using the steepest descent algorithm and subsequently equilibrated under an NVT ensemble for 2 ns. The final trajectory was run under an NPT ensemble with a temperature of 300 K and pressure of 1 bar. We followed the simulation conditions as provided in the CHARMM-GUI [22] solution builder output. For analysis, atomic coordinates and energies were recorded every 10 ps. Binding free energy calculations were carried out using the program gmx_MMPBSA with the following parameters: igb = 5, saltcon = 0, entropy = 2, entropy_seg = 25, entropy_temp = 300. Forty frames were extracted from the last 40 ns of simulation for the calculation. The values of energy differences from the Complex-Receptor-Ligand section in the final output file were collected and analyzed.

2.13. Statistical analysis

Dose-response curve and bar graphs were plotted with Prism 7 (GraphPad Software, La Jolla, CA). All experiments were performed in triplicate. Data are expressed as means ± standard deviation (SD). Statistical differences of the data between control and treatment groups using one-way ANOVA, and the P values <0.05 were considered statistically significant.

3. Results

3.1. Interspecies comparison and structural characterization of V1DP.

V1DP (NKGDTEVMKCIVEVISDTLSKPSPMPVSQECFETLRGDER) has shown high interspecies conservation compared to humans rat, mouse, bovine and pig (Fig. 1A). The N-terminal tripeptide KGD sequence and the Cys17–Cys38 disulfide bridge is highly-conserved in human, rat, mouse, bovine and pig. The C-terminal RGD sequence is replaced by QGD in the rat and mouse, which has raised some arguments against its functional role. The sequence of V1DP has been chemically synthesized with a modification of disulfide bridge at Cys17–Cys38, and mass spectra validated the purity with an observed MW4467.60 (Fig. 1B), and the sequence was validated through MS/MS (Fig. 1C).

3.2. Smooth muscle pharmacology assay and haemolysis assays

Specifically, V1DP induced a dose-dependent relaxation of rat arterial smooth muscle (EC\(_{50}\) 2.34 nM, Fig. 2A) and exhibited contraction of rat uterus smooth muscle preparations (EC\(_{50}\) 0.31 μM, Fig. 2B). The results of the haemolysis assays indicated all of the peptides tested had no observable haemolytic activity in the concentration range investigated (Fig. 2C).

3.3. Cell viability and proliferation assay

The cell proliferation assay was carried out with MITT cell viability assay. HCT116, HKE-3, DLD-1, DKS-8, CCD-18CO and HUVEC were treated with V1DP peptide with different concentration. The data were analyzed to establish comparative IC\(_{50}\) values, and dose–response curves were then constructed using the best-fit algorithm (non-linear regression). Specifically, the comparative IC\(_{50}\) values of V1DP on the human cancer cell lines and normal fibroblast cells were: HCT116 (IC\(_{50}\) 9.6 nM), HKE-3 (IC\(_{50}\) 26 nM), DLD-1 (IC\(_{50}\) 62 nM), DKS-8 (IC\(_{50}\) 35 nM), CCD-18CO (IC\(_{50}\) 2.596 μM), and HUVEC (IC\(_{50}\) 0.154 μM). The data indicated that V1DP has different anti-proliferative efficacy and selective cytotoxicity against cancer cell growth and normal cells with the IC\(_{50}\) magnitude from nanomolar to micromolar (Fig. 3).

| #  | m/z | Charge | Intensity | Mass Error (ppm) | Mass Error (ppm) |
|----|-----|--------|-----------|------------------|------------------|
| 1  | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 2  | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 3  | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 4  | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 5  | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 6  | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 7  | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 8  | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 9  | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 10 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 11 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 12 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 13 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 14 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 15 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 16 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 17 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 18 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 19 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 20 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 21 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 22 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 23 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 24 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 25 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 26 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 27 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 28 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 29 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 30 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 31 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |
| 32 | 810.4777 | 1+     | 50.0528   | 511.3448         | 511.3448         |

![Fig. 1](image-url) (A) Interspecies comparison of known Vasostatin-1 derived peptides, residues (8–47) in human (h), rat (r), mouse (m), bovine (b), and porcine (p). (B) MS spectra of V1DP. (C) MS/MS sequencing data represented in panels which in each case show predicted singly- and doubly-charged b-ions and y-ions arising from MS/MS fragmentation. Observed ions are highlighted in red or blue typeface. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
3.4. Effect of V1DP on HUVEC migration and the anti-angiogenesis in vitro and vivo.

A wound healing assay was performed to determine the potential effect of V1DP on cell migration. After a 24-hour real-time observation, the HUVEC cell migration was effectively inhibited after treated with 10 μM V1DP and selective images were shown in Fig. 4A, while the 1 μM didn’t show a significant inhibitory effect. Similar cases were also observed in the tube formation assay (Fig. 4B). Pretreatment of HUVEC with 10 μM V1DP for 6 h significantly inhibited HUVEC tube formation on matrigel while the inhibitory effect of 1 μM was much weaker. The inhibitory effect of 10 μM V1DP was similar to the positive control Cilengtide, a cyclic RGD pentapeptide that is recognized as a positive anti-angiogenic small molecular in cancer treatment [23]. The anti-angiogenesis effect of V1DP was further validated on the vascular development of zebrafish larvae (Fig. 5). V1DP was able to suppress the formation of intersegmental vessels (ISV) at the concentration of 50–100 μM and subintestinal vessels (SIV) in the zebrafish larvae at the concentration of 25–100 μM.

Fig. 2. Effect of various concentrations of V1DP on smooth muscle contraction frequency in A) rat artery and B) uterus. Each point represents the mean and standard error of six determinations (n = 3, six replicates). C) Haemolytic activities of V1DP following incubation with horse erythrocytes for 2 h.

Fig. 3. MTT cell viability assay of V1DP on HCT116, HKE-3, DLD-1, DKS-8, CCD-18CO and HUVEC, respectively. The IC50 value of the anti-proliferative effects were presented accordingly.

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3.5. V1DP suppressed VEGF-induced ERK activation pathway

VEGFR2 is necessary for VEGF-stimulated endothelial proliferation and angiogenesis [24]. The activation of the VEGF downstream p42/44 MAP kinase pathway can induce cell proliferation and migration and induce the proangiogenic effect. In order to determine the potential effect of V1DP in anti-angiogenesis, the HUVEC cells were pretreated with V1DP at the 10 μM (the effective concentration in vitro test) for 6 h. The phosphorylation of ERK and VEGFR2 was inhibited after V1DP treatment, which suggests that the anti-angiogenesis effect of V1DP might involve the inhibition of VEGF-induced MAPK signaling (Fig. 6).

3.6. Peptide and protein modeling

The SWISS-MODEL structure of the V1DP has a helix-loop-helix fold as shown in Fig. 7. The RMSD analysis of the trajectory showed that the peptide structure was equilibrated in about 90 ns, reaching an average Cα RMSD of 0.41 ± 0.07 nm. Sidechain fluctuations were observed, mainly at the two ends of the peptide and the loop.
The peptide structure with high stability can be attributed to the strong Cys10-Cys31 disulfide bond that tightly told the two helices together. Interestingly, the equilibrated structure exhibits a non-polar surface on one side and a highly negatively charged surface (Glu6, Glu13, Asp17, Glu30, and Glu39) on the other side.

3.7. Identification of peptide binding sites in the kinase domain of VEGFR2

In order to identify potential binding sites in the protein and the initial binding mode of the peptide, we used an ensemble of protein conformations generated from MD to perform docking simulations. For the six representative protein conformations of the JM-in state, the 5 lowest energy predicted poses of the peptide were found to bind to the surface of the protein, except for one that is docked at the catalytic domain (CD). This one has a ZDOCK score of 13.66 kcal/mol, which is the second highest binding energy in the list (refer as S1-CA). For the JM-out state, of the 11 lowest energy predictions, in 6 of them the peptide was docked to the RDP domain. The binding mode of the peptide that has the highest ZDOCK score of 13.68 kcal/mol was identified (refer as S2-RDP). It is worth noting that the peptide was only docked to the CD and the surface of the protein, but not the RDP, when the protein is in the JM-in state. On the contrary, when the protein is in the JM-out state, the peptide was only docked to the RDP and the protein surface, but not the CD. This may hint that the V1DP peptide has a preferential binding location depending on the JM position of the protein. Two predicted complexes, S1-CA and S2-RDP were selected to continue with MD simulation to check for the stability of the binding. Previously, we had also simulated a representative surface-bound peptide, but it was diffused away in a short simulation time.

3.8. Protein-peptide binding analysis

As shown in Fig. 8, the MD results demonstrated that the peptide can stably bind to the CA and RDP of the protein because the peptide did not leave its binding region during the entire 100 ns simulation period. By visual inspection, it can be seen that the peptide is packed tightly at both the CD and RDP binding sites, with its non-polar surface facing to the pocket and the charged surface exposed to the solvent. Indeed, the bound peptide has much lower RMSD than its free form in solution. In S1-CD and S2-RDP, the average Cα RMSD of the peptide (including only residue 7 to 36, i.e. without the terminal coils) is 0.12 ± 0.02 nm and 0.14 ± 0.02 nm, respectively. In contrast, the unbound peptide has the average Cα RMSD of 0.30 ± 0.13 nm. The protein also showed excellent stability in both systems with the average Cα RMSD in S1-CD and S2-RDP being 0.22 ± 0.02 and 0.21 ± 0.04, respectively.

The binding free energies between the protein and peptide, and their energetic components are shown in Table 1. We can see that the van der Waals (E_{vdw}) and electrostatics (E_{elec}) energies between protein and peptide are negative, but the electrostatic contribution to the solvation free energy (E_{gb}) is a large positive value. This reveals that binding of the peptide to the protein involves large energetic penalty on the desolvation of the peptide, however, this loss is overcompensated by the sum of the van der Waals and electrostatics interactions between the protein and peptide. Taken the entropic term into account, the final free energy of binding \( \Delta G \), computed by MM/GBSA, is -12.63 kcal/mol for S1-CD and -21.61 kcal/mol for S2-RDP. Therefore, we can conclude that the peptide binds to the RDP of the protein with a higher affinity than to the CD and a difference them is about 9 kcal/mol.

We examined the hydrogen-bond and hydrophobic interaction types in the bound complex using the protein–ligand interaction profiler (PLIP) [25]. As shown in Fig. 9, the protein was found to form a few highly stable hydrogen bonds (contact frequency =>...
1.0) with the peptide. For the S1-CA system, residues Arg929 and Thr926 of the protein were found always interacted with Gln29 and Met25 of the peptide, respectively. Deeper in the binding site, the protein residues Arg929 and Arg1051 interacted with the residues Val27 and Pro22 over 70% of the time. Interestingly, protein residues at the core of the catalytic site were found to form hydrophobic interactions with the peptide non-polar residues Leu19, Pro24, Leu35, and Val27 in 80% to 40% of the time. It should be pointed out that the DFG-motif residue, Phe1047, was found to form moderate hydrophobic interaction with the peptide residue Pro22 in 60% of the time (also see Fig. 8, upper panel, for the illustration of the aromatic rings interaction of Phe1047-Pro22). This binding analysis suggests that the V1DP peptide may potentially lock the protein in its inactivated state and inhibit the biological function of the protein via binding to its CD.

Similarly, for the S2-RDP system, a number of protein residues formed highly stable hydrogen bond with the peptide, including Arg1027 and Asp1028, which are at the bottom of the RDP pocket. Here, the DFG-motif residue, Asp1046, and the neighboring residue Glu885, together attracted the peptide residue Lys21 to the centre of the RDP. In an alternating fashion, these two residues maintained a close distance of about 0.17 nm to Lys21, thus exerting a very strong attractive force to the peptide (see Fig. 8, lower panel, for the illustration). Regarding the hydrophobic interactions, a
number of pocket residues, Asp1064, Thr1054, Ile888, Ile1053, Arg1066, Ile804, etc. had similar interacting time (40–50%) with the peptide. Therefore, based on the docking and simulation results, it is likely that V1DP preferentially binds to the VEGFR2 kinase domain at the RDP pocket when the protein is in the JM-out state, while it binds to the CD pocket when the protein is in the JM-in state. The strong protein-peptide binding is mainly driven by electrostatics interactions and moderately by van der Waals interactions between the protein and peptide.

4. Discussion

CgA-related polypeptides are considered as serum biomarker for neuroendocrine tumour patients, most of the peptides have
inhibitory roles in endocrine and vascular systems [26]. Neuroendocrine tumours can arise in most parts of the human body and these tumours may produce specific hormones that induce some specific tumour syndromes. The diagnostic approach for neuroendocrine tumours strongly depends on clinical hormone assays, nuclear medicine imaging and radiological imaging [26]. Those hormones and peptide secretions are synthesized as precursors that contain the biologically active peptides [27]. Many of these peptides may contribute to special clinical tumour syndromes or involved in regulating the tumor microenvironment, including fibroblasts and endothelial cells migration and adhesion.

CgA is the member of a family of acidic secretory proteins widely distributed in endocrine and neuroendocrine cells and sympathetic neurons and other neuroendocrine cells distributed along the gastrointestinal tract [28,29]. The significance of the CgA and its derived peptides as a prognostic marker in neuroendocrine tumours has been well recognized [30,31]. They are also active in cardiovascular, immunometabolic regulation such as hypertension, rheumatoid arthritis, heart failure, renal failure and inflammatory bowel disease [6]. Our research teams have been focused on the track research of CgA derived peptides in the last decades. We observed that CgA and its derived peptides are actively processed in pancreatic beta cells of insulinoma, also found in mid-gut carcinoid and phaeochromocytoma where tumors express appropriate prohormone convertases [31]. Among these derived peptides, Vasostatin-1 was named based on its inhibitory effects on blood vessel contractility in 1992 [32]. Later, more inhibitory activities were observed with tumor angiogenesis and a tumour vessel architecture modulator [7]. Vasostatin-1 works as cardio regulatory hormones that can depress myocardial contractility and relaxation, counteract the β-adrenergic induced positive inotropism, and modulate coronary tone mainly via a nitric oxide-dependent mechanism[33]. Meanwhile, it can inhibit the TNF induced endothelial cell activation and permeability, including the phosphorylation of p38 MAP kinase [33].

In this study, V1DP was derived from N-terminal fragment of Vasostatin-1 (residues 8–47). Overviewing the typical primary structure of V1DP, the three typical functional structures, RGD and KGD, and a disulphide bridge formed by two cysteine residues at amino acid positions 17 and 38 might play the main role in determining the functions of the peptide. These three functional domains are also the typical functional structure of Vasostatin-1, which have high degrees of interspecies conservation (Fig. 1A). The interaction of RGD sequences in adhesive glycoproteins were found to be platelet receptor recognition sites on these adhesive ligands and are thought to be crucial to normal platelet function [34,35]. There are coincidentally many cysteine-rich, RGD-containing snake venom peptide disintegrins [35]. The two cysteine residues (Cys17-Cys38) in Vasostatin-1 interact to form an intra-molecular disulphide loop which is required for sorting to secretory granules and homodimerization [36]. The sequence of the disulphide loop, located between the KGD and RGD regions, is a unique hydrophobic region within the entire peptide and has been shown to play an important role in determining its secondary
structural properties and also as a necessity for its biological functions [36]. It also displays a 32% identity and 64% similarity with a region of the fibronectin type III-9 domain of tenasin, an extracellular matrix protein that is conserved in all vertebrates [37].

We found that this novel peptide exerted a dose-dependent relaxation of rat arterial smooth muscle and also induced the contraction activity of rat uterus smooth muscle. It inhibited cancer cell proliferation and HUVEC cell migration and exhibited an anti-angiogenesis effect both in vitro and in vivo. The western blot results in Fig. 6 indicated that the phosphorylation of ERK and VEGFR2 was inhibited after V1DP treatment, which suggests that the V1DP might block the VEGFR signaling pathway. Angiogenesis is involved in tumor growth, macular degeneration, retinopathy and other diseases. VEGF stimulates angiogenesis by binding to specific receptors (VEGFRs) on the surface of vascular endothelial cells. VEGFRs are receptor tyrosine kinases that, like the platelet-derived growth factor receptors (PDGFRs), contain a large insert within the kinase domain [38]. There are many studies on the role of VEGF in new blood vessel formation in these extremely well-vascularized tumors and of its role in metastasis of the tumor. Our docking and simulation results indicated that V1DP might bind to the active site and RDP of the VEGFR2 kinase domain and subsequently inhibited the activation of the VEGF signaling pathway. V1DP exhibited a highly complementary shape to both pockets. However, the free energy of binding of the peptide in the RDP is −21.61 kcal/mol, which is about 9 kcal/mol lower than that of the CA, indicating that the peptide has a stronger binding force in RDP. V1DP showed a similar dose inhibitory effect as cinglitide, a cyclic RGD pentapeptide that is recognized as a positive angiogenic small molecular in cancer treatment. These results further support the structural characterization of V1DP in the anti-angiogenesis effect. In addition, V1DP showed selective cytotoxicity only against cancer cell without any haemolytic toxicity.

Taken together, all the in vitro, in vivo and in silico data obtained in this study indicate that V1DP is a water-soluble and biologically stable peptide and has the potential to develop as an anti-angiogenic drug for cancer treatment after further structural-functional optimization.

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Authors contributions

RW, QW, NA, SS and LW carried out the experiments and performed the MD statistical analysis. RW and SS drafted the manuscript. WG supervised the in vivo work. TC, MZ, CS and HKF participated in the design of the study. HKF and RDY revised the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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