A Heparan Sulfate-Binding Cell Penetrating Peptide for Tumor Targeting and Migration Inhibition

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1. Introduction

Carcinoma is a malignant cancer originating in the ectodermal and endodermal epithelial cells. Interaction between cell surface and microenvironment plays a crucial role in malignant tumor progression. Alterations of cell surface receptor, coreceptor, and adhesive protein expression are reported in various cancer types in vitro and in vivo [1–3]. Abnormal expression of cell surface molecules notably contributes to enhance tumor cell growth, survival, migration, and invasiveness [4]. Characterization of such alterations and development of novel agent for specific targeting are unmet medical need for early cancer diagnosis.

Glycosaminoglycans (GAGs) including heparan sulfate (HS), chondroitin sulfate (CS), keratan sulfate (KS), or dermatan sulfate (DS) are covalently attached to their core proteins to form proteoglycans. HS proteoglycan (HSPG) present in the extracellular matrix (ECM) provides structural frameworks to mediate cell-cell communication and function in growth factor-receptor binding [5, 6]. HSPGs are key players in modulating tumor progression processes including metastasis, angiogenesis, proliferation, and malignant transformation [4]. Thus, upregulation of cell surface HS may play an active and crucial role in directing malignant phenotype of cancer during different developmental stages.

Cell penetrating peptides (CPPs) are short-chain cationic and/or amphipathic peptides which may be internalized into living cells [7]. CPPs are able to mediate translocation of a conjugated cargo (e.g., anticancer therapeutics) across plasma membrane, providing an effective and nontoxic mechanism...
for drug delivery [8]. Most CPPs are rich in positively charged Arg and Lys residues and are internalized after initially interacting with cell surface negatively charged GAGs which cluster CPPs on outer membrane surfaces [9, 10].

CPPs might be potentially used in clinical procedures such as gene therapy and cancer therapy [8, 11]. However, most CPPs are unfeasible for in vivo research due to non-specificity of their highly cationic characteristics. Cell surface negatively charged HS initializes the contact of CPPs, so particular HS binding CPPs might own mysterious sequence to exert multiple functions including HS binding, cellular binding, lipid binding, and in vivo tissue targeting activities. CPPcp is a recently identified CPP not only binding to negatively charged molecules including GAGs and lipids on cell surface in vitro but also targeting mucosal tissues in vivo [12-14]. In this study, we aim to collect and analyze the characteristics of HS-binding cell penetrating peptides derived from natural proteins. Besides, CPPcp itself falling in this classification has demonstrated multiple functions including in vitro tumor binding, tumor migration inhibition and angiogenesis inhibition activities, and in vivo cargo delivery to tumor site. Here, we provide more clues for the design of peptide therapeutics or intratumor delivery strategy by linking of a tumor targeting CPP. Furthermore, CPPcp might be a unique HS probe for cancer diagnosis to facilitate the quality of therapeutic index and molecular imaging in translational medicine.

2. Materials and Methods

2.1. Synthetic Peptides. Peptides CPPcp (NYRWRCKNQN) and EDN32-41 (NYRQRCKNQN) or CPPcp with N-terminal conjugated fluorescein isothiocyanate (FITC) or tetramethylrhodamine (TMR) were synthesized by Genemed Synthesis Inc. and their purities (>90%) were assessed by analytical high-performance liquid chromatography. Peptide sequences were confirmed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry in Genemed Synthesis Inc.

2.2. Flow Cytometry. Cells (3.0 × 10^5/well) were added into six-well plates and cultured in the indicated medium. After 24 h, 5 μM FITC-CPPcp dissolved in medium was added into a well and the samples were incubated for 1 h. Cells were then harvested, washed, and suspended in PBS. The fluorescent intensities of the cell samples were measured using a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) and excitation and emission wavelengths of 488 nm and 515–545 nm, respectively. The relative internalization of FITC-CPPcp was reported as the mean fluorescent signal for 10,000 cells.

2.3. Fluorescence Microscopy. CT-26 cells were cultured on coverslips (5.0 × 10^5/coverslip) in RPMI-1640. After 24 h, cell samples were incubated with FITC or FITC-CPPcp at 37°C for 10 min. Alternatively, CT-26 cells were pretreated with heparinase II (2.5 mU/mL) (Sigma-Aldrich, Missouri, USA) at 37°C for 2 h followed by treatment with 5 μM TMR-CPPcp at 37°C for 10 min. The cells were then washed twice with PBS, fixed with 4% (w/v) paraformaldehyde, and rinsed twice with PBS. The coverslips were mounted in a Vectashield antifade mounting medium with DAPI (Vector Labs). Inverted fluorescent microscopy was performed using Axiovert 135 (Carl Zeiss, Göttingen, Germany) to assess the distribution of the FITC-CPPcp or TMR-ECPPcp in the cells.

2.4. In Vitro Cell Migration Assay. Effect of CPPcp on cell migration was assessed using a 24-well transwell plate inserted with incorporating polyethylene terephthalate filter membrane with 8 μm pores (BD FalconTM Cell Culture Insert System).

Approximately 4 × 10^3 CT-26 cells (obtained from ATCC, number: CRL-2638) were suspended in 200 μL of serum-free RPMI-1640 medium (Sigma-Aldrich, Missouri, USA) and pretreated with 1.25, 2.5, 5, and 12.5 μM CPPcp or EDN32-41 at RT for 30 min, and then seeded on the upper compartment of transwell insert membrane. The lower compartment of membrane containing 300 μL 1% FBS (Gibco/Invitrogen) RPMI-1640 medium was used as chemoattractant. After incubating at 37°C and 5% CO_2 for 18 h, the migrated cells on the lower surface of membrane were fixed with 4% formaldehyde for 15 min and stained with 0.05% crystal violet for 20 min. The nonmigrated cells on the upper surface of membrane were removed by cotton swab. Numbers of migrated cells were counted in a randomly selected microscopic field (100x) using inverted microscope (Olympus CK40, Artisan Technology Group, Mercury Drive Champaign, USA).

Approximately 5 × 10^4 human umbilical vein endothelial cells (HUVECs) (obtained from BCRC, number: H-UV001) were suspended in 200 μL complete EC medium (Gibco) containing 0, 5, or 12.5 μM CPPcp and then seeded on the upper compartment of filter. The lower compartment of filter contains 500 μL complete EC medium with 20 ng/mL VEGF (R&D) as stimulator. After incubating at 37°C and 5% CO_2 for 4 h, the migrated cells on the lower surface of filter were fixed with 4% formaldehyde at RT for 15 min and stained with Hoechst at RT for 15 min. The nonmigrated cells on the upper surface of filter were removed by cotton swab. Filter membrane of transwell insert was cut down and mounted with Fluoromount mounting medium (Sigma Aldrich, Missouri, USA). Numbers of migrated cells were counted in five randomly selected microscopic fields at magnification 100x using inverted fluorescent microscope (TE2000E, Nikon, Kanagawa, Japan) with a cooled CCD (Evolution VF, MediaCybernetics, Bethesda, MD).

The result was represented as mean ± SD (standard deviation) of three independent experiments. Statistically significant differences were analyzed using unpaired Student’s t-test. Asterisks showed level of statistical significance: *P < 0.05; **P < 0.01; ***P < 0.001 compared with control.

2.5. Zebrafish Angiogenesis Model. Tg(kdr:EGFP) zebrafish, a well-studied model for vascular embryogenesis [15], was performed to assess the effects of CPPcp on angiogenesis. The Tg(kdr:EGFP) (kindly provided by Dr. Yung-Jen
Chuang's lab at NTHU) is a transgenic zebrafish line that expresses eGFP driven by the kdr promoter in vasculature endothelial cells during zebrafish embryogenesis, which can serve as an in vivo angiogenesis model for drug screening [16]. Fertilized eggs were generated from adult mating pairs and incubated at 28.5°C in a recirculating aquaculture system. The zebrafish embryos were separately injected with 6.3 or 31.5 ng CPPecp (4.6 nL; 4.56 or 22.8 pmol) into yolk sac at 60 h postfertilization (hpf), and PBS injection was set as control (16–20 zebrafish were used for each treatment condition). After incubating for 24 h, development of subintestinal vessels (SIV) pattern in the zebrafish yolk sac was observed and imaged by inverted fluorescent microscope (TE2000E, Nikon, Kanagawa, Japan) with a cooled CCD (Eclipse VF, MediaCybernetics, Bethesda, MD).

2.6. Animal Model. All work performed with animals was approved by the Institutional Animal Care and Use Committee at the National Tsing Hua University. Five-week-old female Balb/c mice (supplied by National Laboratory Animal Center, Taiwan) were housed in laboratory animal room at National Tsing Hua University and allowed to adapt to new surrounding for about seven to fourteen days. Animal rooms had a twelve-to-twelve-hour light-dark/day-night cycle and were maintained at constant temperature and humidity. For establishment of tumor-bearing mouse model, CT-26, a mouse colon carcinoma cell was suspended at a density of 1 × 10⁶ cells in 100 μL PBS containing 50% Matrigel (BD Biosciences, San Jose, CA) and subcutaneously injected into the right back of each mouse. Once subcutaneous tumor volumes grew up to 100 mm³, all mice were subjected to various treatments. At the end of the experiment, the mice were sacrificed by CO₂ narcosis. All of the organs including kidney, liver, spleen, trachea, lung, intestine, heart, pancreas, stomach, and tumor of these mice were taken, fixed with paraformaldehyde, embedded in paraffin, and sliced into 5 μm tissue slides for Prussian blue staining.

2.7. Magnetic Nanoparticle Conjugated CPPecp and Prussian Blue Staining. To analyze in vivo tissue targeting of CPPecp, we have conjugated CPPecp onto a dextran-coated Fe₃O₄ type of magnetic nanoparticle (MNP) to form MNP-conjugated CPPecp (MNP-CPPecp) with a mean diameter of 59.3 nm (kindly provided by MagQu. Co., Ltd.) [17]. CT-26 tumor-bearing mouse was utilized to investigate biodistribution of MNP-CPPecp and Prussian blue staining was employed to demonstrate ferric iron in mouse tissues. The CT-26 tumor-bearing mouse was intravenously injected with 150 μL MNP-CPPecp (0.06 emu/g) and sacrificed by CO₂ narcosis at a time point of 3, 6, 12, and 24 h after administration. The kidney, heart, liver, spleen, stomach, pancreas, small intestine, large intestine, trachea, lung, and tumor of mice were taken, fixed with paraformaldehyde, embedded in paraffin, and sectioned into 5 μm thick tissue slides, following by deparaffinizing in xylene solution (J. T. Baker Phillipsburg, NJ, USA) and serially rehydrating with 100%, 95%, 85%, 75%, and 50% alcohol. The slides were continuously immersed in working solution (20% hydrochloric acid and 10% potassium ferrocyanide (Sigma, MO, USA) solution mixture, 1:1 volume ratio) at room temperature for 30 min and then counterstained with fast nuclear red (Sigma, MO, USA) at RT for 5 min. After dehydration through 95% and 100% alcohol and clearing with xylene, each slide was finally covered with coverslip. Tissue images were digitized using light microscope (Eclipse E400, Nikon) with digital microscopy camera (AxioCam ICc 5, ZEISS).

3. Results and Discussion

3.1. Heparan Sulfate Binding Cell Penetrating Peptides Derived from Natural Proteins. Heparan sulfate (HS) serves as the initial anchoring site for many CPPs through electrostatic interactions between negatively charged sulfates or carboxyl groups and basic amino acids Arg as well as Lys [18]. Till now 27 CPPs from natural proteins including 14 viral protein-derived peptides, 7 animal homeostatic modulator-derived peptides, 3 antimicrobial peptides, and 3 toxin-derived peptides have been demonstrated or predicted to be able to interact with cell surface HS and penetrate across the plasma membrane. In silico secondary structures of all 27 HS-binding CPPs were predicted by Network Protein Sequence Analysis [19]. As shown in Table 1, 17 peptides including CPPs 2–6, 8–12, 15, and 18–23 exist as α helix (H). Seven peptides including CPPs 1, 7, 13, 14, 16, 17, and 24 form random coil (C). CPP 23 exists as β sheet (E), and CPPs 26 and 27 exist as mixed α helix (H) with β sheet (E) structures. Among 27 CPPs seventeen structures have been validated by in vitro 3D structures deposited in Protein Data Bank (Table 1, underline) [20]. All 14 viral protein-derived CPPs are highly cationic (high pI values) with 10 peptides forming α helix and 4 existing as random coil, penetrating cells through direct translocation [21–24] and lipid raft-mediated endocytosis [25–29]. Most of the 7 animal homeostatic modulator-derived CPPs may be internalized into cytosol through HS-mediated and energy-dependent endocytosis, among which 5 animal protein-derived peptides are demonstrated to possess either α helix or β sheet to interact with the plasma membrane, while our CPPecp and apolipoprotein B binding domain are unique such that they hold random coil structures in this category. As for 3 antimicrobial peptides, all of them are suggested to interact with cell surface HS and penetrate membrane barrier via energy-dependent endocytosis. LL-37 holds high level of α helix, SynB1 possesses β sheet, and SynB3 retains random coil structures [30–32]. For the last category toxin-derived CPPs, bovine prion-derived bPrPp forming α helix and mixed α helix with β strand are distributed in the internal region of venom-derived crotamine, and scorpion toxin-derived maurocalcine [33–36].

Previous researches have shown that the interactions between the positively charged peptide and highly negatively charged membrane components, such as the GAG moieties of cell surface proteoglycans, play a crucial role in the overall process of cellular permeability of highly basic or amphipathic CPPs [37]. Although this investigation may also reflect nonspecific electrostatic interactions between these
### Table 1: pI, sequence, and structures of HS-binding cell penetrating peptides.

| Peptide name | Sequence and predicted secondary structure* | Heparan sulfate binding region | Internalization mechanism | Ref. |
|--------------|---------------------------------------------|--------------------------------|---------------------------|------|
| **Viral protein-derived CPP** | | | | |
| 1 | TAT peptide (49–57) | RKKRRQRRR | RKKRRQRRR | Lipid raft-mediated macropinocytosis | [25, 26] |
| 2 | Nucleoplasmin NLS (153–170) | KRPAAIKKAGQAKKKK | Not reported | Not reported | [58] |
| 3 | HTLV-II Rex (4–16) | TRRQRTTRARRNR | TRRQRT | Direct translocation | [21, 22] |
| 4 | Lambda-N (48–62) | QRARRRERRAEKQAQW | RRERR | Not reported | [22] |
| 5 | Phi21 N (12–29) | TAKTRYKARRAELIAERR | KTRYKARRA | Not reported | [22] |
| 6 | Delta N (1–22) | MDAQTRRRERRRAEKQAQWKAAN | TRRRERRA | Not reported | [22] |
| 7 | FHV coat (35–49) | RRRNRTRRRNRRRVR | RRRNRTRRRNRRRVR | Not reported | |
| 8 | BMV coat (8–26) | KMTAQRRRAARRRWTAR | AARRN | Not reported | |
| 9 | HIV-1 Rev (35–46) | RQARRRNRWRWRERQF | RQARRNRRRRWR | Not reported | [22] |
| 10 | Rev (26–42) | TRQARRNRRRRWRRERQF | TRQARRNRRRRWRRERQF | Energy dependent lipid raft-mediated macropinocytosis | [27, 28] |
| ** CPP from pestivirus envelope glycoprotein (Ems) (994–220) | ENAQGGAARVTSLGQRKLRIAGKRLGSRKSTWFGAYA | Basic residues | Direct translocation | [23] |
| 12 | gp41 fusion sequence | GALFLGWLGAAGSTMGAWSQPKKRRKVK | WSQPKKRRKVK | Direct translocation | [24] |
| 13 | VP22 | DAATATRGRASAERPTERAPARASPRPRPVD | SRPRP | Energy dependent lipid raft-mediated macropinocytosis | [27, 29] |
| **Animal homeostatic modulator-derived CPP** | | | | |
| 15 | Penetratin | RQIKIWFQNRRMKWKK | NRRMKW | Direct translocation | [61] |
| 16 | CPPcep | NNYRWRCKNQN | RWRCR | Endocytosis | |
| 17 | SV40 NLS | PKKKRRKV | PKKKRRKV | Not reported | [59, 60] |
| Peptide name          | Sequence and predicted secondary structure* | Heparan sulfate binding region | Internalization mechanism                              | Ref.       |
|----------------------|---------------------------------------------|--------------------------------|-------------------------------------------------------|------------|
| 17 Apolipoprotein B binding domain | SVCAQQYKKNSDHKRLMRKRGKLCCcccccccccccccccccccccccccccc | Basic residues | Endocytosis | [63, 64] |
| 18 hCT (9–32) | LGTYTQDNKHFHTFPQTAIGVAPHHHHHHHHHHHHHHHHHHHHHHHHHHHHHCC | Not reported | Endocytosis | [63, 65] |
| 19 pVEC (615–632) | LIIILRRIRKQAHASKChhHHHHHHHHHHHhCcC | LRRIRK | Macropinocytosis and clathrin mediated endocytosis | [66–68] |
| 20 hLF peptide | KCFQWQRNMRKVRGPPVSCIKRCCChhHHHHHCcCcC | MRKVRG | Lipid raft-mediated endocytosis | [69] |
| 21 PDX-1-PTD | RHIKWFQNRRMKWKKChhhhhhhhhhhhhhCc | NRRMKWKK | Caveolae-dependent endocytosis and lipid raft-mediated macropinocytosis | [70] |

| Antimicrobial peptide | | | | |
|----------------------|---------------------------------------------|--------------------------------|-------------------------------------------------------|------------|
| 22 LL-37 (1–37) | LLGDFRKSKEIKGKEKRIVQKDFLRNLVPRTESHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHCC | FRKSKEKI | Endocytosis | [30, 31, 71] |
| 23 SynB1 (1–18) | BGGRLSYRRFSTSTGRCCCEEEECC | Basic residues | Endocytosis | [32] |
| 24 SynB3 | RRLSYSRRRFCCC | Basic residues | Endocytosis | [32] |

| Toxin-derived CPP | | | | |
|------------------|---------------------------------------------|--------------------------------|-------------------------------------------------------|------------|
| 25 bPrPp (1–28) | MVKSKIGSWILVFAMWSDVGICCKRPMCCC | Basic residues | Macropinocytosis | [33] |
| 26 Crotamine (1–42) | YKQCHHKGGHCPEKICLPPSSDFGKMDCRWRWKCCKKGS | CCcChhhHHHEEEcccccccccccccccccccccccccccccccccccccccccccccccccccc | RWRWK | Endocytosis | [34] |
| 27 Maurocaine (MCA) (1–33) | GDCLPHKLKCEKDKCCSKKCKRRGTNIEKCCRCCCC | SKKCR and EKCR | Macropinocytosis | [35, 36] |

*The confidence of the prediction is denoted by scaling the predictions from week (lower-case letter) to strong (upper-case letter). “H,” “E,” and “C” refer to α-helical, β-strand, and random coil propensities, respectively.
basic peptides and HS, it has been characterized that negatively charged heparin more effectively blocks uptake of CPPs than other soluble GAGs such as CS and hyaluronic acid [38], likely suggesting that there might be some structural requirements involved in the strong interaction between CPP and HS. In Table 1, 19 of these 27 HS-binding CPPs generally possess conventional heparin binding sequences such as XBBXB and XBDBXXB where B is a basic amino acid and X represents a random amino acid, and they can also be divided into cationic and amphipathic groups. Most viral factor-derived peptides are basic amino acid-rich. For example, cationic TAT is an extensively used CPP rich in Arg and can interact with sulfated proteoglycans and negatively charged phospholipids on the cell membrane [25]. It should be noted that although 10-amino acid CPecep is almost equal size to 9-residue TAT and 10-residue SynB3, the features of TAT and SynB3 are quite different from CPecep. Both TAT derived from viral protein and SynB3 belonging to antimicrobial peptide are highly cationic peptides with high pI values above 12, while our newly identified CPecep containing only 2 Arg and 1 Lys in a total of 10 amino acids is amphipathic with a pI value of 10.05. Interestingly, the proportion of basic residues in amphipathic crotamine (26%) is close to CPecep (30%). “RWRCK” motif of CPecep was previously predicted as a unique functional pattern in all 13 hRNaseA family members employing Reinforced Merging for Unique Segments system (ReMUS) [39]. Another peptide CyLoP-1 (CRWRWKCCKK) derived from crotamine also exhibited efficient intracellular delivery activity. In both cases positively charged residues conducting electrostatic interaction and aromatic Trp exerting transient membrane destabilization were essential to maintain CPP functionality [40, 41]. Taken together, a similar motif “RWRXK” shown on the loop, where X might be a random amino acid, is present in both CPecep and crotamine, suggesting that combination of positively charged residues and nonpolar aromatic residues, especially Trp, might provide a design rationale for novel amphipathic cell penetrating peptides.

3.2. Cellular Binding of CPecep to Tumor Cell with Higher HS Expression Level. Heparan sulfate (HS) is reported to be overexpressed in several tumors [42, 43], while HSPG profiles on different tumor cell surface are largely unclear. Here a mouse colon cancer CT-26 cell line was used for in vitro and in vivo analyses. Cellular binding activity of CPecep and HS expression level on cell surface of CT-26 cells were accessed for quantitative analysis employing flow cytometry and fluorescent microscopy with fluorescence-labeled CPecep FITC-CPecep and an anti-HS monoclonal antibody recognizing an epitope of N-sulfated glucosamine on membrane HS (US Biological, Swampscott, MA, USA). Figure 1(a) showed significant FITC-CPecep binding activity to CT-26 cells, which correlated well with significantly higher HS expression (Figure 1(b)). In addition, 5 μM FITC-CPecep rapidly and efficiently internalized into CT-26 cells within 10 min as analyzed by fluorescent microscopy (Figure 1(c)). To further address the importance of HS for CPecep anchor in the absence of autofluorescence background, removal of cell surface HS by heparinase was carried out along with CPecep labeled with tetramethylrhodamine (TMR). CT-26 cells were incubated in medium with (+) or without (−) heparinase II for 2 h and then treated with 5 μM TMR-CPecep for 10 min. TMR-CPecep rapidly and efficiently bound to CT-26 cell surface (Figure 1(d), upper panel), while removal of cellular HS led to significant reduction in CPecep attachment (Figure 1(d), lower panel). Taken together, our HS-binding CPecep possessed strong binding activity to tumor cell surface with higher HS expression, while depletion of cell surface HS abolished such highly selective binding activity of CPecep to tumor cells.

3.3. Effect of CPecep on Migration of Mouse Colon Carcinoma Cell. It has been shown that HSPGs may modulate cell migration by interacting with growth factors or chemokines and drives cell migrate toward specific stimuli [44]. Since CPecep with a novel heparin-binding motif in ECP has already been identified to possess high recognition activity to cellular surface HSPG and penetration activity into cells [12], here whether CPecep might modulate cancer cell migration through interaction with HSPG was further investigated using in vitro transwell migration assay, while EDN32−41, a 10-amino acid peptide derived from comparable sequence motif of human RNase2 (EDN), possessing a known heparin-binding motif was also analyzed as a control. Figure 2 (black bar) showed that migration activity of CT-26 cell was significantly inhibited by CPecep in a dose-dependent manner such that it decreased to 83%, 71%, 56%, and 54% upon treatment with 1.25, 2.5, 5, and 12.5 μM CPecep, respectively. Yet treatment with 1.25, 2.5 and 5 μM EDN32−41 could not inhibit migration activity of CT-26 cells, and presence of higher concentration of EDN32−41 (12.5 μM) decreased 33% tumor migration (Figure 2 gray bar). These results indicated that CPecep containing core RWRCCK motif, rather than containing known heparin-binding motif, inhibited CT-26 cell migration across the membrane in vitro. It has been reported that cancer migration was inhibited by antagonism of HS side chains. For example, A5G27 peptide derived from laminin α5 globular domain recognizes HS side-chains of CD44 variant 3 and blocks bioactivity of fibroblast growth factor-2 (FGF-2). It significantly inhibits FGF-2-induced WiDr colon cancer cell migration and invasion [45]. Collectively, inhibitory effect of CPecep on cancer cell migration is possibly arisen from interaction with cell surface HS.

3.4. Effects of CPecep on Migration of Vascular Endothelial Cell. Cell surface HS proteoglycan (HSPG) serves as a coreceptor to coordinate binding of vascular endothelial growth factor (VEGF) toward its receptor. It has been reported to be associated with angiogenesis [46, 47]. However, vascular endothelial cell migration is a crucial step in formation of new blood vessel and tumor angiogenesis [48]. To test the hypothesis that CPecep interacting with cell surface HSPGs also affected angiogenesis, a common model cell line human umbilical vein endothelial cell (HUVEC) was used for in vitro transwell migration assay. Figure 3 indicated that VEGF-induced HUVEC migration was restored by cotreatment...
Figure 1: Effect of surface HS level on CPPecp binding to CT-26 cells. (a) CT-26 cells were preincubated at 4°C for 30 min and then incubated with 5 µM FITC-CPPecp for 1 h. The cells were washed twice with 500 µL PBS, trypsinized at 37°C for 15 min, suspended in 500 µL PBS, and subjected to flow cytometry. (b) CT-26 cells were stained with anti-HS monoclonal antibody (10E4) at 4°C for 1 h, washed twice with 500 µL PBS, and hybridized with FITC-conjugated anti-mouse secondary antibody at 4°C for 1 h. After being washed twice with 500 µL PBS, cells were suspended in 500 µL PBS and subjected to flow cytometry. (c) CT-26 cells were treated with 5 µM FITC-CPPecp at 37°C for 10 min. Uptake of FITC-CPPecp by CT-26 cells was examined by fluorescent microscopy. FITC was set as a negative control. DAPI staining of cells indicated intact nucleus. Scale bars in panel represented 10 µm. Green, FITC-labeled CPPecp; blue, DAPI (nucleus). (d) CT-26 cells were pretreated with or without heparinase II (2.5 milliunit/mL) at 37°C for 2 h followed by treatment with 5 µM TMR-CPPecp at 37°C for 10 min. Uptake of TMR-CPPecp by CT-26 cells was examined by fluorescence microscopy. TMR-CPPecp bound on CT-26 tumor cell DAPI staining of cells indicated intact nucleus. Scale bars in panel represented 10 µm. Red, TMR-labeled CPPecp; blue, DAPI (nucleus).
with 5 or 12.5 μM CPPecp, leading to, respectively, 77% and 64% migration activity. This result indicated that CPPecp could inhibit VEGF-induced HUVEC migration. Likewise, the CD44-binding peptide A5G27 derived from laminin α5 globular domain inhibits FGF-induced angiogenesis in Chick CAM Assay [49]. Moreover, an HS-binding peptide 6a-P, corresponding to the HSPG binding domain of VEGF, α could inhibit VEGF-induced HUVEC migration. Likewise, 

CT-26 cells were pretreated with CPPecp or EDN32-41 at indicated concentration in serum-free RPMI-1640 medium at room temperature for 30 min and then seeded onto the upper side of transwell insert membrane at 37°C for 18 h. Number of migrated cells without CPPecp or EDN32-41 treatment was set as 100%. The data represents means ± SD (standard deviation) of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001 compared with control.

3.5. Effects of CPPecp on Angiogenesis during Embryonic Development of Zebrafish. Cell surface HSPGs serve as a coreceptor to coordinate binding of VEGF toward its receptor and have been reported to be associated with angiogenesis [46, 47]. Tg(kdr:EGFP) zebrafish, a well-studied model for vascular embryogenesis, has been used as model for drug screening and angiogenesis studies [51, 52]. It was thus utilized to investigate CPPecp effects on in vivo embryonic angiogenesis by injecting 4.6 nL of 4.56 or 22.8 pmol CPPecp or PBS (control) into yolk sac of zebrafish at 60 h postfertilization (hpf), and the development of subintestinal vessel (SIV) pattern (Figure 4(a), SIV networks are indicated with red rectangle) at 24 h postinjection (hpi) was monitored with images by inverted fluorescent microscope. Here 16–20 zebrafish were tested for each treatment group. The observed SIV patterns of zebrafish were divided into three groups according to growth level of SIV: normal, mildly inhibited, and severely inhibited phenotypes (Figure 4(b)). In the normal phenotype SIV developed as smooth basket-like pattern with 5-6 arcades. Both mild and severe inhibition phenotypes could be further classified as ectopic SIV pattern, in which SIV exhibited tortuous network and was unable to demonstrate complete basket-like pattern that normal phenotype developed. However, severe inhibition phenotype displayed more incomplete SIV network than mild inhibition phenotype did. In contrast, the zebrafish injected with CPPecp appeared to be tortuous, in which SIV pattern shrank significantly as compared with that of PBS control (Figure 4(c)). Figure 4(d) illustrated quantitative analysis data in which percentage of ectopic SIV phenotype (mildly inhibited phenotype plus severely inhibited phenotype) rose from 39.6% up to 49.2% and 52.6% upon injection with 4.56 and 22.8 pmol CPPecp, respectively. Moreover, severely inhibited SIV phenotype increased from 11.1% up to 26.2% and 32.4% upon injection with 4.56 and 22.8 pmol, respectively. In other words, percentage of severely inhibited phenotype in ectopic phenotype of zebrafish increased from 27.3% (control) up to 52.3% and 60.7% upon injection with 4.56 and 22.8 pmol CPPecp, respectively. This data revealed that our CPPecp possessed antiangiogenesis activity in inhibiting SIV growth of zebrafish. As a result, involvement of CPPecp in angiogenesis may be attributed to interaction with cell surface HS. CPPecp is the first antiangiogenic peptide deciphered in embryonic development of zebrafish.

3.6. Time-Dependent Biodistribution of MNP-CPPecp in CT-26 Tumor-Bearing Mouse. To better understand biodistribution of our HS-binding CPPecp in vivo, CPPecp was conjugated with well-dispersed Fe₃O₄ magnetic beads (59.3 nm for diameter) to form magnetic nanoparticle-conjugated CPPecp (MNP-CPPecp). CT-26 tumor-bearing mice were intravenously injected with MNP-CPPecp (0.06 emu/g) and sacrificed at different time point after administration (Figure 5(a)). MNP-CPPecp signal was detected using Prussian blue staining to indicate ferric iron in tissue section (blue color). Figure 5(b) indicated that stainable ferric iron (blue color as indicated by yellow arrow) was barely detectable in trachea, heart, and large intestine at all indicated time points, and so did other tissues including stomach, pancreas and kidney (data not shown). The MNP-CPPecp mainly accumulated in liver tissues from 3 h up to 24 h owing to uptake and removal by macrophages of reticuloendothelial system, which played a role in clearance of external substance in liver [53, 54]. Interestingly, Prussian blue staining signals in CT-26 tumor section suggested MNP-CPPecp accumulation from 12 h to 24 h, whereas MNP signal was only detected in liver at 24 h. One recent report showed that exendin-4 peptide-conjugated superparamagnetic iron oxide nanoparticles were inevitably accumulated in liver tissue, suggesting that a nanoparticle might unavoidably be captured by this metabolic organ [55]. However, it is worth noting that CPPecp has potential to target colon carcinoma in vivo, suggesting...
that CPPecp might be applied for a potent carrier for drug delivery.

3.7. Heparan Sulfate-Binding Cell Penetrating Peptide for Tumor Targeted Strategy. Although CPPs as noninvasive agents have promising biomedical potential for molecular delivery, they are mostly unfeasible for in vivo researches due to nonspecificity of their highly cationic characteristic such as TAT peptide. Due to high uptake rates in vitro and relatively low specificity in vivo of most CPPs, conventional CPPs would be designed for topical applications in CPP-based delivery (Table 1). Further analysis of natural protein-derived CPPs revealed that 5 CPPs exerted in vitro tumor suppression as well as cell internalization activities (Table 2). Although TAT peptide (46–57) demonstrated antiangiogenesis and apoptosis-inducing activities, TAT peptide was proved to show low target specificity in vivo [56]. Distinct from conventional highly cationic CPPs, 4 amphipathic CPPs including CPPecp, crotamine, NFL-TBS (40–63), and p28 peptides demonstrated unique tumor targeting activity in vivo. Even though specific protein receptors for CPPecp and crotamine remain to be investigated, HSPG acting as coreceptor is indispensable for the translocation of CPPecp and crotamine [12, 34]. In addition, both CPPecp and crotamine targeted highly proliferating cells such as tumor tissues [14, 57]. Interestingly, a motif decorating a hydrophilic aromatic amino acid participating in membrane permeation between two arginines (RWR) appeared to be conserved in both CPPecp and crotamine, leading to similar characteristics of these 2 multifunctional HS-binding CPPs. Therefore, amphipathic CPPs might own promising potential to be designed as peptide-based drugs. In particular, HS-binding CPPs are suitable drug carriers for in vivo application in delivery of functional therapeutics.

4. Conclusions

CT-26 colon tumor cells revealed high CPPecp binding activity due to high HSPG expression on cell surface. CPPecp displays not only significantly inhibitory effects on CT-26 cancer migration and angiogenesis in vitro but also antiangiogenesis activity during zebrafish embryogenesis in vivo. Moreover, covalent linkage of CPPecp to magnetic nanoparticle shows potential for in vivo targeting to a subcutaneous CT-26
Figure 4: Inhibitory effect of CPPecp on angiogenesis in Tg(kdr:EGFP) zebrafish. (a) Morphology and green-labeled vessels in Tg(kdr:EGFP) zebrafish. The red rectangle represents the area of subintestinal vessel (SIV) network. Magnification: 100x. Scale bar: 400 μm. (b) Development of SIV network in the zebrafish yolk sac could be classified into three groups: normal, mild inhibition, and severe inhibition pattern. Magnification: 400x. Scale bar: 100 μm. (c) Development of SIV network in the zebrafish yolk sac at 24 h postinjection (hpi). Magnification: 400x. Scale bar: 100 μm. (d) Percentage of different SIV phenotypes in the zebrafish yolk sac at 24 hpi. (e) Percentage of severe inhibited SIV phenotype in ectopic SIV phenotype in the zebrafish yolk sac at 24 hpi. 16–20 zebrafish were used for each treatment group. The data represents means ± SD (standard deviation) of three independent experiments.
Figure 5: Localization of MNP-CPP_{ecp} in CT-26 tumor-bearing mouse. To investigate the biodistribution of CPP_{ecp} in vivo, CT-26 tumor-bearing mice were intravenously injected with 0.06 emu/g MNP-CPP_{ecp} and sacrificed at a time point of 3, 6, 12, and 24 h after injection (a). Signal of MNP-CPP_{ecp} was visualized using Prussian blue staining to indicate ferric iron in tissue section (blue color, yellow arrow). Represented staining patterns of trachea, heart, large intestine, liver, and CT-26 tumor were shown (b). MNP injection was set as negative control. Nuclear fast red staining was set as counterstain (red color). Magnification: 200x and 400x. Scale bar: 100 μm and 50 μm.
| Name/sequence                  | Function                  | Mechanism                                                   | Cell line   | Tumor mouse model      | Ref.  |
|-------------------------------|---------------------------|-------------------------------------------------------------|-------------|------------------------|-------|
| CPPecp/NYWRCKQNQ              | Cell penetrating          | Block putative HS coreceptor for growth factor              | CT-26       | Murine colon carcinoma | [12–14]|
|                               | HS binding                |                                                             | HUVEC       | CT-26                  |       |
|                               | Antimigration              |                                                             |             |                        |       |
|                               | Antiangiogenesis           |                                                             |             |                        |       |
|                               | Tumor targeting           |                                                             |             |                        |       |
| Crotamine/YKQCHKKGGHCFPKEKLPPSSDFGKMDCRWRWKCCCKKGSG | Cell penetrating          | Interact with lysosomes to trigger intracellular Ca"°" transients and alter mitochondrial membrane potential | B16F10      | Murine melanoma (B16F10) | [34, 57]|
|                               | HS binding                |                                                             | CHO-K1      | Murine mammary carcinoma (TS/A-pc, TS/A-pc-pGL3) |       |
|                               | Antiproliferation          |                                                             |             |                        |       |
|                               | Tumor targeting           |                                                             |             |                        |       |
| NFL -TBS. (40–63)/YSSYAPVSSLSVRRYSSSSGS  | Cell penetrating          | Inhibit polymerization of microtubules                      | Human glioblastoma (T98G) | Murine glioblastoma (F98) | [72, 73]|
|                               | Antimigration              |                                                             | Rat glioblastoma (F98) | Murine glioblastoma (F98) |       |
|                               | Apoptosis-inducing         |                                                             | Rat gliosarcoma (9L) | Murine glioblastoma (F98) |       |
|                               | Antitumor growth           |                                                             |             |                        |       |
| TAT peptide (46–57)/SYGRKRRQRRR | Cell penetrating          | Inhibit VEGF binding to HUVEC and inhibit phosphorylation of ERK | HUVEC       | ×                       | [25, 74]|
|                               | HS binding                |                                                             |             |                        |       |
|                               | Antiangiogenesis           |                                                             |             |                        |       |
|                               | Apoptosis-inducing         |                                                             |             |                        |       |
| p28/LSTAADMQGVVTGDGMSGLDKDYLKPDD | Cell penetrating          | Inhibit phosphorylation of VEGFR 2, FAK, and Akt            | HUVEC       | Human melanoma (UISO-Md-6) | [75]  |
|                               | Antiangiogenesis           |                                                             |             |                        |       |
|                               | Antitumor growth           |                                                             |             |                        |       |
tumor site. Moreover, CPPecep containing a core RWRXK sequence demonstrates both cell penetrating and epithelial tumor targeting activities. Taken together, our HS-binding CPPecep might be feasible for further application in molecular imaging for tumor homing and selectively targeting drug delivery system.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors’ Contribution

Chien-Jung Chen and Kang-Chiao Tsai contributed equally to this work.

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