Efficacy of inverso isomer of CendR peptide on tumor tissue penetration

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Abstract  The dense extracellular matrix and high interstitial fluid pressure of tumor tissues prevent the ability of anti-tumor agents to penetrate deep into the tumor parenchyma for treatment effects. C-end rule (CendR) peptides can enhance the permeability of tumor blood vessels and tumor tissues via binding to neuropilin-1 (NRP-1), thus aiding in drug delivery. In this study, we selected one of the CendR peptides (sequence RGERPPR) as the parent L-peptide and substituted D-amino acids for the L-amino acids to synthesize its inverso peptide D(RGERPPR). We investigated the NRP-1 binding activity and tumor-penetrating ability of D(RGERPPR). We found that the binding affinity of D(RGERPPR) with NRP-1 and the cellular uptake was significantly higher than that of RGERPPR. Evans Blue tests revealed that D(RGERPPR) exhibited improved tumor-penetrating ability in C6, U87 and A549 tumor-bearing nude mice. Using nude mice bearing A549 xenograft tumors as a model, we found that the rate of tumor growth in the group co-administered with D(RGERPPR) and gemcitabine (Gem) was significantly lower than the gemcitabine-treated group with a tumor suppression rate (TSR%) of 55.4%. Together, our results demonstrate that D(RGERPPR) is a potential tumor-penetrating peptide.

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1. Introduction

Tumor tissues exhibit specific pathological features, including large amounts of random vasculature with high permeability, the absence of lymphatic drainage, a dense extracellular matrix, high interstitial fluid pressure, and an acidic and anoxic environment in the center of the tumor that is caused by poor elimination of metabolites owing to insufficient blood supply.14 Because of the dense extracellular matrix and high interstitial fluid pressure, antitumor agents are unable to reach deep into the tumor parenchyma, leading to retention of drugs around the tumor blood vessels. This non-uniform distribution of drugs is one of the crucial reasons for relapse and metastasis, which is serious obstacle in the treatment of tumors.

Neuropilin-1 (NRP-1), a transmembrane glycoprotein, was first discovered in the nervous system of Xenopus tadpoles by Takagi et al.7 via hybridoma technology in 1987. As a co-receptor of sema3A and VEGF165, NRP-1 played an important role in tumor occurrence, growth, and metastasis.15-17 NRP-1 was highly expressed on many tumor cells including glioma, non-small cell lung cancer, pancreatic cancer, gastric cancer, colon cancer and breast cancer.13-15 A series of peptides possessing a consensus R/KXXXR/K motif with L-amino acids were identified that showed high binding affinity to NRP-1.15 The C-terminal arginine (or rarely lysine) was important for NRP-1 binding activity. Adding another amino acid or blocking the free carboxyl group of this arginine residue by amidation eliminated the binding activity to NRP-1. This strict requirement for a C-terminal residue was termed as the C-end rule (CendR). By binding NRP-1, the CendR peptides could induce enhanced permeability of tumor blood vessels and tumor tissues, which could enhance the tissue-penetrating ability of free drugs and nanodrug delivery systems.

Despite these initial promising results, these linear L-peptides are susceptible to protease degradation in plasma and readily lose their bioactivity. In our previous study, we selected one of CendR peptides (sequence RGERPPR) as a parent L-peptide and synthesized its retro-inverso isomer, D(RPPREGR)16,17. The retro-inverso peptide was composed of D-amino acids assembled in the reverse order from that in the parent L-sequence.18 Compared with RGERPPR, D(RPPREGR) not only showed improved biological stability but also increased binding affinity to NRP-1. We synthesized D(RPPREGR) modified highly branched polyethyleneimine as gene vector, which significantly enhanced the therapeutic effect of genes on orthotopic tumor-bearing animal models.

The inverse peptide, which is composed of D-amino acids assembled in the same order as that in the parent L-sequence, shows a mirror-image relationship with the parent L-peptide. In general, the inverse peptide might not have similar bioactivity as its parent L-peptide. However, CendR peptides are a series of specific peptides with a consensus R/KXXXR/K motif and exhibited high binding affinity to NRP-1 and improved tumor-penetrating ability. The inverse isomer of CendR peptides also has a consensus D(R/KXXXR/K) motif. Since the C-terminal amino acid of the inverse isomer is arginine or lysine, we speculate that the inverse isomer of CendR peptides also have NRP-1 binding activity and tumor-penetrating ability. To verify our hypothesis, in this paper, we chose RGERPPR as the parent L-peptide and synthesized its inverse isomer, D(RGERPPR). We then investigated the activities of D(RGERPPR), including binding affinity to NRP-1, tumor cell internalization and tumor-penetrating ability.

2. Methods

2.1. Materials

Boc-protected amino acid was purchased from Peptide International (Japan). Hydrogen amine resin and 4-methylbenzene was supplied by Bachem AG (Switzerland). Disopropylethylamine (DIEA) was obtained from Fluka (USA). O-(Benzo triazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluoraphosphate (HBTU) was purchased from Sigma-Aldrich (St Louis, MO, USA).

2.2. Synthesis of peptides and FITC-labeled peptides

RGERPPR, D(RPPREGR), D(RGERPPR), CRGERPPR, D(CRPPREGR) and D(CRGERPPR) were synthesized using Boc-protected solid phase peptide synthesis strategy on 4-methylbenzene and hydrogen amine resin. The inverso peptide, which is composed of D-amino acids with the carboxyl group of the next Boc-protected amino acid was activated by dissolving in HBTU and DIEA for 2 min. The activated amino acid solution was then added to the resin and mixed by stirring for 20 min. The amino group was deprotected by 50% trifluoroacetic acid (TFA) for 10 min. The reaction mixture was then precipitated in diethyl ether and dried under vacuum. The crude product was then purified by high-performance liquid chromatography (HPLC), all synthetic peptides were analyzed by ultraperformance liquid chromatography (UPLC) and electrospray ionization-mass spectrometry (ESI-MS). CRGERPPR, D(CRPPREGR), and D(CRGERPPR) were also characterized by circular dichroism spectrum.

To synthesize FITC-CRGERPPR, a 1.2 × molar excess of Mal-FITC was dissolved in 1 mL of PBS with 5 mg CRGERPPR and stirred for 1 h in the dark. The reaction solution was purified via HPLC. The syntheses of other FITC-labeled peptides were the same as described for FITC-CRGERPPR. All FITC-labeled peptides were analyzed by UPLC and ESI-MS.

2.3. Surface plasmon resonance studies

To evaluate the binding affinity of RGERPPR, D(RPPREGR) and D(RGERPPR) with NRP-1, SPR interaction analyses were conducted using a Biacore T200 system (GE Healthcare, Uppsala, Sweden). First, the NRP-1 protein was coupled to the CM5 sensor chip via the standard amine coupling procedure. The peptides were then dissolved in HBS-EP (pH 4.0) buffer from low to high concentration (31.25 nmol/L to 1 μmol/L) and injected for detect-
HUVECs were seeded in 12-well plates at a density of 8 × 10^4 cells/well (FACSAria, BD, USA). Cellular uptake and A549 cells were seeded into 96-well plates (5 × 2.7 × 10^4 cells/well) with 200 μL/well DMEM containing 10% FBS, 100 μg/mL penicillin and 100 μg/mL streptomycin and incubated at 37 °C, 5% CO₂ for 24 h. After centrifugation (4000 rpm, 10 min, Centrifuge H1650, USA), the HUVECs were trypsinized, centrifuged, and then washed with PBS twice and then overlaid with 10% buffered glycerol solution before observation using an inverted fluorescence microscope. The cells were fixed with 10% formaldehyde for 10 min. The cells were washed with PBS twice and then overlaid with 10% buffered glycerol saline before observation using an inverted fluorescence microscope (Leica, DMI4000B, Wetzlar, Germany).

For flow cytometry evaluation, following the incubation described above, the HUVECs were trypsinized, centrifuged, resuspended in 300 μL PBS and analyzed by flow cytometry (FACSAria, BD, USA). Cellular uptake and flow cytometry tests of C6 cells, U87 cells and A549 cells were performed as the same as described for HUVECs.

2.5. Cell culture

Human lung adenocarcinoma epithelial cells (A549), human umbilical vein endothelial cells (HUVEC), human glioblastoma astrocytoma cells (U87), and C6 rat malignant glioma cells were obtained from the Shanghai Institute of Cell Biology. Cells were cultured in DMEM containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C under 5% CO₂ atmosphere.

2.6. Cellular uptake and flow cytometry tests

HUVECs were seeded in 12-well plates at a density of 8 × 10^4 cells/well in 1 mL of DMEM with 10% FBS and incubated overnight (5% CO₂) at 37 °C. FITC-CRGERPPR, FITC-D(CRP-PREGR) and FITC-Td(CRGERPPR) were added to the plates at a final concentration of 5 × 10⁻⁶ mol/L and incubated for 4 h. HUVECs were washed with PBS (0.01 mol/L, pH 7.4) twice and fixed with 10% formaldehyde for 10 min. The cells were washed with PBS twice and then overlaid with 10% buffered glycerol saline before observation using an inverted fluorescence microscope.

2.7. Cytotoxicity assay

A549 cells were seeded into 96-well plates (5 × 10^3 cells/well) with 200 μL/well DMEM containing 10% FBS, 100 μg/mL penicillin and 100 μg/mL streptomycin and incubated at 37 °C, 5% CO₂ for 24 h. Various concentrations of gemcitabine or carboplatin in PBS were added into the wells, and plates were incubated at 37 °C, 5% CO₂ for 48 h. Control cells were treated with fresh medium. Twenty microliters of 5 mg/mL MTT solution was added to each well and plates were incubated for 4 h. The medium was then removed, and 150 μL of DMSO was added to dissolve the formazan crystals. After 15 min, measurements were performed using a microplate reader (wavelength, 490 nm; PowerWave XS, Bio-TEK, Winooski, VT, USA). The measurements for each sample were conducted in triplicate.

2.8. Animal model establishment

Male nude mice (6–8 weeks old, 18–22 g weight) were purchased from Shanghai Sippr-BK Laboratory Animal Co. Ltd. (Shanghai, China). Mice were kept under specific pathogen-free conditions and provided with sufficient food and water. All animal experiments were conducted in accordance with the guidelines evaluated and approved by the Ethics Committee of Fudan University. The tumor model was established by subcutaneous injection of A549 cells (7 × 10^6 cells), C6 cells (8 × 10^6 cells) or U87 cells (9 × 10^6 cells) in 100 μL PBS into the right flank region of nude mice.

2.10. In vivo treatment study

The A549 tumor-bearing nude mice were randomly divided into four treatment groups (n = 5) when the tumors reached an average volume of 170 mm³. Mice were injected intravenously via a tail vein twice a week for a total of 5 times with the following: 1) equal volume of PBS (the control group); 2) D(RGERPPR) (total dose 75 mg/kg) dissolved in PBS; 3) the free form of gemcitabine (total dose 75 mg/kg) dissolved in PBS; 4) gemcitabine (total dose 75 mg/kg) in PBS combined with D(RGERPPR) (total dose 20 μg/kg) in PBS. Every two days, tumor size was measured across its two perpendicular diameters using a vernier caliper. The volume (V) was calculated according to formula

\[ V = 0.5 \times a \times b^2 \]  

where a and b are the longest and shortest diameters of the tumors, respectively. Tumor suppression rate was calculated as follows:

\[ \text{TSR\%} = \left( \frac{V_c - V_t}{V_c} \right) \times 100 \]  

where V_c is the tumor volume of the control group and V_t is the tumor volume of the treatment group.

2.11. Statistical analysis

All data are represented as mean ± SEM for all quantitative data. Statistical analysis was performed with the GraphPad Prism software. Two-tailed Student's t-test was used to determine significant differences between two groups (P < 0.05). Two-way analysis of variance (ANOVA) was used to determine significant differences between multiple groups (P < 0.05, *** P < 0.0001). ns indicates no significant differences (P > 0.05).
3. Results

3.1. Characterization and evaluation of peptides

Using a solid phase peptide synthesis strategy, we successfully obtained adequate quantities of highly pure RGERPPR, D(RPPREGR), D(RGERPPR) and FITC-labeled peptides. The HPLC and ESI-MS data (Supplementary Information Fig. S1) confirmed high purity levels and the molecular weight fit the theoretical value. RGERPPR, D(RPPREGR) and D(RGERPPR) were also characterized by circular dichroism spectrum (Fig. 1). All three peptides had an absorption peak at about 200 nm, indicating that the three peptides are disordered linear peptides. Moreover, the absorption peak of D(RGERPPR) was at about 200 nm, indicating that the three peptides are disordered linear peptides. The computational analyses

3.2. Computational analyses

Here we designed a peptide comprised of D-amino acids, D(RGERPPR). Notably, although the side chain of residues in D(RGERPPR) and D(RPPREGR) adopted opposite orientations compared with the reported active l-peptide, D(RGERPPR) and D(RPPREGR) showed higher binding affinity to the NRP-1. We performed a molecular docking study with the Schrodinger software package to explore the binding modes of the active peptides and determine the reason for the higher binding affinity of the two peptides comprised of D-amino acids. The three peptides were docked into the binding pocket of NRP-1 with the peptide docking package in Schrodinger. Examination of the binding modes of the peptides revealed that RGERPPR adopted the classic binding mode of the C-end peptide, in which the Arg7 at the C-terminal formed an ion interaction with Asp320 in NRP-1, and the COO\(^{-}\) at the C-terminus formed a hydrogen bond network with Tyr353, Ser346, Thr349 and Trp301. Arg1 and Arg4 formed hydrogen bonds with the backbone of NRP-1. Arg7 and Arg1 formed cation–π interactions with Tyr353 and Tyr297 (Fig. 2 A and B).

In the binding mode of D(RPPREGR), the DArg1 at the N-terminal formed an ion interaction with Glu319 in NRP-1, and the NH\(^+_3\) at the N-terminal interacted with Asp320. DArg4 formed a cation–π interaction with Tyr297 and Tyr353. DArg7 formed an ion interaction with Glu348 (Fig. 2 C and D).

In the binding mode of D(RGERPPR), the three DArg residues also formed a strong hydrogen network and ion interaction with the residues of NRP-1. DArg7 interacted with negatively charged Glu324 and Glu319. DArg4 formed hydrogen bonds with the backbone of NRP-1. DArg1 in D(RGERPPR) formed ion interactions with Asp320. DArg1 and DArg4 also formed cation–π interactions with Tyr353 and Tyr297 (Fig. 2E and F).

In the sequence of the peptides, the Arg or DArg residues contributed to the primary binding affinity of the peptides. Comparing the binding modes of the three peptides, the three DArg residues in D(RGERPPR) and D(RPPREGR) fit well with the binding pocket and effectively interact with residues in NRP-1 by hydrogen bond, ion interaction and cation–π interaction. In the sequence RGERPPR, only Arg7 at the C-terminal could form strong interaction with the residues of NRP-1, and Arg1 and Arg4 formed weak interaction with the backbone of NRP-1. We predicted the binding affinity of the three peptides (Table 1), which indicated that D(RGERPPR) and D(RPPREGR) were more potent than RGERPPR, and D(RGERPPR) was slightly stronger than D(RPPREGR) in terms of binding affinity. The computational predicted result was also in good agreement with SPR-determined results. These results demonstrate that D(RGERPPR) and D(RPPREGR) possessed stronger binding affinity to NRP-1 compared with RGERPPR.

3.3. In vitro cellular uptake of peptides

The cellular uptake and flow cytometry data for FITC-CRGERPPR, FITC-D(CRPPREGR), and FITC-D(CRGERPPR) were shown in Fig. 3. In the cell uptake experiments, higher fluorescent signals of FITC-D(CRGERPPR) were observed in HUVEC, C6, U87 and A549 cells compared with FITC-D(CRPPREGR) and FITC-D(CRGERPPR). The mean fluorescent intensity of FITC-D(CRGERPPR) in HUVECs was nearly 13.1 times higher than that of FITC-CRGERPPR, and 5.7 times higher than that of FITC-D(CRPPREGR). In C6 and U87 cells, the mean fluorescent intensity of FITC-D(CRGERPPR) was 5.4 times and 3.3 times higher than that of FITC-CRGERPPR, and 2.9 times and 3.5 times higher than FITC-D(CRPPREGR), respectively. FITC-positive

| Table 1 | Experimentally determined and predicted free energies of binding of the peptides with NRP-1 (ΔG = 1.3636 logKₐ). |
|---------|---------------------------------------------------------------|
| Peptide | Kₐ (nmol/L) | Experimental ΔG (kcal/mol) | Predicted ΔG (kcal/mol) |
| RGERPPR | 580 | −8.50 | −8.03 |
| D(RPPREGR) | 88.5 | −9.61 | −8.58 |
| D(RGERPPR) | 44.3 | −10.02 | −9.31 |
ratio of FITC-\(\psi\)CRGERPPR in A549 cells was 1.3 times and 1.6 times higher than that of FITC-D(RPPREGR) and FITC-CRGERPPR. These data indicated that the cellular uptake efficiencies of D(RGERPPR) by HUVEC, C6, U87 and A549 cells were higher than that of both RGERPPR and D(RPPREGR), which was consistent with the result of SPR interaction analyses above. As HUVECs can simulate tumor neovascularization\(^{23}\), we speculated that D(RGERPPR) possessed the capacity to target tumor vessels. Thus, D(RGERPPR) might have broad-spectrum tumor-penetrating ability.

### 3.4. Tumor-penetrating ability of D(RGERPPR)

Based on aforementioned investigations in vitro, we can conclude that D(RGERPPR) possessed higher NRP-1 binding affinity and better cellular uptake efficiencies than other two peptides. Next, we investigated the tumor-penetrating ability of D(RGERPPR) in vivo.

Evans blue is a water-soluble albumin-binding dye, which was used to assess the integrity of blood vessels\(^{24}\) as well as the tumor-penetrating effect mediated by NRP-1. Sugahara et al.\(^{25}\) designed a cyclic peptide containing the CendR motif, iRGD (CRGDKGPDC), possessing tumor-penetrating ability. The iRGD peptide was cleaved by protease to expose the cryptic CendR motif: CRGDK. The CendR peptide then mediated binding to NRP-1 resulting enhanced permeability of tumor blood vessels and tumor tissues, which was confirmed via quantification of tumor-specific entry of Evans blue into tumor tissues. In this study, D(RGERPPR) co-administered with the Evans blue dye, caused tumor-specific accumulation of the dye in all 3 tumor models we tested (Fig. 4). Compared with the control PBS group, D(RGERPPR) increased the Evans blue permeation by 36.4%, 30.5% and 46.4% in C6, U87 and A549 subcutaneous tumor models, respectively. By contrast, iRGD increased the Evans blue permeation by 49.7%, 17.7% and 32.9% in C6, U87 and A549 subcutaneous tumor models, respectively. According to a previous report, iRGD could remarkably increase the permeability of tumor tissues\(^{25}\). In our research, we found that the tumor-penetrating ability of D(RGERPPR) was comparable to that of iRGD in C6, U87 and A549 tumor-bearing nude mice. Furthermore, the Evans blue average permeation of PBS group in A549 tumor model was 3.04 times and 12.25 times higher than that of C6 tumor model and U87 tumor model, indicating that tumor tissue of A549 tumor model has better permeability. And the tumor-penetrating ability of D(RGERPPR) was the highest in A549 tumor-bearing mice among three tumor models. Based on the above considerations, the in vivo treatment study was then performed on the A549 subcutaneous tumor model.

### 3.5. In vivo anti-tumor efficiency

We examined gemcitabine and carboplatin, as these are mainly used as first-line therapy in clinical treatment for non-small-cell lung cancer. The in vitro cytotoxicity of gemcitabine and carboplatin were measured by MTT assays. The IC\(_{50}\) values were 4.83 × 10\(^{-6}\) mol/L for gemcitabine and 1.38 × 10\(^{-4}\) mol/L for carboplatin after 48 h treatment (Supplementary Information Fig. S3), indicating that gemcitabine exhibited higher growth inhibitory effects in A549 cells than carboplatin.

The in vivo antitumor efficiency was next investigated on nude mice bearing A549 cell-derived xenograft tumors. PBS, D(RGERPPR), gemcitabine, or gemcitabine combined with D(RGERPPR) were injected via the tail vein. To evaluate tumor growth, tumor volumes (\(V_n\)) were divided by their primary tumor volume (\(V_0\)) (Fig. 5). Compared with the PBS treatment group, the gemcitabine combined with D(RGERPPR) treatment was effective.
Figure 3  Representative images of cellular uptake and flow cytometry data for FITC-CRGERPPR, FITC-D(CRPPREGR), and FITC-D(CRGERPPR) in various cell lines as indicated. Percentages and numbers below in the flow cytometry data represent FITC-positive ratios and mean fluorescent intensities, respectively.
in retarding tumor growth, as shown in Fig. 5. The rate of tumor growth in the co-administration group was significantly lower than that of the gemcitabine alone group ($P < 0.05$), with a tumor suppression rate (TSR%) of 55.4%. The enhancement of anti-tumor effects could be attributed to the tumor-penetrating ability of D(RGERPPR), as evidenced above.

4. Discussion

Peptides possess low immunogenicity and high specificity, and are typically easy to synthesize with high quality. Consequently, peptides are widely applied in the treatment of tumors as well as cardiovascular, immune, and hematological diseases, and are also used as recognition molecules for drug delivery systems. However, natural bioactive peptides are susceptible to protease degradation and readily lose bioactivity in vivo. In addition, the chains of natural peptides are able to freely rotate and change shapes. Hence, they can completely or partially bind to various receptors, leading to low selectivity. The modification of the structure of bioactive peptides to enhance their stability, specificity, and selectivity has been a research hotspot. Generally, structural transformation of peptides mainly involves the following approaches: 1) substitution of unnatural amino acid, including D-amino acid, N-methyl-a-amino acid, $\beta$-amino acid, etc.; 2) cyclization of the peptide sequence; 3) isosteric, or not, amide bond replacement between two amino acids; 4) modification of N- or C-terminal ends; and 5) retro-inverso peptides. Among these methods, substitution of D-amino acids has remarkable superiority. First, because of the lack of protease that can degrade D-peptides in vivo, substitution of D-amino acid can enhance the biological stability and prolong the plasma half-life. Second, the substitution of D-amino acid also changes the conformation of peptides, which is beneficial to screen the bioactive peptides with high specificity and affinity to target protein.

In 1979, Goodman and Chorev established a retro-inverso peptide method that involved D-amino acids assembled in the reverse order from that in the parent L-sequence. Since the side chain topology of the retro-inverso peptide was similar to that of the parent L-peptide, the retro-inverso peptide might have similar bioactivities as the parent L-peptides. In our previous studies, we selected a CendR peptide (sequence RGERPPR) as the parent L-peptide, and designed its retro-inverso isomer, D(RGERPPR). Our results confirmed that D(RGERPPR) not only exhibited improved cellular uptake efficiency, but also possessed higher tumor-penetrating ability and better biological stability compared to RGERPPR.

Since D-peptide shows opposite chirality to the parent L-peptide with the same sequence, the inverso peptide might not have similar bioactivity. However, CendR peptides were a series of specific peptides with R/KXXR/K sequence, of which inverso isomer owned a D(R/KXXR/K) sequence. Since the C-terminal amino acid of the inverso isomer of CendR peptides was also arginine or lysine, we thus speculated that the inverso isomer also had NRP-1 binding activity and tumor-penetrating ability. In this study, we selected RGERPPR as the parent L-peptide and assembled D-amino acid residues in the order of the parent L-peptide sequence to synthesize the inverso peptide D(RGERPPR). As expected, the D(RGERPPR) possessed improved bio-stability (Supplementary Information Fig. S4). And the circular dichroism spectrum results showed that the absorption peak of D(RGERPPR) was enantiomorphous with RGERPPR, indicating that the optical rotation of D(RGERPPR) was opposite to RGERPPR. Surprisingly, the binding affinity of D(RGERPPR) with NRP-1 was significantly higher than that of
RGERPPR. This result could be explained by computer simulation analysis as following: the three DArg residues in ω(RGERPPR) fit well with the binding pocket and effectively interact with residues in NRP-1 by hydrogen bond, ion interaction and cation–π interaction. However, in the sequence RGERPPR, only Arg7 at C-terminal could interact well with the residues of NRP-1, and Arg1 and Arg4 formed weak interaction with the backbone of NRP-1. Therefore, ω(RGERPPR) possessed stronger binding affinity to NRP-1 than RGERPPR. In the cellular uptake test, the cell uptake amount of ω(RGERPPR) was significantly higher than that of iRGD-labeled RGERPPR. We also investigated the tumor-penetrating ability by Evans blue test and found that the tumor-penetrating ability of ω(RGERPPR) was comparable to iRGD in C6, U87 and A549 tumor-bearing nude mice. Due to its excellent biological activity, ω(RGERPPR) could accumulate at tumor tissues and bind to NRP-1 on the surface of tumor cells. By binding NRP-1, ω(RGERPPR) enhanced the permeability of tumor blood vessels and tumor tissues, which could facilitate anti-tumor agents to infiltrate into the tumor tissues.

5. Conclusions

By binding NRP-1, the CendR peptides can induce enhanced permeability of tumor blood vessels and tumor tissues. In this study, we selected a CendR peptide (sequence RGERPPR) as the parent 1- peptide and synthesized its inverse isomer ω(RGERPPR). The results indicated that ω(RGERPPR) possessed high binding affinity to NRP-1 and improved tumor cell internalization, which could enhance the permeability of tumor blood vessels and tumor tissues, as well as facilitate anti-tumor agents to penetrate deep into tumor parenchyma. In conclusion, our findings demonstrate that ω(RGERPPR) is a potential tumor-penetrating peptide and should be explored in further studies.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.apsb.2018.06.006.

References

1. Lu P, Weaver VM, Werb Z. The extracellular matrix: a dynamic niche in cancer progression. J Cell Biol 2011;196:385–406.
2. Gilkes DM, Semenza GL, Wirtz D. Hypoxia and the extracellular matrix: drivers of tumour metastasis. Nat Rev Cancer 2014;14:430–9.
3. Baxter LT, Jain RK. Transport of fluid and macromolecules in tumors. I. Role of interstitial pressure and convection. Microvasc Res 1989;37:77–104.
4. Roostad EK, Galupathii K, Mathiesen BS. Tumor interstitial fluid pressure—a link between tumor hypoxia, microvascular density, and lymph node metastasis. Neoplasia 2014;16:586.
5. Mohammadi M, Chen P. Effect of microvascular distribution and its density on interstitial fluid pressure in solid tumors: a computational model. Microvasc Res 2015;101:26–32.
6. Tiéblé O, Galmarini CM, Patel K, Tannock IF. Drug resistance and the solid tumor microenvironment. J Natl Cancer Inst 2007;99:1441–54.
7. Minchinton AI, Tannock IF. Drug penetration in solid tumours. Nat Rev Cancer 2006;6:583–92.
8. Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis?. Nat Rev Cancer 2004;4:891–9.
9. Takagi S, Tsuji T, Amagai T, Takamatsu T, Fujisawa H. Specific cell surface labels in the visual centers of Xenopus laevis tadpole identified using monoclonal antibodies. Dev Biol 1987;127:90–100.
10. Kolodkin AL, Levengood DV, Rowe EG, Tai YT, Giger RJ, Ginty DD. Neuritin is a semaphorin III receptor. Cell 1997;90:753–62.
11. Oh H, Takagi H, Otani A, Koyama S, Kemmochi S, Uemura A, et al. Selective induction of neurophin-1 by vascular endothelial growth factor (VEGF): a mechanism contributing to VEGF-induced angiogenesis. Proc Natl Acad Sci U S A 2002;99:383–8.
12. Miao HQ. Neurophin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell 1998;92:735–45.
13. Teesalu T, Sugahara KN, Kotamraju VR, Ruoasliati E. C-end rule peptides mediate neuplin-1-dependent cell, vascular, and tissue penetration. Proc Natl Acad Sci U S A 2009;106:16157–63.
14. Osada H, Tokunaga T, Nishi M, Hatanaka H, Abe Y, Tsuji A, et al. Overexpression of the neuropilin-1 (NRP1) gene correlated with poor prognosis in human glioma. Anticancer Res 2004;24:547–52.
15. Nasarre C, Roth M, Jacob L, Roth L, Koncina E, Thien A, et al. Peptide-based interference of the transmembrane domain of neuropilin-1 inhibits glioma growth in vivo. Oncogene 2010;29:2381–92.
16. Jing W, Yang L, Cao X, Lu W, Wagner E, Xie Z, et al. Retro-inverso CendR peptide-mediated polyethyleneimine for intracranial glioblastoma targeting gene therapy. Bioconjugate Chem 2014;25:414–23.
17. Liu M, Li X, Xie Z, Xie C, Zhan C; Hu X, et al. Peptides as recognition molecules and therapeutic agents. Chem Rev 2016;116:1772.
18. Goodman M, Chorev M. On the concept of linear modified retropeptide structures. Acc Chem Res 1979;12:1–7.
19. Schrödinger. Glide, version 6.6. New York: Schrödinger, LLC, 2013.
20. Halgren TA, Murphy RB, Friesner RA, Beard HS, Pollard WT, et al. Glide: a new approach for rapid, accurate docking and scoring. 2. Enrichment factors in database screening. J Med Chem 2004;47:1750–9.
21. Friesner RA, Murphy RB, Repasky MP, Frie LL, Greenwood JR, Halgren TA, et al. Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. J Med Chem 2006;49:6177.
22. Friesner RA, Banks JL, Murphy RB, Halgren TA, Klick RJ, Mainz DT, et al. Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. J Med Chem 2004;47:1739–49.
23. Lim HN, Jang JP, Han JM, Jung JH, Ahn JS, Jung HJ. Antiangiogenic potential of microbial metabolite elaiophyllin for targeting tumor angiogenesis. Molecules 2018;23:563.
24. Murohara T, Horowitz JR, Silver M, Tsurumi Y, Chen D, Sullivan A, et al. Vascular endothelial growth factor/vascular permeability factor enhances vascular permeability via nitric oxide and prostacyclin. Circulation 1998;97:99–107.
25. Sugahara KN, Teesalu T, Karmali PP, Kotamraju VR, Agemy L, Greenwald DR, et al. Coadministration of a tumor-penetrating peptide enhances the efficacy of cancer drugs. Science 2010;328:1031–5.
26. Vlieghje P, Lisowski V, Martinez J, Khrestchatisky M. Synthetic therapeutic peptides: science and market. Drug Discov Today 2010;15:40–56.
27. Wei X, Zhan C, Shen Q, Fu W, Xie C, Gao J, et al. A n-peptide ligand of nicotinamide acetaldehyde receptors for brain-targeted drug delivery. Angew Chem 2015;54:3023–7.