Peptide Probes with Aromatic Residues Tyr and Phe at the X Position Show High Specificity for Targeting Denatured Collagen in Tissues

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ABSTRACT: The construction of potent peptide probes for selectively detecting denatured collagen is crucial for a variety of widespread diseases. However, all of the denatured collagen-targeting peptide probes found till date primarily utilized the repetitive \( (\text{Gly-}X-\text{Y})_n \) sequences with exclusively imino acids Pro and Hyp in the X and Y positions, which stabilized the triple helical conformation of the peptide probes, resulting in severe obstacles for their clinical applications. A novel series of peptide probes have been constructed by incorporating nonimino acids at the X position of the \( (\text{GPO})_3\text{GXO}(\text{GPO})_4 \) sequence, while the X-site residue is varied as Tyr, Phe, Asp, and Ala, respectively. Peptide probes FAM-GYO and FAM-GFO containing aromatic residues Tyr and Phe at the X position showed similarly high binding affinity and tissue-staining efficacy as the well-established peptide probe FAM-GPO, while peptide probes FAM-GDO and FAM-GAO with the corresponding charged residue Asp and the hydrophobic residue Ala indicated much weaker binding affinity and tissue-staining capability. Furthermore, FAM-GYO and FAM-GFO could specifically detect denatured collagen in different types of mouse connective tissues and efficiently stain various human pathological tissues. We have revealed for the first time that the incorporation of nonimino acids, particularly aromatic residues at the X and Y positions of the repetitive \( (\text{Gly-}X-\text{Y})_n \) sequences, may provide a convenient strategy to create novel robust collagen-targeting peptide probes, which have promising diagnostic applications in collagen-involved diseases.

1. INTRODUCTION

Collagen is a group of extracellular matrix proteins with a unique triple helical structure. As the principal component of an extracellular matrix, collagen is ubiquitous in connective tissues such as skin, bone, tendon, and ligaments.\(^1\)\(^\text{−}\)\(^3\) It provides a vital structural scaffold for tissue development and mediates various cellular behaviors such as cell adhesion, proliferation, migration, and differentiation.\(^4\)\(^\text{−}\)\(^6\) Abnormal collagen remodeling has been found to be intimately correlated with various critical diseases such as cancer and fibrosis.\(^7\)\(^\text{−}\)\(^13\) Collagen is widely considered as the key constituent of a tumor microenvironment, and it can influence tumor cell behavior, increase tumor tissue stiffness, and regulate tumor immunity. Extensive clinical data have identified collagen as a prognostic factor for cancer differentiation, cancer invasion, lymph node metastasis, and the stage of cancer.\(^14\)\(^\text{−}\)\(^15\) Imbalanced collagen synthesis and degradation have also been reported as the leading causes of fibrosis in the lung and liver.\(^16\)\(^\text{−}\)\(^17\)

To decipher the pathogenesis and develop novel treatments of these collagen-related diseases, extensive research has been conducted to discover antibodies and peptide probes for detecting collagen. An ELISA-based screening has discovered two monoclonal antibodies E1E5 and E4A11 for specifically recognizing type II collagen.\(^18\) Two peptides WREPSFCALS and LRELHLNNNN derived from the collagen-binding proteins have been identified to target type I collagen.\(^19\)\(^\text{,}\)\(^20\) A novel class of peptide probes consisting of the repetitive \( (\text{Gly-Pro-Hyp})_n \) sequences have recently been found to specifically recognize denatured collagen.\(^21\) However, their collagen-targeting capability has been revealed to fully rely on the strict maintenance of monomer conformation, and heating or UV pretreatments are required to dissociate the peptide probes into the monomer state prior to the assay.\(^22\)\(^\text{,}\)\(^23\) Side-chain modifications of \( (\text{Gly-Pro-Hyp})_n \) sequences have been introduced to weaken the triple-helix stability as well as to maintain the specificity for collagen detection.\(^24\)}
The amino acid content has been shown as a determinant factor in the triple helical stability of collagen mimetic peptides. The close packing of the three chains of collagen requires Gly to be every third residue, leading to the characteristic (Gly-X-Y)n amino acid sequence pattern.25–30 The Gly-Pro-Hyp triplet has been found to be the most stabilizing sequence for the triple helical structure.31,32 Studies on a series of host–guest peptides have indicated that the identity of the residue in the X and Y positions of the (Gly-X-Y)n sequence significantly influenced their triple helical stability. It revealed that Pro and Arg were the most favored residues in the X position to stabilize the triple helical structure, while aromatic residues Y, F, and W were the most destabilizing residues in the X position.33–39

To discover novel collagen-targeting peptides with weaker triple helical propensity, we have constructed a series of peptide probes consisting of the (GPO)3GXO(GPO)4 sequences (O, (2S,4R)-4-hydroxyproline), while X represents different types of amino acids. We have systematically investigated the effect of the identity of the X residue on the collagen-targeting capability and have found two peptide probes FAM-GYO and FAM-GFO with the aromatic residues Tyr and Phe in the X position displaying superior binding affinity toward denatured collagen. It provided a new convenient strategy to construct collagen-targeting peptides by incorporating appropriate nonimino acids at the X and Y positions, which have promising diagnostic applications in collagen-involved diseases.

Table 1. Construction of Collagen-Targeting Peptide Probes

| name       | sequence                     | m/z calculated | m/z found |
|------------|------------------------------|----------------|-----------|
| FAM-GPO    | FAM-(GPO)₃GPO(GPO)₄         | 2536.6         | 2536.8    |
| FAM-GYO    | FAM-(GPO)₃GYO(GPO)₄         | 2586.7         | 2587.0    |
| FAM-GFO    | FAM-(GPO)₃GFO(GPO)₄         | 2602.7         | 2602.0    |
| FAM-GDO    | FAM-(GPO)₃GDO(GPO)₄         | 2531.6         | 2531.3    |
| FAM-GAO    | FAM-(GPO)₃GAO(GPO)₄         | 2484.5         | 2484.8    |
| FAM-Control| FAM-PPPGGOOGOPPGGGOOOGPG    | 2536.6         | 2536.9    |

“Sequences and Mass Characterization of the Peptide Probes. FAM, 5(6)-carboxyfluorescein; O, (2S,4R)-4-hydroxyproline.

2. RESULTS AND DISCUSSION

2.1. Design of Fluorescent Collagen-Targeting Peptide Probes

All of the reported peptide probes that could specifically recognize denatured collagen are composed of the (Gly-X-Y)n sequences, with the residues at the X and Y positions as purely imino acids (Pro or Hyp). The inclusion of imino acids at the X and Y positions is known to strongly stabilize the triple helical structure, which could pose severe drawbacks of the peptide probes to target denatured collagen, which required a single-stranded conformation. Herein, we have constructed a novel series of peptide probes by introducing nonimino acids at the X positions of the collagen-targeting sequences (GPO)₃GXO(GPO)₄ (Table 1). FAM (5(6)-carboxyfluorescein), a widely used fluorescent dye, is conjugated with the N-terminal of the peptide sequences. Peptide probe FAM-GPO containing Pro at the X position is constructed as a control, which has been shown to bind denatured collagen with high selectivity. Peptide probes containing different types of imino acids (Tyr, Phe, Asp, and Ala) at the X position are denoted as FAM-GYO, FAM-GFO, FAM-GDO, and FAM-GAO, respectively (Table 1). The substitution of Pro by aromatic residues Tyr and Phe, the charged residue Asp, and the hydrophobic residue Ala has been reported to weaken the triple helical stability of collagen mimetic peptides in the context of (GPO)₃GXO(GPO)₄ by 13, 13.8, 7.2, and 5.6 °C, respectively.34

Furthermore, peptide probes FAM-2GYO and FAM-2GAO are designed to contain Tyr and Ala at two X positions of the FAM-(GPO)₂GXOGPXO(GPO)₃ sequences, respectively, to evaluate if the introduction of multiple nonimino acids affects the collagen-binding affinity (Table 1). Peptide probe
Peptide probes FAM-GYO and FAM-2GYO both displayed ability (Figure 1a). The collagen-targeting capability of the three peptide probes may modulate their collagen-binding affinity differently.

2.2. Collagen-Targeting Capability of the Fluorescent Peptide Probes. The collagen-targeting capability of the peptide probes FAM-GPO, FAM-GYO, FAM-GFO, FAM-GDO, FAM-GAO, FAM-2GYO, FAM-2GAO, and FAM-control was evaluated by the binding experiments (Figure 1a). As expected, peptide probe FAM-GPO showed high binding affinity toward gelatin, while the negative control probe FAM-control showed little binding. Notably, FAM-GYO and FAM-GFO showed similarly high fluorescence intensity as FAM-GPO, suggesting that the inclusion of aromatic residues Tyr and Phe at the X position did not alter their collagen-binding affinity. However, FAM-GDO and FAM-GAO showed much weaker fluorescence intensity, indicating that the presence of charged residue Asp and hydrophobic residue Ala significantly reduced their collagen-binding ability.

Furthermore, the introduction of Tyr or Ala at two X positions led to much weaker binding affinity than that at only one X position, suggesting that two or more substitutions of Pro by other types of residues in the (Gly-Pro-Hyp)₈ sequence may significantly interfere in the collagen-binding capability. Peptide probes FAM-GYO and FAM-2GYO both displayed much higher binding affinity than their counterparts FAM-GAO and FAM-2GAO, indicating that Tyr is a more favorable residue at the X position than Ala in terms of collagen-binding ability (Figure 1a).

Since peptide probes FAM-GYO and FAM-GFO showed similarly strong binding affinity as FAM-GPO, their specificity to recognize denatured collagen was further examined (Figure 1b). Wells of a 96-well plate were coated with denatured type I collagen, pepsin, trypsin, and hemoglobin, respectively. Similarly as FAM-GPO, FAM-GYO and FAM-GFO showed little binding with all other proteins besides denatured type I collagen (Figure 1b). It demonstrated that peptide probes FAM-GYO and FAM-GFO could detect denatured collagen with high specificity.

2.3. Tissue Staining by Fluorescent Peptide Probes. The tissue-staining capabilities of the peptide probes were further examined. Fluorescence micrographs of stained normal intestine sections all showed a little green fluorescence, indicating the inability of the three peptide probes to target intact collagen (Figure 3a,d,g). In contrast, the fluorescence micrographs of the stained impaired intestine sections all showed a significant green fluorescence, demonstrating the high specificity of the three peptide probes to recognize denatured collagen (Figure 3b,e,h). The co-staining of 4′,6-diamidino-2-phenylindole (DAPI) for the cell nuclei (blue) confirmed the distinct collagen distribution in intestine tissues (Figure 3).

Inhibition experiments were performed using peptide G(POG)₁₀ as an inhibitor to evaluate the binding pattern of the three peptide probes FAM-GPO, FAM-GYO, and FAM-GFO (Figure 3c,f,i). Peptide G(POG)₁₀ was applied onto the intestine tissues prior to the staining with the peptide probes. In the presence of G(POG)₁₀, the fluorescence micrographs of the impaired intestine tissues all showed a weak fluorescence, indicating that G(POG)₁₀ efficiently blocked the staining of denatured collagen by the peptide probes. Similarly as FAM-GPO, peptide probes FAM-GYO and FAM-GFO probably shared the same mechanism by hybridizing with the unfolded sites and specifically binding to denatured collagen.

Peptide probes FAM-GYO and FAM-GFO were further applied to stain impaired mouse heart, cornea, and cartilage, which was consistent with previous reports (Figure 2a). Fluorescence micrographs of stained mouse ear tissues showed that FAM-GPO selectively recognizes denatured collagen, while FAM-GYO and FAM-GFO displayed the strongest fluorescence and thus highest staining efficacy (Figure 2b,c). In contrast, FAM-GDO, FAM-GAO, and FAM-2GYO showed weaker fluorescence and less staining efficacy, while FAM-2GAO and FAM-control showed a little green fluorescence and complete loss of the collagen-targeting capability (Figure 2d–h). These results demonstrated that peptide probes FAM-GYO and FAM-GFO possessed similarly strong tissue-staining capability as FAM-GPO.

2.4. Specific Recognition of Denatured Collagen by Peptide Probes FAM-GYO and FAM-GFO. The collagen-targeting features of the two most promising peptide probes FAM-GYO and FAM-GFO were further examined (Figure 3). Normal and impaired mouse intestine tissue sections were stained with FAM-GPO, FAM-GYO, and FAM-GFO. Fluorescence micrographs of the stained normal intestine sections all showed a little green fluorescence, indicating the inability of the three peptide probes to target intact collagen (Figure 3a,d,g). In contrast, the fluorescence micrographs of the stained impaired intestine sections all showed a significant green fluorescence, demonstrating the high specificity of the three peptide probes to recognize denatured collagen (Figure 3b,e,h). The co-staining of 4′,6-diamidino-2-phenylindole (DAPI) for the cell nuclei (blue) confirmed the distinct collagen distribution in intestine tissues (Figure 3).

Fluorescence micrographs of impaired mouse ear tissues stained with FAM-GPO showed a strong green fluorescence, confirming that FAM-GPO selectively recognizes denatured collagen, which was consistent with previous reports (Figure 2a). Fluorescence micrographs of impaired mouse ear tissues stained with other peptide probes showed different levels of green fluorescence, while FAM-GYO and FAM-GFO displayed the strongest fluorescence and thus highest staining efficacy (Figure 2b,c). In contrast, FAM-GDO, FAM-GAO, and FAM-2GYO showed weaker fluorescence and less staining efficacy, while FAM-2GAO and FAM-control showed a little green fluorescence and complete loss of the collagen-targeting capability (Figure 2d–h). These results demonstrated that peptide probes FAM-GYO and FAM-GFO possessed similarly strong tissue-staining capability as FAM-GPO.

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the stained tissues showed a strong green fluorescence, demonstrating the robustness of both peptide probes to target denatured collagen in a broad variety of connective tissues. DAPI was applied to stain the nuclei (blue) and colocalize collagen (Figure 4).

### 2.5. Human Pathological Tissue Staining by Peptide Probes FAM-GYO and FAM-GFO

The applicability of peptide probes FAM-GYO and FAM-GFO to specifically target denatured collagen in human pathological tissues was further examined (Figures 5 and 6). Fluorescence micrographs of formalin-fixed paraffin-embedded (FFPE) tissues of human liver fibrosis, liver cancer, rectal cancer, and esophageal cancer stained with FAM-GYO all showed an intense green fluorescence, indicating that FAM-GYO could detect degenerated collagen in various pathological tissues (Figure 5). DAPI costaining of the nuclei confirmed the unique collagen distribution in a diseased connective tissue (Figure 5). The fluorescence micrographs of FFPE tissues of human liver fibrosis, liver cancer, rectal cancer, and esophageal cancer stained with FAM-GFO and DAPI demonstrated that FAM-GFO could highly sensitively and selectively target denatured collagen in different types of pathological connective tissues (Figure 6). It suggested that the two novel peptide probes FAM-GYO and FAM-GFO were similar broad-spectrum biosensors as FAM-GPO for detecting denatured collagen.

### 3. CONCLUSIONS

The discovery of novel peptide probes to specifically detect denatured collagen has received increasing attention since denatured collagen is intimately correlated with various critical diseases such as cancer.\(^{40−42}\) Recent progress of the collagen-targeting peptide probes is predominantly dependent on the inclusion of the repetitive (Gly-Pro-Hyp)\(\_n\) sequences, whose high triple helical stability may hinder their clinical applications.\(^{43,44}\) Previous studies have indicated that the presence of nonimino acids in the X and Y positions of the (Gly-X-Y)\(\_n\) sequences would weaken the triple helical stability, while the extent of the destabilization depended on the identity of the nonimino acids.\(^{35,46}\)

Herein, we have constructed a series of peptide probes by introducing nonimino acids at the X position of the (GPO\(\_3\)GXO(GPO\(\_4\)) sequence, while the X-site residue is varied as Tyr, Phe, Asp, and Ala, respectively. We have systematically investigated the effect of the identity of the X residue on the collagen-targeting capability by utilizing protein-binding assays and tissue-staining experiments. Compared with Pro at the X position, the inclusion of aromatic residues Tyr and Phe did not significantly affect the binding affinity and tissue-staining efficacy of the peptide probes, while the presence of a charged residue Asp and a hydrophobic residue Ala largely reduced their binding affinity and tissue-staining capability. In addition, the replacement of Pro by Tyr or Ala at two X positions significantly interfered in the binding and staining of collagen by the peptide probes, suggesting that the inclusion of multiple nonimino acids may impair their collagen-targeting efficiency.

The two peptide probes FAM-GYO and FAM-GFO containing aromatic residues Tyr and Phe at a single X position have further been shown to specifically target denatured collagen in various types of mouse connective tissues and they have been successfully applied for efficiently staining different human pathological tissues. We have revealed for the first time that aromatic residues could be incorporated in the construction of robust collagen-targeting peptides. Previous studies have shown that aromatic interactions could promote the self-association of triple helical peptides and fibrillogenesis of type I collagen.\(^{38,39}\) Our results suggested that aromatic interactions may facilitate the peptide probes to bind with denatured collagen. Among all types of imino acids at the X position, aromatic residues have been shown to be the most destabilizing residues for a triple-helix conformation. The incorporation of nonimino acids, particularly aromatic residues at the X and Y positions, may provide a convenient strategy to create novel potent collagen-targeting peptide probes, which have promising diagnostic applications in collagen-involved diseases.

### 4. EXPERIMENTAL SECTION

#### 4.1. Materials

Rink amide resin (200−400 mesh, loading = 0.345 mmol/g), Fmoc-Gly-OH, Fmoc-Pro-OH, Fmoc-
Hyp(tBu)-OH, Fmoc-Asp(Otbu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Ala-OH, Fmoc-Phe-OH, O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluorophosphate (HBTU), and N-hydroxybenzotriazole (HOBt, anhydrous) were purchased from GL Biochemical Company (Shanghai, China). N,N-Diisopropylethylamine (DIEA) was obtained from Hanhong Chemical Technology Co. Ltd (Shanghai, China). 5(6)-Carboxyfluorescein (FAM) and piperazine (anhydrous) were provided by Aladdin Industrial Corporation (Shanghai, China). Trifluoroacetic acid (TFA) and triisopropylsilane (Tis) were purchased from Tokyo Chemical Industry Co. Ltd (Tokyo, Japan). Bovine serum albumin (BSA), goat serum, and 4',6-diamidino-2-phenylindole (DAPI) were obtained from Solarbio Science & Technology Co. Ltd (Beijing, China). Hemoglobin, trypsin, and pepsin were obtained from Yuanye Biological Technology Co. Ltd (Shanghai, China). Gelatin was obtained from Biotop Science & Technology Co. Ltd (Beijing, China). All of the commercial reagents were of analytical grade and were used without further purification.

4.2. Peptide Synthesis. Peptides were synthesized in-house by a standard Fmoc solid-phase synthesis (SPPS) method. Fmoc-amino acids (Fmoc-Gly-OH, Fmoc-Pro-OH, Fmoc-Hyp(tBu)-OH, Fmoc-Asp(Otbu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Phe-OH, and Fmoc-Ala-OH) (5 equiv), HBTU (5 equiv), and DIEA (8 equiv) in N,N-dimethylformamide (DMF) were applied during each step of amino acid coupling. Resin was washed by DMF (3 × 10 mL) and dichloromethane (DCM, 2 × 10 mL). Fmoc protection groups were then eliminated by 5% piperazine in DMF. The status of coupling and deprotection reactions was monitored by the chloranil test. After the completion of coupling all amino acids, FAM was conjugated to the N-terminal of the peptide using FAM (5 equiv), HBTU (5 equiv), and DIEA (8 equiv) in DMF. At the end of peptide synthesis, the resins were treated with TFA/TIS/H2O (95:2.5:2.5) for 3 h to remove the tBu and Otbu groups.
groups and release the peptide from the resin. The peptides were harvested by precipitation with cold Et2O. Crude peptides were collected after resuspension in cold Et2O, sonication, and centrifugation. All of the peptides were purified using reverse-phase high-performance liquid chromatography (HPLC) on a C18 column, and the purity of the peptides was confirmed by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (Bruker, Germany). The peptide was lyophilized and stored at −20 °C for future use.

4.3. Protein-Binding Assay. Gelatin (1 mg/mL) was prepared in 10 mM PBS (pH 7.4) at 70 °C. Seventy microliters of gelatin was added into each well of a 96-well plate and air-dried. After coating, the gelatin film was washed with 400 μL of 10 mM PBS (pH 7.4) for 3 min three times. Hundred microliters of the solution of BSA in 10 mM PBS (pH 7.4) (1% v/v) was added and incubated at room temperature for 1 h to block nonspecific binding. The plate was washed with 400 μL of 10 mM PBS (pH 7.4) for 3 min three times. Seventy microliters of the solution of 20 μM fluorescent peptide probes (FAM-GPO, FAM-GYO, FAM-GFO, FAM-GDO, FAM-GAO, FAM-2GYO, FAM-2GAO, or FAM-control) in 10 mM PBS (pH 7.4) was added to each well and incubated at 4 °C for 4 h to ensure their binding to the gelatin films. All fluorescent peptide probes were heated at 80 °C for 15 min and quenched in ice water for 30 s prior to the usage. The wells were washed with 400 μL of 10 mM PBS (pH 7.4) for 5 min three times. Fluorescence (ex: 495 nm, em: 541 nm) was measured on an Infinite M200 (TECAN Corporation, Switzerland). Each measurement was repeated three times.

The binding affinity of the peptide probes FAM-GPO, FAM-GYO, and FAM-GFO toward denatured collagen, hemoglobin, pepsin, and trypsin was similarly determined. Type I collagen was dissolved in a 0.5 M acetic acid solution and denatured by heating at 70 °C for 15 min. Solutions of collagen, hemoglobin, pepsin, and trypsin with a concentration of 1 mg/mL were prepared in 10 mM PBS (pH 7.4). The protein solutions were added into the wells of a 96-well plate and air-dried. After coating, the protein film was washed with 400 μL of 10 mM PBS (pH 7.4) for 3 min three times. FAM-GPO, FAM-GYO, and FAM-GFO were heated at 80 °C for 15 min and quenched in ice water for 30 s. The three peptide probes were then added to each well, respectively, and incubated at 4 °C for 4 h. The wells were washed with 400 μL of 10 mM PBS (pH 7.4) for 5 min three times. Fluorescence (ex: 495 nm, em: 541 nm) was measured on an Infinite M200 (TECAN Corporation, Switzerland). Each measurement was repeated three times.

4.4. Tissue Staining and Imaging. The ear, intestine, heart, cartilage, and eye tissues were obtained from 7–8 week old KM mice (18–22 g). All tissues were fixed with 4% paraformaldehyde in 10 mM PBS (pH 7.4) for 1 h and embedded in paraffin. The tissues were sectioned to 4 μm thickness on poly-lysine-treated glass slides. Paraffin was washed away using xylene, 100% ethanol, 95% ethanol, 50% ethanol, and deionized water for two cycles of 5 min for each solvent in a consecutive order. Goat serum solution (0.5 mL) in PBS (5% v/v) was added onto each tissue slide and incubated at room temperature for 30 min to block nonspecific binding. The blocking solution was removed with a paper towel.

The solutions of peptide probes (FAM-GPO, FAM-GYO, FAM-GFO, FAM-GDO, FAM-GAO, FAM-2GYO, FAM-2GAO, and FAM-control) were heated at 80 °C for 15 min and quenched in ice water for 30 s prior to the usage. Normal and thermally impaired tissue sections were treated with 100 μL of solutions of preheated peptide probes (15 μM) in 10 mM PBS (pH 7.4), and incubated at 4 °C for 4 h. The slides were covered with parafilm to prevent drying during the incubation. After the staining using the peptide probes, the paraffin was removed and excess solution was wiped away by a paper towel. Two hundred microliters of DAPI (5 μg/mL) solution in 10 mM PBS was applied to each tissue slide and incubated at room temperature for 1 min. After DAPI staining, the slides were immersed in a staining tank in 10 mM PBS buffer for 5 min 5 times to wash off the unbound DAPI. Denatured collagen (FAM channels) and nuclei (DAPI channels) in the tissue slides were imaged on a Leica DM4000B metallurgical upright microscope (Leica Microsystems Inc., Wetzlar, Germany).

For the inhibition experiments, a solution of an inhibitor peptide G(POG)10 (15 μM) was prepared and heated at 85 °C for 30 min to make the peptide in the single-stranded conformation. Two hundred microliters of the G(POG)10 solution was immediately applied onto the impaired intestine tissues prior to staining the tissue slides using fluorescent peptide probes. The tissue slides were incubated at 4 °C for 4 h to ensure complete binding of the inhibitor with denatured collagen. PBS buffer (10 mM, pH 7.4) was applied five times to rinse the tissue slides and eliminate the unbound inhibitors.

4.5. Pathological Human Tissue Staining and Imaging. Pathological human tissues of liver fibrosis, liver cancer, rectal cancer, and esophageal cancer were provided by The First Hospital of Lanzhou University. The tissues were fixed with 4% paraformaldehyde in a 10 mM PBS solution (pH 7.4) for 1 h and embedded in paraffin. The tissues were sectioned to 4 μm thickness on poly-lysine-treated glass slides. Paraffin was washed away using xylene, 100% ethanol, 95% ethanol, 50% ethanol, and deionized water for two cycles of 5 min for each solvent in a consecutive order. Goat serum solution (0.5 mL) in PBS (5% v/v) was added onto each tissue slide and incubated at room temperature for 30 min to block nonspecific binding. The blocking solution was removed with a paper towel.

The solutions of peptide probes FAM-GYO and FAM-GFO (15 μM) were heated at 80 °C for 15 min and quenched in ice water for 30 s prior to the usage. The tissue sections were treated with 100 μL of solutions of preheated peptide probes in 10 mM PBS (pH 7.4), and incubated at 4 °C for 4 h. The slides were covered with parafilm to prevent drying during the incubation. After the staining using the peptide probes, the paraffin was removed and excess solution was wiped away by a paper towel. Two hundred microliters of a DAPI (5 μg/mL) solution in 10 mM PBS was applied to each tissue slide and incubated at room temperature for 1 min. After DAPI staining, the slides were immersed in a staining tank in 10 mM PBS buffer for 5 min 5 times to wash off the unbound DAPI. Denatured collagen (FAM channels) and nuclei (DAPI channels) in the tissue slides were imaged on a Leica DM4000B metallurgical upright microscope (Leica Microsystems Inc., Wetzlar, Germany).
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This manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

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REFERENCES

(1) Exposito, J. Y.; Valcourt, U.; Cluzel, C.; Lehias, C. The fibrillar collagen family. Int. J. Mol. Sci. 2010, 11, 407–426.
(2) Ricard-Blum, S. The collagen family. Cold Spring Harbor Perspect. Biol. 2011, 3, No. a004978.
(3) Shoulders, M. D.; Raines, R. T. Collagen Structure and Stability. Annu. Rev. Biochem. 2009, 78, 929–958.
(4) Liotta, L. A.; Tryggvason, K.; Garbisa, S.; Hart, I.; Foltz, C. M.; Shafie, S. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature 1980, 284, 67–68.
(5) Prockop, D. J.; Kivirikko, K. I. Collagens: molecular biology, diseases, and potentials for therapy. Annu. Rev. Biochem. 1995, 64, 403–434.
(6) Sorushanova, A.; Delgado, L. M.; Wu, Z.; Shologu, N.; Kharisgar, A.; Raghunath, R.; Mullen, A. M.; Bayon, Y.; Pandit, A.; Raghunath, M.; Zeugolis, D. I. The Collagen Suprafamily: From Biosynthesis to Advanced Biomaterial Development. Adv. Mater. 2019, 31, No. e1801651.
(7) Tlsty, T. D.; Coussens, L. M. Tumor stroma and regulation of cancer development. Annu. Rev. Pathol.: Mech. Dis. 2006, 1, 119–150.
(8) McClneroy, W.; Lee, T. H.; Atabai, K. Always cleave up your mess: targeting collagen degradation to treat tissue fibrosis. Am. J. Physiol.: Lung Cell. Mol. Physiol. 2013, 304, L709–L721.
(9) Myllyharju, J.; Kivirikko, K. I. Collagens and collagen-related diseases. Ann. Med. 2001, 33, 7–21.
(10) De Palma, M.; Biziato, D.; Petrovta, T. V. Microenvironmental regulation of tumour angiogenesis. Nat. Rev. Cancer 2017, 17, 457–474.
(11) Kalluri, R. Basement membranes: Structure, assembly and role in tumor angiogenesis. Nat. Rev. Cancer 2003, 3, 422–433.
(12) Chu, G. C.; Kimmelman, A. C.; Hezel, A. F.; DePinho, R. A. Stromal biology of pancreatic cancer. J. Cell. Biochem. 2007, 101, 887–907.
(13) Payne, L. S.; Huang, P. H. The Pathobiology of Collagens in Glioma. Mol. Cancer Res. 2013, 11, 1129–1140.
(14) Xu, S.; Xu, H.; Wang, W.; Li, S.; Li, H.; Li, T.; Zhang, W.; Yu, X.; Liu, L. The role of collagen in cancer: from bench to bedside. J. Transl. Med. 2019, 17, 1–22.
(15) Kessnbrook, K.; Plaks, V.; Web, Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 2010, 141, 52–67.
(16) Seandel, M.; Noack-Kunnmann, K.; Zhu, D.; Aimes, R. T.; Quigley, J. P. Growth factor-induced angiogenesis in vivo requires specific cleavage of fibrillar type I collagen. Blood 2001, 97, 2323–2332.
(17) Biasin, V.; Wogreczka, M.; Marsh, L. M.; Becker-Pauli, C.; Bracic, L.; Ghanim, B.; Klepetko, W.; Olschewski, A.; Kwapiszewski, G. Meprin β contributes to collagen deposition in lung fibrosis. Sci. Rep. 2017, 7, No. 39969.
(18) Srivivas, G. R.; Barrach, H. J.; Chichester, C. O. Quantitative immunoassays for type II collagen and its cyanoan bromide peptides. J. Immunol. Methods 1993, 159, 53–62.
(19) Federico, S.; Pierce, B. F.; Piluso, S.; Wischke, C.; Lendlein, A.; Neffe, A. T. Design of Decorin-Based Peptides That Bind to Collagen I and their Potential as Adhesion Moieties in Biomaterials. Angew. Chem., Int. Ed. 2015, 54, 10980–10984.
(20) Takagi, J.; Asai, H.; Saijo, Y. A collagen/ gelatin-binding decapetide derived from bovine propolypeptide of von Willebrand factor. Biochemistry 1992, 31, 8530–8534.
(21) Wang, A. Y.; Mo, X.; Chen, C. S.; Yu, S. M. Facile modification of collagen directed by collagen mimetic peptides. J. Am. Chem. Soc. 2005, 127, 4130–4131.
(22) Li, Y.; Foss, C. A.; Summerfield, D. D.; Doyle, J. J.; Torok, C. M.; Dietz, H. C.; Pomper, M. G.; Yu, S. M. Targeting collagen strands by photo-triggered triple-helix hybridization. Proc. Natl. Acad. Sci. U.S.A. 2012, 109, 14767–14772.
(23) Li, Y.; Yu, S. M. Targeting and mimicking collagens via triple helical peptide assembly. Curr. Opin. Chem. Biol. 2013, 17, 968–975.
(24) Cai, X.; Liu, Z.; Zhao, S.; Song, C.; Dong, S.; Xiao, J. A single stranded fluorescent peptide probe for targeting collagen in connective tissues. Chem. Commun. 2017, 53, 11905–11908.
(25) Beck, K.; Chan, V. C.; Shenoy, N.; Kirkpatrick, A.; Ramshaw, J. A.; Brodsky, B. Destabilization of osteogenesis imperfecta collagen-like model peptides correlates with the identity of the residue replacing glycosylation. Proc. Natl. Acad. Sci. U.S.A. 2000, 97, 4273–4278.
(26) Bella, J.; Eaton, M.; Brodsky, B.; Berman, H. M. Crystal and molecular structure of a collagen-like peptide at 1.9 Å resolution. Science 1994, 266, 75–81.
(27) Cowan, P. M.; McGavin, S.; North, A. C. The polypeptide chain configuration of collagen. Nature 1955, 176, 1062–1064.
(28) Rich, A.; Crick, F. H. The molecular structure of collagen. J. Mol. Biol. 1961, 3, 483−506.
(29) Fraser, R. D.; MacRae, T. P.; Miller, A. Molecular packing in type I collagen fibrils. J. Mol. Biol. 1987, 193, 115−125.
(30) Hulmes, D. J.; Miller, A.; Parry, D. A.; Pier, K. A.; Woodhead-Galloway, J. Analysis of the primary structure of collagen for the origins of molecular packing. J. Mol. Biol. 1973, 79, 137−148.
(31) Bella, J.; Brodsky, B.; Berman, H. M. Hydration structure of a collagen peptide. Structure 1995, 3, 893−906.
(32) Privalov, P. L. Stability of proteins: Proteins which do not present a single cooperative system. In Advances in Protein Chemistry; Academic Press, 1982; Vol. 35, pp 1−104.
(33) Persikov, A. V.; Ramshaw, J. A.; Brodsky, B. Prediction of collagen stability from amino acid sequence. J. Biol. Chem. 2005, 280, 19343−19349.
(34) Persikov, A. V.; Ramshaw, J. A.; Kirkpatrick, A.; Brodsky, B. Amino acid propensities for the collagen triple-helix. Biochemistry 2000, 39, 14960−14967.
(35) Chan, V. C.; Ramshaw, J. A.; Kirkpatrick, A.; Beck, K.; Brodsky, B. Positional preferences of ionizable residues in Gly-X-Y triplets of the collagen triple-helix. J. Biol. Chem. 1997, 272, 31441−31446.
(36) Shah, N. K.; Ramshaw, J. A.; Kirkpatrick, A.; Shah, C.; Brodsky, B. A host-guest set of triple-helical peptides: stability of Gly-X-Y triplets containing common nonpolar residues. Biochemistry 1996, 35, 10262−10268.
(37) Yang, W.; Chan, V. C.; Kirkpatrick, A.; Ramshaw, J. A.; Brodsky, B. Gly-Pro-Arg confers stability similar to Gly-Pro-Hyp in the collagen triple-helix of host-guest peptides. J. Biol. Chem. 1997, 272, 28837−28840.
(38) Cejas, M. A.; Kinney, W. A.; Chen, C.; Leo, G. C.; Toungue, B. A.; Vinter, J. G.; Joshi, P. P.; Maryanoff, B. E. Collagen-related peptides: Self-assembly of short, single strands into a functional biomaterial of micrometer scale. J. Am. Chem. Soc. 2007, 129, 2202−2203.
(39) Cejas, M. A.; Kinney, W. A.; Chen, C.; Vinter, J. G.; Almond, H. R.; Balss, K. M.; Maryanoff, C. A.; Schmidt, U.; Breslav, M.; Mahan, A.; Lacy, E.; Maryanoff, B. E. Thrombogenic collagen-mimetic peptides: Self-assembly of triple helix-based fibrils driven by hydrophobic interactions. Proc. Natl. Acad. Sci. U.S.A. 2008, 105, 8513−8518.
(40) Jugdutt, B. I.; Joliat, M. J.; Khan, M. I. Rate of collagen deposition during healing and ventricular remodeling after myocardial infarction in rat and dog models. Circulation 1996, 94, 94−101.
(41) Motola, D. L.; Caravan, P.; Chung, R. T.; Fuchs, B. C. Noninvasive Biomarkers of Liver Fibrosis: Clinical Applications and Future Directions. Curr. Pathobiol. Rep. 2014, 2, 245−256.
(42) Lotta, L. A.; Kohn, E. C. The microenvironment of the tumour-host interface. Nature 2001, 411, 375−379.
(43) Hwang, J.; Huang, Y.; Burwell, T. J.; Peterson, N. C.; Connor, J.; Weiss, S. J.; Yu, S. M.; Li, Y. In Situ Imaging of Tissue Remodeling with Collagen Hybridizing Peptides. ACS Nano 2017, 11, 9825−9835.
(44) Li, Y.; Foss, C. A.; Pomper, M. G.; Yu, S. M. Imaging denatured collagen strands in vivo and ex vivo via photo-triggered hybridization of caged collagen mimetic peptides. J. Visualized Exp. 2014, 83, No. e51052.
(45) Persikov, A. V.; Ramshaw, J. A.; Brodsky, B. Collagen model peptides: Sequence dependence of triple-helix stability. Biopolymers 2000, 55, 436−450.
(46) Persikov, A. V.; Ramshaw, J. A.; Kirkpatrick, A.; Brodsky, B. Peptide investigations of pairwise interactions in the collagen triple-helix. J. Mol. Biol. 2002, 316, 385−394.