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Targeting Gelatinases with a Near-Infrared Fluorescent Cyclic His-Try-Gly-Phe Peptide

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Abstract

Purpose—To synthesize and characterize near-infrared (NIR) fluorescence imaging probes targeted to gelatinases.

Procedures—A phage display-selected cyclic peptide containing the His-Try-Gly-Phe (HWGF) motif was used as the lead compound. Structure-activity relationship analysis was used to identify stable and potent gelatinase inhibitors suitable for NIR imaging applications.

Results—Replacing the S-S bond in cyclic peptide c(CTTHWGFTLC)NH₂ (**C1**) with an amide bond between the ε-amino group of Lys and the side chain of Asp resulted in a significant increase in stability and a 4-fold increase in gelatinase inhibition of the resulting peptide, c(KAHWGFTLD)NH₂ (**C6**). Conjugation of Cy5.5 to **C6** led to Cy5.5-C6, which was selectively taken up by MMP-2 expressing human glioma U87 cells. *In vivo*, selective accumulation of Cy5.5-C6, but not Cy5.5-C1 or a Cy5.5-scrambled peptide conjugate, was visualized in intratibial prostate PC-3 tumors 48 hr after their intravenous injection. Moreover, Cy5.5-C6 was readily visualized in orthotopically inoculated U87 brain tumors.

Conclusions—Cy5.5-C6 may be a useful agent for molecular imaging of gelatinases. The approach of producing stable cyclic peptides through side chain amide linkage should be applicable to other peptide-based imaging agents.

Keywords

Near-infrared fluorescent imaging; gelatinases; Optical imaging; Cyclic peptides

Introduction

Optical imaging is increasingly used to probe protein function and gene expression in live animals. Of the various optical imaging techniques investigated to date, near-infrared (NIR,

700- to 900-nm wavelength) fluorescence (NIRF) imaging is of particular interest for noninvasive *in vivo* imaging because of the relatively low tissue absorbance and minimal autofluorescence of NIR light.^{1–3} A number of NIRF contrast-enhanced optical imaging probes have been developed and evaluated in small animals.^{4–13} These studies have established the use of NIR optical imaging in diagnosis, molecular characterization, and monitoring of treatment response in a number of disease models.

The matrix metalloproteinases (MMPs) are a family of enzymes capable of degrading the constituents of the extracellular matrix and the basement membrane. In cancer, MMP levels can be abnormally elevated, and high MMP levels are associated with poor prognosis.^{14, 15} Among the MMPs, gelatinase A (MMP-2) and gelatinase B (MMP-9) are the most closely correlated with metastatic potential.¹⁶ Gelatinases are also involved in the migration of endothelial cells in angiogenic blood vessels.^{17–19} The importance of MMP-2 and MMP-9 in tumor progression, angiogenesis, and metastasis suggests that targeting imaging agents to MMP-2 and MMP-9 would be a useful strategy for noninvasive detection and characterization of solid tumors. Scintigraphic imaging of metalloproteinase activity has been reported using radiolabeled ligands.^{20, 21}

Bremer et al.^{22, 23} constructed an MMP-2-selective, optical imaging probe that can be used to image MMP-2 activity both *in vitro* and in tumor xenografts. The probe is based on a novel fluorogenic polymer, polyethylene glycol grafted polylysine, containing the NIRF dye Cy5.5, that can be cleaved selectively by MMP-2 and MMP-9 and activated by means of MMPs induced cleavage of the substrate peptide Pro-Leu-Gly-Val-Arg. To distinguish the fluorescence signal resulting from dequenching of the fluorogenic polymer from fluorescence signal resulting from the residual fluorescence in intact polymeric conjugate, conjugates that include a noncleavable internal reference signal to provide for detection of both cleaved and uncleaved reagent were proposed.²⁴ In this design, each of the signals from MMP activity and the substrates can be visualized and optically discriminated based on the ratio of the two fluorescence signals, with one of the fluorescence signals serving as an internal reference.

An alternative approach to targeting MMP-2 and MMP-9 is to develop small peptide-based imaging agents that directly bind to the target enzyme. Small peptides regulate many physiological processes. Compared to high-molecular-weight polymers, small peptides are structurally well defined, and they are normally cleared from the blood circulation much faster, which may lead to a higher target-to-background ratio. Random libraries of peptides can be screened *in vivo* for affinity and selectivity for molecular targets using the powerful technique of phage display. Using *in vivo* phage display, Koivunen et al.¹⁹ identified a cyclic decapeptide, c(CTTHWGFTLC)NH₂ (**C1**), containing a His-Try-Gly-Phe (HWGF) motif that exhibited potent inhibitory activity against MMP-2 and MMP-9. They showed that phages expressing **C1** homed better to the tumor vasculature than did phages expressing RGD-containing peptides, which target integrin $\alpha v \beta 3$ receptors. **C1** contains a disulfide bond that can be readily degraded *in vivo*. In fact, Kuhnast et al.²⁵ showed that ¹²⁵I-labeled **C1** was almost completely degraded *in vivo* in serum after intravenous injection. For *in vivo* imaging applications, it is necessary that the peptidyl imaging probes remain intact in blood circulation so that they can bind to their targets with full capacity.

In this report, we describe the design and synthesis of a stable, MMP-2-targeted NIRF probe, Cy5.5-c(KAHWGFTLD)NH₂ (Cy5.5-C6), identified through structure-activity relationship (SAR) analysis of **C1**. We found that Cy5.5-C6 was taken up preferentially by tumor cells expressing MMP-2 *in vitro*, and that human prostate PC-3 tumor inoculated intratibially and human brain U87 tumors inoculated intracranially could be readily visualized with NIRF optical imaging after intravenous injection of this imaging agent.

Materials and Methods

Materials

All *N*^α-Fmoc-amino acids, 1-hydroxybenzotriazole (HOBt), benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), and solid support linker [4-(4-hydroxymethyl-3-methoxyphenoxy)butyric acid] (HMPB) and Fmoc-Rink linker were purchased from Novabiochem (San Diego, CA). Trifluoroacetic acid (TFA) was purchased from Chem-Impex International, Inc. (Wood Dale, IL). PL-DMA resin was purchased from Polymer Laboratories (Amherst, MA). 4-Dimethylamino-pyridine, ammonium acetate (NH₄OAc), 1,3-diisopropylcarbodiimide (DIC), ethylenediamine, *N*-hydroxysuccinimide, *N,N*-diisopropylethylamine (DIPEA), triethylsilane (TES), doxycycline, *p*-aminophenylmercuric acetate (APMA), and β-casein were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Monofunctional hydroxysuccinimide ester of Cy5.5 dye (Cy5.5-NHS) was purchased from Amersham Biosciences (Piscataway, NJ). All solvents were purchased from VWR (San Dimas, CA).

Analytical Methods

Analytical high-performance liquid chromatography (HPLC) was carried out on an Agilent 1100 system (Wilmington, DE) equipped with a 4.6×250 mm Vydac C-18 Peptide and Protein column (Anaheim, CA). Preparative HPLC was carried out on a Rainin Rabbit HP system (Walnut Creek, CA) equipped with a 25 × 2.14 cm Vydac C-18 Peptide and Protein column. Peptides were eluted with gradients of acetonitrile in H₂O containing 0.1% TFA. Matrix-assisted laser desorption ionization (MALDI) mass spectrometry was performed in the Proteomics Core Laboratory in the Department of Molecular Pathology at The University of Texas M. D. Anderson Cancer Center (Houston, TX). Fluorescence spectra were obtained on a Fluorolog-3 fluorometer (Jobin Yvon Inc., Edison, NJ).

Synthesis of Cyclic Peptides C1, C2, and C4–C7

Cyclic peptides **C1**, **C2**, and **C4–C7** were synthesized on PL-DMA resin using Fmoc solid phase chemistry (Table 1). The protecting groups of the amino acid side chains were 4-methyltrityl for Lys, *t*-butyl for Thr, trityl for His and Cys, *t*-butyloxycarbonyl for Trp, and 2-phenylisopropyl for Asp. Fmoc-Rink linker (3 eq) was attached to PL-DMA resin that had been treated with ethylenediamine overnight. The amino acids (3 eq) were then sequentially coupled on solid support using DIC and HOBt (3/3 eq) as coupling agents. The cyclization for the different peptides was achieved using different conditions. For c(CTTHWGFTLC)NH₂ (**C1**), cyclization between Cys and Cys was achieved in NH₄OH solution (pH 9.5) after the corresponding linear peptide was cleaved from the solid support using TFA, H₂O, and TES (94/2/4). For the other cyclic peptides (**C2**, **C4–C7**), cyclization of each peptide between amine and carboxyl groups was achieved in the presence of DIC and HOBt (3/3 eq) on solid support. The cyclization of head-side chain for **C2**, **C4**, and **C5** was carried out after separately removing N-terminal Fmoc group and carboxyl side chain protecting group (2-phenylisopropyl), while the side chain-side chain connection for **C6** and **C7** was performed after one step deprotection of methyltrityl group from Lys and 2-phenylisopropyl groups from Asp with 1% TFA in CH₂Cl₂. The N-terminal Fmoc group was removed after cyclization. Cyclic peptides were cleaved from the solid support using TFA, H₂O, and TES (94/2/4).

Synthesis of Cyclic Peptide C3

For synthesis of **C3**, linker HMPB (3 eq) was attached to PL-DMA resin that had been treated with ethylenediamine overnight. The amino acids (3 eq) were then sequentially coupled on solid support using DIC and HOBt (3/3 eq). The linear peptide was cleaved from

the support using 1% TFA in dichloromethane with all side-chain protecting groups intact. The head-to-tail cyclization was then carried out in the presence of PyBOP, HOBt, and DIPEA (3/3/6 eq). All side-chain protecting groups were removed by treatment with TFA, H₂O, and TES (94/2/4) in the last step.

Peptide Purification and Validation

All peptides were purified by reverse phase HPLC and validated by analytic HPLC and MALDI mass spectrometry. The structures and physicochemical properties of peptides **C1-C7** are summarized in Table 1.

Stability Study

Each peptide was incubated at 25°C in Dulbecco's modified Eagle's medium and nutrient mixture F-12 Ham (DMEM/F-12) containing 10% fetal bovine serum (GIBCO, Grand Island, NY). At various time intervals, aliquots were removed and injected into an HPLC system with a diode array UV/Vis detector at 230 nm. Peak areas for the intact peptides were quantified, and the data were expressed as a percentage of the peak area at 0 min of incubation.

Conjugation of Cy5.5 to C1, C6, and C7

A solution of Cy5.5-NHS (1 eq) and **C1**, **C6**, or **C7** (1.3 eq) in dimethylformamide and DIPEA (10/1) was stirred at room temperature overnight. After the solvent was removed under vacuum, the residual was purified by reverse-phase HPLC, eluted with a 0.01 M solution of NH₄OAc in water and acetonitrile, and lyophilized. The products were validated by analytical HPLC and MALDI mass spectrometry.

Cell Lines

Human prostate cancer PC-3 cells, human lung cancer A549 cells, and human glioma U87 cells were obtained from American Type Cell Culture (Manassas, VA). A549 cells exhibit minimal MMP-2 activity, and U87 cells exhibit strong MMP-2 activity. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂ in Dulbecco's modified Eagle's medium and nutrient mixture F-12 Ham (DMEM/F-12) containing 10% fetal bovine serum (GIBCO, Grand Island, NY).

Fluorescence Microscopy and Confocal Microscopy

For fluorescence microscopy, cells were seeded on cover slips in 24-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) and incubated in DMEM/F-12 (0.5 ml/well) overnight. Cy5.5-C6, Cy5.5-C1, or unconjugated Cy5.5 was added into each well at a concentration of 10 µM and incubated at 37°C for 5 min. For the blocking experiment, cells were first treated with doxycycline (5, 25, and 100 µM) or **C6** (5, 25, and 100 µM) and then with Cy5.5-C6 (10 µM). Cells were washed twice with phosphate-buffered saline (PBS) and incubated in a solution of Sytox Green in 95% ethyl alcohol (1 µM, Molecular Probes, Eugene, OR) for 15 min to fix and stain cell nuclei. The cover slips were mounted for microscopic examination using a DMR microscope (Leica Microsystems, Bannockburn, IL). The microscope was equipped with a 75-W Xenon lamp, differential interference contrast optical components, 560 nm/645 nm and 480 nm/535 nm (excitation/emission) filter sets (Chroma Technology, Brattleboro, VT), a Hamamatsu black and white chilled charge-coupled device camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan), and Image-Pro Plus 4.5.1 software (Media Cybernetics, Silver Spring, MD). Cy5.5 was pseudocolored red, and Sytox Green was green.

For confocal microscopy, U87 cells (1×10^5) were seeded on cover slips in 24-well plates and treated with Cy5.5-C6 or Cy5.5 as described in the preceding paragraph. Cells were incubated at 4°C or 37°C for 5 min in a dark environment, washed twice with PBS, and incubated in 500 μ l of 1 μ M Sytox Green. Finally, the cells were washed twice with PBS, and cover slips were mounted onto slides and examined with confocal microscopy (Olympus 1 $\times$ 81, Miami, FL). The emission wavelengths used were 693 nm for Cy5.5 and 488 nm for Sytox Green. Cy5.5 was pseudocolored green, and Sytox Green was pseudocolored red.

***In Vitro* MMP-2 Inhibition Assay**

In vitro inhibition of MMP-2 was measured using the Chemicon gelatinase activity assay kit according to manufacturer-provided procedures (Chemicon, Temecula, CA). The kit utilizes a biotinylated gelatinase substrate, which is cleaved by active MMP-2 enzyme. Remaining biotinylated fragments are then added to a biotin-binding plate and detected with streptavidin-enzyme complex. Addition of enzyme substrate results in a colored product, detectable by its optical density at 450 nm. For the assay, MMP-2 (Chemicon) was activated by APMA. Each test compound (10 μ l) was preincubated in a 96-well plate with activated MMP-2 (10 μ l, 40 ng/200 μ l) for 1 hr at 37°C, after which 200 μ l of diluted biotinylated gelatinase substrate was added and incubated for an additional hour. The mixture (100 μ l) was transferred to a rehydrated biotin-binding plate and incubated for 30 min at 37°C. The wells were washed extensively to remove unattached biotinylated substrate. This was followed by the addition of streptavidin-enzyme conjugate to each well. After incubation and washing steps, 100 μ l of an enzyme substrate was added. Finally, the optical density at 450 nm was measured on a microplate reader to obtain relative gelatinase activity in test samples. The data were analyzed using Graph-Pad PRISM software (San Diego, CA).

***In Vitro* β -Casein Degradation Assay**

The *in vitro* β -casein degradation assay was performed according to the method of Pirila et al.²⁶ Briefly, APMA-activated MMP-2 (2 μ l, 50 ng/ μ l) was incubated with 4 μ l of Cy5.5-C1 or Cy5.5-C6 at concentrations of 0, 10, 50, and 200 μ M for 30 min at room temperature, after which samples were further incubated with β -casein (4 μ l, 0.25 mg/ml) for 2 h at 37°C. Doxycycline, a broad-spectrum MMP inhibitor,²⁷ was used as a positive control for MMP-2 inhibition. The reaction was stopped by boiling in Laemmli-buffer (Bio-Rad Laboratories, Hercules, CA). β -Caseinolysis was analyzed by separating proteins on a 10% sodium dodecyl sulfate-polyacrylamide gel stained with 0.2% SERVA blue R for 1 hr. The disappearance of a 24-kDa band and the appearance of two lower-molecular-weight bands were regarded as confirmation of MMP-2 activity. The gels were destained with 40% methanol and 10% acetic acid and photographed, and quantitation was done with a Kodak 4000 imaging system using Kodak 1D 3.6 imaging analysis software (New Haven, CT).

Gelatin Zymography

Zymogram gel was purchased from Bio-Rad. MMP activities were determined by zymography according to the method of Mook et al.²⁸ Cells were lysed with the extraction buffer [1% (w/v) Triton X-100, 50 mM Tris-HCl, pH 7.6, 200 mM NaCl, and 10 mM CaCl₂]. U87 tumor tissue excised from tumor grown in the brain of nude mice was homogenized in RIPA buffer. Cell lysates or homogenized tissue was centrifuged for 10 min at 9700 $\times$ g. Supernatants (15 μ l) were diluted 1:4 with sample buffer (0.1 M Tris-HCl, pH 6.8), 4% (w/v) sodium dodecyl sulfate, 20% (w/v) glycerol, 0.005% (w/v) bromophenol blue, and 10 mM EDTA and electrophoresed, together with molecular weight markers, on a 10% polyacrylamide gel containing 1 mg/ml gelatin. An equal amount of protein was loaded onto the zymography gel. After electrophoresis, gels were washed for 4 hr in a buffer containing 50 mM Tris-HCl (pH 7.5), 2.5% (w/v) Triton X-100, and 5 mM CaCl₂ and

incubated overnight in development buffer [1% (w/v) Triton X-100, 50 mM Tris-HCl, 5 mM CaCl₂, and 0.02% (w/v) NaN₃, pH 7.6] at 37°C. Gels were stained with Coomassie blue and destained with acetic acid in methanol and dH₂O (1:3:6), both for 60 min, to permit visualization of bands with gelatinolytic activity. To characterize the enzyme(s) responsible for gelatin breakdown, doxycycline (100 µM) was added to the incubation buffer.

***In Vivo* Imaging**

Four- to six-week-old athymic nude mice (18–22 g, Harlan Sprague Dawley, Inc., Indianapolis, IN) were maintained in a pathogen-free mouse colony in the Department of Veterinary Medicine at M. D. Anderson Cancer Center. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee. Tumor cells ($1-2 \times 10^6$ cells/site) were implanted intratibially (PC-3) or intracranially (U87). In the case of PC-3 tumor model, the contralateral legs were injected with saline as a control in PC-3 tumor model. For screening study, two mice were used in for each imaging agent. Imaging studies were performed 3 days after PC-3 inoculation. For imaging of U87 tumors, mice were divided into 2 groups consisting of 3 mice each. The first group of mice were inoculated with U87 cells, and the second group was injected with PBS (25 µl) as a control. Image studies were initiated 10 days after U87 inoculation. In all *in vivo* imaging studies, images were acquired 48 hr after injection of each imaging agent administered intravenous at a dose of 15 nmol/mouse.

In vivo fluorescence imaging was accomplished by illuminating the animal with light from a laser diode (35 mW) expanded to an approximately 8-cm-diameter circular area. The re-emitted fluorescent light was collected by an image intensifier (model FS9910C, ITT Night Vision, Roanoke, VA) lens-coupled to a charge-coupled device camera (model CH350, Photometrics, Tucson, AZ). The lens was fitted with a holographic notch-plus filter (660-nm center wavelength for Cy5.5, Kaiser Optical Systems, Inc., Ann Arbor, MI) and a bandpass filter (710-nm center wavelength) for Cy5.5 to reject back-scattered and reflected excitation photons. Images were acquired using V++ software (Digital Optics, Auckland, New Zealand), and the obtained images were stored in uncompressed tagged image file format. Data processing and analysis were accomplished using Matlab software (The MathWorks, Inc., Natick, MA). The acquisition parameters were kept constant for all imaging sessions. The integration time for each image was 800 ms.

After *in vivo* imaging studies, brains were excised, and the excised tissues were imaged again. For quantitative comparison of target-to-background ratio, region-of-interest (ROI) was selected in the tumor and normal brain areas (or the site of PBS inoculation and normal brain for the control group) and fluorescence intensity in each ROI was measured. Three mice were used in each group to derive the quantitative data. Statistical comparisons were made using the Student's t-test with $P < 0.05$ considered to be statistically significant. Tissues were then fixed in 10% phosphate-buffered formalin, processed, and sectioned into 6-µm slices. Slides were stained with hematoxylin-eosin for histologic evaluation.

Results

Improvement of Peptide Stability and SAR Analysis

The structures and physicochemical properties of Cy5.5-conjugated peptides are summarized in Table 1.

To improve peptide stability, Cys¹ and Cys¹⁰ were substituted with Ala and Asp, respectively, and the disulfide bond was replaced with an amide linkage formed between the α-NH₂ of Ala¹ and the side chain COOH of Asp¹⁰ to give c(ATTHWGFTLD)NH₂ (**C2**,

Scheme 1). While **C1** was completely degraded when incubated in serum-containing cell culture medium at 25°C over a period of 3 hr, **C2** remained intact under the same conditions (Fig. 1). Furthermore, **C2** exhibited increased inhibitory activity against MMP-2 compared to **C1** (Table 1, Fig. 2A).

To examine the effect of the α -CONH₂ group of Asp¹⁰ on the inhibitory activity of **C2**, we synthesized c(ATTHWGFTL- β -Ala) (**C3**), in which the Asp-NH₂ was replaced with β -Ala (Scheme 1). **C3** had reduced potency compared to **C2**: the concentration required for 50% inhibition of gelatinase (IC₅₀) was 11 μ M for **C2** compared to 84 μ M for **C3** (Table 1, Fig. 2A). These results and the fact that the HWGF motif in **C1** is necessary for selective inhibition of MMP-2¹⁹ suggested that the HWGFTLD-NH₂ sequence of **C2** is necessary for its enzyme inhibition activity and selectivity towards MMP-2.

To introduce a conjugation site suitable for the introduction of a fluorophore, a series of operations were conducted on **C2**. We focused on the Ala-Thr-Thr motif to leave the HWGF motif and Asp-NH₂ intact. First, Thr³ of **C2** was substituted with Ala to give c(ATAHWGFTLD)NH₂ (**C4**). Next, Ala¹ of **C4** was substituted with Lys to give c(KTAHWGFTLD)NH₂ (**C5**). These two operations did not impair the inhibitory activity of the resulting peptides against MMP-2 (Table 1). Note that in **C5** the side chain NH₂ of Lys¹ was available for coupling to dye. Finally, Thr² in **C5** was deleted and the cyclization between Lys and Asp was carried out on the side chains of both amino acids to yield c(KAHWGFTLD)NH₂ (**C6**, Scheme 1). **C6** was stable in serum-containing culture medium (Fig. 1) and had almost the same inhibitory activity as **C2** (Table 1, Fig. 2A). This may be explained by the fact that **C6** possesses the same ring size as **C5**. **C6** had the α -NH₂ of Lys¹ available for conjugation to a NIR fluorophore. Moreover, a part of unnatural backbone - (CH₂)₄- was introduced to the peptide, which is expected to improve the stability of the resulting peptide.

Conjugation to Cy5.5 and Effect of Cy5.5-Conjugates on β -Casein Degradation by MMP-2

Cy5.5 was conjugated to **C1** and **C6** in solution to give the corresponding NIRF agents Cy5.5-C1 and Cy5.5-C6, respectively, using activated monofunctional ester Cy5.5-NHS. A scrambled peptide composed of the same amino acids as **C6** was also synthesized and conjugated to Cy5.5 to give Cy5.5-c(KHGLTWFAD)NH₂ (Cy5.5-C7). All peptide conjugates had fluorescence emission spectra similar to that of Cy5.5 (data not shown).

In vitro evaluation of inhibitory activity of Cy5.5-C1 and Cy5.5-C6 was not possible because the Cy5.5 fluorophore in these conjugates interferes with the fluorescence readout in the Chemicon assay. We therefore evaluated the ability of Cy5.5-C1 and Cy5.5-C6 to inhibit degradation of β -casein by MMP-2. Both conjugates inhibited the degradation of β -casein in a dose-dependent manner with a similar activity, albeit they were less active than doxycycline (Fig. 2B).

Probe Binding to Cancer Cells *In Vitro*

Figure 3 shows fluorescence microscopic images of U87 cells incubated with peptide conjugates and unconjugated Cy5.5. While Cy5.5-C6, and to a less degree Cy5.5-C1, was rapidly taken up by U87 tumor cells (Fig. 3A and D), unconjugated Cy5.5 did not bind to the cells (Fig. 3E). Coincubation with the MMP inhibitor doxycycline partially blocked the binding of Cy5.5-C6 to U87 cells in a dose-dependent manner (Fig. 3B and C).²⁷ Cy5.5-C6 was also taken up by PC-3 cells (Fig. 3F) but not by A549 lung cancer cells that have minimal MMP-2 activity (Fig. 3G).

Figure 4 shows confocal fluorescence images of U87 cells after 5 min of incubation with Cy5.5-C6 and Cy5.5. At 37°C, Cy5.5-C6 was distributed to cell cytosol and nuclei (Fig. 4A–C). At 4°C, cell uptake of Cy5.5-C6 was substantially reduced (Fig. 4D–F). Cy5.5 had minimal uptake in the cells at 37°C (Fig. 4G–I).

***In Vivo* Imaging**

To confirm selective tumor uptake of Cy5.5-C6 and not the other conjugates, we compared NIRF images of mice bearing intratibially inoculated human prostate PC-3 tumors 48 hr after intravenous injection of Cy5.5-C1, Cy5.5-C6, and Cy5.5-C7. Cy5.5-C7 was not taken up by the tumors, and was quickly cleared from the body through renal route (Fig. 5A). Cy5.5-C1 exhibited increased blood-pool activity, but tumor targeting could not be detected (Fig. 5B). In contrast, accumulation of Cy5.5-C6 in PC-3 tumors was clearly visualized (Fig. 5C). The contralateral leg injected with saline did not show uptake of Cy5.5-C6, indicating that the fluorescence signal observed at the tumor inoculation site was not a result of wound injury caused by injection.

To determine the utility of Cy5.5-C6 in noninvasive detection of solid tumors, we used the human glioma cell line U87. Zymography confirmed gelatinase activity in U87 cell lysates and U87 tumor tissue, which could be blocked by the MMP inhibitor doxycycline (Fig. 6A). Twenty-four hours after intravenous administration of Cy5.5-C6 in mice with orthotopically inoculated U87 gliomas, localization of the imaging agent to tumors was readily visible (Fig. 6B). Histologic examination and *ex vivo* optical imaging of excised tumor-bearing brain clearly demonstrated localization of Cy5.5-C6 to the tumor. The signal-to-background ratios were 3.30 ± 0.64 for U87 tumors, and 1.30 ± 0.05 for mice inoculated with PBS as a control after intravenous injection of Cy5.5-C6. The signal-to-background ratio was significantly higher in tumor-bearing mice than in mice inoculated with PBS control ($P = 0.01$).

Discussion

In this study, SAR analysis allowed us to develop an MMP-2-targeted NIRF imaging agent, Cy5.5-C6, that was significantly more stable than a disulfide-linked cyclic peptide. Cy5.5-C6 targeted MMP-2 activity *in vitro*. It was also preferentially taken up by tumors *in vivo*. Although inhibitory activity against MMP-9 was not evaluated in this study, Cy5.5-C6 might also be a suitable probe for imaging MMP-9 activity because the original peptide reported by Koivunen et al.¹⁹ was an inhibitor of both MMP-2 and MMP-9.

Our initial efforts were focused on improving the stability of **C1**, optimizing its MMP-2-inhibitory activity, and introducing a proper conjugation site for the introduction of a NIRF fluorophore. We found that replacing the two Cys residuals with Ala and Asp cyclizing through an amide bond formed between α -NH₂ of the former and the side chain COOH of the latter resulted in a significantly more stable peptide (**C2**) with preserved activity against MMP-2. However, replacing Asp with a β -Ala unit and cyclization through an Ala- β -Ala linkage resulted in a peptide (**C3**) with a higher IC₅₀ value, indicating that the C-terminal α -CONH₂ group in **C2** was critical for efficient MMP-2 inhibition.

In **C2**, replacing the Thr³ with Ala and Ala¹ with Lys did not affect the inhibitory activity of the resulting peptides **C4** and **C5**, suggesting that the Ala-Thr-Thr motif in **C2** can be modified without adverse effects on the biological activity of cyclic HWGF peptides. Finally, elaboration of **C5** to introduce a functional group and to reduce the number of amino acids led to **C6**, which was stable in serum-containing culture medium and had the same inhibitory activity as **C5**. The NIRF fluorophore Cy5.5 was readily conjugated to **C6** via activated ester hydroxysuccinimide ester.

Several of our findings suggest that uptake of the fluorophore-peptide conjugate Cy5.5-C6 in tumors cells is mediated by MMP-2. *In vitro*, Cy5.5-C6, but not unconjugated Cy5.5, was taken up by U87 tumor cells, which exhibit strong MMP-2 activity (Fig. 3A). Cy5.5-C6 did not bind to A549 cells, which exhibit minimal MMP-2 activity (Fig. 3G). Moreover, coincubation of Cy5.5-C6 with doxycycline, a broad-spectrum MMP inhibitor²⁷, partially blocked the binding of Cy5.5-C6 to the tumor cells (Fig. 3A–C). MMP-2 is known to localize to the cell surface and cytoplasmic compartment but is not known to internalize inhibitors upon binding.^{29, 30} However, we observed that Cy5.5-C6 was taken up by U87 tumor cells as soon as 5 min after incubation at 37°C and was distributed to the cytoplasmic compartment and the cell nuclei (Fig. 4A–C). The cellular uptake of Cy5.5-C6 was energy dependent, as indicated by the observation of significantly reduced cellular uptake at 4°C (Fig. 4D–F). Further studies are needed to clarify the role of gelatinases in cellular internalization of Cy5.5-C6.

In an intratibial prostate PC-3 tumor model, fluorescence signal was visualized only in the tumor of mice injected with Cy5.5-C6, whereas in mice injected with Cy5.5-C1, fluorescence signal was distributed throughout the whole body, suggesting that extensive degradation of the conjugate occurred after its intravenous injection. When a control peptide Cy5.5-C7, a NIRF conjugate of a scrambled peptide was injected, no tumor accumulation of the fluorescence agent was visualized (Fig. 5). Interestingly, high fluorescence signal was seen throughout the whole body in mice injected with Cy5.5-C1. The cause of such a phenomenon is not clear. It may be caused by disulfide binding with other serum proteins. Nevertheless, the initial screening imaging findings suggest that tumors could be imaged *in vivo* using small-molecular-weight peptidyl gelatinase inhibitor. To establish the *in vivo* specificity, it is necessary to demonstrate the blockage of tumor uptake of Cy5.5-C6 by MMP-2 specific inhibitors. However, *in vivo* uptake of Cy5.5-C6 in PC-3 tumors could not be blocked by co-injection with doxycycline (data not shown). Since the specificity of Cy5.5-C6 in MMP-2-mediated uptake was demonstrated *in vitro*, it is speculate that mechanism(s) other than MMP-2 mediated binding to tumor cells or tumor stroma may also exist *in vivo*. Clearly, further studies are needed to establish the specificity of Cy5.5-C6 in imaging MMP-2 *in vivo*. In the brain tumor model, we further showed that Cy5.5-C6 allowed clear visualization of intracranially inoculated U87 tumors (Fig. 6). The signal-to-background ratios obtained at 24 hr after intravenous injection of Cy5.5-C6 were 3.30 and 1.30 for mice inoculated with U87 tumors and PBS, respectively. These data again confirm that Cy5.5-C6 was preferentially taken up by tumors cells.

Conclusion

The major finding of this study is that by improving the stability of cyclic peptide, it is possible to achieve significantly improved imaging properties. SAR studies allowed us to identify a NIRF imaging agent, Cy5.5-C6, that possesses greater stability than a related disulfide-linked cyclic peptide. Cy5.5-C6 targets tumor cells with MMP-2 activity both *in vitro* and *in vivo*. Further evaluation of Cy5.5-C6's specificity of targeting to MMPs and other tumor-associated proteinases are necessary for validation of this agent as an *in vivo* marker of gelatinase activity.

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Figure 1. Stability of MMP-2-targeted cyclic peptides in serum-containing culture medium at 25°C. Peptides were analyzed by HPLC. Sample was eluted with gradients of acetonitrile in H₂O containing 0.1% TFA.

Figure 2.

(A) Inhibition of MMP-2 activity by cyclic peptides according to the Chemicon gelatinase activity assay. The data are expressed as mean and standard deviation of percentage change in absorption ($n = 3$). (B) Effect of cyclic peptides on β -casein degradation by activated MMP-2. Activated MMP-2 was incubated with β -casein (0.25 mg/ml) in the absence or presence of each test compound. Proteins were separated on 11% sodium dodecyl sulfate-polyacrylamide gel and detected with SERVA blue R.

Figure 3.

Fluorescence micrographs showing uptake of cyclic peptides in U87 cells (A–E), PC-3 cells (F), and A549 cells (G). Cells were incubated with Cy5.5-C6 (A, F, G), doxycycline at a concentration of 25 μ M followed by Cy5.5-C6 (B), doxycycline at a concentration of 100 μ M followed by Cy5.5-C6 (C), Cy5.5-C1 (D), or Cy5.5 (E). Cells were incubated with each compound at a concentration of 10 μ M at 37°C for 5 min. Note the strong fluorescence signal in U87 and PC-3 cell lines treated with Cy5.5-C6, but not in U87 cells treated with Cy5.5 or A549 treated with Cy5.5-C6. Also note partial blocking of fluorescence signal in U87 cells by doxycycline. Red: Cy5.5 fluorophore; Green: Sytox Green-labeled cell nuclei. Original magnification: x60.

Figure 4. Confocal fluorescence micrographs of U87 cells exposed to Cy5.5-C6 at 37°C (A–C), Cy5.5-C6 at 4°C (D–F), and Cy5.5 (G–I). The corresponding merged images are also shown (C, F, I). Note the strong fluorescence signal in U87 cells treated with Cy5.5-C6 at 37°C, but not in those treated with Cy5.5-C6 at 4°C or with Cy5.5 at 37°C. Red: Cy5.5 fluorophore; Green: Sytox Green-labeled cell nuclei. Original magnification: x40.

Figure 5.

NIR images of mice with PC-3 tumors inoculated intratibially 3 days before the injection of Cy5.5-C7 (A), Cy5.5-C1 (B), or Cy5.5-C6 (C). Images were acquired 48 hr after intravenous injection of Cy5.5-peptide conjugates at a dose of 15 nmol/mouse. Images were normalized to the same scale. Arrows indicate sites of tumor inoculation or saline injection.

Figure 6.

(A) Zymograph of cell lysate and tumor tissue extract from U87 cells. The cell lysate was either treated or not treated with 100 $\mu\text{g/ml}$ doxycycline (Doxy) before being applied to the gel. Equal amounts of proteins were applied. (B) Representative images of a mouse with an intracranially implanted U87 brain tumor. (i) *In vivo* NIRF image acquired 24 hr after intravenous injection of Cy5.5-C6 at a dose of 15 nmol/mouse. Fluorescence (ii), bright light (iii), and the corresponding merged images of the excised brain (iv) are also shown. The fluorescence signal in NIRF images corresponded to the presence of tumor (v & vi) in the brain. Arrows indicate tumor. Scale bar: 0.5 cm.

Scheme 1.
Design of cyclic peptides targeted to MMP-2.

Table 1

Physicochemical Properties of Peptidyl MMP-2 Inhibitors

MMP-2 Inhibitor	Mass Spectrometry	HPLC	MMP-2 Inhibition	
	Molecular formula	Observed (M+1)	Retention Time (min) ^a	IC ₅₀ (μM)
c(CTTHWGFTLC)NH ₂ (C1) ^b	C ₅₂ H ₇₂ N ₁₄ O ₁₃ S ₂	1165.78	15.64	30.30
c(ATTHWGFTLD)NH ₂ (C2) ^c	C ₅₃ H ₇₂ N ₁₄ O ₁₄	1129.56	14.49	10.87
c(ATTHWGFTL-β-Ala) (C3) ^d	C ₅₂ H ₇₁ N ₁₃ O ₁₃	1086.55	17.26	83.90
c(ATAHWGFTLD)NH ₂ (C4) ^c	C ₅₂ H ₇₀ N ₁₄ O ₁₃	1099.50	15.22	9.28
c(KTAHWGFTLD)NH ₂ (C5) ^c	C ₅₅ H ₇₇ N ₁₅ O ₁₃	1156.52	14.00	7.97
c(KAHWGFTLD)NH ₂ (C6) ^b	C ₅₁ H ₇₀ N ₁₄ O ₁₁	1055.55	13.88	7.56
c(KHGLTWFD)NH ₂ (C7) ^b	C ₅₁ H ₇₀ N ₁₄ O ₁₁	1055.54	14.02	N/A
Cy5.5-c(CTTHWGFTLC)NH ₂ (Cy5.5- C1)	C ₉₃ H ₁₁₃ N ₁₆ O ₂₆ S ₆	2064.72	17.24	N/A
Cy5.5-c(KAHWGFTLD)NH ₂ (Cy5.5- C6)	C ₉₂ H ₁₁₃ N ₁₆ O ₂₄ S ₄	1954.74	15.32	N/A
Cy5.5-c(KHGLTWFD)NH ₂ (Cy5.5- C7)	C ₉₂ H ₁₁₃ N ₁₆ O ₂₄ S ₄	1954.67	17.26	N/A

MW, molecular weight.

^aSample was eluted with water and acetonitrile containing 0.1% TFA varying from 10% to 50% over 30 minutes. The flow rate was 1.5 mL/min.

^bSide chain to side chain cyclization.

^cSide chain to tail cyclization.

^dHead to tail cyclization.