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Near-infrared triple-helical peptide with quenched fluorophores for optical imaging of MMP-2 and MMP-9 proteolytic activity *in vivo*

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Abstract

The gelatinase members of the MMP family have consistently been associated with tumor invasiveness, which make them an attractive target for molecular imaging. We report new activatable proteolytic optical imaging agents that consist of triple-helical peptide (THP) conjugates, with high specificity to the gelatinases, bearing quenched cypate dyes. With quenching efficiencies up to 51%, the amplified fluorescence signal upon cypate₃-THP hydrolysis by the gelatinases (k_{cat}/K_M values of $6.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ to $9.1 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ for MMP-2 and MMP-9, respectively) in mice bearing human fibrosarcoma xenografted tumors was monitored with fluorescence molecular tomography. There was significant fluorescence enhancement within the tumor and this enhancement was reduced by treatment with pan-MMP inhibitor, Ilomastat. These data, combined with the gelatinase substrate specificity observed *in vitro*, indicated the observed fluorescence at the site of the tumor was due to gelatinase mediated hydrolysis of cypate₃-THP.

Keywords

Matrix metalloproteinase; Gelatinase; Triple-helical peptides; Near-infrared (NIR) optical imaging; Fluorescence molecular tomography (FMT)

Molecular optical fluorescence imaging is an attractive technique for diagnostic imaging and therapeutic applications. Molecular optical imaging is non-invasive, uses non-ionizing radiation, is capable of multiplexed imaging, and is highly translatable to clinical settings,

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Supplementary Material

Supplementary material that may be helpful in the review process should be prepared and provided as a separate electronic file. That file can then be transformed into PDF format and submitted along with the manuscript and graphic files to the appropriate editorial office.

such as intraoperative fluorescence guided surgery and endoscopy procedures.^{1, 2} One technology being developed in pre-clinical imaging is fluorescence molecular tomography (FMT). FMT is a three dimensional quantitative fluorescent imaging technique. FMT offers improvements over conventional reflectance optical imaging because it allows for quantification of fluorochrome concentration and distribution in much deeper tissue compared to the depths that can be acquired in reflectance imaging.

The identification of molecular targets and development of probes specific for these targets is necessary for effective diagnostic imaging and therapy targeting. A family of proteins hypothesized to be prognostic markers in cancer is the gelatinases. The gelatinases, matrix metalloproteinase (MMP)-2 and -9, are part of an extensive family of zinc dependent endopeptidases capable of degrading extracellular matrix (ECM) components and other extracellular proteins. Gelatinase expression and activity is generally low and the gelatinases are synthesized as inactive zymogens (proMMPs) that are either secreted into the extracellular space or anchored to the cell membrane.³ However, increased expression and gelatinase activation occurs during tissue remodeling events, such as inflammation and wound healing, where proteolysis removes the propeptide domain, exposing the active site within the catalytic domain.⁴ Tumors have been referred to as “wounds that do not heal”⁵ and overexpression and activation of the gelatinases is observed in many cancer types, including breast,^{6, 7} colorectal,^{8, 9} prostate,^{10, 11} and gastric.^{12, 13} The gelatinases are thought to promote tumor progression through degradation of the ECM to result in tumor cell migration, stromal invasion, intravasation into the blood or lymph, and extravasation from these vessels at the metastatic site.^{14, 15} The gelatinases further promote cancer through regulation of growth, apoptosis, and angiogenesis.¹⁶

Various substrates, including PLG~VR and PLG~LA have been investigated as activatable fluorescent reporter probes.¹⁷⁻²² However, the high promiscuity of these substrates for multiple MMPs within the MMP family limits their application as molecular imaging agents. Overall, there is a need for molecular imaging probes that are specifically recognized by the gelatinases since multiple MMP family members are active within the tumor microenvironment. In efforts to achieve MMP selectivity, homotrimeric fluorescent triple-helical peptides (THPs) have been developed.^{23, 24} These sequences display much higher selectivity for the gelatinases. For example, gelatinase hydrolysis of the 1(V)436–447 fTHP yielded k_{cat}/K_M values more than 10 fold greater than the catalytic efficiencies observed with other MMP family members.^{23, 24}

In this study, we have reported the development of two activatable cypate-conjugated THPs, cypate₃-THP and the polyethylene glycol (PEG) modified probe, cypate₃-(PEG)₂-THP. These THPs incorporate sequences based off of a native collagen sequence (residues 437–447 of the α chain of type V collagen), with repeating Gly-Pro-4-hydroxyproline (GPO) triplets at both the N- and C-termini to result in self-assembly of three single-stranded peptides into one THP,²³ to create activatable reporter probes for the selective imaging of gelatinase activity in HT-1080 fibrosarcoma xenografted tumors. Cypate, a cyanine-based NIR dye was chosen for its facile synthesis, high extinction coefficient, high absorption peak in the NIR region, where tissue absorption and light scattering are at a minimum, and overall biocompatibility. Further, cypate is compatible with the acidic conditions of solid-phase

peptide synthesis and the photo-physical properties of cypate conjugated THPs can be easily tuned by structure modifications and/or bio-conjugation.²⁵ Cypate also incorporates carboxylic acid groups for conjugation to amino groups of resin-bound peptides and is also reported to have good binding affinity to albumin.²⁶

The synthesis of cypate-labeled collagen peptide was straightforward by using standard peptide synthesis and coupling methods (Figure 1)²⁷. For cypate₃-THP, the carboxylic acid group on cypate was conjugated to the ϵ -amino groups of Lys in the THP backbone. Through self-assembly of three cypate-labeled collagen sequences, the THP was formed. Complete substitution of cypate-THP was confirmed by negative Kaiser test and mass spectrometric analysis of the purified cypate-THP conjugates. Self-assembly of three cypate-conjugated collagen peptides led to the quenching of the fluorescence from the cypate molecules until hydrolysis of the THPs by MMP-2 or MMP-9. The ratio of cypate to THP was determined to be 3:1. The calculated mass of cypate₃-THP (M+H)⁺ was 4713 and the observed mass peaks were 1571 and 1178, which correspond to mass peaks of (M+H)³⁺ and (M+H)⁴⁺ ions (Figure S1). Cypate₃-(PEG)₂-THP was synthesized and characterized in the same manner (Figure S1).

The absorbance spectra of cypate₃-THP conjugates at room temperature (Figure 2) revealed the presence of an additional absorption peak at 710 nm along with the main absorption peak at 780 nm. This indicated significant intramolecular H-aggregation since the concentration of cypate₃-THP was too low for significant intermolecular aggregates (300 nM) and the absorption band did not increase as the concentration of THP was increased to 1.5 μ M. Further, variable temperature absorbance spectra showed that as the temperature was increased and the triple-helix started to unwind due to melting, the intensity of the absorption band at 710 nm remained unchanged while the absorption band at 780 nm increased, suggesting that the aggregation of dyes was decreased accordingly.^{28, 29} We have previously observed these temperature dependent changes in absorption.²⁴ This phenomenon indicated that the quenching of conjugates was possibly due to the stacking of the fluorophores.

The first derivatives of the fluorescence melting curves estimated the melting points (T_m) of cypate₃-THP and cypate₃-(PEG)₂-THP at 50.5 °C and 45.5 °C, respectively (Figure 3), which are similar to the T_m values of previously reported fluorogenic THPs (41°C - 45 °C) possessing the same 1(V)437-447 sequence.^{23, 30} This indicated that the addition of the cypate molecules did not significantly affect the triple-helical content. Along with the variable temperature absorbance spectra and variable temperature fluorescence melting curves, these data confirmed the triple-helical content of the cypate-conjugated THPs. Maintaining triple-helicity is an important feature for recognition of the THPs by MMP-2 and -9, as the single-stranded sequence is not hydrolyzed by the MMP-2 or MMP-9 efficiently.²³ Quenching studies revealed quenching efficiencies³¹ up to 51% for the cypate-THPs (Table 1).

Enzyme kinetic parameters (k_{cat} , K_M , and k_{cat}/K_M) of MMP-2 and MMP-9 hydrolysis of cypate₃-THP and cypate₃-(PEG)₂-THP were derived from Michaelis-Menten analyses ($\lambda_{excitation}$ = 780 nm and $\lambda_{emission}$ = 810 nm; Figure 4). The k_{cat}/K_M values for hydrolysis of

cypate₃-THP with MMP-2 and MMP-9 were determined to be $6.8 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ and $6.4 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, respectively, while the $k_{\text{cat}}/K_{\text{M}}$ values for MMP-2 and MMP-9 hydrolysis of cypate₃-(PEG)₂-THP were calculated to be $9.1 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ for both enzymes (Table 1). These catalytic efficiencies are on the same order of magnitude previously reported for MMP hydrolysis of other THP conjugates, indicating that dye conjugation to the triple-helix was not detrimental for efficient gelatinase hydrolysis.²³⁻²⁵ This suggests that the THPs can accommodate a variety of dyes in the visible or NIR regions to enable the wide usage of these probes *in vivo*, without sacrificing gelatinase substrate specificity (Table 1). These data further suggest that the THP can be hydrolyzed efficiently prior to wash-out from the target site for good signal to noise ratios in *in vivo* imaging.

The purpose of the PEG spacer was to increase the distance of the dyes from the triple-helical backbone for more efficient intramolecular stacking of the cypate dyes and concomitant quenching of the dyes. However, the quenching efficiencies, determined from variable temperature absorbance spectra and variable temperature fluorescence melting curves, for cypate₃-THP and cypate₃-(PEG)₂-THP were similar (Figures 2 and 3). The apparent rigidity of the THPs limits the collisional quenching of the conjugated dyes.³² Ultimately for translation, more efficient quenching of the activatable fluorescent reporter probe is desired to minimize background signal. Future work will incorporate non-radiative quenching mechanisms (FRET) with either a non-fluorescing absorber that will serve as the quencher for the cypate or with a donor acceptor system.³³

To determine whether cypate₃-THP could visualize gelatinase activity *in vivo*, the quenched probe was administered i.v. to mice bearing HT-1080 xenografts³⁴, which express high levels of the gelatinases, with and without the pan-MMP inhibitor Ilomastat ($K_i = 0.5 \text{ nM}$, MMP-2; $K_i = 0.2 \text{ nM}$, MMP-9). Since no improvements to the quenching or kinetic efficiency were observed with the PEG-ylated probe, only cypate₃-THP was used in FMT studies. Ilomastat was administered at both 24 hr and 1 hr pre-administration of cypate₃-THP and again 4 hr post cypate₃-THP administration to account for the putative widely different pharmacokinetics of a ~15 kDa THP and a 0.3 kDa inhibitor. Normalized fluorescence intensity values from MMP-2 and -9 mediated hydrolysis of cypate₃-THP (pmol cypate/mm³ tumor volume) were relatively low initially at 1 hour post-cypate₃-THP injection, which peaked at 4 hours post-cypate₃-THP injection, and slowly cleared from most tissues after 24 h (Figure 5A,C). The fluorescence intensity was significantly reduced when Ilomastat was co-administered ($p < 0.05$; Figure 5B,C). The average tumor to background ratio at 4 hours post-injection was 10.2. Some of the fluorescent signal observed in mice treated with Ilomastat was attributed to non-quenched cypate₃-THP probe. However, the Ilomastat treated group serves as a good control, allowing for visualization of the fluorescence signal from cypate₃-THP in the absence of MMP activity. The significantly different results between the cypate₃-THP group and the cypate₃-THP plus MMP inhibitor group demonstrate the efficient and selective hydrolysis of cypate₃-THP by the gelatinases.

Amersham MMP-2 and MMP-9 Biotrak Activity Assay Systems quantified levels of MMP2 and -9 in disaggregated tumor tissue. The levels of MMP-2, extrapolated from a standard curve, ranged from 32 ng/ml to 176 ng/ml, while MMP-9 levels ranged from 4.1 ng/ml to 10.2 ng/ml. When MMP levels were normalized to protein content, an average of $9.08 \pm$

0.50 ng MMP-2/mg protein and an average of 0.88 ± 0.16 ng MMP-9/mg protein were observed. While quantitative zymography is a useful method of evaluating gelatinase activity, it cannot distinguish between tissue inhibitors of metalloproteinase (TIMP)-inactivated MMP-2 or MMP-9 and the active forms. In the assay we utilized, an activation step allowed for quantitation of total MMP-2 and MMP-9 levels separately. These results confirm the functional expression of MMP-2 and MMP-9 within tumor tissue.

While we have documented the development of multiple substrates for MMP, our THP is unique in its specificity towards the two MMP family members, MMP-2 and -9. For example, MMPsense (Perkin-Elmer) is a widely used probe. MMPsense is comprised of a poly-Lysine-PEG co-polymer, with a relatively high molecular weight (450KDa), conjugated to a peptide that is a substrate for MMP-2 (PLGVR) that bears NIR-fluorophores.^{18,19} Since the fluorophores are in close proximity, their natural fluorescence is highly quenched. While PLGVR is efficiently hydrolyzed by MMP-2 ($k_{cat} = 4.1 \text{ sec}^{-1}$ and $K_M = 290 \text{ }\mu\text{M}$, $k_{cat}/K_M = 1.4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$), it also a substrate for MMP-1 and MMP-7.^{19,35} HT-1080 human fibrosarcoma cells express, in addition to MMP-2 and MMP-9, MMP-1 and MMP-7, among other MMPs.³⁶ This cell line, which was used in our xenograft mouse model was also used to evaluate MMPsense for visualizing proteolytic activity in an animal model of human cancer.

MMPsense enabled the visualization of the HT-1080 xenografts by NIR reflectance imaging. Co-administration of the pan-MMP inhibitor prinomastat resulted in a 2.5-fold reduction of tumor-associated fluorescence.¹⁹ Prinomastat is a potent inhibitor of the gelatinases (MMP-2, $K_i = 0.08 \text{ nM}$, MMP-9, $K_i = 0.26 \text{ nM}$), but also has inhibitory capacity toward both MMP-1 ($K_i = 8 \text{ nM}$) and MMP-7 ($K_i = 54 \text{ nM}$).³⁷ The observed fluorescence in the HT-1080 xenografts and reduction of signal with prinomastat could have been an amalgamated result from the action of all of the MMPs that were functional in the tumor. MMPsense is therefore suitable for imaging total MMP activity; however, it would not be suitable for clinical applications where the goal of the study is to ascertain the molecular signature of the tumor to guide therapeutic decisions. The gelatinase-selective THPs described here would be the ideal tool for diagnostic imaging of MMP-2 and MMP-9 activity and we have demonstrated functionality in an animal model.

In summary, we have reported the design, synthesis, and *in vitro* and *in vivo* characterization of a THP bearing quenched cypate NIR dyes. These cypate-conjugated THPs displayed quenched fluorescent signal from the cypate molecules through the self-assembly of three cypate-conjugated single-stranded collagen peptide sequences until hydrolysis of the THP by MMP-2 and MMP-9 at the site of the tumor. The cypate₃-THP conjugates retained the kinetic parameters of previously described fluorogenic THPs and were efficiently hydrolyzed selectively by MMP-2 and -9. Further, cypate₃-THP enabled the selective visualization of gelatinase activity in mice bearing human tumors, and the fluorescence signal was diminished with a known MMP inhibitor.. Imaging of gelatinase activity is of great interest due to the prognostic capability of these enzymes in cancer since levels of MMP-2 and -9 are hypothesized to be implicated in clinical outcome.^{8, 16, 38-40} Work is in progress to increase the quenching levels to affect greater *in vivo* contrast enhancement for

sensing tumor-related gelatinase activity in hopes of mediating improved diagnosis and treatment of cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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27. The single-stranded collagen peptides ((GPO)5GPKGPPG~VVGEK(cypate)GEQ(GPO)5 and (GPO)5GPKGPPG~VVGEK(PEG2-cypate)GEQ(GPO)5) were synthesized as previously described (Figure 1)²⁴ to generate cypate₃-THP and cypate₃-(PEG)₂-THP, respectively. For cypate₃-THP, after removal of the 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) protecting groups (4% hydrazine in DMF), rinsing (3 × 5 mL DMF, 10% aqueous DMF, CH₂Cl₂, CH₃OH), and drying (in vacuo), cypate was conjugated to the ε-amino groups of Lys (25 mg resin, resin: cypate: DIC: HOBt-Cl = 1: 5: 5: 5) in DMF. After overnight reaction, the mixture was filtered and rinsed (3 × 5 mL DMF, CH₂Cl₂, CH₃OH). The Kaiser test was negative, indicating complete coupling of cypate. The peptide was cleaved from the resin (water: TFA, 1:19, 3 h), diluted with water, and lyophilized. The crude product was re-dissolved (0.1% acetic acid) and purified by size exclusion gel chromatography (G-25). For cypate₃-(PEG)₂-THP, the ε-amino groups of Lys from the resin-bound peptide were first acylated with Fmoc-NH-(PEG)₂-COOH using standard coupling conditions (30 mg resin: Fmoc-NH-(PEG)₂-COOH: HCTU: HOBt-Cl: DIEA = 1: 3: 3: 6) in DMF. After LC-MS confirmation of complete coupling of the PEG group, Fmoc was removed by 20% piperidine/DMF, and then cypate was conjugated to the free amines following the same procedure described above for conjugate cypate₃-THP. The purification of cypate₃-(PEG)₂-THP used the same method as described above. Substitution levels of the conjugates were determined by absorption (780 nm, ε = 200,000 cm⁻¹M⁻¹)²⁷ and the molecular weights of both compounds were confirmed by ESI-TOF-MS (Figure S1).
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34. All animal experiments were conducted in compliance with the Guidelines for the Care and Use of Research Animals established by The University of Pittsburgh's Animal Studies Committee. Human fibrosarcoma xenografts were grown by subcutaneous injection of 2×10^6 HT-1080 cells (ATCC) in the neck region of 6-week old female athymic nude mice (Taconic). Two to three weeks after implantation, tumor-bearing mice were divided into two groups. One group received cypate₃-THP (i.v., 2 nmol in 100 μL PBS, n = 3) and a second group (n = 4) was treated with Ilomastat (1 mg/kg in DMSO, i.p.) 24 hr and 1 hr prior to cypate₃-THP injection and again 4 hr after cypate₃-THP injection. Mice were anesthetized with 2% isoflurane and oxygen mixture and placed in an imaging cassette (VisEN Medical) for image acquisition. Mice were imaged using the FMT2500 fluorescence molecular tomography system (PerkinElmer) at 1, 4, and 24 h after cypate₃-THP (or cypate₃-THP + Ilomastat) injection. Fluorescence images were acquired using $\lambda_{\text{excitation}} = 790 \text{ nm}$ and $\lambda_{\text{emission}} = 805 \text{ nm}$. Prior to imaging of tumor-bearing mice, the FMT system was calibrated with the cypate dye. Region of interest (ROI) analyses were conducted using the FMT system software TrueQuant. ROIs were selected as the tumor location according to its location in the brightfield image. The picomoles of cypate were then normalized to tumor volume (pmol/mm³; calculated as $1/2 L \times W^2$, length (L) and the width (W) measured with

calipers). Data was analyzed with P-values determined from two-way ANOVA analysis to determine statistical differences between experimental groups.

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Cypate₃-THP: X = Boc, Y = NH₂, Dye = Cypate

(PEG)₂-Cypate₃-THP: X = Boc, Y = NH₂, Dye =(PEG)₂-Cypate

Figure 1.

(a) Solid phase synthesis of cypate-THP conjugates. O = 4-hydroxyproline. (b) The structure of cypate.

Figure 2.
Variable temperature absorbance spectra of cypate3-THP in PBS.

Figure 3.
Fluorescence-derived melting curve of cypate₃-THP.

Figure 4.
Michaelis-Menten plot of MMP-2 hydrolysis of cypate₃-THP.

Figure 5.

(A) FMT images of a representative mouse injected with 2 nmol cypate₃-THP. From left to right: brightfield image with the scan area highlighted, fluorescence images at 1 hour, 4 hours, and 24 hours post-injection of cypate₃-THP. (B) FMT images of a representative mouse injected with 2 nmol cypate₃-THP with Ilomastat administered 24 hours and 1 hour pre-cypate₃-THP injection and again at 4 hours post-cypate₃-THP injection. From left to right: brightfield image with the scan area highlighted, and fluorescence images at 1, 4, and 24 hours post-injection of cypate₃-THP. (C) ROI analysis in pmol cypate₃-THP/mm³ of tumors from mice injected with cypate₃-THP ± Ilomastat.

Table 1

Properties of cypate-THP conjugates

Compound	k_{cat} (s^{-1})	K_{M} (M) ^a	$k_{\text{cat}}/K_{\text{M}}$ ($\text{s}^{-1}\text{M}^{-1}$)	% quenching	T_{M} (°C)
cypate ₃ -THP (MMP-2)	0.056	8.19E-6 ($\pm 1.96\text{E-6}$)	6.8×10^3	N/A	50.5 ^b
cypate ₃ -THP (MMP-9)	0.142	2.23E-5 ($\pm 1.70\text{E-6}$)	6.4×10^3	0.49	
cypate ₃ - (PEG) ₂ -THP (MMP-2)	0.099	1.09E-5 ($\pm 1.22\text{E-6}$)	9.1×10^3	N/A	45.5 ^b
cypate ₃ - (PEG) ₂ -THP (MMP-9)	0.202	2.22E-5 ($\pm 7.32\text{E-6}$)	9.1×10^3	0.51	
fTHP (MMP-2) ^c	0.061	4.40E-6	14×10^3	N/A	45.0
fTHP (MMP-9) ^c	0.044	8.10E-6	5.4×10^3	N/A	

^aError values are $\pm$ standard error of the mean^bValues from fluorescence melting curves^cEnzyme kinetics and melting temperature values for fTHP were obtained from ref. 23, where fTHP is (GPO)5GPK(Mca)GPPG~VVGEEK(Dnp)GEN(GPO)5-NH₂, Mca is (7-methoxycoumarin-4-yl)acetyl and Dnp is 2,4-dinitrophenyl