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Detection of MMP-2 and MMP-9 activity *in vivo* with a triple-helical peptide optical probe

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Abstract

We report a novel activatable NIR fluorescent probe for *in vivo* detection of cancer-related matrix metalloproteinase (MMP) activity. The probe is based on a triple-helical peptide substrate (THP) with high specificity for MMP-2 and MMP-9 relative to other members of the MMP family. MMP-2 and MMP-9 (also known as gelatinases) are specifically associated with cancer cell invasion and cancer-related angiogenesis. At the center of each 5 kDa peptide strand is a gelatinase sensitive sequence flanked by 2 Lys residues conjugated with NIR fluorescent dyes. Upon self-assembly of the triple-helical structure, the 3 peptide chains intertwine, bringing the fluorophores into close proximity and reducing fluorescence via quenching. Upon enzymatic cleavage of the triple-helical peptide, 6 labeled peptide chains are released, resulting in an amplified fluorescent signal. The fluorescence yield of the probe increases 3.8-fold upon activation. Kinetic analysis showed a rate of LS276-THP hydrolysis by MMP-2 ($k_{\text{cat}}/K_M = 30,000 \text{ s}^{-1}\text{M}^{-1}$) similar to that of MMP-2 catalysis of an analogous fluorogenic THP. Administration of LS276-THP to mice bearing a human fibrosarcoma xenografted tumor resulted in a tumor fluorescence signal more than 5-fold greater than muscle. This signal enhancement was reduced by treatment with the MMP inhibitor Ilomostat, indicating that the observed tumor fluorescence was indeed enzyme mediated. These results are the first to demonstrate that triple-helical peptides are suitable for highly specific *in vivo* detection of tumor-related MMP-2 and MMP-9 activity.

Introduction

MMPs are a family of zinc dependent proteases capable of degrading extracellular matrix (ECM) components and other extracellular proteins. MMPs are synthesized as inactive zymogens (proMMPs) and are either secreted into the extracellular space or anchored to the cell membrane. Activation requires removal of the propeptide domain by proteolysis to expose the active site within the catalytic domain (1).

A number of MMPs are overexpressed in various human cancers. Among these are MMP-2 (gelatinase A, 72 kDa gelatinase, or 72 kDa type IV collagenase) and MMP-9 (gelatinase B, 92 kDa gelatinase, or 92 kDa type IV collagenase), both referred to collectively as

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Supporting information available: ES-MS of LS276-THP. This material is available free of charge via the Internet at <http://pubs.acs.org>.

gelatinases. MMP-2 and MMP-9 are secreted as zymogens usually by stromal cells such as fibroblasts (2, 3). Cancer types that have shown increased expression of MMP-2 and MMP-9 include breast (4, 5), colorectal (6, 7), prostate (8, 9), and gastric cancer (10, 11). MMP-2 overexpression is strongly linked to melanoma progression whereas relatively little MMP-2 expression is observed in normal tissues of the skin (12). These tissues are highlighted because they are accessible to the penetration depth of NIR light or are accessible with endoscopes fitted with NIR reflectance imaging sensors (13).

Gelatinases are important imaging targets due to their possible prognostic capability. For example, increased MMP-2 expression predicts decreased disease free survival in human prostate cancer (14). Additionally, MMP-2 in breast carcinoma correlated with shortened recurrence-free survival and relative overall survival (15). Therefore, the development of specific gelatinase imaging probes would be of great interest in unraveling their role in tumor biology in animal models or diagnosing, directing, or monitoring therapy in humans. Imaging probes have been reported for detecting MMPs with nuclear imaging (either PET or SPECT), optical imaging, and magnetic resonance imaging [reviewed in (16)]. At present, peptide substrates for gelatinase activity, while efficiently hydrolyzed by MMP-2, are relatively promiscuous and are subject to hydrolysis by other members of the MMP family (17-19). An understanding of molecular events, such as enzyme activity, requires high specificity of the quenched probe for the substrate. The current lack of specificity of peptide-based substrates is hindering imaging of gelatinase activity *in vivo* and is thus limiting an understanding of how gelatinases could serve as markers for cancer progression or for monitoring the severity of other diseases (20, 21).

For this reason, a triple-helical peptide (THP) that is highly specific for MMP-2 and MMP-9 has been chosen for NIRF imaging. The self-assembling THP incorporates a native collagen sequence (residues 437-447) from the $\alpha 1$ chain of type V collagen (22, 23). This natural collagen sequence, modified to contain a pair of Lys residues flanking the hydrolysis site, contains at both the N- and C-termini repeating Gly-Pro-4-hydroxy-L-proline (GPO) triplets that result in self-assembly of three single-stranded peptides into one triple-helical peptide. The support for triple-helicity is evident in the strong molar ellipticity at $\lambda = 225$ nm which diminishes upon thermal denaturation of the helix. The type V collagen sequence GPPG~VVGEKGEG (the scissile bond lies between G and V), as a single-stranded peptide (i.e., without the GPO triplets), was hydrolyzed extremely slowly by either MMP-2 or MMP-9 (24). Therefore, it is the triple-helical structural feature, along with the sequence, that imparts the substrate specificity amongst the various MMPs (24). We hypothesized that the THP backbone would serve as the core of a quenched fluorescent probe for detection of MMP activity in cancer and other diseases. We have constructed an activatable molecular probe by covalently conjugating NIR fluorescent dyes (LS276) to ϵ -amino groups of Lys that flank the hydrolysis site (Figure 1 A). LS276 is a highly fluorescent, mono-functional, water-soluble heptamethine cyanine dye (Figure 1 B) (25). The close proximity of dyes renders them relatively non-fluorescent. The single-stranded peptides were synthesized entirely on the solid phase and LS276 was incorporated while the peptide was resin-bound. After cleavage from the resin, the resulting triple-helical peptide, LS276-THP, was evaluated *in vitro* to determine whether the addition of the fluorophores impeded the formation of the triple-helix resulting in detrimental increases of the K_M value to MMP-2. Additionally, LS276-THP was tested *in vivo* for its ability to visualize gelatinase activity in a tumor bearing animal model.

EXPERIMENTAL PROCEDURES

Materials

Ilomastat was purchased from Calbiochem (La Jolla, CA). Dimethyl sulfoxide (DMSO) was purchased from Sigma Aldrich (St. Louis, MO). MMPsense™ 680 was purchased from VisEn Medical (Bedford, MA) and prepared according to manufacturer's instructions.

Synthesis

The THP was synthesized as previously described (Figure 1) (22). After removal of the 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) protecting groups (2% hydrazine, DMF), rinsing (3 × 5 mL DMF, 10% aqueous DMF, CH₂Cl₂, CH₃OH), and drying (in vacuo), LS276 was conjugated to the ε-amino groups of Lys (25 mg resin, resin:LS276:DIEA:HATU 1:6:24:12) in DMF. After reaction (3.5 h), the mixture was filtered and rinsed (3 × 5 mL DMF, CH₂Cl₂, CH₃OH). The Kaiser test was negative indicating complete coupling of LS276. The peptide was cleaved from the resin (water:TFA 1:19, 3 h), diluted with water, and lyophilized. The crude mixture was re-dissolved (0.0001% ammonia, pH 8) and purified by size exclusion gel chromatography (G-25). Substitution levels were determined by absorption ($\lambda = 780$ nm, $\epsilon = 220,000$ cm⁻¹ M⁻¹) and the ninhydrin method utilizing bovine insulin as a standard (25, 26). The molecular weight was confirmed by ESI-TOF-MS (Figure S1).

Quenching

To determine the amount of quenching, the fluorescence of unhydrolyzed LS276-THP (f_{intact}) was compared with the fluorescence of completely hydrolyzed LS276-THP ($f_{\text{hydrolyzed}}$), utilizing $[(f_{\text{hydrolyzed}} - f_{\text{intact}})/f_{\text{hydrolyzed}}] \times 100 = \% \text{ quenched}$. Three concentrations of LS276-THP (0.25, 0.5, and 1.0 μ M, $n=3$ per concentration) were digested with MMP-2 (20 nM) until fluorescence reached a plateau with no further increases (3.75 h).

Enzyme assays

The proenzyme of MMP-2 was activated using 4-aminophenylmercuric acetate (APMA). APMA (10 mM, 3.5 mg/mL) in assay buffer (50 mM tricine pH 7.4, 50 mM NaCl, 10 mM CaCl₂, 0.05% Brij-35) was diluted to 2 mM prior to addition to the proenzyme solution. ProMMP-2 was activated by adding APMA (2 mM) to a proMMP-2 solution in equal parts by volume for a final solution of 1 mM (1 h, 37°C) of each component. Enzyme kinetics were carried out using activated MMP-2 at nominal concentrations of 5 nM at 37°C. The rates of hydrolysis were determined by liberated fluorescence units in a microtiter plate reader ($\lambda_{\text{excitation}} = 780$ nm, $\lambda_{\text{emission}} = 810$ nm). The relative fluorescent units were converted to molar units utilizing LS276 as an external standard (25). The error values are standard error of the mean. The amount of active enzyme (E_T) was taken from published values of the % active enzyme generated under identical conditions (70%, MMP-2) (27, 28). K_M values were determined by non-linear regression utilizing $V_o = V_{\text{MAX}}[S]/(K_M + [S])$ (GraphPad Prism version 5.04 for Windows, GraphPad Software, San Diego, CA), and k_{cat} was determined from E_T and V_{MAX} .

Immunohistochemistry

Ten μ m thick sections were cut from tumor tissue snap-frozen in OCT media for routine staining with hematoxylin and eosin (H&E) and IHC staining with MMP-9 and MMP-2. The polyclonal antibodies used were Anti-mouse MMP-9 (5 μ g/ml) and Anti-mouse/rat MMP-2 (5 μ g/ml) from R&D Systems, Inc. (Minneapolis, MN). Immunohistochemical analysis staining was done with the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). The reaction products were visualized using diaminobenzidine (DAB)

(Vector Laboratories, Burlingame, CA) as a chromogen. The sections were counterstained with hematoxylin. As negative controls, primary antibody was replaced with 1X PBS.

In vivo imaging

For imaging studies, human fibrosarcoma xenografts were grown by subcutaneous injection of 200,000 HT1080 cells (ATCC) in the flanks of 6-week old male NCR nude mice (Taconic Farms, Hudson, NY). Tumor-bearing mice received 1 mg/kg LS276-THP probe *i.p.* (20 nmol in 250 μ L PBS) (n=4). A second group (n=3) was treated with Ilomastat (1 mg/kg in DMSO *i.p.*) 2 h before LS276-THP injection and again 4 and 20 h after injection. A third group (n=3) received 2 nmol MMPsense™ 680 *i.v.* Mice in the LS276-THP groups were imaged using the Kodak IS4000MM multimodal imaging system (Carestream Health, New Haven, CT) immediately and at 1, 4, and 24 h after LS276-THP injection, followed by *ex vivo* fluorescence biodistribution imaging of organ tissues. Fluorescence images were acquired using $\lambda_{\text{excitation}} = 755 \pm 35$ nm and $\lambda_{\text{emission}} = 830 \pm 75$ nm detection, 60 sec exposure with 2x2 binning. Mice in the MMPsense™ 680 group were imaged with the Pearl NIR fluorescence imaging system (LiCor Biosciences, Lincoln, NE) with $\lambda_{\text{excitation}} = 685$ nm and $\lambda_{\text{emission}} = 720$ nm collection. Region of interest (ROI) analysis was performed using ImageJ software (LS276-THP groups) or Pearl Cam Software (MMPsense™ 680). Mean fluorescence intensity values for tumor and contralateral flank ROIs were plotted versus time to analyze biodistribution and activation kinetics. Fluorescence values for *ex vivo* tissues were normalized to equalize blood fluorescence levels due to differences in absolute values between imaging systems and detection wavelengths. Statistical significance was calculated with a one-tailed, unpaired *t*-test (GraphPad Prism version 5.04 for Windows, GraphPad Software, San Diego, CA). Outlying data were analyzed with the Grubb's test. All animal studies were conducted by protocols approved by the Animal Studies Committee at Washington University School of Medicine.

RESULTS AND DISCUSSION

Probe development and characterization

Near infrared fluorescent THP probes were synthesized for specific detection of MMP-2 and MMP-9 activity *in vivo*. We hypothesized that full substitution of the two Lys ϵ -amino groups per peptide strand would result in high efficiency quenching of fluorescence upon self-assembly of the triple-stranded peptide structure. Fluorescence would then be regenerated by gelatinase catalyzed hydrolysis, reporting MMP-2/-9 activity in aggressive tumors.

Currently, carbocyanine analogs, especially indocyanine green (ICG), are widely used as optical reporters for *in vivo* imaging of tumors because of their established safety profile in humans and known photo-physical properties (29, 30). The carbocyanine dyes absorb and emit light in the NIR and therefore are suitable for imaging superficial or even deep-tissue disease states. Because our ultimate goal is to develop enzyme activatable probes for preclinical drug testing and diagnostic use in humans, we have utilized the NIR dye LS276. LS276 is compatible with relatively harsh basic conditions of solid-phase peptide synthesis and incorporates a single carboxylic acid group for conjugation to amino groups of resin-bound peptides (25). Mass spectrometric analysis of the purified LS276-THP indicated disubstituted single-stranded peptide: calculated $(M+H)^+ = 5666$, observed $(M+H)^+ = 5667$ (Figure S1), which was corroborated by ninhydrin analysis that showed a substitution level of 6 LS276: 1 THP indicating complete conjugation of LS276 to the ϵ -amino groups of Lys while the peptide was resin-bound. The emission spectrum of LS276-THP shifted slightly to the red by ~6 nm relative to unconjugated LS276, but otherwise the spectral properties of LS276-THP were unchanged indicating stability during peptide synthesis (Figure 2).

By comparing the fluorescence of a fully proteolyzed sample of LS276-THP with an undigested sample, an average value of quenching of $73.5\% \pm 0.5\%$ (standard deviation) of LS276 fluorescence was observed in the THP. By comparison, more flexible single-stranded peptides, such as the MMP-7 substrate GVPLSLTMGC bearing a heterologous donor and acceptor pair of near infrared dyes on the *N*- and *C*-terminus, yielded a level of 85% quenching with an approximate 7-fold increase after digestion with MMP-7 (31). In flexible peptides, a mechanism of quenching has been attributed to the formation of intramolecular dimers between the donor and quencher fluorophores (32). Examination of the absorption profile of LS276-THP under conditions too dilute for intermolecular aggregation (33 nM) provided evidence of H-aggregation by the absorption at $\lambda = 715$ nm relative to the absorption of the non-aggregated dye ($\lambda = 815$ nm) (Figure 3 A) (33, 34). Furthermore, as the triple-helix started to unwind during melting, the absorption band due to H-aggregation decreased while that of the non-aggregated dye increased (Figure 3 B). This indicated that quenching, at least in part, was due to sandwich-style stacking of the fluorophores. The triple-helical backbone is somewhat rigid, limiting the possibility of intramolecular collisional quenching. The level of quenching determined for the LS276-THP is lower than that reported for other NIR fluorogenic probes that have been used *in vivo* (26, 35, 36); however, these molecular probes were based on short peptide sequences that have lower selectivity for MMP-2/-9. Moreover, achieving visualization of protease activity *in vivo* relies on a complex interplay of factors including extravasation of the probe into the tumor, enzyme-mediated proteolysis, and retention of the proteolyzed fragments within the tumor.

Plotting the change in absorbance of the LS276-THP at $\lambda = 815$ nm as a function of temperature yielded a sigmoidal plot with a midpoint transition temperature of 41°C ($T_M = 41^\circ\text{C}$) (Figure 3 B). While determining the melting temperature in this fashion does not take into account the conformational state of the triple-helical backbone, this value compares well with that previously determined for the fluorogenic THP [(GPO)₅GPK((7-methoxycoumarin-4-yl)acetyl)GPPG~VVGEK(2,4-dinitrophenyl)GEQ(GPO)₅]₃ ($T_M = 45^\circ\text{C}$) (24), showing that there was no destabilization of the triple-helix upon incorporation of LS276.

Kinetics of proteolysis

To determine whether the addition of the fluorophores perturbed the triple-helix and altered substrate avidity for the gelatinases, enzyme kinetic parameters were determined (Figure 4). LS276-THP was efficiently hydrolyzed by MMP-2 ($K_M = 2.2 \pm 0.24$ μM , $k_{\text{cat}} = 0.066$ s^{-1} , $k_{\text{cat}}/K_M = 30,000$ $\text{s}^{-1}\text{M}^{-1}$). The kinetic constants and catalytic rate were of similar magnitude to those observed for MMP-2 hydrolysis of a fluorogenic THP with the sequence of [(GPO)₅GPK((7-methoxycoumarin-4-yl)acetyl)GPPG~VVGEK(2,4-dinitrophenyl)GEQ(GPO)₅]₃ ($K_M = 4.4$ μM , $k_{\text{cat}} = 0.062$ s^{-1} , $k_{\text{cat}}/K_M = 14,000$ $\text{s}^{-1}\text{M}^{-1}$) (24). The comparable kinetic constants indicate that LS276 incorporation was not detrimental to efficient MMP-2 hydrolysis (37). This result suggests that the THP could accommodate a variety of near infrared dyes with differing sizes and chemical properties (such as varying hydrophobicity or ionizable functional groups). A prior study examining MMP-2 hydrolysis of fluorogenic THPs possessing methoxycoumarin analogs reached a similar conclusion(38).

The k_{cat}/K_M value provides an important criterion in the evaluation of a substrate with quenched fluorophores for molecular imaging. Those substrates with the highest k_{cat}/K_M values would be expected to be the most rapidly hydrolyzed before wash-out from the target site, resulting in greater signal to noise. *In vivo* protease imaging is in a nascent stage but the feasibility of these studies in small animals has been reported. For example, MMP-2 activity in mouse models was visualized with a poly-lysine-PEG co-polymer, of relatively high molecular weight (450 kDa), when conjugated to a peptide with a sequence of -Pro-Leu-

Gly~Val-Arg-Gly-(35, 39). This peptide itself was a substrate for MMP-2 ($k_{\text{cat}} = 4.1 \text{ sec}^{-1}$ and $K_M = 290 \text{ }\mu\text{M}$, $k_{\text{cat}}/K_M = 1.4 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) (19). Presumably, conjugation to the polymer did not change these kinetic parameters. In another study, a fluorescein-linear peptide bearing a MMP-7 hydrolysis site and tetramethylrhodamine (TMR) were conjugated to a polyamido-amino dendrimeric polymer (14 kDa) (26). Fluorescein was the FRET donor and TMR the acceptor. The dendrimeric peptide was efficiently ($k_{\text{cat}}/K_M = 1.9 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$) and selectively (MMP-2 $k_{\text{cat}}/K_M = 3.4 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, MMP-3 $k_{\text{cat}}/K_M = 1.5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) cleaved by the target, MMP-7. While the endogenous fluorescence was competing with fluorescein fluorescence, a subcutaneous tumor (MMP-7 positive) was visualized relative to a control (MMP-7 negative). An additional example was *in vivo* tumor imaging with a polymeric quenched-fluorescent probe activated by cathepsin D (40). The peptide substrate bearing the Cy5.5 quenched probes had a relatively high efficiency for cathepsin D mediated hydrolysis ($k_{\text{cat}}/K_M = 7 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$). Together, these examples suggest that there is a range of k_{cat}/K_M values of $\sim 1 \times 10^4$ to $7 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ for substrates that successfully imaged *in vivo* proteolytic activity (35, 39, 40). The k_{cat}/K_M value for LS276-THP hydrolysis by MMP-2 falls inside this range and, due to its kinetic parameters, it is suitable for *in vivo* imaging of gelatinase activity.

***In vivo* Imaging of MMP activity with LS276-THP and MMPsense™ 680**

Planar fluorescence imaging was performed in mice bearing HT1080 sub cutaneous xenografts after contrast agent injection. The HT1080 human fibrosarcoma is well known for its high expression of gelatinases (18, 41, 42). Intravenous administration of LS276-THP resulted in rapid clearance of the conjugate with no observable visualization of gelatinase activity in the tumor (data not shown). Unlike large molecular weight polymers, such as MMPsense™ 680, LS276-THP is below the renal filtration threshold leading to fast clearance via the kidneys. Intravenous administration resulted in low tumor-specific contrast, likely due to rapid washout of the relatively small probe ($\sim 15 \text{ kDa}$, data not shown). To slow the uptake and clearance of LS276-THP and maintain a steady concentration of the gelatinase sensing probe in blood, it was administered intraperitoneally.

Planar fluorescence imaging after injection of the LS276-THP probe demonstrated low initial fluorescence intensity followed by high peak fluorescence at 4 h post-injection and cleared from most tissues after 24 h (Figure 5). While tumor fluorescence was partially obscured by overwhelming signal from the ipsilateral kidney, the fluorescence intensity from the tumor region was higher than that of the contralateral flank at 4 and 24 h. This rapid increase in fluorescence intensity was not observed in mice treated with Ilomastat, a pan MMP inhibitor. Ilomastat is a potent inhibitor of gelatinase activity ($K_i = 0.5 \text{ nM}$, MMP-2, $K_i = 0.2 \text{ nM}$, MMP-9) (43). Instead, a steady increase in fluorescence intensity occurred, indicating incomplete inhibition of tumor-associated MMP-2/MMP-9 activity. The fluorescence intensities in the tumor region relative to contralateral thigh shows that LS276-THP had maximum contrast at 4 h relative to mice receiving LS276-THP and inhibitor (Figure 5 A and B). Normalized fluorescence biodistribution measured from *ex vivo* tissues confirmed higher tumor accumulation for LS276-THP relative to LS276-THP in Ilomastat-treated controls (Figure 5 E). The *ex vivo* tumor-to-muscle ratio of fluorescence was 5.36 ± 2.32 for LS276-THP versus 1.99 ± 0.42 for LS276-THP + inhibitor ($P < 0.05$).

Because planar imaging is surface weighted, the relatively high fluorescence from the skin obscured fluorescence signal from the tumor. To obtain a better understanding of whether the observed tumoral fluorescence was mediated by enzyme hydrolysis, an *ex vivo* fluorescence imaging biodistribution was performed. High fluorescence intensity was observed from the liver and kidneys, indicating a mixed clearance pathway for the probe and possibly for the hydrolyzed fragments (Figure 5). Fluorescence was also observed in non-target tissues including lungs, spleen, and skin that was not reduced with the inhibitor ($P >$

0.05). On the other hand, the tumor mediated accumulation was reduced by the inhibitor Ilomastat ($P < 0.05$). While Ilomastat is a pan-MMP inhibitor, the THP sequence used in this work has already been shown to be impervious to the proteolysis of other tumor specific MMPs such as MMP-1, MMP-13, and MMP-14. Other tumor MMPs that are activated in cancer such as MMP-7 need not have been investigated because they do not proteolyze collagen in the $\alpha 1(V)436-447$ region (24). Additionally, examination of the dissected HT1080 tumors by immunohistochemistry and fluorescence imaging showed strong expression at the tumor periphery, consistent with previous observations of gelatinase expression (Figure 6) (44, 45). The correlation of intense fluorescence and as well as intense staining at the tumor periphery co-localizes gelatinase expression with NIR fluorescence and supports the conclusion of gelatinase mediated proteolysis of LS276-THP. Taken together, these results strongly support MMP-2 mediated activation of LS276-THP.

To place these results in perspective, the tumor associated fluorescence of MMPsense™ 680 was investigated in the same tumor model. This is a commercially available pan-matrix metalloproteinase imaging agent that has been utilized in a variety of animal models to investigate MMP activity as a function of disease (20, 21). MMPsense™ 680 has molecular weight of 450 kDa and consists of relatively short peptides bearing fluorophores conjugated to a polymer backbone. Within the peptide is an MMP sensitive sequence. The close proximity of the fluorophores results in quenching of the fluorescence. Fluorescence dequenching is observed after enzyme mediated hydrolysis as the peptide fragments bearing the fluorophores are liberated from the polymer. The molecular weight of MMPsense™ 680 is relatively large; therefore it was administered intravenously rather than impose the additional barrier of peritoneal absorption with *i.p.* administration.

The tumor-specific fluorescence contrast with MMPsense™ 680 increased over time for 24 h, indicating that the higher molecular weight resulted in greater residence time in the tumor tissue and therefore greater activation. By 24 h post-injection, the tumor contrast for MMPsense™ 680 was about two-fold higher than the contralateral thigh while the contrast ratio for LS276-THP remained at about 1.5, unchanged from the 4 h time point. Another factor that could have contributed to the higher tumoral activation of MMPsense™ 680 was the lack of MMP selectivity. The peptide bearing the quenched fluorophores that is conjugated to the polymeric backbone of MMPsense™ 680 contains the sequence -PLGVR- which is subject to proteolysis by members of the MMP family that include, in addition to the gelatinases, MMP-1, MMP-3, and MMP-7 (35, 46, 47). These MMPs are included in those that have been shown to be expressed by the human fibrosarcoma cell line HT1080 (48) but are not always associated with tumor invasiveness or aggressive phenotypes (11, 14, 15, 49, 50). In fact, the correlation of MMP-2/-9 activity with tumor aggressiveness is a driving force in the development of new inhibitors (51-53). These contrasting results indicate that both increased tumor residence time and increased quenching efficiency are needed to improve the contrast enhancement due to MMP-2 activation in triple-helical peptides. While the tumor-specific fluorescence enhancement is slightly higher with MMPsense™ 680 relative to that of LS276-THP in this study, the utilization of triple-helical peptides represents a leap forward in selectively visualizing gelatinase activity by replication of the natural enzyme substrate.

Conclusions

Overall, we have presented the synthesis and characterization of a THP bearing NIR dyes with a relatively high degree of quenching. This probe, LS276-THP, retained the kinetic parameters of the previously described fluorogenic THP and was efficiently hydrolyzed by MMP-2. LS276-THP enabled the visualization of MMP-2 activity in mice bearing human tumors which was diminished with a known inhibitor. This work represents the first report of the use of a THP for *in vivo* imaging of proteolytic activity. Work is in progress to

increase the quenching levels and serum half-life to affect greater *in vivo* contrast enhancement for sensing tumor-related MMP-2 activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A) Solid-phase synthesis of LS276-THP ("O" represents 4-hydroxy-L-proline). B) Chemical structure of LS276.

Figure 2. Fluorescence spectra of LS276 (solid) and LS276-THP (dashed), where $\lambda_{\text{excitation}} = 780$ nm and $\lambda_{\text{emission}} = 760\text{-}850$ nm.

Figure 3.

A) Absorbance of LS276-THP with varying temperature. B) Thermal transition curve for LS276-THP.

Figure 4. Proteolysis of increasing concentrations of LS276-THP by MMP-2. Lineweaver-Burk analysis is provided in the inset. Error bars indicating standard deviation are too narrow to be visualized on this plot.

Figure 5.

Representative *in vivo* whole-body images of mice bearing HT1080 tumor xenografts 24 h after injection of (A) LS276-THP; n=4, (B) LS276-THP and inhibitor; n=3, or (C) MMPSense™ 680; n=3. Tumors (arrows) and kidney (K) regions are marked. (D) The ratio of tumor and contralateral thigh ROI fluorescence with respect to time show the time dependent activation of the molecular probes. (E) *Ex vivo* fluorescence biodistribution confirmed the high fluorescence in the non-inhibited tumors and the high retention of LS276-THP in the mouse kidneys relative to the larger MMPSense™ 680. Error bars represent standard deviation; au = arbitrary units.

Figure 6. Immunohistochemistry for MMP-2 (A), MMP-9 (B), and secondary antibody control (C) with hematoxylin counterstain in HT1080 xenograft cryosections. (D) Fluorescence imaging of HT1080 tumor xenograft 24 h after injection of the LS276-THP probe.