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TITLE: Dual PET/Fluorescence Imaging of Glioma with an MMP-14-Activatable Peptide Probe

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14. ABSTRACT The purpose of the project is to develop and test, in preclinical models, a sensitive, specific imaging peptide probe to distinguish GBM tissue from healthy tissue. The novel imaging probe targets a cell-surface marker, matrix metalloproteinase 14 (MMP-14) that is expressed to a much greater degree in GBM cells than in healthy cells. Upon binding to MMP-14, a dual-modality fluorescent tag is activated. This tag is detectable by positron emission tomography (PET) for pre-operative tumor assessment, and by near infrared fluorescence (NIRF) imaging for real-time surgical guidance in distinguishing tumor cells from healthy cells. Results from the project indicate that GBM cells with MMP-14 activity showed activation and retention of NIRF signal from the cleaved peptide probes. Resected mouse brains with patient derived xenograft (PDX) GBM tumors showed tumor-to-background NIRF ratios of 7.6-11.1 at 4 h after <i>i.v.</i> injection of the peptides. PET images showed localization of activity in orthotopic PDX tumors after <i>i.v.</i> injection of radiolabeled peptide probes; uptake of the radiolabeled probes in tumors was significantly reduced (p<0.05) by blocking with the non-labeled peptide. PET and NIRF signals correlated linearly in the orthotopic PDX tumors. Immunohistochemistry showed co-localization of MMP-14 expression and NIRF signal in the resected tumors.					
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1. INTRODUCTION: Malignant glioma (GBM) is the most common brain tumor that occurs in adults, yet the 5-year survival rate is only 5%, with the majority of patients surviving less than 14 months after diagnosis. It is a rapidly growing, aggressive tumor that grows diffusely into neighboring areas of the brain. Exposure to ionizing radiation is the main external risk factor associated with GBM, and individuals exposed to nuclear weapons testing or other types of ionizing radiation are at an increased risk of developing GBM compared to the general population. There are known survival benefits associated with surgical removal of the GBM tumor. However, the diffuse growth of the tumor makes it difficult for a surgeon to clearly distinguish tumor cells from adjacent, healthy cells. As a result, GBM cells remain in the brain post-surgery, leading to tumor recurrence and the death of the patient. Therefore, there is an urgent need for novel tools that improve tumor detection and the prognosis for individuals diagnosed with GBM. The purpose of the project is to develop and test, in preclinical models, an imaging probe that is both sensitive and specific in distinguishing GBM tissue from healthy tissue. The novel imaging probe will target a cell-surface marker, matrix metalloproteinase 14 (MMP-14) that is expressed to a much greater degree in GBM cells than in healthy cells. Upon binding to its target, a dual-modality fluorescent tag will be activated. This tag will be both detectable by positron emission tomography (PET) to allow for pre-operative assessment of tumor burden and localization, and by near infrared fluorescence (NIRF) imaging to allow for real-time surgical guidance in distinguishing tumor cells from healthy cells. Once developed, the use of this probe will be validated in mouse models of GBM to determine *in vivo* pharmacokinetics, biodistribution, signal retention, sensitivity, and specificity for imaging the GBM tumors. The successful development of this innovative tool will provide a crucial foundation to address a critical gap in managing GBM patient care.

2. KEYWORDS: Glioma, GBM, molecular imaging, PET, NIRF, preclinical, MMP-14, dual modality.

3. ACCOMPLISHMENTS:

○ **What were the major goals of the project?**

Specific Aim 1: Design and characterize an MMP-14 activatable dual PET/NIRF peptide probe.

Major goal 1: Develop novel MMP-14 activatable peptide probes.

Milestone achieved: Identification of initial candidate MMP-14 peptide probes at Stanford and shipment to UAB.

Target completion from SOW: February 2019

Actual completion date: Prior to July 2018

Specific Aim 2: Establish the sensitivity and specificity of the MMP-14 activatable dual PET/NIRF peptide probe for *in vivo* imaging of GBM models.

Major goal 2: Perform *in vivo* studies (using the peptides in mice with GBM tumors) to identify a lead peptide probe.

Milestone to achieve: Identify a lead substrate-peptide conjugate for further *in vivo* studies. Criteria for identification will be: candidate with highest tumor uptake with lowest background tissue accumulation.

Target completion from SOW: August 31, 2019

Percentage of completion as of August 1, 2020: 90%

Major goal 3: Determine the optimal dose and imaging time points for *in vivo* imaging with the lead peptide probe, with corresponding sensitivity and specificity metrics.

Milestone to achieve: Identification of optimal dose and imaging time point for the lead peptide probe.

Target completion from SOW: November 31, 2019

Percentage of completion as of August 1, 2020: 50%

Milestone to achieve: Sensitivity, specificity, PPV (positive predictive value), NPV (negative predictive value) of lead peptide probe determined.

Target completion from SOW: December 31, 2019

Percentage of completion as of August 1, 2020: 75%

Major goal 4: Test lead peptide probe in a panel of GBM models *in vivo*.

Milestone to achieve: Global sensitivity, specificity, PPV, NPV of lead peptide probe determined across multiple PDX models.

Target completion from SOW: June 30, 2020
Percentage of completion as of August 1, 2020: 25%

Milestone to achieve: Submit additional extramural research proposals (DOD, NIH) for subsequent stages of investigation (surgical resection in mice with PDX tumors).

Target completion from SOW: August 31, 2020
Percentage of completion as of August 1, 2020: 25%

○ **What was accomplished under these goals?**

The following accomplishments were part of the prior reporting period:

Major activity: Dr. Jianghong Rao at Stanford has been synthesizing and characterizing the novel MMP-14 activatable peptide probes for the project. The objective is to produce MMP-14 activatable peptide probes that could be used for both NIRF and PET imaging of MMP-14 activity and expression (see Figure 1 of the attached manuscript in the appendix). Methods have included a combination of solid phase peptide synthesis and solution phase chemistry to produce the peptides, followed by standard chemical characterization techniques (high performance liquid chromatography and mass spectrometry). Major accomplishments: As shown in Supplementary Figures S2, S3, S4, S5, S6, and S7 (see attached Supplementary Material in the appendix), their work has generated an MMP-14 activatable “substrate peptide” that can be used for NIRF imaging, an MMP-14 “binding peptide” that can be used for PET imaging, and a “substrate-binding peptide” (a covalently joined construct of the substrate and binding peptide moieties) that can be used for both NIRF and PET imaging. These peptides have been sent to Dr. Jason Warram at UAB and used in the following in vitro and in vivo experiments.

Key outcome achieved: an initial series of novel, MMP-14 peptide probes has been successfully produced to fulfill Major Goal 1 above.

Major activity: Characterized MMP-14 expression and activity in several human glioma tumor tissues and cells lines. The objective was to validate the overexpression of MMP-14 in glioma and glioma cell lines that would be suitable in assays that employ peptide probes as enzymatic substrates for MMP-14. Methods have included immunofluorescence staining of a human glioma tissue microarray, GBM cell lines cultured in vitro, and GBM tumor xenografts grown in athymic nude mice in vivo; Western blot analyses of MMP-14 and MMP-2 expression in GBM cell lines cultured in vitro; and gel zymography analyses of soluble MMP-2 activity of GBM cell lines cultured in vitro. Major results: Human glioma tissues, GBM xenografts, and GBM cell lines express MMP-14 on the cell surface, and expression levels are significantly higher in GBM tissues relative to normal brain tissue ($p < 0.05$). Different GBM cell lines display different levels of MMP-14 expression and activity (see Figure 2 of the attached manuscript in the appendix, and Supplementary Figure S1 of the attached Supplementary Material in the appendix), and are suitable for assays with novel MMP-14 peptide probes.

Major activity: Determine peptide probe binding, cleavage, and NIRF signal activation in vitro with MMP-14 enzyme, GBM cell lines. The objective is to determine that the novel peptides are substrates for MMP-14 to activate the NIRF signal, and that GBM cells show retention of the NIRF and PET signals from the peptides. Methods have included NIRF activation and microscopy studies using the peptides and recombinant MMP-14 enzyme or GBM cell lines cultured in vitro, and binding assays with the radiolabeled binding peptide or substrate-binding peptide and GBM cell lines cultured as adherent monolayers in vitro. Major results: Both the substrate peptide and substrate-binding peptides showed NIRF signal activation within the first several minutes and up to 2 h after incubation with MMP-14 enzyme or with GBM cells. GBM cells incubated for 4 h with the substrate-binding peptide showed significantly more cell-associated NIRF signal than cells incubated for 4 h with the substrate peptide ($p < 0.05$) (see Figure 3 of the attached manuscript in the appendix). These results are consistent with the anticipated mechanism of the substrate-binding peptide, where the MMP-14 binding moiety mediates cellular retention of the residual fluorophore-containing product following cleavage of the substrate sequence by MMP-14. Ongoing studies with the peptides are being performed to determine the enzyme selectivity and kinetics of peptide cleavage in the presence of MMP inhibitors. Cell binding studies showed that the radiolabeled ^{64}Cu -binding peptide binds to GBM cells cultured in vitro, with increased cell-associated activity present at 2 h than at 30 min (Figure A). As expected, lower activity was associated with the cells when the non-labeled binding peptide was used to block MMP-14 binding sites. Separate studies showed that the radiolabeled ^{64}Cu -substrate-binding peptide also bound to GBM cells cultured in vitro in a temperature-dependent manner, where more activity was present following incubation at 37 °C relative to 4 °C (Figure B). This result is consistent with internalization of the radiolabeled peptide after binding to MMP-14 on the cell surface. Ongoing studies with the radiolabeled peptides are being performed to determine the affinities and further define the specific binding of the peptides to MMP-14 on GBM cells cultured in vitro.

Key outcomes achieved: MMP-14 and human GBM cells are capable of cleaving the novel peptide substrates in vitro to activate the NIRF signal. The cell-associated NIRF and PET signals from the peptides indicate that the peptides bind to the GBM cell lines.

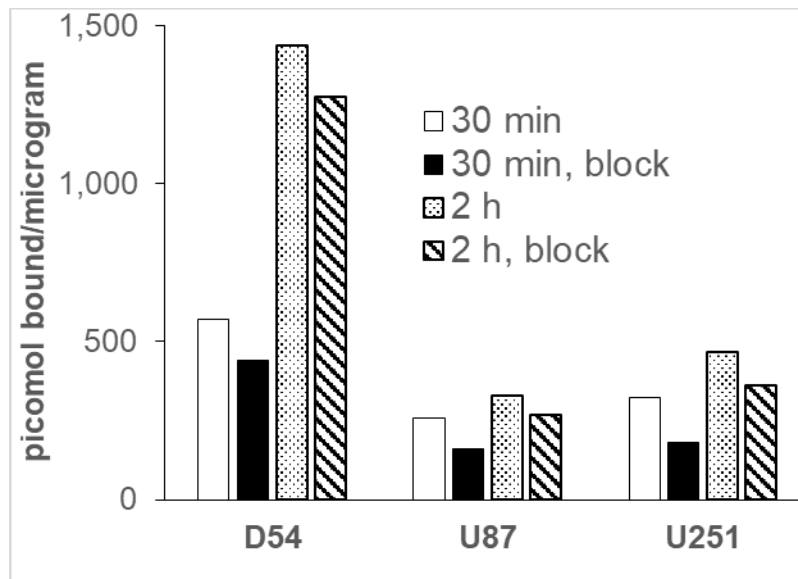


Figure A. Radiolabeled binding peptide binds to human GBM cells in vitro. Adherent GBM cells in 24-well plates (0.4 million cells/well) were incubated at 37 °C for 30 min or 2 h with 46 nM ^{64}Cu -binding peptide. Excess non-labeled binding peptide (1 μM) was added as a block to selected wells. Following incubation, cells were rinsed with PBS and lysed with 1 M NaOH, and the solution was counted on a gamma counter to calculate the cell-associated ^{64}Cu -binding peptide signal, expressed as picomol bound per microgram of protein present in the lysed cell solution.

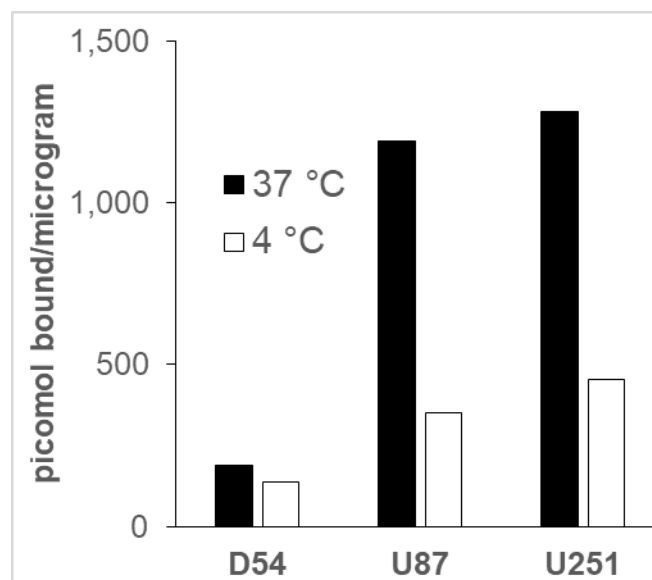


Figure B. Radiolabeled substrate-binding peptide binds to human GBM cells in vitro. GBM cells in suspension (0.5 million cells/tube) were incubated at 37 °C or 4 °C for 10 h with 20 nM ^{64}Cu -substrate-binding peptide. Following incubation, cells were rinsed with PBS, then cell surface-bound activity was collected by rinsing the cells with pH 4 buffer for 10 min. The cells were then lysed with 1 M NaOH, and the collected solutions were counted on a gamma counter to calculate the cell-associated ^{64}Cu -substrate-binding peptide signal, expressed as picomol bound per microgram of protein present in the collected solutions.

Major activity: Perform NIRF imaging and PET/CT pharmacokinetic and biodistribution studies with the first candidate substrate, binding, and substrate-binding peptide probes in mice with orthotopic PDX GBM tumors (one xenoline). The **objective** is to characterize the GBM xenograft tumor uptake, specificity and pharmacology of the imaging candidates in mice. **Methods** have included NIRF imaging studies performed 1-24 h after i.v. injection of the peptides in mice with subcutaneous (s.c.) flank xenografts of GBM cell lines, or in mice with orthotopic (intracranial) patient-derived xenograft (PDX) GBM tumors. PET/CT imaging and biodistribution studies with the peptides have been performed 1-6 h after i.v. injection of the radiolabeled peptides in mice with orthotopic PDX GBM tumors. **Major results:** At 24 h after i.v. injection of the substrate-binding peptide, *in vivo* NIRF imaging showed a low tumor-to-background ratio (TBR, 1.3 ± 0.2 relative to muscle) in s.c. D54 tumors and significantly higher TBR in s.c. U87 tumors (2.2 ± 0.4 ; $p < 0.001$) (see Figure

4A,B of the attached manuscript in the appendix). Low NIRF signal was observed in all normal tissues except the kidneys, indicating predominantly renal clearance of the peptide. Subcutaneous U87 tumors from groups of mice injected *i.v.* with either the substrate peptide or the substrate-binding peptide showed no significant difference in TBR or mean NIRF intensity at the 24 h time point (see Supplementary Figure S8 of the attached Supplementary Material in the appendix). These initial studies demonstrated that glioma tumors show uptake of NIRF signal after administering the quenched peptide substrates.

High contrast of the NIRF signal in orthotopic PDX JX12 tumors relative to adjacent normal brain was apparent in gross slices of resected brains at 1, 4, and 24 h after *i.v.* injection of either the substrate peptide (see Figure 4C of the attached manuscript in the appendix) or the substrate-binding peptide (see Figure 4D of the attached manuscript in the appendix). NIRF analyses of gross brain slices from the 4 and 24 h time points (see Table 1 of the attached manuscript in the appendix) showed significantly higher ($p < 0.001$) mean NIRF signal in the tumor relative to adjacent normal brain from mice given the substrate-binding peptide. While the NIRF mean tumor signals and TBRs were higher in mice given the substrate-binding peptide relative to the substrate peptide (see Table 1 of the attached manuscript in the appendix), the TBRs were not significantly different at the time points examined ($p > 0.05$, ANOVA). NIRF microscopy showed dispersion of the activated NIRF signal from the peptides throughout the tumors, including near the leading edge of tumor progression (see Figure 4C, D of the attached manuscript in the appendix). Both peptides also yielded positive NIRF signals in regions of diffuse glioma cell growth beyond the bulk tumor. Comparing histological tissue sections with confirmed glioma growth by H+E to NIRF signals measured in tissue sections yielded sensitivities above 83% for the substrate peptide and above 85% for the substrate-binding peptide (see Supplementary Table S1 of the attached Supplementary Material in the appendix) at the 4 h and 24 h time points. These results confirmed that the novel MMP-14 peptides could be used for NIRF imaging of orthotopic models of human glioma.

A preliminary study was performed using the binding peptide labeled with ^{68}Ga (^{68}Ga -binding peptide) in mice with orthotopic PDX JX12 tumors. At 2 h after *i.v.* dosing, the tumors were visible during PET/CT imaging while normal brain showed low uptake of activity (see Figure 5A of the attached manuscript in the appendix). *Ex vivo* biodistribution analyses indicated significantly more uptake of radioactivity in brains of mice injected with ^{68}Ga -binding peptide (0.16 ± 0.02 %ID/g) compared to brains of mice injected with ^{68}Ga -binding peptide and 60-fold excess non-labeled binding peptide as a blocking agent (0.07 ± 0.02 %ID/g, $p < 0.01$) (see Figure 5B of the attached manuscript in the appendix), thus supporting the specific retention of the radiolabeled peptide in the PDX tumors. The amount of ^{68}Ga in resected brains correlated with qualitative tumor burden determined by H+E tissue analyses (see Supplementary Figure S10 of the attached Supplementary Material in the appendix). PET images and *ex vivo* biodistribution analyses showed high accumulation of activity in the liver, spleen, and kidneys (see Supplementary Figure S11, S12 of the attached Supplementary Material in the appendix), which was likely due to the relative hydrophobicity of the peptide at physiological pH. A separate cohort of mice bearing orthotopic PDX JX12 tumors were used for PET imaging and biodistribution analyses with ^{64}Cu -labeled substrate-binding peptide (^{64}Cu -substrate-binding peptide). At 4 h after *i.v.* dosing, PET/CT imaging showed significant contrast in the tumor relative to normal brain (see Figure 5C of the attached manuscript in the appendix), yielding a standardized uptake value ratio (SUVR: ratio of tumor SUV_{mean} to normal brain SUV_{mean}) of 3.9 ± 0.5 . The SUVR was lower in a group of tumor-bearing mice (2.5 ± 1.3) that had been co-injected with 80-fold excess of the non-labeled binding peptide as a blocking agent, although the difference between the two groups of mice was not significant ($p = 0.056$). The activity present in whole resected brains from mice in the ^{64}Cu -substrate-binding peptide group (0.43 ± 0.13 %ID/g) was significantly higher than that in the blocked group (0.23 ± 0.07 %ID/g; $p < 0.05$) (see Figure 5D of the attached manuscript in the appendix). This result suggests that the binding peptide is able to partially block binding of the substrate-binding peptide to PDX glioma tumors *in vivo*. Biodistribution analyses of resected tissues was consistent with the PET images, indicating predominantly hepatobiliary accumulation of activity (see Supplementary Figure S14, S15 of the attached Supplementary Material in the appendix). Future studies planned include pharmacology studies of the radiolabeled substrate-binding peptide from 0-6 h after *i.v.* injection in mice with orthotopic PDX GBM tumors.

Key outcomes achieved: The first candidate peptide probes show specific localization and favorable

retention of the NIRF and PET signals in a human PDX GBM tumor model in vivo.

Major activity: Perform histological and zymography analyses of MMP-14 in resected tumors and brain sections from biodistribution studies. The objective is to correlate MMP-14 target expression and probe signal accumulation. Methods include NIRF imaging, histology, MMP-14 immunofluorescence staining, and gel zymography of resected tissues from orthotopic PDX tumor-bearing mice injected with the radiolabeled peptide probes. Correlations between PET, NIRF, and MMP-14 levels in the tumors will be generated and compared to values obtained from normal tissues. Major results: The resected brains from mice injected with the ^{64}Cu -substrate-binding peptide in the above studies were sectioned and used for NIRF analyses. Gross imaging showed high contrast between the NIRF signal in PDX tumor regions compared to contralateral normal brain, yielding a TBR of 7.2 ± 1.3 ($p < 0.001$). The summed NIRF signal from tumor regions in these tissue sections correlated linearly ($R^2 = 0.84$, $p < 0.0001$) with the *in vivo* PET %ID/cm³ signal present in the tumor-bearing brain regions (see Figure 6A of the attached manuscript in the appendix). Microscopic imaging of hematoxylin and eosin (H+E) stained tissue sections confirmed that the NIRF signal co-localized in the PDX glioma tumors, which showed high expression of MMP-14 relative to normal brain (see Figure 6B-G of the attached manuscript in the appendix). These results support the hypothesis that the NIRF signal from the cleavable peptide was specifically retained in the PDX glioma tumors due to the expression of MMP-14 in the tumors. Future studies will compare tissue MMP activity (as determined by gel zymography) and quantified MMP-14 expression with the PET and activated NIRF signals in the tissues.

Key outcomes achieved: The PET and NIRF signals from the first candidate peptide probes show a positive, linear correlation in tumor tissues. Signals from the probes show high spatial congruity with MMP-14 expression in tumor tissues. Based on the cumulative results, the initial candidate peptides meet criteria as “lead” probes for the remaining studies.

○ **What opportunities for training and professional development has the project provided?**

No updates since last reporting period: Two different postdoctoral scholars (Benjamin Kasten and Hailey Houson) have worked significantly on the project under the mentorship of Dr. Jason Warram and other senior investigators at UAB. Training activities resulting from the project have provided these postdoctoral scholars with greater proficiency in designing and executing multi-modality imaging experiments, NIRF image processing, PET image processing, histological analyses, tumor biology, writing scientific research grants, and manuscript preparation. Their professional development activities have included the opportunity to attend and present at annual scientific conferences (as specified further below). Four different medical students (Aditi Jani, Denzel Cole, Andrew Prince, Neha Udayakumar) completed a 2-4 month mentored scholarly research activity rotation in Dr. Warram's laboratory. Three different undergraduate students (Savannah Ferch, Himani Modi, Morgan Richardson) also participated in directed research activities in Dr. Warram's laboratory as part of their degree credits. These individuals received training in techniques related to the project, including cell assays, histological tissue processing and analyses, and fluorescence imaging.

○ **How were the results disseminated to communities of interest?**

Initial results from the project have been disseminated to the scientific research community through presentation at an annual, national meeting (Society of Nuclear Medicine and Molecular Medicine, 2019 Annual Meeting, from prior reporting period), and through publication of a manuscript in a peer-reviewed journal (*European Journal of Nuclear Medicine and Molecular Imaging* (published since the last reporting period)).

○ **What do you plan to do during the next reporting period to accomplish the goals?**

Planned activity: Perform blood and tissue pharmacokinetic studies with the initial candidate peptide probes in mice with orthotopic PDX GBM tumors. The objective is to determine the in vivo stability of the peptide and to identify optimal time points to perform in vivo NIRF or PET imaging analyses. Anticipated completion date: January 31, 2021.

Originally planned activity: Identification of additional candidate MMP-14 peptide probes at Stanford and shipment to UAB. This originally planned activity may not be pursued in the next reporting period, because the initial candidate peptides meet criteria as “lead” probes for the remaining studies. If further studies (e.g., pharmacology) do not support further development of the initial candidate peptides developed at the current stage of the project, additional candidate peptides will be generated and tested. Anticipated date to decide if this activity will or will not be further pursued: December 31, 2020.

Originally planned activity: NIRF and PET/CT pharmacokinetic and biodistribution studies with additional candidate substrate, binding, and substrate-binding peptide probes in mice with orthotopic PDX GBM tumors (one xenoline). This task will be performed to characterize the specificity and pharmacology of additional imaging candidates, if they are developed as indicated based on the above criteria. If this activity is pursued, the anticipated target date for completion will be: June 30, 2021.

Milestone Achieved: Lead substrate-peptide conjugate identified for further in vivo studies. The cumulative in vitro and in vivo data from the above studies will be assessed to determine the lead peptide probe for subsequent studies. The probe with the highest specificity for imaging tumor relative to normal brain within 6 h of dosing. Target completion from SOW: August 31, 2019. Anticipated completion date: January 31, 2021.

Planned activity: Explore 1 nmol and 10 nmol doses of the peptides at 3 time points in mice with orthotopic PDX GBM tumors (one xenoline); use peptide block and non-tumor groups as controls. These in vivo PET and NIRF imaging studies will be performed to assess the effects of various doses on signal accumulation and target specificity. Target completion from SOW: November 31, 2019. Anticipated completion date: March 31, 2021.

Planned activity: Histological and zymography analyses of MMP-14 in resected tumors and brain sections from dose optimization studies, according to established methods used in studies to date. This task is performed to correlate target MMP-14 expression and probe signal accumulation. Target completion from SOW: November 31, 2019. Anticipated completion date: March 31, 2021.

Projected Milestone: Identification of optimal dose and imaging time point for the lead peptide probe. Target completion from SOW: November 31, 2019. Anticipated completion date: March 31, 2021.

Planned activity: Fluorescence imaging with 5-ALA, and PET/CT imaging with ^{18}F [FDG] and ^{18}F [FET] in mice with orthotopic PDX GBM tumors (one xenoline). This task is performed to compare the lead candidate imaging performance with current gold standards for fluorescence or PET imaging. Target completion from SOW: December 31, 2019. Anticipated completion date: June 30, 2021.

Projected Milestone: Sensitivity, specificity, PPV, NPV of lead peptide probe determined. Target completion from SOW: December 31, 2019. Anticipated completion date: June 30, 2021.

Planned activity: Explore *in vivo* imaging of the lead peptide probe at optimal dose/time point in 4 additional PDX models (four xenolines). NIRF and PET imaging studies with the probe will be performed in groups of mice that have orthotopic PDX GBM tumors from xenolines with varying levels of MMP-14 expression and molecular characteristics. This task is performed to assess the broad application of the imaging agent among different GBM tumors. Target completion from SOW: June 30, 2020. Anticipated completion date: July 30, 2021.

Planned activity: Histological and zymography analyses of MMP-14 in resected tumors and brain sections from additional PDX model (one xenoline), according to established methods used in studies to date. This task is performed to correlate target MMP-14 expression and probe signal accumulation. Target completion from SOW: June 30, 2020. Anticipated completion date: July 31, 2021.

Projected Milestone: Global sensitivity, specificity, PPV, NPV of lead peptide probe determined across multiple PDX models. Target completion from SOW: June 30, 2020. Anticipated completion date: August 31, 2021.

Projected Milestone: Submit additional extramural research proposals (DOD, NIH) for subsequent stages of investigation (surgical resection in mice with PDX tumors). Target completion from SOW: August 31, 2020. Anticipated completion date: September 31, 2021.

4. IMPACT:

○ What was the impact on the development of the principal discipline(s) of the project?

Projected impact (included in the prior reporting period): The project, if successful in the long-term, has the potential to significantly impact the prognosis for patients with malignant glioma. The proposed strategy is anticipated in the long-term to positively impact both neurosurgeons and patients by mediating rational, individualized, synergistic assessment of GBM for pre-surgical biopsy, marking tumor boundaries, and enhancing successful maximal safe resection in a timely and seamless manner. These factors would overcome significant limitations of currently approved imaging agents to distinguish GBM from normal brain tissue. The proposed project is anticipated to reveal novel cross-correlations between biological parameters (genomic, transcriptomic, kinomic, histologic) of the GBM xenografts and clinically relevant sensitivity, specificity, and predictive metrics that will be defined with the novel MMP-14 activatable PET/NIRF imaging probes. Synergistically employing these novel parameters and integrating them into imaging systems using alternative modalities (e.g., MRI) would foreseeably enable rational, personalized planning for surgical resection or alternative therapeutic interventions in future stages of research. If successful, these advancements are anticipated to improve the prognosis for individuals with GBM.

○ What was the impact on other disciplines?

Projected impact (included in the prior reporting period): The project aims to validate a novel molecular imaging approach, where the same enzyme is responsible for both activation of the imaging signal and binding to the cancer cells. Other activatable probes in existing research target separate cellular or physiological pathways. As a result, it can be difficult to distinguish the primary mechanism of action that results in target enhancement. The proposed strategy aims to simplify the straightforward co-registration of NIRF and PET signals as a result of both components detecting a single molecular target. Many different types of cancer (e.g., breast cancer, melanoma) overexpress MMP-14 relative to normal tissues; therefore, the project has the potential to significantly impact management of clinical care across a variety of cancers.

○ What was the impact on technology transfer?

Nothing to report.

○ What was the impact on society beyond science and technology?

Projected impact (included in the prior reporting period): The proposed project has the potential to impact the lives and well-being of individuals diagnosed with brain tumors, as well as their family members and friends. Currently, GBM is understood to be essentially non-curable and has a very short time frame for survival after

diagnosis (less than 2 years). People who hear this news understandably face fear and anxiety. As the proposed strategy may significantly impact the prognosis and extend the survival of people with GBM, these negative emotional and psychological impacts could foreseeably be diminished. The result would positively contribute to society, as the individuals could perform normally in their daily lives and work activities.

5. CHANGES/PROBLEMS:

○ Changes in approach and reasons for change

As noted above, the results with the initial candidate peptides are consistent with a “lead” candidate for further evaluation. Therefore, it is likely that the originally planned activities to develop “additional candidate” peptides for evaluation will not be necessary. Forthcoming results from planned experiments in the next several months will determine whether or not additional peptide constructs will be developed. This is not a significant change that would alter the course of the project.

No other significant changes have been made to the approach since the last reporting period.

○ Actual or anticipated problems or delays and actions or plans to resolve them

The work indicated above was significantly delayed because the fluorescence dyes were on back-order from the company (LI-COR) at the time more peptides were being prepared for planned experiments, and because of mandatory shutdowns to research activities at our institutions (University of Alabama at Birmingham, and sub-contract site Stanford University). The mandatory closures, due to Covid19, caused delays during the closure period and also the months before and after laboratories were closed. Additionally, we’ve had changes in non-essential laboratory personnel that were assisting with the project, and have now trained incoming personnel to continue working on the tasks indicated above.

○ Changes that had a significant impact on expenditures

Due to the delays specified above, we filed a no-cost extension for the project. No other significant changes have been made to expenditures.

○ Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

○ Significant changes in use or care of human subjects.

Nothing to report.

○ Significant changes in use or care of vertebrate animals.

Nothing to report.

○ Significant changes in use of biohazards and/or select agents.

Nothing to report.

6. PRODUCTS:

○ Publications, conference papers, and presentations.

▪ Journal publications.

Peer-reviewed article: Benjamin B. Kasten, Ke Jiang, Denzel Cole, Aditi Jani, Neha Udayakumar, G. Yancey Gillespie, Guolan Lu, Tingting Dai, Eben L. Rosenthal, James M. Markert, Jianghong Rao, Jason M. Warram; “Targeting MMP-14 for dual PET and fluorescence imaging of glioma in preclinical models;” *European Journal of Nuclear Medicine and Molecular Imaging*; 47(6), 2020; 1412-1426; Published; acknowledgment of federal support: Yes.

Published abstract (from prior reporting period): Hailey Houson, Benjamin Kasten, Ke Jiang, Jianghong Rao, Jason Warram; “MMP-14 as a noninvasive marker for PET and NIRF imaging of glioblastoma multiforme;” *Journal of Nuclear Medicine*; 60(no. supplement 1):2019;1033; published.

▪ Books or other non-periodical, one-time publications.

Nothing to report.

▪ Other publications, conference papers, and presentations.

Presentation at international meeting: Hailey Houson, Benjamin Kasten, Ke Jiang, Jianghong Rao, Jason Warram; “MMP-14 as a noninvasive marker for PET and NIRF imaging of glioblastoma multiforme;” poster presentation; SNMMI 2019 Annual Meeting; 2019 Jun 22-25; Anaheim, CA.

▪ Website(s) or other Internet site(s)

Nothing to report.

▪ Technologies or techniques

Nothing to report.

▪ Inventions, patent applications, and/or licenses

Nothing to report.

▪ Other Products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Jason Warram
Project Role:	Principal Investigator
ORCID ID:	000-0001-5230-0011
Nearest person month worked:	1
Contribution to Project:	Dr. Warram has supervised and oversee lab personnel, conducted and analyzed data, reported results.
Funding Support:	Partial salary support from the NIH as Co-Investigator on unrelated projects.

Name: Jianghong Rao
Project Role: Site PI
ORCID ID: 0000-0002-5143-9529
Nearest person month worked: 1
Contribution to Project: Dr. Jianghong oversees the subtask 1.1 delivered to the UAB team.
Funding Support: Partial salary support from NIH on unrelated projects.

Name: Catherine Langford “no change from initial submission”
Project Role:
Nearest person month worked:
Contribution to Project
Funding Support:

Name: Yolanda Hartman “no change from initial submission”
Project Role:
Nearest person month worked:
Contribution to Project
Funding Support:

Name: Min Chen
Project Role: Postdoctoral Scholar
Nearest person month worked: 3
Contribution to Project: Dr. Chen contributed to the subtask 1.1 in developing and testing the imaging probe delivered to the UAB team.
Funding Support: Partial salary support from NIH on unrelated projects

Name: Mingxi Fang
Project Role: Postdoctoral Scholar
Nearest person month worked: 5
Contribution to Project: Dr. Fang contributed to the subtask 1.1 in developing and testing the imaging probe delivered to the UAB team.
Funding Support: Partial salary support from NIH on unrelated projects

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Warram

Dr. Jason Warram

Newly Extended Projects:

W81XWH1810160 Warram (PI) 09/01/2018 - 08/31/2021 1.2 Cal Mo
DOD PRCRP \$206,530 (No Cost Extension)

Dual PET/Fluorescence Imaging of Glioma with an MMP-14 Activatable Peptide Probe

Specific Aims:

1. To design and characterize an MMP-14 activatable dual PET/NIRF peptide probe.
2. To establish the sensitivity and specificity of the MMP-14 activatable dual PER/NIRF peptide probe for in vivo imaging of GBM models.

Overlap: This is the current project

Newly Pending Projects:

R01CA245157

Warram (PI)

04/01/2021 – 03/31/2026

NIH

Reduction of Tumor-Positive Margins in Oncology Surgery

Specific Aims:

1. To determine if fluorescence imaging more accurately identifies close or tumor-positive margins during the surgical procedure compared to standard of care.
2. To determine the clinical benefit of FGS for the identification of close or tumor-positive margins by ex vivo fluorescence imaging of the specimen.
3. To localize fluorescence in resected tissues and correlate with molecular markers including tumor size, grading, vascular density, and epidermal growth factor receptor (EGFR) expression levels.

The major goal of this project is to determine if optical imaging during surgery can improve detection of tumor-positive margins in real-time.

Overlap: There is no overlap in scope, funding, or effort with the current grant.

Dr. George Y. Gillespie

Newly Extended Projects:

U01CA224151

Chambers (PI)

9/30/2017 - 8/31/2021

NCI

\$428,817

Canine Immuno Neurotherapeutics

The goal of this project is to evaluate the use of a clinical grade oncolytic herpes simplex virus that expresses human interleukin-12 for the treatment of canine malignant glioma with or without an IDO inhibitor.

Role: Co-Investigator

Grants Management Specialist: Marianne Galczynski of the National Cancer Institute (NCI)

RIVER5 BG RM 4000

8490 Progressive Dr.

Frederick, MD 21701

W81XWH1810160 Warram (PI)

09/01/2018 - 08/31/2021

DOD PRCRP

\$206,530 (No Cost Extension)

Dual PET/Fluorescence Imaging of Glioma with an MMP-14 Activatable Peptide Probe

Specific Aims:

1. To design and characterize an MMP-14 activatable dual PET/NIRF peptide probe.
2. To establish the sensitivity and specificity of the MMP-14 activatable dual PER/NIRF peptide probe for in vivo imaging of GBM models.

Overlap: This is the current project

Newly Awarded Projects:

R21CA252382-01

Gillespie (PI)

7/01/2020-06/30/2022

1.2 Cal Mo

NIH/NCI

\$381,264 (Total Dollars)

GBM Avatar Screening Paradigm for Rational Pharmacokinomic Glioma Therapy

The goal of this research is to rationally improve the effectiveness of small molecule inhibitor-based therapies for individual patients' malignant brain tumors by identifying and targeting key protein kinases that are actively driving tumor cell survival, migration, invasiveness and proliferation signaling pathways.

Role: Principal Investigator

Grants Management Specialist: Kerry Gastley of the National Cancer Institute (NCI)

RIVER5 BG RM 4078
Frederick, MD 21701

Newly Pending Grants:

P50CA257908 (Markert, PI) 04/01/2021-03/31/2026 0.6 Cal Mo
NIH/NCI \$ 1,674,491 (Total Dollars)
NIH/NCI \$ 1,889,709 (Total Dollars)
UAB SPORE in Brain Cancer - Project 1: OVICIT: Oncolytic Viral and Immune Checkpoint Inhibitor Therapy for Glioma

The goal of this project is to demonstrate the safety and improved efficacy of this novel combinatorial treatment approach and identify systemic biomarkers of response in patients.

Role: Co-Investigator

Scientific Review Administrator (SRO): Anita Tandle, Ph.D. of the National Cancer Institute
Meidcal Center Dr.
BG 9609 RM 7W122
Rockville, MD 20850

Program Official (PO): Leah Hubbard, Ph.D. of the National Cancer Institute
9609 Medical Center Dr.
BG 9609 RM 3W212
Rockville, MD 20850

TBD (Friedman, PI) 06/01/2021-05/31/2024 0.6 Cal Mo
Department of Defense \$ 742,502 (Total Dollars)
Targeting Leptomenigeal Disease in Pediatric Brain Tumors with Immunovirotherapy
The goal of this project is to determine the best way of giving the virus into the fluid that surrounds the brain and spinal cord, called 'cerebrospinal fluid', so that we can conduct a clinical trial that avoids the brain surgery and enables us to give repeat injections of the virus so that we can treat leptomenigeal disease.
Role: Co-Investigator
Grants Specialist: To Be Determined

TBD (Beierle- Chen, PI) 08/01/2021-07/31/2024 0.6 Cal Mo
Department of Defense \$1,485,000 (Total Dollars)
First in Children Phase I Study of Genetically Engineered HSV-1 for Treatment of Solid Tumors
The goal of this project is to conduct a Phase I single institution, open-label clinical trial of M032 for the treatment of children with solid tumors to assess the safety and tolerability of M032.
Role: Co- Investigator
Grants Specialist: To Be Determined

Markert

Dr. James M. Markert

Newly Active Projects:

R01FD006368 (Friedman, PI) 09/15/2019-08/31/2022 0.36 Cal Mo
Food & Drug Administration \$750,000 (Total Dollars)
Ph1 Study of HSV G207 in Pediatric Malignant Cerebellar Tumors IND 16294 01/05/18
The goal of this project is to assess the safety and tolerability of G207 in children with recurrent or progressive malignant cerebellar brain tumors.

Role: Co-Investigator

Grants Management Specialist: Daniel Lukash of the Food and Drug Administration

W81XWH2010340 (Cassady, PI) 07/01/2020-06/30/2023 1.2 Cal Mo
 The Research Institute at Nationwide Children's Hospital \$98,664 (UAB Portion- Total Dollars)
 The goal of this project is to examine the impact our oHSV in these *in vivo* models.
 Role: Subaward Principal Investigator
 Grants Specialist: Christopher L. Meinberg of the Department of Defense

Newly Extended Projects:

W81XWH1810160 Warram (PI) 09/01/2018 - 08/31/2021
 DOD PRCRP \$206,530 (No Cost Extension)
Dual PET/Fluorescence Imaging of Glioma with an MMP-14 Activatable Peptide Probe
 Specific Aims:
 1. To design and characterize an MMP-14 activatable dual PET/NIRF peptide probe.
 2. To establish the sensitivity and specificity of the MMP-14 activatable dual PER/NIRF peptide probe for *in vivo* imaging of GBM models.

Overlap: This is the current project

Newly Pending Projects:

P50CA257908 (Markert, PI) 04/01/2021-03/31/2026 1.56 Cal Mo
 NIH/NCI \$ 1,674,491 (Total Dollars)
 NIH/NCI \$ 1,889,709 (Total Dollars)
UAB SPORE in Brain Cancer - Project 1: OVICIT: Oncolytic Viral and Immune Checkpoint Inhibitor Therapy for Glioma

The goal of this project is to demonstrate the safety and improved efficacy of this novel combinatorial treatment approach and identify systemic biomarkers of response in patients.

Role: Co-Investigator

Scientific Review Administrator (SRO): Anita Tandle, Ph.D. of the National Cancer Institute
 9609 Medical Center Dr.
 BG 9609 RM 7W122
 Rockville, MD 20850

Program Official (PO): Leah Hubbard, Ph.D. of the National Cancer Institute
 9609 Medical Center Dr.
 BG 9609 RM 3W212
 Rockville, MD 20850

P50CA257908 (Markert, PI) 04/01/2021-03/31/2026 0.0 Cal Mo
 NIH/NCI \$ 2,079,000 (Total Dollars)
UAB SPORE in Brain Cancer

The goals of this project are to foster advancements in the treatment of malignant gliomas, new discoveries about the molecular and cellular biology of malignant gliomas, and expansion of brain tumor research opportunities and activities through productive Developmental Research and Career Enhancement Programs.

Role: Principal Investigator

Scientific Review Administrator (SRO): Anita Tandle, Ph.D. of the National Cancer Institute

9609 Medical Center Dr.
BG 9609 RM 7W122
Rockville, MD 20850

Program Official (PO): Leah Hubbard, Ph.D. of the National Cancer Institute
9609 Medical Center Dr.
BG 9609 RM 3W212
Rockville, MD 20850

P50CA257908 (Markert, PI) 04/01/2021-03/31/2026 0.84 Cal Mo
NIH/NCI \$ 1,674,491 (Total Dollars)
UAB SPORE in Brain Cancer - Administrative and Clinical Trials Core
The goal of this project is to aid in understanding brain tumors and advance treatment to improve patient outcomes and survival
Scientific Review Administrator (SRO): Anita Tandle, Ph.D. of the National Cancer Institute
9609 Medical Center Dr.
BG 9609 RM 7W122
Rockville, MD 20850

Program Official (PO): Leah Hubbard, Ph.D. of the National Cancer Institute
9609 Medical Center Dr.
BG 9609 RM 3W212
Rockville, MD 20850

P50CA250990 (Zhou, PI) 07/01/2021 – 06/30/2026 0.6 Cal Mo (Years 4-5)
NIH/NCI \$1,936,132 (Total Dollars- Project 2 only)
SPORE in Neuroendocrine Cancer at the University of Alabama at Birmingham- Project 2. A Novel Mitochondrial Chemo-Optogenetics to Cure Metastatic Neuroendocrine Cancers
The goal of the SPORE in Neuroendocrine Cancer at UAB is to advance the translational research that will improve treatment options and outcomes for patients with neuroendocrine cancers.
Role: Clinical Co-Leader
Grants Management Specialist: To Be Determined

TBD (Friedman, PI) 06/01/2021-05/31/2024 0.36 Cal Mo
Department of Defense \$ 742,502 (Total Dollars)
Targeting Leptomeningeal Disease in Pediatric Brain Tumors with Immunovirotherapy
The goal of this project is to determine the best way of giving the virus into the fluid that surrounds the brain and spinal cord, called 'cerebrospinal fluid', so that we can conduct a clinical trial that avoids the brain surgery and enables us to give repeat injections of the virus so that we can treat leptomeningeal disease.
Role: Co-Investigator
Grants Specialist: To Be Determined

TBD (Beierle- Chen, PI) 08/01/2021-07/31/2024 0.6 Cal Mo
Department of Defense \$1,485,000 (Total Dollars)
First in Children Phase I Study of Genetically Engineered HSV-1 for Treatment of Solid Tumors
The goal of this project is to conduct a Phase I single institution, open-label clinical trial of M032 for the treatment of children with solid tumors to assess the safety and tolerability of M032.

Role: Co- Investigator
Grants Specialist: To Be Determined

Newly Completed Projects:

UFRA-154 (Markert, PI) 01/01/2019 – 06/30/2020 Completed
University of California San Francisco \$22,703 (Total Dollars)

Wolfe Meningioma Program Project

The goal of this project is to assess viral efficacy in select meningioma cell lines

Role: Principal Investigator

Assistant Director: Rachel Sievert at the University of California San Francisco

333 California St. Suite 315

San Francisco, CA 94143-3273

U01NS093663 (Griguer and Markert, PIs) 11/21/2018 – 06/30/2020 Completed
NIH/NINDS

Cytochrome C Oxidase: Biomarker In Newly Diagnosed Glioblastoma Multiforme

The goal of this study is to validate Cytochrome c Oxidase (CcO) as a novel prognostic marker and its associations with other classical markers in primary GBM patients in a Phase II prospective trial

Role: Principal Investigator

Grants Management Specialist: Yvonne C. Talley of the National Institute of Neurological Disorders and Stroke (NINDS)

6001 Executive Blvd

NSC BG RM 3256

Rockville, MD 20852

N/A (Markert, PI) 09/30/2016-06/30/2020 Completed

Massachusetts General Hospital

NN106 Cytochrome C Oxidase Activity In Newly Diagnosed Glioblastoma Multiforme (GBM)

Role: Principal Investigator

Project Manager: Tim Leonard of the Partners Organization

tpleonard@partners.org

Clinical Trial Assistant Project Manager: Christina Desir of the Partners Organization

Dr. Joshua S. Richman

01/01/2019-12/31/2021

1.2 Cal

Department of Veterans Affairs \$81,750

Title: *Genetics of Osteoarthritis and Joint Replacement Recovery: Key to Precision Rehabilitation*

Time Commitments: 1.2CM

Supporting Agency: Department of Veterans Affairs Million Veterans Program (MVP)

Address: MVP Program

810 Vermont Ave NW,

Washington, DC, 20420

Contracting/Grants Officer:

Performance period: 01/01/2019-12/31/2021

Level of funding: \$81,750

Project Goals: The goal of this project to harness the large sample size of the MVP to identify genetic variants associated with osteoarthritis to improve quality of life for Veterans.

Role: Co-Investigator

Specific Aims:

1: To identify genetic variants associated with OA.

2: To identify genetic variants prognostic of progression to end-stage OA

Exploratory Aim: To identify genetic variants prognostic of THA/TKA recovery

Overlap: None

No Number Johnston (PI) 07/01/2019-06/30/2022 0.18 Cal

Conquer Cancer Foundation of ASCO \$33,000

Title: *Hospice Use at End-of-Life in Children with Cancer*

Time Commitments: 0.18 calendar

Supporting Agency: Conquer Cancer; The ASCO Foundation

Address: 2318 Mill Road, Suite 800

Alexandria, VA 22314

Contracting/Grants Officer: Thomas G. Roberts, Jr.

Performance Period: 7/1/19 – 6/30/22

Level of funding: \$33,000

Project goals: The major goal of this study is to better understand drivers of variability in pediatric hospice and what models may help agencies provide high quality pediatric hospice services.

Specific Aims:

1: Determine rates of hospice use in pediatric oncology and identify clinical and sociodemographic factors associated with hospice use

Methods: We will use Truven (national insurance claims data) to examine clinical and sociodemographic disparities in hospice use

Hypothesis: Less than 30% of pediatric oncology patients will enroll in hospice and hospice enrollment will be associated with white race, solid tumor diagnosis, and private insurance

2: Obtain diverse perspectives on home death and hospice use in pediatric oncology

Sub Aim 1: Determine racially/ethnically diverse bereaved families' perspective on home death and hospice use

Sub Aim 2: Determine providers' perspectives on home death and hospice, including barriers and facilitators of hospice enrollment

Methods: Semi-structured interviews with 1) racially/ethnically diverse bereaved families and 2) pediatric oncology and hospice healthcare provider teams

Hypothesis: Hypothesis generating

Measurable Objective: Qualitative themes around pediatric oncology hospice enrollment

Learning Objective: Deepen qualitative skills and coordination of multisite studies

Overlap: None

No Number Wadhwa (PI) 02/01/2019-01/31/2021 0.60 Cal

Kaul Pediatric Research Institute \$35,000

Title: *Body Composition and Adverse Outcomes in Childhood Cancer*

Time Commitments: 0.60 calendar

Supporting Agency: Kaul Pediatric Research Institute

Address: 1600 7th Avenue South

Birmingham, LA 35233

Contracting/Grants Officer: Coke Matthews

Performance Period: 02/01/2019 – 01/31/2021

Level of funding: \$35,000

Project goals: The major goal of this project is to identify a method to personalize the chemotherapy dose for each child and minimize serious side-effects but at the same time, maximize cure rates. Specific Aims:

Primary 1: Characterize skeletal muscle mass (using SMI) and height-adjusted total adipose tissue (hTAT) in children with HL, NHL, RMS, NBL from diagnostic CT scans.

Aim 1.1: Model the relation of SMI with BMI%ile and BSA at cancer diagnosis, overall and by specific cancer type; **Aim 1.2:** Model the relation of hTAT with BMI%ile and BSA at cancer diagnosis, overall and by specific cancer type. Hypothesis 1: SMI and hTAT will show modest correlation with BMI%ile and BSA with a $r^2 < 0.5$.

Primary 2: Examine the association between SMI, hTAT and toxicities experienced in the first 6 months after cancer diagnosis in children with HL, NHL, RMS and NBL. **Aim 2.1:** Model the association between SMI and the following outcomes within the first 6 months after cancer diagnosis: occurrence of any grade 3-5 toxicity, >1 grade 3-5 toxicity, toxicity-related treatment delays, and any toxicity-related dose reduction or dose omission. **Aim 2.2:** Model the association between hTAT and same outcomes as in Aim 2.1 within the first 6 months after cancer diagnosis. Hypothesis 2: We hypothesize that patients with a lower SMI (Ho2.1) or higher hTAT (Ho2.2) will experience greater toxicities, treatment delays and dose reductions or omissions.

Exploratory Aim 1: Estimate the association of SMI and hTAT at cancer diagnosis with progression-free and overall survival in patients with HL, NHL, RMS and NBL.

Overlap: None

Recently Completed

No Number	Wolfson (PI)	07/01/2018-06/30/2020	0.3 Cal
The Rally Foundation		\$100,000	

Title: *Understanding Causes of Outcome Disparities in AYA with ALL*

Time Commitments: 0.3 calendar

Supporting Agency: The Rally Foundation

Address: 5775 Glenridge Drive
Building B, Suite 370
Atlanta, GA 30328

Contracting/Grants Officer: Dean Crowe

Performance Period: 7/1/18 – 6/30/20

Level of funding: \$100,000

Project goals: The major goal of this project is to develop an AYA consortium to investigate a broad range of factors on AYA disparities in AYAs with ALL

Specific Aims:

COHORT A: Children (10-14y) and AYA (15-39y) enrolled at diagnosis of ALL:

1: Evaluate the difference in leukemia biology and germline genetic variants between AYA and children. Ho1: There will be an over-representation of high-risk somatic mutations and germline alleles in AYA.

2: Evaluate difference in therapeutic approach (pediatric vs. adult-inspired) and enrollment on clinical trials between AYA and children. Aim 2.1: Describe the role of sociodemographics (race/ethnicity, income, education, material hardship, insurance), in therapeutic approach and enrollment on clinical trials. Ho2: A smaller proportion of AYA will be treated with pediatric-inspired therapy/enrolled on clinical trials, after adjustment for sociodemographics.

COHORT B: Children (10-14y) and AYA (15-39y) with ALL enrolled during maintenance:

3: Evaluate the difference in 6MP adherence between AYA and children using electronic medication monitoring (MEMS). Aim 3.1: Describe the role of sociodemographics (race/ethnicity, income, education, material hardship, and HoE: Genetic variants in telomere homeostasis, DDR,

inflammation, myostatin and/or altered chemotherapy PKs (oxaliplatin as example) will mediate the association between myopenia & cumulative grades 3-5 chemotoxicity.

Overlap: None

MMSG-18-020-01-CPPB Kenzik (PI) 07/01/2018-06/30/2023 0.3 Cal
American Cancer Society \$145,800

Title: *Health Care Utilization and Associated Costs Among Older Cancer Survivors*

Time Commitments: 0.3 calendar

Supporting Agency: American Cancer Society

Address: 250 Williams Street
Atlanta, GA 30303

Contracting/Grants Officer: Elvan Daniels

Performance Period: 7/1/18 – 6/30/23

Level of funding: \$145,800

Project goals: The major goal of this project is to utilize the evidence generated to inform survivorship care delivery and to develop strategies and infrastructure for older cancer survivors requiring long-term follow-up care.

Specific Aims:

1: Examine patterns of high-intensity healthcare use associated with cancer survivorship in the elderly

Aim 1.1 Determine trajectories of high-intensity healthcare use during survivorship and compare to age-, sex-, and race-matched non-cancer population to establish excess use. H1.1: Survivors will demonstrate excess healthcare use compared with age-, sex-, and race-matched non-cancer population

Aim 1.2 Among survivors, examine trajectories of high-intensity healthcare utilization by adherence to standardized healthcare recommendations. H1.2: Non-adherent survivors will demonstrate greater utilization of high-intensity healthcare when compared with adherers, accounting for new-onset morbidities

2: Determine the costs of healthcare utilization among older cancer survivors

Aim 2.1 Estimate the healthcare utilization costs (high-intensity; PCP/specialty care) to Medicare for older cancer survivors when compared with age-, sex-, and race-matched non-cancer population and establish excess cost. H2.1 The healthcare utilization costs will be significantly higher for cancer survivors when compared matched non-cancer population

Aim 2.2 Among survivors, compare healthcare utilization costs by adherence to standardized healthcare guidelines. H2.2: Healthcare costs will be lower among adherers, accounting for sociodemographic predictors; the lower healthcare costs in adherers will be due to a lower incidence of new-onset morbidities and less high-intensity healthcare use.

Aim 2.3 Among survivors, determine healthcare utilization costs attributable to key new-onset morbidity. H2.3: Healthcare costs will vary by type of specific new-onset morbidity; healthcare costs will be higher for non-adherers

3: Examine self-reported financial hardship among older cancer survivors

H3.1: Financial hardship will be greater among those with morbidities when compared with those without, accounting for sociodemographics. H3.2: Financial hardship will be greater among non-adherers, explained by risk of new morbidity.

Dr. James R. Hackney

Newly Extended Projects:

W81XWH1810160 Warram (PI) 09/01/2018 - 08/31/2021

DOD PRCRP \$206,530 (No Cost Extension)

Dual PET/Fluorescence Imaging of Glioma with an MMP-14 Activatable Peptide Probe

Specific Aims:

1. To design and characterize an MMP-14 activatable dual PET/NIRF peptide probe.
2. To establish the sensitivity and specificity of the MMP-14 activatable dual PER/NIRF peptide probe for in vivo imaging of GBM models.

Overlap: This is the current project

Recently Completed Projects:

U01NS093663 Markert (PI) 09/01/16 – 08/31/20 1.2 Mo

NIH/NINDS \$591,993

Prospective Study of Cytochrome C. Oxidase Activity as a Novel Biomarker in Subjects with Newly Diagnosed Primary Glioblastoma Multiforme (GBM)

Specific Aims:

1. To determine the correlation between CcO activity and Progression Free Survival
2. To compare the prognostic value of tumor CcO activity with the value of other commonly used prognostic markers.

This biomarker trial is designed to prospectively evaluate the hypothesis that the overall survival (OS) time of a subject with newly diagnosed primary GBM tumors, treated by standard of care (SOC), is a function of the CcO enzymatic activity in the tumor (OS; time interval from date of first diagnosis to death from any cause, irrespective of post-SOC therapies, assessed up to 24 months from accrual). In particular, tumors with high CcO activity, defined as CcO/citrate synthase (CcO/CS) ratio >4 , are associated with shorter OS time as compared to tumors with low CcO activity ($\text{CcO/CS} \leq 4$). SOC consists of post-surgical radiation therapy with concurrent Temozolomide followed by up to 12 cycles of adjuvant Temozolomide.

Role: Collaborator ended 09/2018

Grant Administrator: Janice Cordell of the National Institute of Neurological Disorders and Stroke

6001 Executive Boulevard
Suite 3309
Bethesda, MD 20892-9531

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

Collaborative Awards: N/A

Quad Charts: N/A

9. APPENDICES:

- Supplementary Materials and Methods
- Abstract “MMP-14 as a noninvasive marker for PET and NIRF imaging of glioblastoma multiforme” presented at the Society of Nuclear Medicine and Molecular Medicine, 2019 Meeting

- Accepted Publication “Targeting MMP-14 for dual PET fluorescence imaging of glioma in preclinical models” accepted by Eur J Nucl Med Mol Imaging

SUPPLEMENTARY MATERIAL

Supplementary Materials and Methods

Cell lines and cell culture

Human GBM cell lines and animal models were maintained and generated by the UAB Brain Tumor Animal Models Core Facility. D-54MG (D54 cells) and U-251MG (U251 cells) were gifts from Darell D. Bigner (Duke University, Durham NC), and U-87MG (U87 cells) was obtained from the American Tissue Type Collection (Manassas, VA). Cell lines were genetically modified with a luciferase-expressing construct (Addgene, Cambridge, MA). Cells were cultured as adherent monolayers in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and prophylactic plasmocin. Experiments with cell lines were performed within 10-12 months of thawing frozen stocks. Cell line authentication analyses were not performed during the time of these studies. For *in vitro* NIRF activation studies, cells were seeded in 96-well plates at 5,000-40,000 cells/well 1-2 days before the experiment. For immunocytochemistry, Western blot, and gel zymography analyses, cells were seeded near confluency in 12-well or 6-well plates the day before the experiment.

Western blot

Adherent monolayers of glioma cells were rinsed 2x with ice-cold PBS and lysed with RIPA buffer containing protease inhibitors on ice for 5 min. The supernatants were clarified by centrifugation at 4 °C, analyzed for protein concentration, aliquoted, and frozen at -20 °C until use. Cell lysates (20-25 µg) were separated by SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked with 3% BSA in TBST, probed with primary antibodies overnight at 4 °C (1/2000 dilution for anti-MMP-14 mAb, Abcam ab51074; 1/1000 dilution for anti-MMP-2 antibody, Abcam ab37150; 1/2000 dilution for anti-β-actin antibody, Santa Cruz Biotechnology sc-47778), rinsed, probed with HRP-conjugated secondary antibody (Santa Cruz Biotechnology sc-2004, 1/1000 dilution), rinsed, and visualized by enzymatic chemical luminescence substrate (ECL) through exposure to X-ray film. Relative band intensities on the developed film were quantified by ImageJ. The experiment was performed in triplicate.

Animal subjects and husbandry, and *in vivo* tumor xenografts

Animal studies were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (20366) and performed in compliance with guidelines from the Public Health Service Policy and Animal Welfare Act of the United States. 5-8 week old female athymic nude mice from Envigo (Indianapolis, IN) or from Charles River (Wilmington, MA) were used for all *in vivo* studies. Standard show and water was available *ad libitum*. All *i.v.* injections were performed *via* the lateral tail vein. For NIRF imaging of flank GBM xenografts, anesthetized mice were implanted *s.c.* in the right hind flank with 2 million D54 or U87 cells (n=5 mice per tumor type). NIRF imaging experiments with the MMP-14 peptide probes were performed 3-4 weeks later, when tumors were 5-8 mm in diameter. Orthotopic PDX GBM xenografts were generated as previously described [1]. Briefly, PDX JX12 tumors passaged in the flanks of athymic nude mice were harvested and dissociated into single cell suspensions. Anesthetized mice were implanted intracranially with 500,000 dissociated PDX tumor cells (5 µL methylcellulose suspension) 2 mm anterior and 1 mm lateral to the bregma at a depth of 2 mm over 2 min. Mice were monitored until fully recovered from anesthesia. Imaging and biodistribution studies were performed 13-17 days after PDX tumor cell implantation. Mice were anesthetized with isoflurane during all live imaging procedures.

General chemistry methods

All chemicals were purchased from commercial sources (such as Aldrich, Synpeptide). Commercially available reagents were used without further purification, unless noted otherwise. All chemicals were reagent grade or better. The rink amide MBHA resin (100-200 mesh) was purchased from Millipore Sigma. The peptides were synthesized according to standard solid-phase peptide synthesis (SPPS); The NHS ester or maleimide coupling reactions were according to thermos fisher scientific coupling protocols. MALDI-MS spectrometric analyses were performed at the Mass Spectrometry Facility of Stanford University. HPLC was performed on a Dionex HPLC System (Dionex Corporation) equipped with a GP50 gradient pump and an in-line diode array UV-Vis detector. A reversed-phase (RP) C18 (Phenomenex, 5 µm, 4.6x250 mm, 5 µm, 10x250 mm or 21.2x250 mm) column was used for analysis and semi-preparative purification. UV absorbance of the probe was recorded on an Agilent 8453 UV spectrophotometer. Fluorescence was recorded on a Fluoromax-3 spectrofluorometer (Jobin Yvon). Unless noted, the compounds were analyzed by RP-HPLC (1 mL/min flow rate) or purified by semi-preparative RP-HPLC (3 mL/min flow rate), using water containing 0.1% trifluoroacetic acid (TFA) (solvent A) and acetonitrile containing 0.1% TFA (solvent B) as the eluates, or using 50 mM pH 5.5 triethylammonium acetate buffer (solvent C) and acetonitrile (solvent D) as the eluates, according to the following gradient: 0-21 min linear gradient from 0% B (or D) to 50% B (or D), 21-25 min linear gradient to 100% B (or D), 25-27 min hold 100% B (or D), 27-31 min linear gradient to 0% B (or D) and equilibrate in 0% B (or D).

Synthesis of substrate peptide: The peptide 1 (Figure S2) was synthesized using rink amide MBHA resin (100-200 mesh) according to standard SPPS methods. Peptide X (1.27 mg, 1 µmol, 1 eq), IRDye800CW-maleimide (1.19 mg, 1 µmol, 1 eq) and 0.5 mL of pH 7.4 PBS buffer were combined in a 1 mL vial and stirred at room temperature for 2 h. Then the quencher QC-1-NHS ester (1.24 mg, 1 µmol, 1 eq) was added to solution and stirred overnight. The reaction was purified directly by semi-preparative RP-HPLC using solvents C and D as eluates to afford light blue substrate-peptide 2 (1.1 mg, 32%). >99% purity was achieved after purification. MS: calcd. for C₁₆₀H₂₀₅ClN₂₃O₄₃S₉ [(M+H)⁺]: 3459.17; found Maldi-Tof MS: m/z 3460.32 (Figure S5).

Synthesis of binding peptide: The peptide 2 (Figure S3) was synthesized using rink amide MBHA resin (100-200 mesh) according to standard SPPS methods. Peptide Y (1.9 mg, 1 μ mol, 1 eq), NOTA-maleimide (0.85 mg, 2 μ mol, 2 eq) and 0.5 mL of pH 7.4 PBS buffer were combined in a 1 mL vial and stirred at room temperature for 2 h. The reaction was purified directly by semi-preparative RP-HPLC using solvents A and B as eluates to afford white binding peptide 1 (1.65 mg, 71%). >99% purity was achieved after purification. MS: calcd. for $C_{105}H_{157}N_{34}O_{26}S$ [(M+H)⁺]: 2342.16; found Maldi-Tof MS: m/z 2342.30 (Figure S6).

Synthesis of substrate-binding peptide: To a 1 mL glass vial was added 0.34 mg of substrate peptide (~0.1 μ mol), 150 μ L of pH 7.4 PBS buffer, 100 μ L of *N,N*-dimethylformamide, 0.46 mg of binding peptide (~0.2 μ mol), a premixed solution of 25 μ L of 20 mM $CuSO_4$ and 50 μ L of 50 mM THPTA (tris(3-hydroxypropyltriazolylmethyl)amine), 100 μ L of 500 mM sodium ascorbate, and 100 μ L of 500 mM aminoguanidine hydrochloride. The vial was capped tightly and stirred at room temperature for 1 h. Excess EDTA was added to the stirred solution and then the mixture was purified directly by preparative RP-HPLC using solvents C and D as eluates to afford the substrate-binding peptide (0.31 mg, 54%). >99% purity was achieved after purification. MS: calcd. for $C_{265}H_{361}ClN_{57}O_{69}S_{10}$ [(M+H)⁺]: 5800.33; found Maldi-Tof MS: m/z 5803.03 (Figure S7).

Radiolabeling and radio HPLC analyses

Radioactive RP-HPLC analyses were performed on an Agilent 200 liquid chromatography system outfitted with an Agilent Zorbax SB-C18 RP-HPLC column (3x250 mm, 5 μ m particle size) and a matching guard cartridge (3x7 mm). Gradient elutions at a flow rate of 0.5 mL/min were performed with solvent A and solvent D according to the following method: 0-15 min linear gradient from 90% A/10% B to 60% A/40% B, 15-17 min linear gradient to 5% A/95% B, 17-20 min hold at 5% A/95% B, 20 min return to 90% A/10% B and equilibrate. UV-Vis was monitored at 254 and 775 nm. An attached radiodetector (model 105-S, Carroll & Ramsey Associates) was used to monitor elution of radioactive compounds.

⁶⁸Ga-binding peptide: 135 MBq ⁶⁸Ga eluate (0.18 mL) in a screw-cap vial was adjusted to pH ~4.5 with 2 M NaOH and 1 M sodium acetate. To this solution (0.2 mL) was added 7.5 nmol binding peptide and the solution was heated at 90 °C for 20 min, after which it was cooled on ice and an aliquot was analyzed by radio RP-HPLC (Supplementary Figure S5). Aliquots of the crude radiolabeling reaction were diluted with PBS, or with PBS containing 50 nmol unlabeled binding peptide (block dose), to give 0.2 mL (2.2-4.5 MBq) per mouse dose. Molar activity at the time of dosing was approximately 6.1 GBq/ μ mol (non-blocked group).

⁶⁴Cu-substrate-binding peptide: 198 MBq ⁶⁴Cu (7 μ L), 0.5 M pH 6.0 sodium acetate buffer containing 1% gentisic acid (50 μ L), acetonitrile (25 μ L), and 5 nmol substrate-binding peptide were combined in a plastic microcentrifuge tube. After sitting at room temperature for 20 min, and aliquot was analyzed by radio RP-HPLC (Supplementary Figure S9). Nitrogen gas was blown into the reaction tube for 20 min to evaporate residual acetonitrile, and aliquots of the residual crude solution were diluted with PBS containing 4.3% EtOH, or with PBS containing 4.3% EtOH and 22 nmol unlabeled binding peptide (block dose), to give 0.2 mL (6.3-6.5 MBq) per mouse dose. Molar activity at time of dosing was approximately 46.6 GBq/ μ mol (non-blocked group).

Supplementary Figures

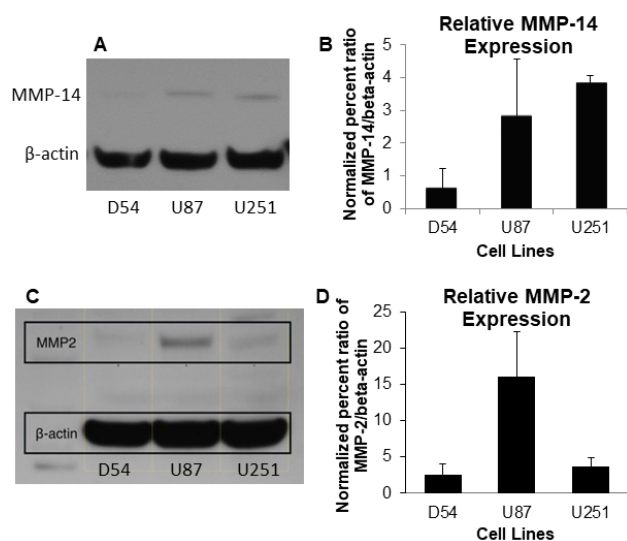


Figure S1. Western blots of MMP-14 (A) or MMP-2 (C) with quantification of respective blot intensities (B,D) from lysates of adherent GBM cells grown *in vitro* (average $\pm$ SD, n=3/group).

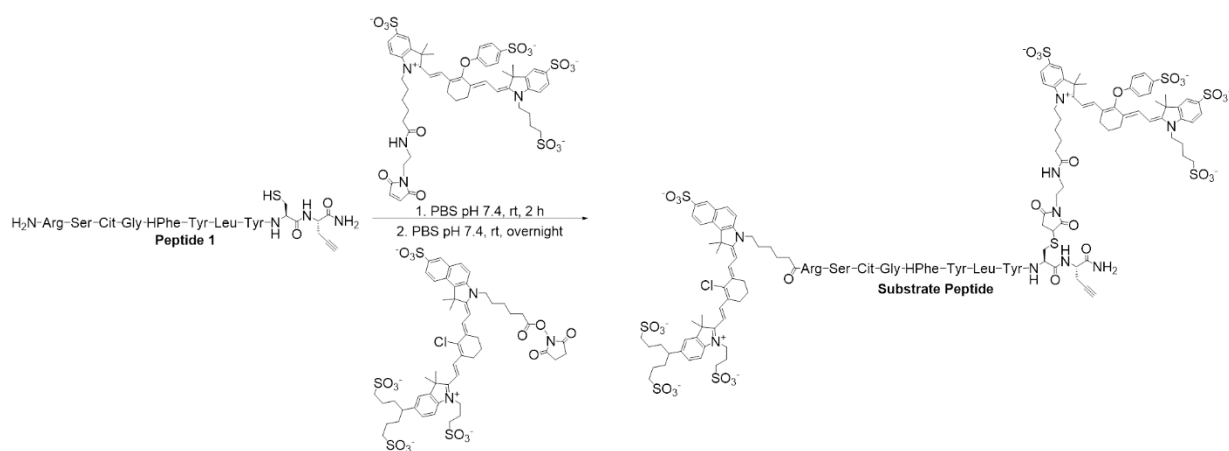


Figure S2. Synthetic scheme and structure of MMP-14 substrate peptide.

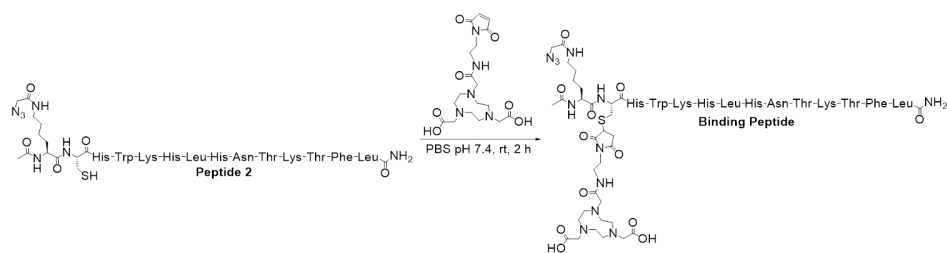


Figure S3. Synthetic scheme and structure of MMP-14 binding peptide.

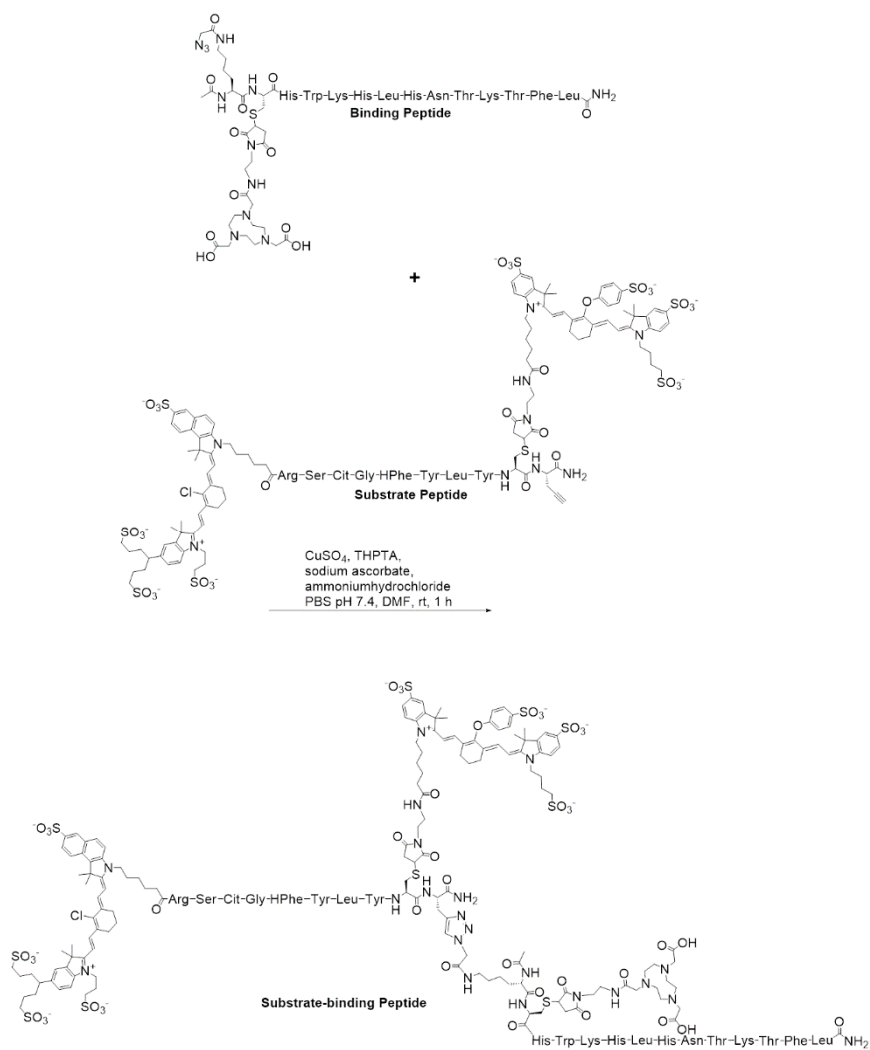
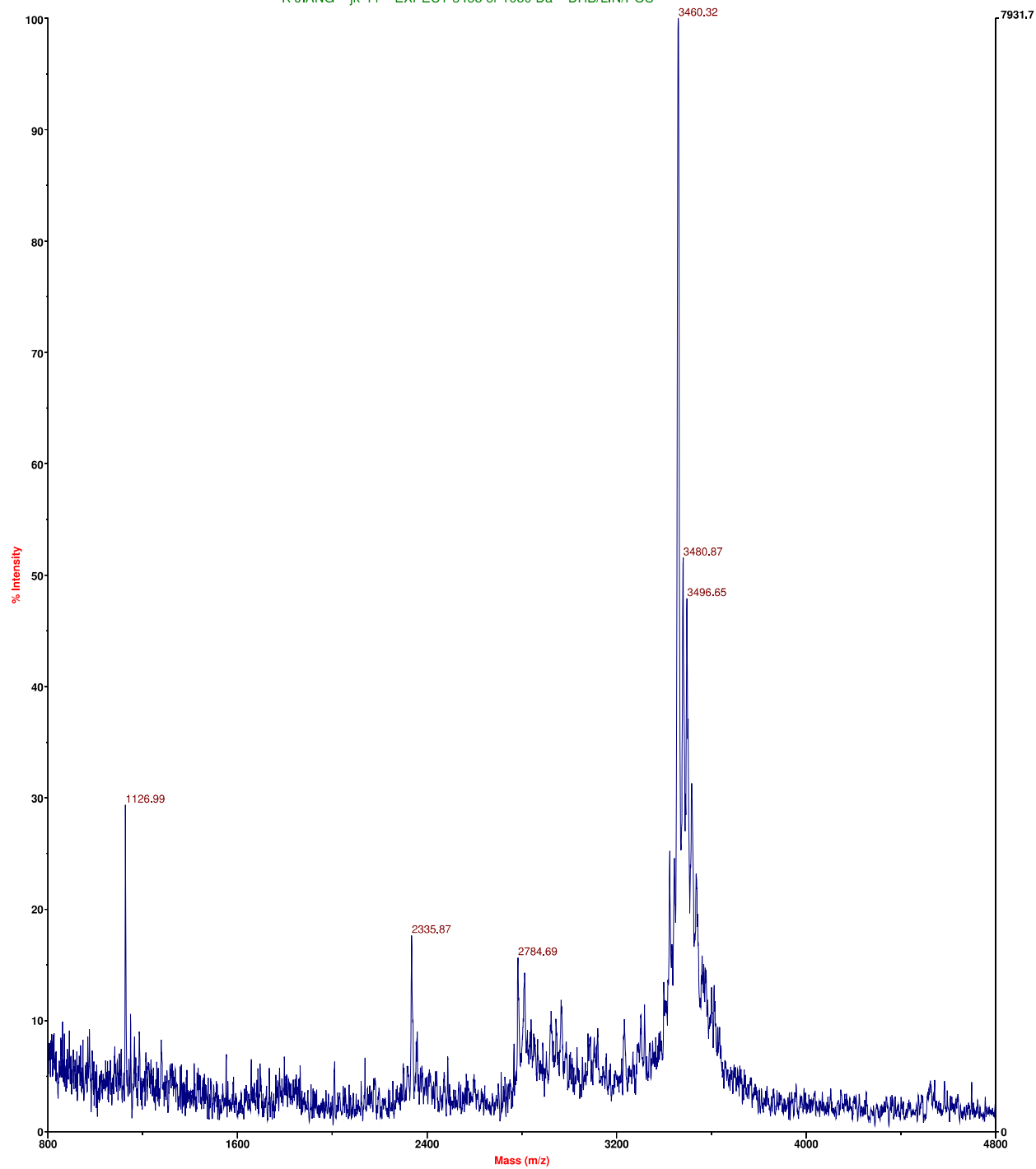


Figure S4. Synthetic scheme and structure of MMP-14 substrate-binding peptide.

Voyager Spec #1=>SM5[BP = 3460.6, 7932]

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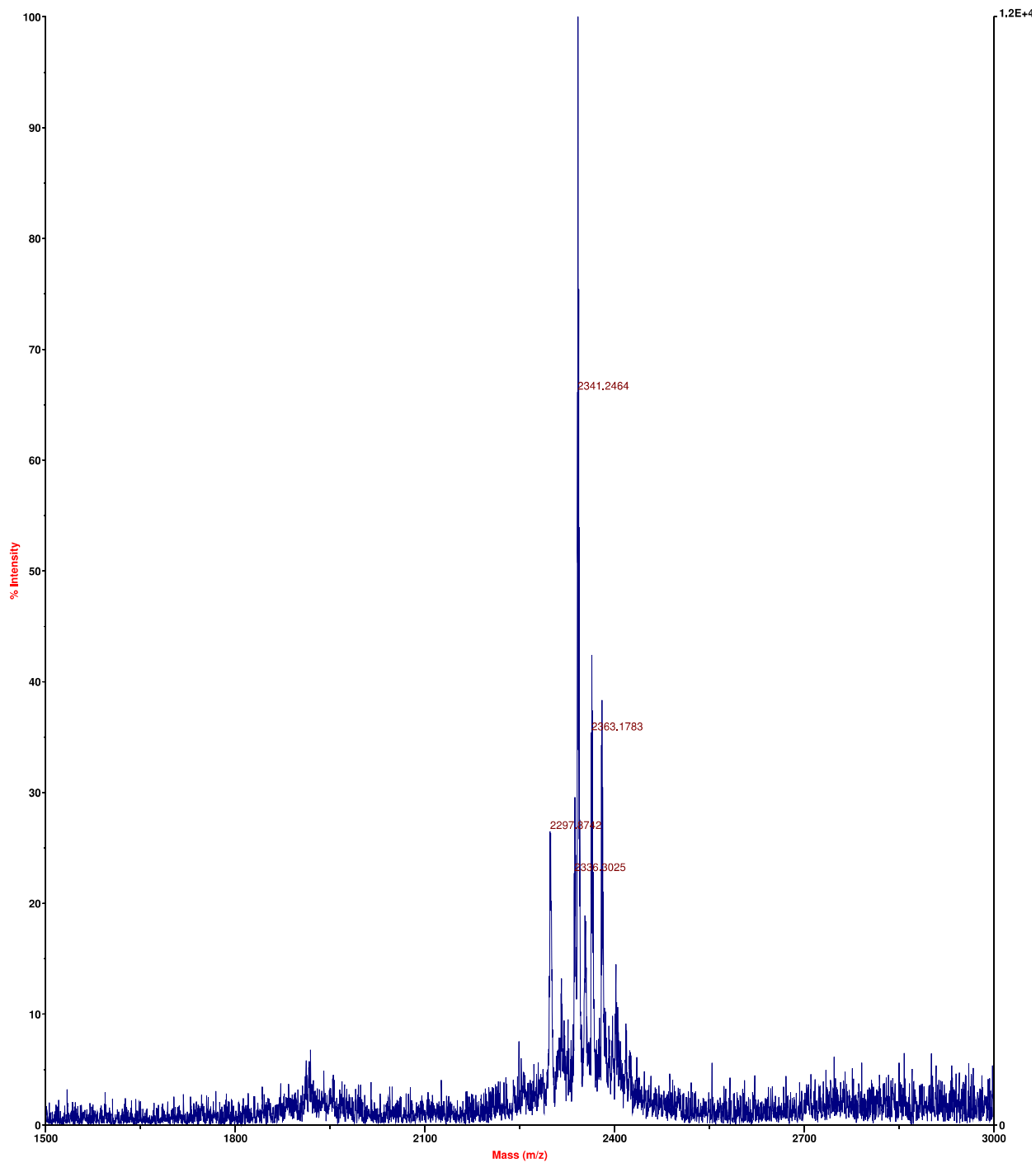


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Figure S5. Maldi-Tof MS spectrum of substrate peptide.

Voyager Spec #1[BP = 2342.3, 12381]

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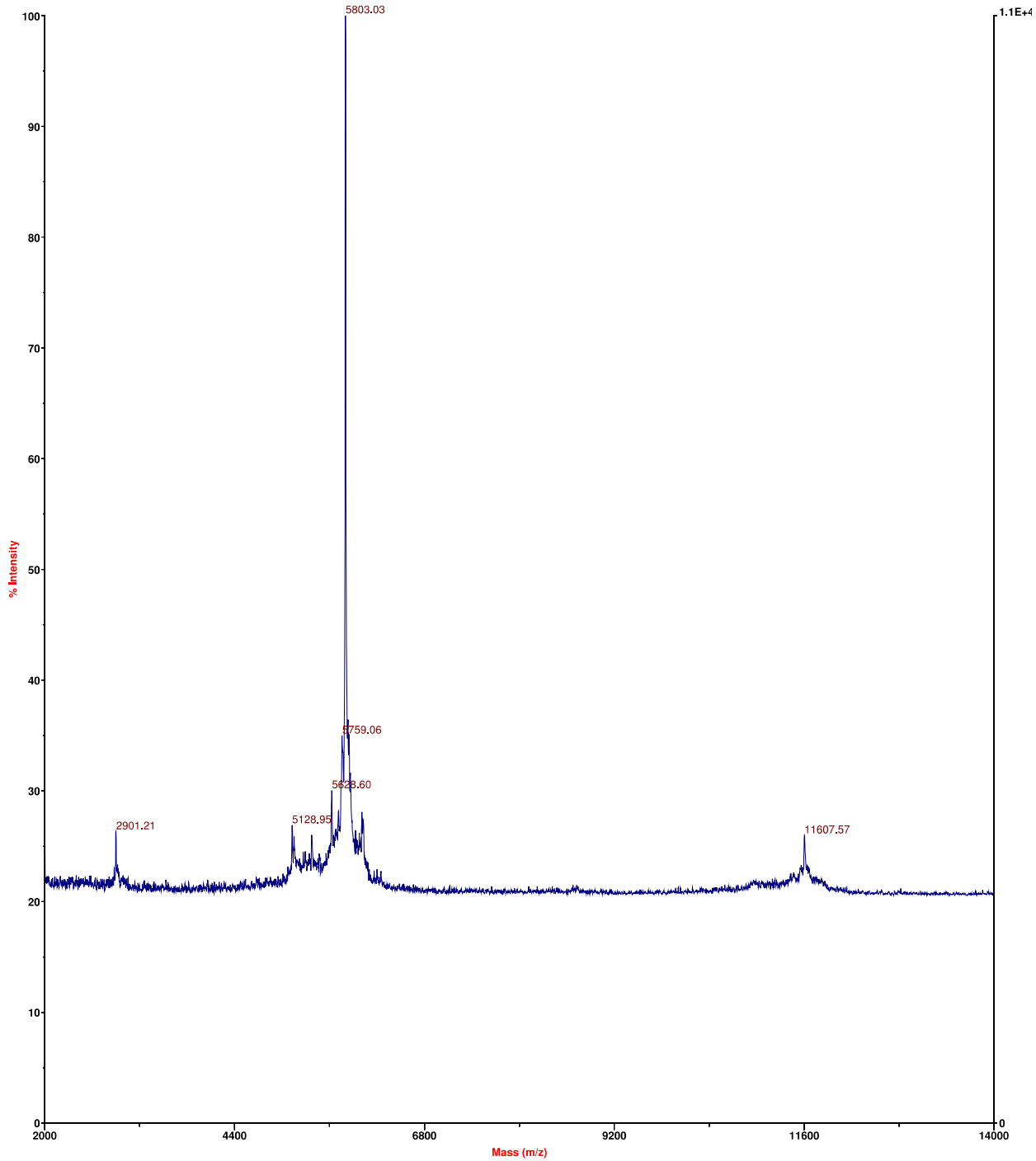


J Kiang jk-27 Expect 2341 Da DHB/ref/pos
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Figure S6. Maldi-Tof MS spectrum of binding peptide.

Voyager Spec #1=>SM5[BP = 5802.6, 10799]

K JIANG JK-MMP-14 Probe EXPECT 5804 Da SA/LIN/POS



K JIANG JK-MMP-14 Probe EXPECT 5804 Da SA/LIN/POS
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Acquired: 13:54:00, March 06, 2018

Figure S7. Maldi-Tof MS spectrum of substrate-binding peptide.

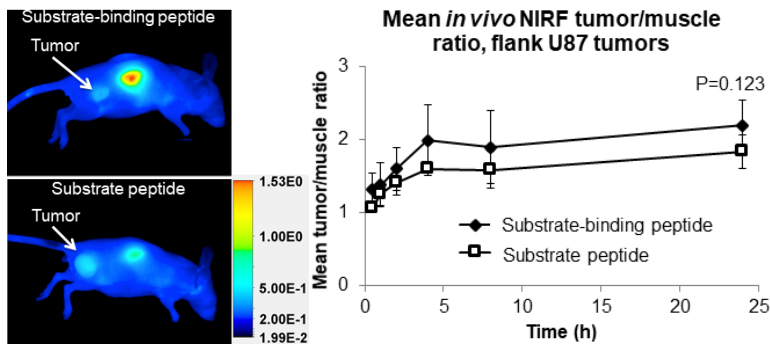


Figure S8. Mice bearing *s.c.* U87 xenografts were injected *i.v.* with 10 nmol of the substrate peptide or the substrate-binding peptide (n=5 mice/group) and imaged from 0-24 h on a Pearl imaging system (LI-COR). Left, representative *in vivo* NIRF images of mice at the 24 h time point after injection of the substrate-binding peptide (top) or the substrate peptide (bottom). Tumors are indicated by arrows. Right, mean *in vivo* NIRF tumor/muscle ratios at various time points (0.5-24 h) after injection of the substrate-binding peptide. No significant difference in NIRF tumor/muscle ratios between groups of mice was observed (p=0.123 at 24 h p.i.).

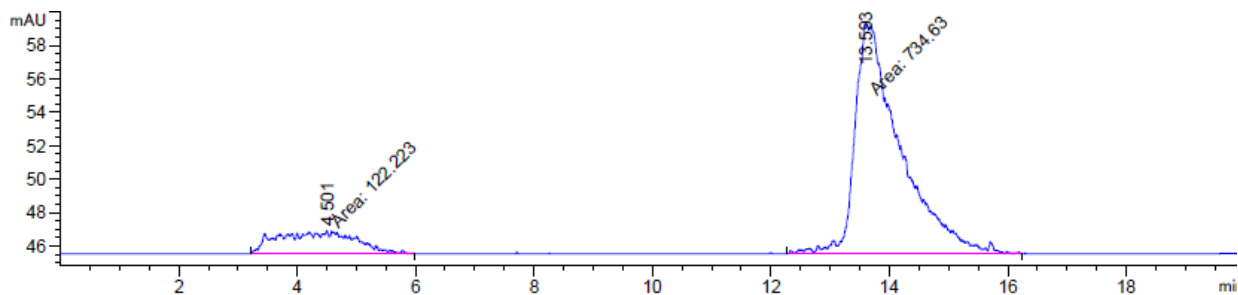


Figure S9. Radio RP-HPLC analysis of ^{68}Ga -binding peptide reaction. Product retention time: 13.6 min. Retention time of the non-labeled binding peptide: 13.4 min.

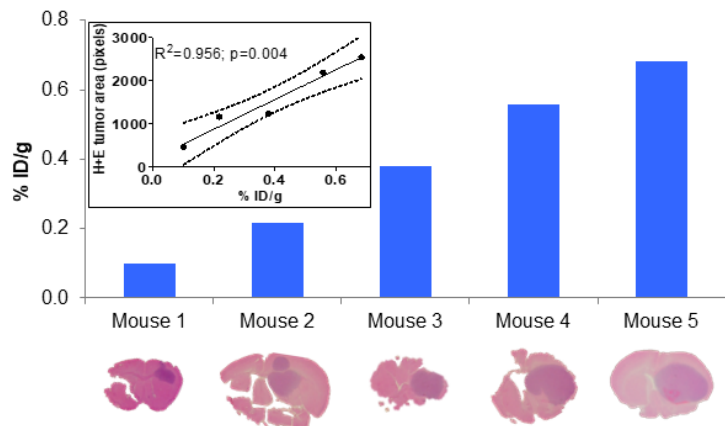


Figure S10. Correlation between the activity of ^{68}Ga -binding peptide in brains of mice bearing orthotopic PDX JX12 GBM tumors and tumor size. Athymic nude mice (n=5) with orthotopic PDX JX12 GBM tumors were injected *i.v.* with the ^{68}Ga -labeled binding peptide (4.4-9.6 MBq, 5.8 nmol peptide), imaged by PET/CT at 1 h, and euthanized for biodistribution analyses at 1.5 h p.i. The graph shows the % injected dose per gram (%ID/g) of radioactivity in individual mouse brains after resection. Inset, correlation between %ID/g in resected brains and quantification of tumor area in H+E stained sections (5 μm) containing the widest tumor diameter (as shown in lower panels). Tumor area was quantified in ImageJ by calculating the pixel area from pictures of H+E stained sections.

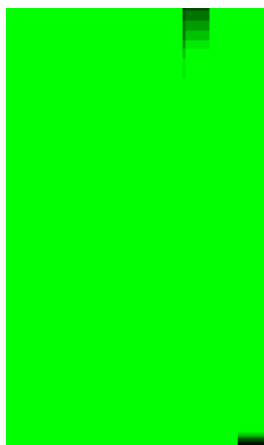


Figure S11. Representative maximum intensity projection PET/CT image of a mouse bearing an orthotopic PDX JX12 GBM tumor at 2 h after *i.v.* injection of ^{68}Ga -binding peptide. (PET SUV scale 0.1 to 3.0)

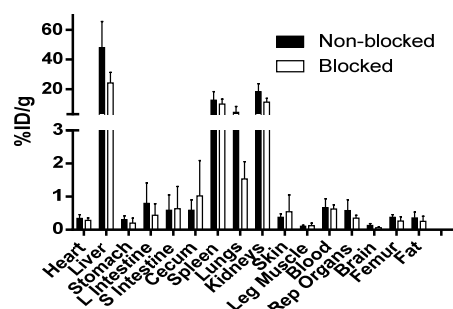


Figure S12. Biodistribution results (average $\pm$ SD) of ^{68}Ga -binding peptide from resected tissues of mice bearing orthotopic PDX JX12 GBM tumors. Mice ($n=5$ mice for non-blocked group, $n=4$ mice for blocked group) were euthanized at 3.5 h *p.i.* of ^{64}Cu -substrate-binding peptide.

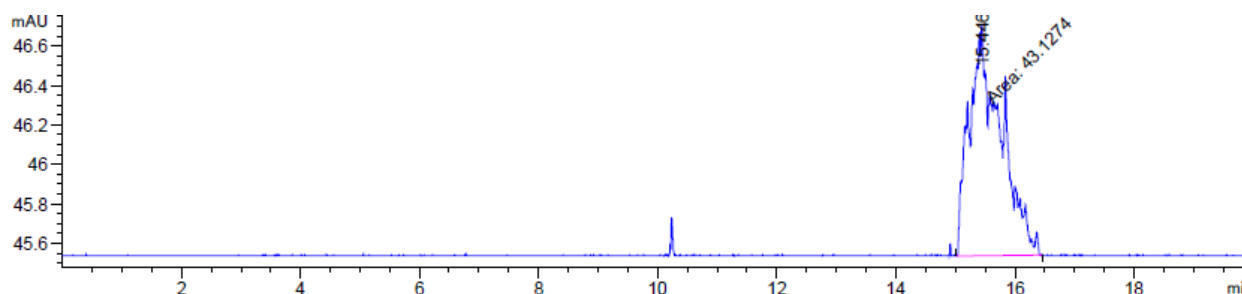


Figure S13. Radio RP-HPLC analysis of ^{64}Cu -substrate-binding peptide reaction. Product retention time: 15.4 min. Retention time of the non-labeled substrate-binding peptide: 15.3 min.

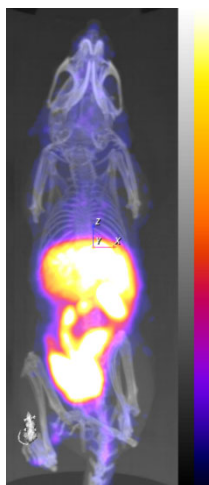


Figure S14. Representative maximum intensity projection PET/CT image of a mouse bearing an orthotopic PDX JX12 GBM tumor at 2 h after *i.v.* injection of ^{64}Cu -substrate-binding peptide. (PET SUV scale 0.25 to 3.15)

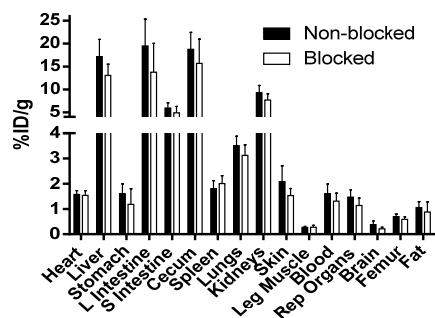


Figure S15. Biodistribution results (average $\pm$ SD) of ^{64}Cu -substrate-binding peptide from resected tissues of mice bearing orthotopic PDX JX12 GBM tumors. Mice (n=8 mice for non-blocked group, n=7 mice for blocked group) were euthanized at 5.5 h *p.i.* of ^{64}Cu -substrate-binding peptide.

Table S1. *Ex vivo* NIRF signal (measured by the Odyssey scanner) metrics of positive predictive value, negative predictive value, sensitivity, and specificity from the substrate peptide or the substrate-binding peptide, relative to histologic verification of tumor (by H+E), measured in 5 μm sections of paraffin-embedded brain slices from mice bearing orthotopic PDX glioma tumors.

	Positive predictive value	Negative predictive value	Sensitivity	Specificity
Substrate peptide, 4 h	67.9%	100.0%	100.0%	58.3%
Substrate peptide, 24 h	50.0%	91.7%	83.3%	73.3%
Substrate-binding peptide, 4 h	85.7%	50.0%	85.7%	100.0%
Substrate-binding peptide, 24 h	83.3%	100.0%	100.0%	60.0%

Supplementary References

1. Warram, J. M.; de Boer, E.; Korb, M. L.; Hartman, Y. E.; Kovar, J.; Markert, J. M.; Gillespie, G. Y.; Rosenthal, E. L., Fluorescence-guided resection of experimental malignant glioma using cetuximab-IRDye 800CW. *British Journal of Neurosurgery* **2015**, 29 (6), 850-858.

MMP-14 as a noninvasive marker for PET and NIRF imaging of glioblastoma multiforme

Hailey Houson, Benjamin Kasten, Ke Jiang, Jianghong Rao, Jason Warram

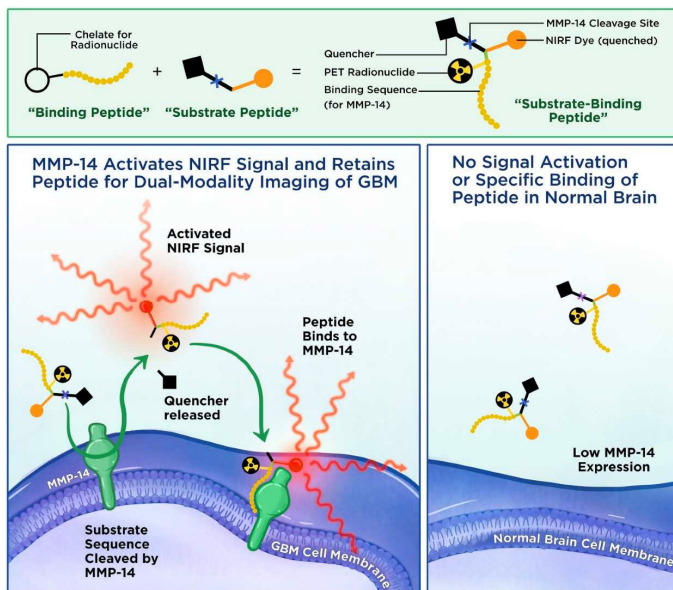
Background: Glioblastoma multiforme (GBM) is a rapidly proliferating and invasive cancer originating from the glial cells of the brain. GBMs are diffuse, with indistinct tumor margins leading to incomplete surgical resection and subsequent recurrence. Matrix metalloproteinase 14 (MMP-14) is an enzyme that degrades the extracellular matrix. MMP-14 is highly expressed in GBM, and is involved in the invasion of the cancerous cells into the surrounding tissue. The primary treatment for GBM is surgery, with an increasing percentage of the tumor removed correlating to improved survival of the patient. However, current imaging of GBM pre-surgery is difficult to translate during surgery due to shift in the tissue. Combinatorial imaging of MMP-14 pre-surgery with PET and intra-operatively with near infrared fluorescence (NIRF) would allow for improved surgical resection of the tumor.

We have developed peptides to bind MMP-14, which are suitable for both PET and NIRF imaging.

Methods: Several peptide constructs were developed and used for imaging in mice bearing intracranial implants of patient derived xenograft GBMs. Construct 1 exhibited binding affinity to MMP-14, construct 2 was a substrate for MMP-14, and construct 3 was a combination of 1 and 2. Constructs 1 and 3 were labeled with ^{68}Ga or ^{64}Cu and used for PET imaging. Constructs 2 and 3 were fluorescent and were used for NIRF imaging.

Results: Immunohistochemistry showed the presence of MMP-14 in the tumor areas, which was co-localized with fluorescence signal from probes 2 and 3. Radiolabeled probes 1 and 3 showed accumulation in the tumor, which could be significantly reduced with the addition of non-labeled blocking peptides ($p < 0.01$ and $p < 0.05$ respectively). Additionally, in vivo PET and ex vivo NIRF was well correlated as shown using construct 3 ($R^2 = 0.80$).

Conclusions: All 3 constructs showed accumulation in the tumor area. Results warrant further investigation of probes in additional preclinical GBM models. Development of probes to image MMP-14 could improve noninvasive detection of the tumor area before surgery, guided tumor resection, and surveillance for recurrence.





Targeting MMP-14 for dual PET and fluorescence imaging of glioma in preclinical models

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Abstract

Purpose There is a clinical need for agents that target glioma cells for non-invasive and intraoperative imaging to guide therapeutic intervention and improve the prognosis of glioma. Matrix metalloproteinase (MMP)-14 is overexpressed in glioma with negligible expression in normal brain, presenting MMP-14 as an attractive biomarker for imaging glioma. In this study, we designed a peptide probe containing a near-infrared fluorescence (NIRF) dye/quencher pair, a positron emission tomography (PET) radionuclide, and a moiety with high affinity to MMP-14. This novel substrate-binding peptide allows dual modality imaging of glioma only after cleavage by MMP-14 to activate the quenched NIRF signal, enhancing probe specificity and imaging contrast.

Methods MMP-14 expression and activity in human glioma tissues and cells were measured in vitro by immunofluorescence and gel zymography. Cleavage of the novel substrate and substrate-binding peptides by glioma cells in vitro and glioma xenograft tumors in vivo was determined by NIRF imaging. Biodistribution of the radiolabeled MMP-14-binding peptide or substrate-binding peptide was determined in mice bearing orthotopic patient-derived xenograft (PDX) glioma tumors by PET imaging.

Results Glioma cells with MMP-14 activity showed activation and retention of NIRF signal from the cleaved peptides. Resected mouse brains with PDX glioma tumors showed tumor-to-background NIRF ratios of 7.6–11.1 at 4 h after *i.v.* injection of the peptides. PET/CT images showed localization of activity in orthotopic PDX tumors after *i.v.* injection of ⁶⁸Ga-binding peptide or ⁶⁴Cu-substrate-binding peptide; uptake of the radiolabeled peptides in tumors was significantly reduced ($p < 0.05$) by blocking with the non-labeled-binding peptide. PET and NIRF signals correlated linearly in the orthotopic PDX tumors. Immunohistochemistry showed co-localization of MMP-14 expression and NIRF signal in the resected tumors.

Conclusions The novel MMP-14 substrate-binding peptide enabled PET/NIRF imaging of glioma models in mice, warranting future image-guided resection studies with the probe in preclinical glioma models.

Keywords MMP-14 · Glioma · NIRF · PET · Molecular imaging · Dual modality

Benjamin B. Kasten and Ke Jiang contributed equally to this work.

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Introduction

Malignant glioma is the most common and deadly primary brain malignancy in adults. The current standard of care, comprised of maximal safe surgical resection followed by radio-chemotherapy, is associated with a median survival of less than 18 months [1]. Clinical trials are testing novel therapeutic strategies in attempts to improve the current prognosis of patients with glioma. Studies have shown that the extent of surgical resection correlates with patient outcomes [2, 3]. Unfortunately, malignant tissues are frequently difficult to differentiate from normal brain parenchyma, making complete surgical resection of all glioma while sparing vital healthy brain a significant clinical challenge. Developing agents that specifically target aggressive glioma cells for both non-invasive and intraoperative imaging is an attractive strategy to guide effective therapeutic intervention. Glioma is known for its invasive and diffuse growth pattern, which is indicative of matrix metalloproteinase (MMP) activity [4–7]. Activated MMP-14, also called MT1-MMP, is overexpressed in glioma while it is not expressed at significant levels in normal brain (cerebral) tissue. MMP-14 expression is known to increase with the grade of glioma and correlates with poor patient outcome [8–11]. Preclinical studies have demonstrated that MMP-14 can be exploited as a biomarker for molecular imaging of glioma with various non-invasive or intraoperative reporting modalities [12–14].

Non-invasive imaging of glioma patients through positron emission tomography (PET) with radiolabeled amino acid analogs has enabled preoperative planning for biopsy or surgery, mapping for radiotherapy, and assessing therapeutic response [15–22]. However, clinical PET suffers from low spatial resolution (> 3 mm) and does not permit real-time surgical guidance. Problems with brain shift further hamper co-registration of PET and anatomical images. While clinical PET complements the information from MRI [23], these modalities remain insufficient to guide real-time surgical resection of occult glioma. The US Food and Drug Administration (FDA) recently approved 5-aminolevulinic acid (5-ALA), which is converted into the fluorescent metabolite protoporphyrin IX (PpIX) in rapidly proliferating tumor cells (e.g., glioma), for fluorescence-guided surgical resection of glioma. Phase III trials have shown 5-ALA fluorescence-guided resection in glioma patients was well tolerated and mediated greater rates of complete resection compared to MRI alone [24, 25]. Nonetheless, the optical properties of PpIX are not ideal for in vivo imaging. Significant tissue autofluorescence and photon attenuation at the wavelengths of excitation (~ 405 nm) and emission (635 nm) reduce spatial resolution and tumor-to-background ratios (TBRs) associated with 5-ALA imaging of glioma [26–28]. Molecular imaging strategies that use near-infrared fluorescence (NIRF) reporters are attractive for in vivo imaging and fluorescence-guided resection due to

the minimal tissue autofluorescence and significantly less photon attenuation in the NIR window (700–900 nm) [29–31]. Several molecular targeted compounds with NIRF reporters are currently being evaluated for resecting glioma in preclinical and clinical studies [14, 29, 30, 32–34].

The purpose of the present work was to design a novel, MMP-14-activatable dual PET/NIRF peptide probe for imaging and guiding resection of glioma (Fig. 1). The peptide probe combined (1) a NIRF reporter and quencher pair separated by a peptide sequence (MMP-14 “substrate peptide”) that is cleaved specifically by activated MMP-14 to release the quencher and allow visualization of the NIRF dye and (2) a chelate for radionuclides attached to a peptide sequence that binds to MMP-14 (MMP-14 “binding peptide”) and enables PET imaging. The following in vitro and in vivo studies tested the ability of these MMP-14-targeted imaging probes to detect preclinical models of human glioma. Glioma cells in vitro and orthotopic xenograft tumors in mice in vivo cleaved the substrate and the substrate-binding peptides to activate the NIRF signal of the initially quenched peptide probes, yielding favorable imaging contrast in tumors relative to the normal brain. In vivo PET/CT imaging showed notable activity in orthotopic glioma tumors relative to normal brain after *i.v.* injection of the radiolabeled-binding or substrate-binding peptides. PET and NIRF signals from the substrate-binding peptide correlated linearly in the orthotopic PDX tumors and co-localized with MMP-14 expression in the resected tumors. The results from these initial studies indicate the success of the proposed dual-modality imaging strategy to detect MMP-14 in glioma models with the first-generation substrate-binding peptide probe.

Results

Human glioma tumor tissues and cell lines express varying levels of MMP-14

Immunofluorescence staining of a human glioma tissue microarray was performed to determine the relative protein expression of MMP-14 in glioma and normal brain specimens. Consistent with previous reports [8–11], the immunofluorescence results showed significant overexpression of MMP-14 in all grades of glioma relative to normal cerebral tissue (1.6 ± 0.6 , 2.2 ± 0.7 , 2.5 ± 0.8 , 3.0 ± 1.2 glioma/normal brain ratios for grade 1, grade 2, grade 3, and grade 4, respectively; $p < 0.05$), with highest expression in grade 4 glioma (Fig. 2A). Western blot, immunofluorescence, and gel zymography studies were then performed to characterize the in vitro expression and activity of MMP-14 and MMP-2 in immortalized human glioma cell lines. Absolute expression of MMP-14 does not necessarily correlate to MMP-14 activity, as the latter is regulated partly through tissue inhibitors of metalloproteinases (TIMPs). For instance, TIMP-2 binds to the catalytic domain

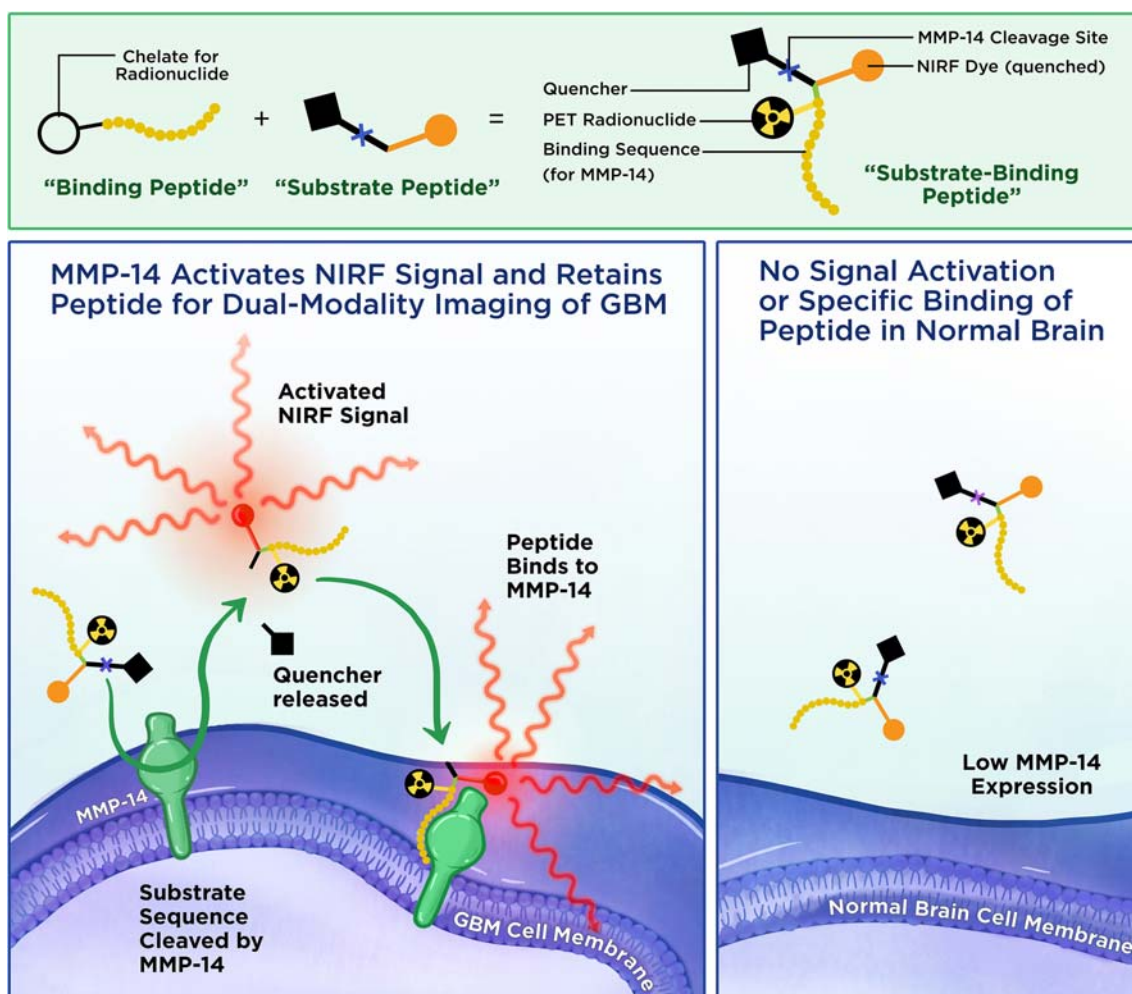


Fig. 1 Diagram showing general scheme for dual-modality PET/NIRF imaging of glioma (GBM) with an MMP-14 activatable peptide

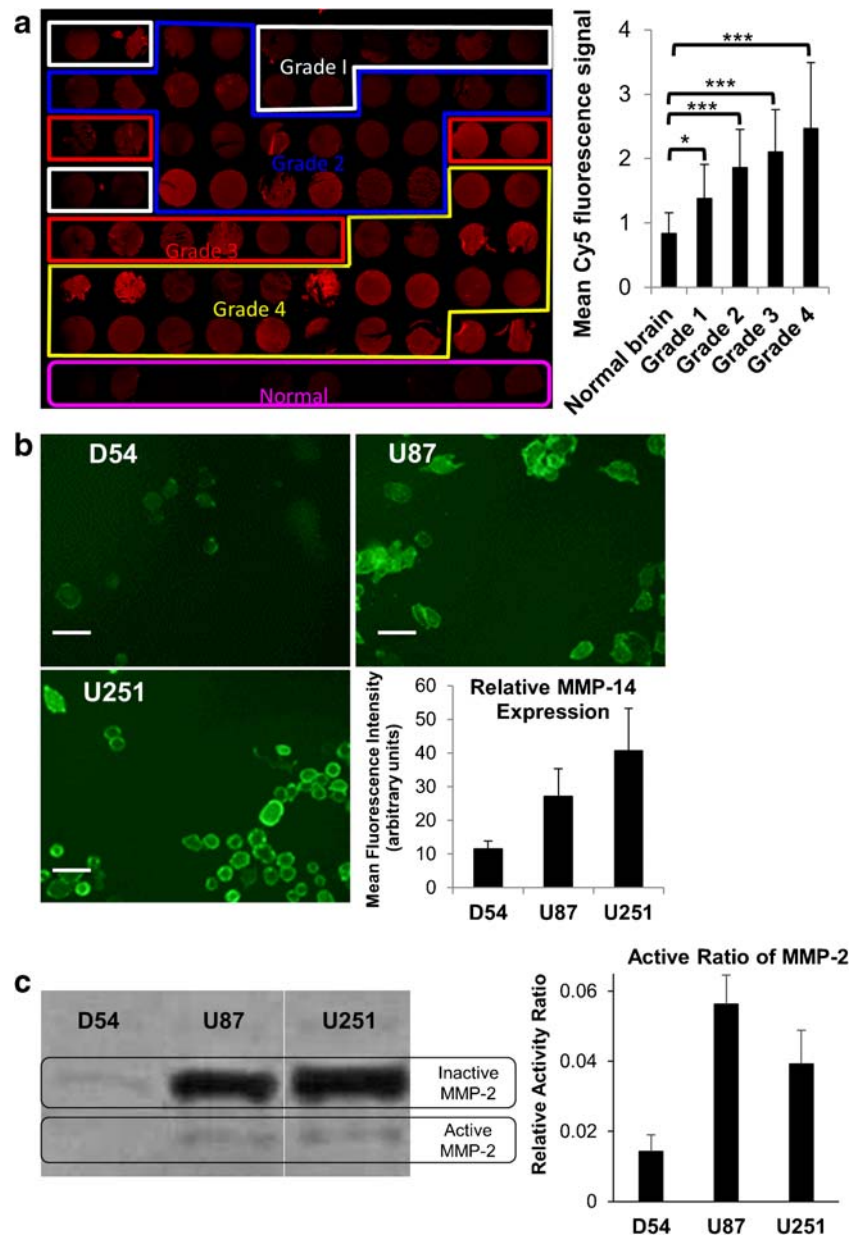
of MMP-14, thus blocking enzymatic activity. TIMP-2 is also required in a ternary complex with MMP-14 and proMMP-2 prior to enzymatic cleavage by a second MMP-14 enzyme to activate MMP-2 [7]. U251 cells in our studies displayed the highest expression of MMP-14 by immunofluorescence (Fig. 2B) and Western blot analyses (Fig. S1A,B), while U87 cells displayed the highest level of MMP-14 activity as indirectly determined through gel zymography analyses of MMP-2 activity (Fig. 2C), which is catalyzed by MMP-14 as described [5, 35]. U87 cells showed the highest MMP-2 expression by Western blot (Fig. S1C,D). Although TIMP expression was not determined in our studies, the zymography assay confirmed MMP-14 activity in the glioma cells. These results indicate the cell lines would be suitable in assays that employ peptide probes as enzymatic substrates for MMP-14.

Description of the novel MMP-14 activatable peptide probes

We and others have used an MMP-14 substrate peptide sequence (RSCitG-HPhe-YLY) to generate peptide probes for

imaging and therapy studies in human xenograft tumors that over-express MMP-14 [36–38]. For the present studies, this sequence was used between the NIRF IRDye800 and quencher IR QC-1 pair [39, 40] to generate the MMP-14 activatable NIRF substrate peptide (Supplementary Fig. S2). This strategy allows the initially quenched signal from IRDye800, which is suitable for NIRF imaging *in vivo*, to become activated upon cleavage of the peptide by MMP-14. For the second peptide component, an MMP-14-binding peptide sequence (HWKHLHNTKTFL) was selected that has previously been described with an apparent k_d of 47.4 nM for MMP-14 [41]. This binding peptide sequence has been joined to various reporting moieties and used in a rat orthotopic glioma model [12] as well as human xenograft tumors that overexpress MMP-14 [41–43]. For the present studies, a derivative of the NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid) chelate for coordination with $^{64}\text{Cu}(\text{II})$ or $^{68}\text{Ga}(\text{III})$ was attached to the peptide sequence to generate the MMP-14-binding peptide (Supplementary Fig. S3) for *in vivo* PET imaging of MMP-14 expression. The two peptide precursors were synthesized separately by standard solid phase synthesis, NHS, and

Fig. 2 Human glioma patient specimens and cell lines grown in vitro express different levels of MMP-14. **A** MMP-14 immunofluorescence (left) and quantification of fluorescence signal (right) in a human tissue microarray (mean $\pm$ SD, $n = 10$ –24 tissue sections/group); **B** MMP-14 immunofluorescence of adherent GBM cells grown in vitro and quantification of fluorescence signal (bottom right; mean $\pm$ SD, $n = 25$ –30/group); **C** MMP-2 gel zymography (left) and quantification of relative active/latent MMP-2 band intensity (right) from supernatants of adherent GBM cells grown in vitro (mean $\pm$ SD, $n = 3$ /group); scale bar in B, 20 μ m; * $p < 0.05$; *** $p < 0.001$



maleimide coupling reactions and then joined via a cycloaddition reaction between the azide and alkyne moieties present on the corresponding precursor peptides to generate the combined MMP-14 substrate-binding peptide (Supplementary Fig. S4). Mass spectroscopic analyses were consistent with the anticipated peptide structures (Supplementary Figs. S5, S6, S7).

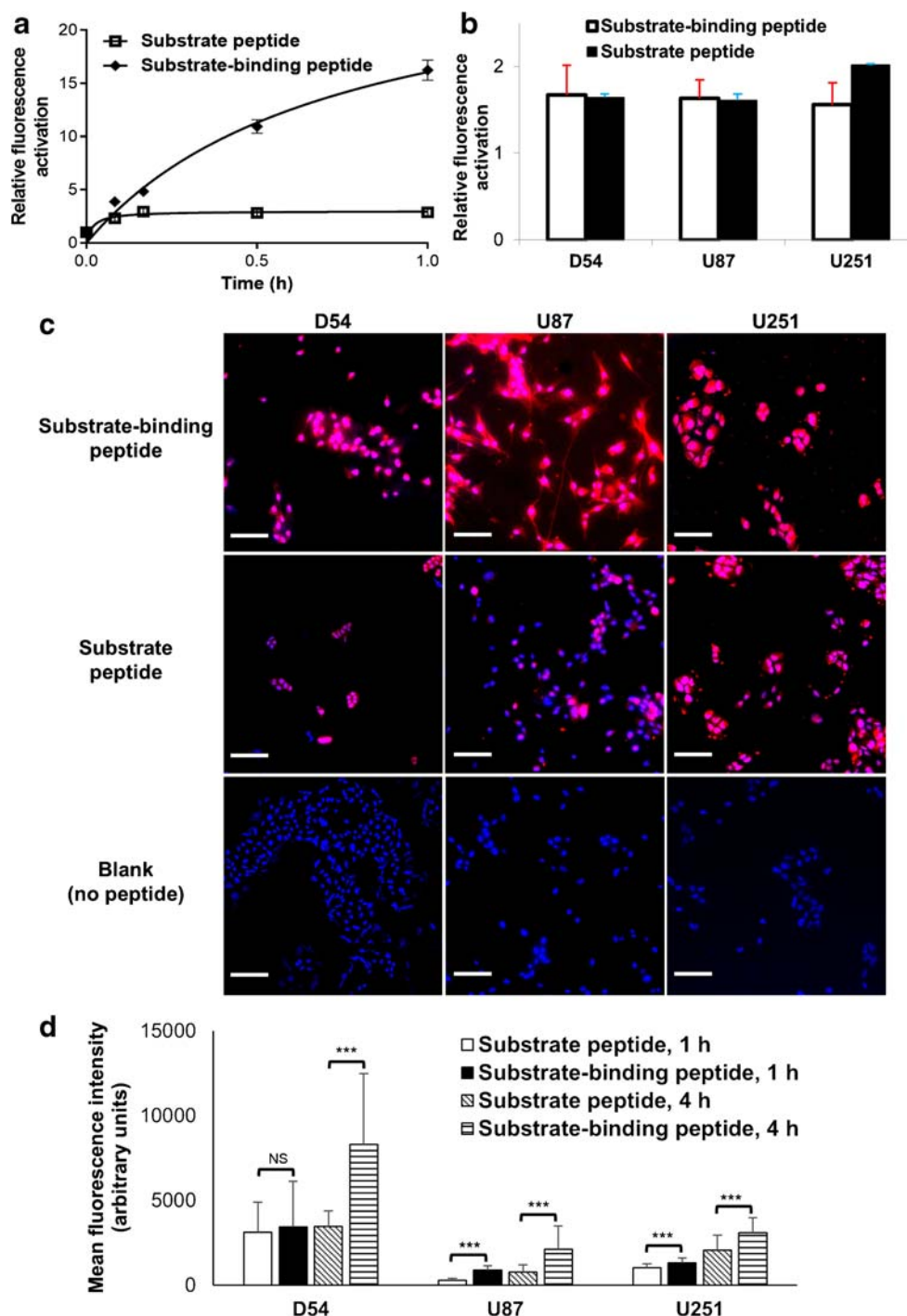
MMP-14 and human glioma cells activate the NIRF signals of the MMP-14 peptide probes in vitro

With the novel MMP-14-targeted peptides in hand, in vitro NIRF activation studies with the catalytic domain of MMP-14 were performed. Previous studies have shown that appending

fluorescence dyes or other moieties to the N- and C-termini of the core substrate peptide sequence does not abrogate cleavage of the peptide by MMP-14 [37, 44]. However, studies to date have not determined if incorporation of an MMP-14-binding ligand into the same scaffold as an MMP-14 substrate sequence affects cleavage of the substrate peptide by the enzyme.

As presented in Fig. 3A, both the substrate and the substrate-binding peptides showed NIRF activation over time relative to the quenched starting moieties during incubation with MMP-14. The relative NIRF activation of the substrate peptide appeared to stabilize within the first 10 min, while NIRF activation of the substrate-binding peptide continued to increase beyond 1 h. The fold of increase in the

Fig. 3 Human MMP-14 enzyme and glioma cells activate the NIRF signals of the MMP-14 peptides in vitro. **A**, relative increase in NIRF activation at various time points (0–1 h) after incubating the substrate-binding peptide or the substrate peptide (0.5 μ M) with the recombinant catalytic domain of MMP-14 at 37 °C. **B**, relative increase in NIRF activation released into the supernatant after incubating the substrate-binding peptide or the substrate peptide (0.5 μ M) with human glioma cell lines (D54, U87, U251) at 37 °C for 2 h (mean $\pm$ SD, $n = 2$ –4/group). **C**, NIRF microscopy showing the cell-associated NIRF signal (red) in glioma cells (D54, U87, U251) 1 h after incubation with the substrate-binding peptide (top), substrate peptide (middle), or buffer control without peptide (bottom); cell nuclei were counter-stained with DAPI (blue); scale bar: 50 μ m. **D**, quantification of cell-associated NIRF signal at 1 or 4 h after incubating glioma cells in vitro with the substrate or substrate-binding peptide (mean $\pm$ SD, $n = 30$ /group). *** $p < 0.001$; NS, not significant



fluorescence with the substrate-binding peptide was much higher than that with the substrate peptide. Based on these initial results, it is possible that the binding sequence may influence the kinetics of the substrate-binding peptide cleavage reaction by MMP-14.

NIRF signal activation of the substrate and substrate-binding peptides was apparent in supernatant solutions (Fig. 3B) and associated with the cells (Fig. 3C) during in vitro incubation with the glioma cell lines. Similar increases in

NIRF intensities released into the supernatant were observed during incubation of the peptides with all three glioma cell lines (Fig. 3B) regardless of their relative expression of active MMP-14 (Fig. 2C). NIRF microscopy showed that U87 and U251 cells had significantly higher cell-associated mean NIRF signal at both 1 and 4 h after incubation with the substrate-binding peptide relative to the substrate peptide ($p < 0.001$) at the respective time points (Fig. 3D). In D54 cells, which had lower MMP-14 expression and activity relative to

the other cells (Fig. 2B,C), the cell-associated NIRF signal was significantly higher for the substrate-binding peptide compared to the substrate peptide at 4 h ($p < 0.001$) but not at 1 h ($p > 0.05$; Fig. 3D). These results are consistent with the anticipated mechanism of the substrate-binding peptide, where the MMP-14 binding moiety mediates cellular retention of the residual fluorophore-containing product following cleavage of the substrate sequence by MMP-14. The observed NIRF signal associated with cells incubated with the substrate peptide, which lacks the MMP-14-binding component, likely indicates nonspecific cell uptake of the substrate peptide or the fluorophore-containing product following cleavage by MMP-14. Collectively, these results indicate that glioma cells, including those with relatively low MMP-14 activity, are capable of cleaving the novel peptide substrates *in vitro*.

NIRF signals of the MMP-14 activatable peptide probes localize in human glioma xenografts in nude mice *in vivo*

Having confirmed that glioma cells activate the NIRF signal of the peptides *in vitro*, NIRF imaging studies were performed to determine if the peptide probes showed uptake in glioma xenograft tumors *in vivo*. At 24 h after *i.v.* injection of the substrate-binding peptide, *in vivo* NIRF imaging showed a low TBR (1.3 ± 0.2 relative to muscle) in subcutaneous

(*s.c.*) D54 tumors and significantly higher TBR in *s.c.* U87 tumors (2.2 ± 0.4 ; $p < 0.001$) (Fig. 4A, B). Low NIRF signal was observed in all normal tissues except the kidneys (Fig. 4A), indicating predominantly renal clearance of the peptide. *S.c.* U87 tumors from groups of mice injected *i.v.* with either the substrate peptide or the substrate-binding peptide showed no significant difference in TBR or mean NIRF intensity at the 24 h time point (Supplementary Fig. S8). These initial studies demonstrated that glioma tumors show uptake of NIRF signal after administering the quenched peptide substrates. The moderate NIRF TBRs observed for the flank tumors could be due to endogenous expression of MMP-14 in the tissues surrounding the tumors (e.g., muscle, skin) [45].

NIRF imaging studies with the substrate or substrate-binding peptides were subsequently performed in mice bearing orthotopic patient-derived xenograft (PDX) glioma tumors. Relative to flank xenografts of immortalized cell lines, these orthotopic PDX tumors more accurately retain characteristics of clinical glioma [46, 47]. Furthermore, orthotopic tumors were anticipated to better demonstrate the signal contrast from the peptide probes in glioma tumors relative to the endogenous surrounding tissue (e.g., normal brain). The low expression of MMP-14 in normal brain was anticipated to result in a low background NIRF signal, resulting in a higher and more appropriate TBR in the orthotopic glioma tumor model than was observed in

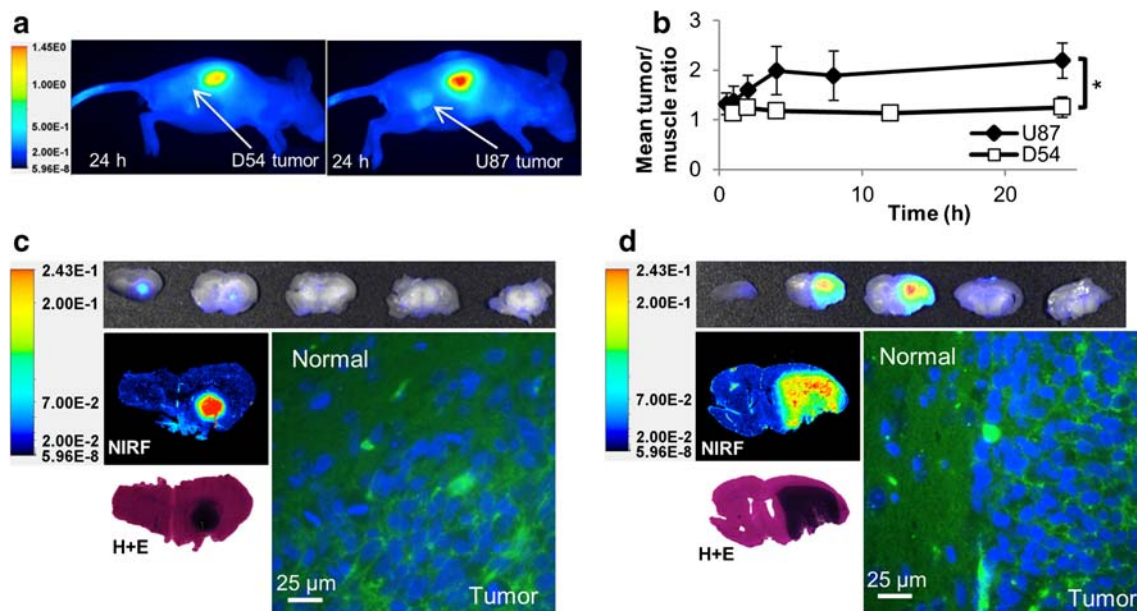


Fig. 4 NIRF signals from the MMP-14 peptide probes localize in human glioma xenograft tumors in mice *in vivo*. **A**, *in vivo* NIRF images of live mice bearing flank D54 or U87 glioma tumors at 24 h after *i.v.* injection of the substrate-binding peptide. **B**, mean *in vivo* tumor/muscle NIRF ratios at various time points (0.25–24 h) after injection of the substrate-binding peptide in mice bearing flank D54 or U87 glioma tumors (mean $\pm$ SD, $n = 5$ mice/group). **C,D**, mice bearing orthotopic PDX JX12 glioma tumors were injected *i.v.* either with the substrate peptide (**C**) or with the substrate-binding peptide (**D**). Mice were euthanized 1 h later, when the

brains were resected, fixed in formalin overnight and serially sectioned (2 mm). Gross tissue NIRF imaging (Pearl system) showed localization of NIRF signals in the serial tumor-bearing sections (2 mm sections, top panels), as confirmed by higher resolution NIRF imaging (Odyssey system) of 5 μ m tissue sections and H + E stained 5 μ m tissue sections. NIRF microscopy of DAPI-stained tissue sections (5 μ m, right panels, 630 $\times$ magnification) showed NIRF signal accumulation (yellow channel) in the leading tumor edge but not adjacent normal brain (blue channel, DAPI). $*p < 0.05$

the flank tumor studies. High contrast of the NIRF signal in PDX JX12 tumors relative to adjacent normal brain was apparent in gross slices of resected brains at 1, 4, and 24 h after *i.v.* injection of either the substrate peptide (Fig. 4C) or the substrate-binding peptide (Fig. 4D). NIRF analyses of gross brain slices from the 4 and 24 h time points (Table 1) showed significantly higher ($p < 0.001$) mean NIRF signal in the tumor relative to adjacent normal brain from mice given the substrate-binding peptide. This result is consistent with retention of the cleaved peptide moiety containing the fluorophore within the tumor and low accumulation of the cleaved peptide in normal brain, which has negligible expression of MMP-14. The NIRF signal in the tumor was significantly higher than in normal brain of mice at 4 h ($p < 0.001$), but not at 24 h ($p = 0.074$), after injection of the substrate peptide. The group of mice analyzed at 24 h after injecting the substrate peptide had fewer evaluable tumors compared to the other groups, which could have impacted the statistical outcome of the NIRF signal comparison in these mice. While the NIRF mean tumor signals and TBRs were higher in mice given the substrate-binding peptide relative to the substrate peptide (Table 1), the TBRs were not significantly different at the time points examined ($p > 0.05$, ANOVA). NIRF microscopy showed dispersion of the activated NIRF signal from the peptides throughout the tumors, including near the leading edge of tumor progression (Figs. 4C, D). Both peptides also yielded positive NIRF signals in regions of diffuse glioma cell growth beyond the bulk tumor (data not shown). Comparing histological tissue sections with confirmed glioma growth by H + E to NIRF signals measured in tissue sections yielded sensitivities above 83% for the substrate peptide and above 85% for the substrate-binding peptide (Supplementary Table S1) at the 4 h and 24 h time points. These results confirmed that the novel MMP-14 peptides could be used for NIRF imaging of orthotopic models of human glioma. These findings support future studies that utilize the peptides for intraoperative resection of preclinical glioma tumors *in vivo*.

PET signals of the radiolabeled MMP-14 peptide probes localize in human glioma orthotopic xenografts in nude mice *in vivo*

The second goal of this work was to determine if the peptides could be used for *in vivo* PET imaging of orthotopic models of human glioma tumors in mice. A preliminary study was performed using the binding peptide labeled with ^{68}Ga (^{68}Ga -binding peptide) in mice with orthotopic PDX JX12 tumors. The radiolabeled peptide was obtained in 87–91% radiochemical conversion after heating with ^{68}Ga at 90 °C for 20 min; further heating did not improve the yield of the product ^{68}Ga -binding peptide (Supplementary Fig. S9). Tumor-bearing mice were dosed *i.v.* with 0.4–0.8 nmol ^{68}Ga -binding peptide from the diluted reaction solution (molar activity approximately 6.1 GBq/ μmol at time of dosing). At 2 h after dosing, the tumors were visible during PET/CT imaging, while normal brain showed low uptake of activity (Fig. 5A). *Ex vivo* biodistribution analyses indicated significantly more uptake of radioactivity in brains of mice injected with ^{68}Ga -binding peptide (0.16 ± 0.02 %ID/g) compared to brains of mice injected with ^{68}Ga -binding peptide and 60-fold excess non-labeled-binding peptide as a blocking agent (0.07 ± 0.02 %ID/g, $p < 0.01$) (Fig. 5B), thus supporting the specific retention of the radiolabeled peptide in the PDX tumors. The amount of ^{68}Ga in resected brains correlated with qualitative tumor burden determined by H + E tissue analyses (Supplementary Fig. S10). PET images and *ex vivo* biodistribution analyses showed high accumulation of activity in the liver, spleen, and kidneys (Supplementary Figs. S11, S12), which was likely due to the relative hydrophobicity of the peptide at physiological pH.

A separate cohort of mice bearing orthotopic PDX JX12 tumors were used for PET imaging and biodistribution analyses with ^{64}Cu -labeled substrate-binding peptide (^{64}Cu -substrate-binding peptide). ^{64}Cu (12.7 h half-life) was used as the radionuclide with the substrate-binding peptide to allow PET imaging analyses at later time points (e.g., 4 h) that were used in the NIRF imaging studies. The relatively short half-

Table 1 *Ex vivo* NIRF signal intensities and TBRs from the substrate peptide or the substrate-binding peptide measured in 2 mm gross brain slices from mice bearing orthotopic PDX glioma tumors

	Mean NIRF signal in tumor	Mean NIRF signal in normal brain	<i>p</i> value (tumor vs. normal brain NIRF signal)	Mean $\pm$ SD TBR (range)
Substrate peptide, 4 h	0.012 ± 0.007	0.002 ± 0.001	< 0.001	7.6 ± 2.9 (2.7–10.9)
Substrate peptide, 24 h	0.011 ± 0.010	0.001 ± 0.00	0.074	8.7 ± 6.3 (2.9–17.1)
Substrate-binding peptide, 4 h	0.024 ± 0.011	0.002 ± 0.001	< 0.001	11.1 ± 4.5 (7.8–18.9)
Substrate-binding peptide, 24 h	0.021 ± 0.010	0.002 ± 0.001	< 0.001	13.3 ± 3.7 (6.0–17.6)

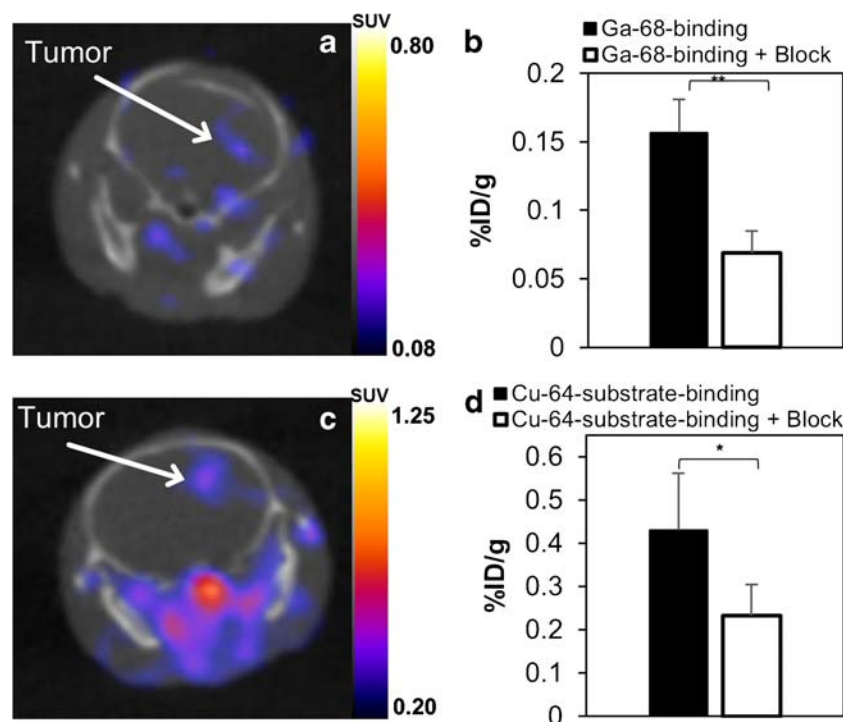


Fig. 5 PET imaging and biodistribution show specific localization of radiolabeled peptide probes in orthotopic PDX glioma tumors in vivo. **A**, representative transverse PET/CT image at 2 h after *i.v.* injection of ^{68}Ga -binding peptide in athymic nude mice bearing orthotopic PDX JX12 glioma tumors, showing localization of activity in the tumor. **B**, ex vivo biodistribution showing whole-brain activity at 3.5 h after *i.v.* injection of ^{68}Ga -binding peptide or of ^{68}Ga -binding peptide + block (non-labeled-binding peptide) in athymic nude mice bearing orthotopic PDX JX12 glioma tumors. Full biodistribution analyses are available in the Supplementary Data. **C**, representative transverse PET/CT image at

4 h after *i.v.* injection of ^{64}Cu -substrate-binding peptide in athymic nude mice bearing orthotopic PDX JX12 glioma tumors, showing localization of activity in the tumor. Activity in soft tissues of the neck (e.g., blood vessels, muscle, lymph nodes) was apparent at the scaling intensity shown in the PET image. **D**, ex vivo biodistribution showing whole-brain activity at 5.5 h after *i.v.* injection of ^{64}Cu -substrate-binding peptide or ^{64}Cu -substrate-binding peptide + block (non-labeled-binding peptide) in athymic nude mice bearing orthotopic PDX JX12 glioma tumors. Full biodistribution analyses are available in the Supplementary Data. * $p < 0.05$; ** $p < 0.01$; SUV, standardized uptake value

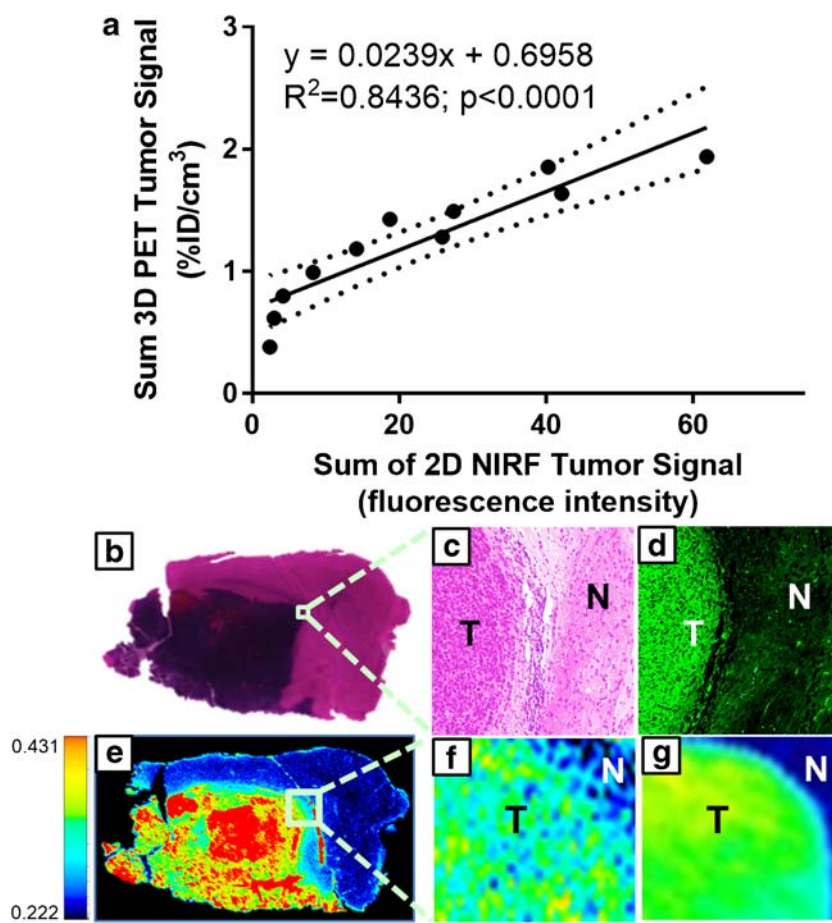
life of ^{68}Ga (67.7 min) precludes PET imaging analyses beyond 3 h. The ^{64}Cu -substrate-binding peptide was generated quantitatively after labeling with ^{64}Cu at room temperature for 20 min (Supplementary Fig. S13). Tumor-bearing mice were dosed *i.v.* with 0.13 nmol ^{64}Cu -substrate-binding peptide from the diluted reaction solution (molar activity approximately 46.6 GBq/ μmol at time of dosing). At 4 h after dosing, PET/CT imaging showed significant contrast in the tumor relative to normal brain (Fig. 5C), yielding a standardized uptake value ratio (SUVr: ratio of tumor SUV_{mean} to normal brain SUV_{mean}) of 3.9 ± 0.5 . The SUVr was lower in a group of tumor-bearing mice (2.5 ± 1.3) that had been co-injected with 80-fold excess of the non-labeled binding peptide as a blocking agent, although the difference between the two groups of mice was not significant ($p = 0.056$). The activity present in whole resected brains from mice in the ^{64}Cu -substrate-binding peptide group (0.43 ± 0.13 %ID/g) was significantly higher than that in the blocked group (0.23 ± 0.07 %ID/g; $p < 0.05$) (Fig. 5D). This result suggests that the binding peptide is able to partially block binding of the substrate-binding peptide to PDX glioma tumors in vivo. Biodistribution analyses of resected tissues were consistent

with the PET images, indicating predominantly hepatobiliary accumulation of activity (Supplementary Figs. S14, S15).

NIRF signals from the ^{64}Cu -substrate-binding peptide correlate with PET signals and co-localize with MMP-14 expression in human glioma orthotopic xenografts

The resected brains from mice injected with the ^{64}Cu -substrate-binding peptide in the above studies were sectioned and used for NIRF analyses. Gross imaging showed high contrast between the NIRF signal in PDX tumor regions compared to contralateral normal brain, yielding a TBR of 7.2 ± 1.3 ($p < 0.001$). The summed NIRF signal from tumor regions in these tissue sections correlated linearly ($R^2 = 0.84$, $p < 0.0001$) with the in vivo PET %ID/ cm^3 signal present in the tumor-bearing brain regions (Fig. 6A). Microscopic imaging of hematoxylin and eosin (H + E) stained tissue sections confirmed that the NIRF signal co-localized in the PDX glioma tumors, which showed high expression of MMP-14 relative to normal brain (Fig. 6B–G). These results support the hypothesis that the NIRF signal from the cleavable peptide was

Fig. 6 NIRF signals from the radiolabeled substrate-binding peptide show high concordance with PET signal, orthotopic PDX glioma localization, and MMP-14 expression. **A**, linear correlation between in vivo PET (%ID/cm³) and ex vivo NIRF signals from brains from mice bearing orthotopic PDX JX12 glioma tumors after *i.v.* injection of the ⁶⁴Cu-substrate-binding peptide (0.13 nmol); dotted lines show the 95% confidence interval of the correlation. Co-localization of H + E stained tumor (**B, C**), IRDye800 fluorescence on Odyssey scanner (**E, F**), and MMP-14 expression (**D, G**) in brain sections from mice bearing orthotopic PDX JX12 glioma tumors after *i.v.* injection of the ⁶⁴Cu-substrate-binding peptide. Separate sections were probed using an anti-MMP-14 antibody conjugated to either dye Alexa Fluor 488 for microscopy (**D**) or dye Cy5 for Odyssey scanner (**G**, 21 μm resolution). Microscopic images (**C, D**) are 100x. T, tumor; N, normal brain



specifically retained in the PDX glioma tumors due to the expression of MMP-14 in the tumors. The absolute NIRF signal from brains of mice injected with the ⁶⁴Cu-substrate-binding peptide was lower than that from mice in the study with the non-labeled substrate-binding peptide. This result was anticipated due to the different mass dose of the peptides used for the two studies. These studies support the feasibility of dual-modality PET and NIRF imaging with the radiolabeled peptide probe in a PDX glioma model. A goal of future work will be to determine the effect of the labeled and non-labeled peptide doses on the TBR for the PET and NIRF signals separately.

Discussion

The membrane-bound collagenase MMP-14 is a key enzyme in initiating and propagating the invasive phenotype associated with malignant glioma progression. MMP-14 processes other MMPs (e.g., proMMP-2) to their active state and cleaves adhesion proteins (e.g., CD44, integrins, etc.) in addition to extracellular collagen [5, 35, 48–50]. MMP-14 is also expressed on tumor-associated glial cells and macrophages,

which can comprise up to 30% of the tumor bulk and have been implicated in promoting glioma cell invasion, expansion, and pathogenesis [51–53]. These combined factors formed the rationale for exploiting MMP-14 as a biomarker for imaging glioma. The peptide probes developed here may also be relevant for imaging other malignancies (e.g., breast cancer, melanoma) that overexpress MMP-14 [54–57].

Our imaging approach is unique in that the substrate-binding peptide probe exploits MMP-14 for both enzymatic NIRF signal activation and specific localization of the PET and activated NIRF signals. Most existing PET and activatable fluorescence dual-imaging probes utilize different molecular targets for fluorescence signal activation and binding to the tumor cells. The proposed approach exploits enzymatic amplification of the NIRF signal to enable high-contrast intraoperative imaging of tumors that express activated MMP-14. Exploiting a single biological target for both PET and NIRF reporters can potentially enable straightforward co-registration of the imaging signals for intraoperative fluorescence imaging with high spatial resolution [58–62], which is otherwise challenging when employing separate probes that have differing pharmacokinetic and localization patterns [63]. It remains to be determined if the substrate-binding peptide

first binds to MMP-14, thus preventing the substrate from being activated by the same MMP-14. Mechanistic studies to determine factors affecting peptide cleavage were beyond the scope of our proof-of-concept experiments discussed above. Future experiments will more fully characterize peptide cleavage kinetics and potential mechanisms of inhibition caused by the peptides on MMP-14 enzymatic activity. Since MMP-14 is located on the cell surface, the kinetics *in vivo* may not be the same as that determined in solution. As endogenous substrates of MMP-14 are large tertiary protein complexes (e.g., proMMP-2, TIMP-2, MMP-14), it is likely a second MMP-14 on the cell surface could process the substrate sequence in the peptide-MMP-14 complex to produce fluorescence activation. Other MMP-14 activatable fluorescence imaging probes do not include a targeted binding moiety, resulting in diffusion of the activated probe away from the target cells [64, 65]. Incorporating the binding peptide into the substrate-binding probe in our studies was anticipated to cause specific retention of the PET and activated NIRF signals on glioma cells, which would enhance signal contrast of the tumor cells relative to adjacent healthy brain. Glioma cell lines showed up to 2.8-fold higher cell-associated NIRF signal at 4 h after *in vitro* incubation with the substrate-binding peptide relative to the substrate peptide (Fig. 3D; $p < 0.001$), thus supporting the hypothesis that the binding peptide component enhances cell retention of the activated NIRF signal. In addition to ligand-protein interactions that result in signal retention on target cells, factors such as probe extravasation and clearance from tumors also influence TBRs *in vivo*. The above NIRF imaging results of PDX glioma tumors in resected brain tissues showed comparable TBRs for the substrate-binding peptide and substrate peptide at 4 and 24 h p.i. (Table 1). At the 24 h time point, however, the substrate peptide resulted in the lowest NIRF signal among the groups examined, which is consistent with lower specific retention of the cleaved substrate peptide due to its lack of a binding component.

The results from this work are in agreement with prior studies that have targeted MMP-14 as a biomarker for imaging glioma in preclinical animal models. Favorable PET SUVR (3.9) and NIRF TBR (13.3) signals from the substrate-binding peptide probe were observed in the present studies with an orthotopic PDX glioma model. Previous studies in glioma models or in humans with glioma have used dual-modality imaging probes targeted to other proteins that are overexpressed in glioma relative to normal brain tissue. For instance, preclinical studies using a dual PET/NIRF peptide that binds to EphB4 (Cy5.5-TNYL-RAWK- ^{64}Cu -DOTA) in rodents with orthotopic human glioma xenografts indicated TBRs of 9 for PET imaging and 6–7 for fluorescence imaging [58]. A clinical trial using a dual PET/NIRF probe that targets the gastrin releasing peptide receptor (^{68}Ga -BBN-IRDye800) in glioma patients showed a PET SUVR of 13.4 and a NIRF TBR of 4.9 [66].

A primary goal of future studies is to assess if the activatable NIRF probes are suitable for optical-guided resection of glioma models *in vivo*. The TBRs and specific localization of the NIRF signals in the resected PDX glioma tumors from the present studies suggest the probes would be worthwhile candidates for NIRF-guided resection of glioma. Several preclinical studies in rodents with orthotopic brain tumors have shown TBRs ranging from 3 to 16 for various established (e.g., 5-ALA) or experimental fluorescence imaging agents [14, 32, 33, 63, 67]. Clinical therapy trials in patients with glioma are in progress to evaluate fluorescence-guided resection using imaging agents that include 5-ALA (NCT02119338, NCT01502280, NCT02632370, NCT00752323, NCT02191488, NCT01811121, NCT02379572), ABY-029 (NCT02901925), BLZ-100 (recently completed NCT02234297), panitumumab-IRDye800 (NCT03510208), ^{68}Ga -BBN-IRDye800 (NCT03407781), and fluorescein (NCT03291977, NCT02691923, [68]). These studies highlight the continued thrust to incorporate fluorescence-guided resection to improve outcomes for patients with glioma. Conceptually, it would be ideal to perform fluorescence-guided resection using an imaging probe with minimal photon attenuation in human tissue. While the spectral properties of NIRF fluorophores result in less photon attenuation relative to that of fluorescein or 5-ALA when imaging in human tissues, safe resection is the most critical factor during intraoperative brain tumor surgery. Maintaining the patient's quality of life governs the extent of surgical resection in critical brain tissues, even if those regions contain detectable tumor infiltration, regardless of the sensitivity or specificity of the imaging probe employed during fluorescence-guided resection.

In our pilot studies above, we observed relatively low PET signal in the orthotopic glioma tumors. This result may be due to moderate expression of MMP-14 relative to alternative biomarkers, restricted extravasation of the peptide into tumor parenchyma, moderate molar activity of the radiolabeled probes, sub-optimal mass doses of the probes, or other pharmacokinetic factors. Many small molecule PET probes show moderate accumulation and contrast (TBR 2–5 [69–73]) in glioma, partly due to rapid blood clearance kinetics and short PET radionuclide half-life, while macromolecule-based PET probes often show high accumulation due to significantly longer circulation times that allow the tracer to access tumors through disrupted blood-brain barrier (BBB) regions [13, 74]. The results from the *in vivo* blocking experiment support the conclusion that the radiolabeled peptide probes showed specific binding in the tumor. It was beyond the scope of these proof-of-concept studies to quantify nonspecific pooling of the probes in glioma tumors due to a disrupted BBB. In human patients, glioma often contains significant portions of tumor cell infiltration in non-contrast-enhancing regions of the brain. Therefore, it would be worthwhile in future experiments to investigate the

accumulation of the novel probes in spontaneous glioma models with tumor invasion in intact BBB regions. The PET studies presented here did not contain an additional method to confirm the size or location of the orthotopic PDX glioma tumors in vivo, although the observed probe uptake in brains during PET imaging was consistent with the histologically confirmed tumor regions identified by H + E and MMP-14 staining of resected brain tissue sections. Due to this limitation, it is possible that the tissue regions selected for in vivo PET measurements could have over- or underestimated the tumor volume and impacted the corresponding SUV results. Having an independent modality, such as MRI, to assess the in vivo tumor volume would increase the rigor of the PET analyses in the orthotopic xenografts. While MMP-14 is expressed in systemic tissues, the presence of tissue inhibitor of MMP-14 (TIMPs) may help minimize its activity in the circulation. Future studies to determine the specificity of glioma-mediated NIRF activation would benefit from comparing NIRF imaging with the probes above to control peptides that either lack the quencher molecule or that have a non-cleavable sequence separating the dye and quencher pair. It would also be useful to determine if different tumor infiltrating cells (e.g., tumor associated glial cells, macrophages) besides glioma cells contribute to the NIRF signal localization from the MMP-14 peptides within the tumor microenvironment [49, 51, 53].

Conclusion

The novel MMP-14 targeted and activatable peptide probes enabled dual PET and NIRF imaging of glioma in preclinical studies. High NIRF signal TBRs were observed in the resected brain sections of mice bearing PDX glioma tumors. Correlations between in vivo PET and ex vivo NIRF signals support the concept for dual-modality imaging of glioma with a single, MMP-14-targeted probe scaffold. The colocalization of NIRF signals and MMP-14 expression in the tumors observed by tissue staining confirmed the specific localization of the peptide probes. These results support future preclinical studies designed to test the efficacy of surgical resection of glioma with the MMP-14-targeted probes.

Materials and methods

General reagents

All general reagents were from commercial suppliers (Thermo Fisher, Waltham, MA, USA; Sigma, St. Louis, MO, USA) unless specified otherwise. Primary antibodies for MMP-14 (rabbit anti-MMP-14 monoclonal antibody (mAb), clone EP1264Y), MMP-2 (rabbit anti-MMP-2 polyclonal,

ab37150), or isotype control (rabbit mAb, clone EPR25A) were from Abcam (Cambridge, MA). Fluorophore-conjugated goat anti-rabbit polyclonal secondary antibodies were from Invitrogen (Thermo Fisher). HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology, Inc. (Dallas, TX). The recombinant catalytic enzyme domain of MMP-14 was from Invitrogen. ^{64}Cu was obtained in 0.1 M HCl from the *Mallinckrodt Institute of Radiology PET Nuclear Pharmacy and Cyclotron Facility of the Washington University Medical Center* or from the University of Alabama at Birmingham (UAB) Cyclotron Facility. ^{68}Ga , eluted in 0.1 M HCl from a $^{68}\text{Ge}/^{68}\text{Ga}$ generator, was obtained from the UAB Cyclotron Facility.

Gel zymography

Gel zymography was performed as previously described [75]. Briefly, adherent monolayers of glioma cells were rinsed with PBS and incubated in serum-free medium for 24 h. Supernatants were collected, analyzed for protein content, and loaded on zymogram gels (10% gelatin, Novex® Zymogram Gels, Thermo Fisher) according to the manufacturer's recommendations. Relative band intensities were quantified by ImageJ.

Immunofluorescence

Immunocytochemistry: Glioma cells were seeded at 100,000 cells/well in a 12-well plate and allowed to attach overnight. Adherent cells were rinsed in PBS, blocked with 1% BSA in PBS at room temperature for 45 min, incubated with the anti-MMP-14 mAb (Abcam ab51074) or isotype control mAb (Abcam ab172730) at 1 $\mu\text{g}/\text{mL}$ in 0.1% BSA in PBS at room temperature for 30 min, rinsed 4 times with 0.1% BSA in PBS, incubated with Ready Probes Alexa Fluor 488-goat anti-rabbit antibody solution (R37116, Molecular Probes, Thermo Fisher) at room temperature for 30 min, rinsed 3 times with 0.1% BSA in PBS, and imaged on an inverted fluorescence microscope at 200 $\times$ magnification. Mean fluorescence intensity per cell was quantified using ImageJ by randomly selecting 20–25 cells over four fields of view and measuring the mean fluorescence intensity.

Immunohistochemistry: A slide with a formalin-fixed paraffin-embedded (FFPE) human glioma tissue microarray (BS17016b, US Biomax, Inc, Derwood, MD) was deparaffinized and antigen was retrieved by heating for 10 min at 90 $^{\circ}\text{C}$ in citrate buffer pH 6 with 1 mM EDTA. Tissues were blocked in 5% BSA/TBST at room temperature, incubated overnight at 4 $^{\circ}\text{C}$ with anti-MMP-14 mAb at 1 $\mu\text{g}/\text{mL}$ in 5% BSA/TBST, washed in TBST, incubated for 2 h at room temperature with Cy5-secondary antibody (A10523, Invitrogen, Thermo Fisher) at 1/1000 dilution in 5% BSA/TBST, rinsed in TBST, and mounted in DAPI-Fluoromount-

G (Southern Biotech, Birmingham, AL). Microarray tissues were imaged on an Odyssey scanner (LI-COR Biosciences, Lincoln, NE) using the 700 nm channel. Integrated instrument software (Image Studio, LI-COR) was used to determine mean fluorescence intensity as total counts/region of interest (ROI) pixel area. 5–6 μm FFPE sections from resected brains of mice bearing orthotopic PDX JX12 glioma tumors were processed using the same protocol, except Alexa Fluor 488-conjugated secondary antibody (A11008, Invitrogen, Thermo Fisher) was used (1/2000 dilution).

Production of peptide probes

The MMP-14 binding, substrate, and substrate-binding peptide probes were synthesized by solid phase techniques using protected amino acids and commercially available IRDye800CW-maleimide and quencher QC-1-NHS ester (LI-COR). The synthesized peptides were purified by semi-preparative reverse-phase high-performance liquid chromatography (RP-HPLC) and characterized by mass spectrometry. Additional details are provided in the Supplementary Materials.

In vitro NIRF activation and NIRF microscopy studies

The catalytic enzyme domain of MMP-14 (5 nM, 5 ng/well) or adherent glioma cells in 96-well plates (Corning Costar, Corning, NY) were incubated with 0.5 μM solutions of the substrate or substrate-binding peptide in 50 μL MMP-14 assay buffer (PBS with 1 mM CaCl_2 , 0.5 mM MgCl_2 , 10 μM ZnCl_2) at 37 °C, 5% CO_2 for 0–24 h. At designated time points, aliquots (1.5 μL) were removed and spotted on Whatman 1 chromatography paper. After drying at room temperature, NIRF signal of the blots was quantified by the Pearl imaging system (LI-COR). Alternatively, for quantification of the NIRF signal from the substrate peptide, the NIRF signal in the 96-well plates containing glioma cells was directly imaged on the Pearl system. As controls to determine relative fluorescence signal activation at each time point, solutions were used that contained the respective peptide solution without MMP-14 or glioma cells. Cell studies were performed in duplicate wells. For NIRF microscopy studies, glioma cell lines were seeded in 8-chamber slides (10^4 cells/chamber) 2 days before the experiment. On the day of the experiment, cells were rinsed with PBS and incubated at 37 °C, 5% CO_2 with the substrate or substrate-binding peptides (1 μM) in cell medium containing 1% FBS. Chambers were placed on ice at designated time points and medium was aspirated; chambers on ice were washed with ice-cold buffer (PBS, 1% BSA), fixed in formaldehyde at room temperature for 15 min, washed to remove formaldehyde, and mounted in Fluoromount-G prior to NIRF microscopy imaging at 100 $\times$ magnification. Mean fluorescence intensity per cell was quantified using ImageJ

by randomly selecting 30 cells over three fields of view and measuring the mean fluorescence intensity.

In vivo NIRF imaging studies

Mice bearing flank D54 or U87 xenografts ($n = 5/\text{group}$) were injected *i.v.* with 10 nmol substrate peptide or substrate-binding peptide (0.2 mL in PBS). Live, anesthetized mice were imaged on the Pearl system (LI-COR) from 0–24 h *p.i.* Mice bearing orthotopic PDX JX12 xenografts were injected *i.v.* with 0.8–0.9 nmol substrate peptide or substrate-binding peptide (0.2 mL in PBS) and euthanized at 1 h ($n = 1$ mouse/group), 4 h ($n = 4–6$ mice/group), or 24 h ($n = 3–5$ mice/group) *p.i.* Immediately after euthanizing anesthetized mice by cervical dislocation, the skin over the skull was removed, and NIRF signal was determined by the Pearl system. The whole brains were resected, fixed in 10% formalin overnight, and imaged by the Pearl system. Brains were then serially sectioned (1 or 2 mm coronal slices), and all slices were imaged by the Pearl system. Three or four slices containing tumor and adjacent normal brain without tumor were then dehydrated in 70% EtOH and processed for embedding in paraffin and further sectioning (5–6 μm). Unstained sections were imaged on an Odyssey system (LI-COR) or stained for H + E or immunofluorescence (anti-MMP-14 mAb or isotype control) as described above. NIRF signals from the Odyssey scans were defined as positive if they were above the following threshold: three-times the standard deviation above the pooled mean NIRF signal in negative regions (no tumor cells present by H + E histology). Odyssey images and H + E images at the same resolution were compared by ImageJ to define true positive, true negative, false positive, or false negative NIRF signal regions.

In vivo PET/CT imaging studies and biodistribution studies.

Mice bearing orthotopic PDX JX12 xenografts were used for PET/CT imaging and subsequent biodistribution studies with the radiolabeled peptide probes. Mice injected *i.v.* with ^{68}Ga -binding peptide (0.7–1.0 nmol, 2.2–4.5 MBq) with or without unlabeled binding peptide (50 nmol) ($n = 3–4$ mice/group) were imaged at 1 and 2 h time points *p.i.* Mice injected *i.v.* with ^{64}Cu -substrate-binding peptide (0.3 nmol, 6.3–6.5 MBq) with or without unlabeled binding peptide (22 nmol) ($n = 5–6$ mice/group) were imaged at 1 and 4 h time points *p.i.* PET (energy window 350–650 KeV; 15 min acquisition for ^{64}Cu studies, 20 min acquisition for ^{68}Ga studies) and CT (voltage 80 kVp, current 150 μA , 720 projections) images were acquired on a GNext PET/CT small animal scanner (Sofie Biosciences, Culver City, CA). The PET images were reconstructed using a 3D ordered-subset expectation maximization algorithm (24 subsets and 3 iterations), with random,

attenuation, and decay correction. The CT images were reconstructed using a modified Feldkamp algorithm. Reconstructed images were analyzed using VivoQuant software (version 3.5patch 2, Invicro, LLC, Boston, MA). Following the last imaging time point, anesthetized mice were euthanized by cervical dislocation. Whole brains and other selected tissues were resected, weighed, and counted on a gamma counter (1480 Wizard², Perkin Elmer, Shelton, CT). Percent uptake of the injected dose per gram (% ID/g) was calculated by comparing the tissue activity to solutions with known activity of the radionuclide of interest. Resected brains were then fixed in 10% formalin overnight, serially sectioned (1 or 2 mm coronal slices), and processed for further NIRF imaging, paraffin embedding, and tissue sectioning for H + E or immunofluorescence analyses as described above.

Statistical analyses

Data were analyzed using Microsoft Excel or GraphPad Prism (Version 6.1, GraphPad Software, La Jolla, CA, USA). Student's *t*-test was used when comparing two groups. When comparing multiple groups, one-way ANOVA tests, followed by Bonferroni corrections for multiple comparisons, were performed. All *p*-values correspond to two-tailed tests; significance was considered to be at *p* < 0.05.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All applicable international, national, and institutional guidelines for the care and use of animals were followed. Animal studies were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (20366) and performed in compliance with guidelines from the Public Health Service Policy and Animal Welfare Act of the United States. This article does not contain any studies with human participants performed by any of the authors.

Abbreviations 5-ALA, 5-aminolevulinic acid; ANOVA, analysis of variance; BBB, blood-brain barrier; BSA, bovine serum albumin; CT, computed tomography; FDA, Food and Drug Administration; FFPE, formalin-fixed paraffin-embedded; GBM, glioblastoma multiforme; %ID/g, percent injected dose per g; i.v., intravenous; mAb, monoclonal antibody; MMP, matrix metalloproteinase; NIR, near-infrared; NIRF, near-infrared fluorescence; NOTA, 1,4,7-triazacyclononane-1,4,7-triacetic acid; PDX, patient-derived xenograft; PET, positron emission tomography; p.i., post-injection; PpIX, protoporphyrin IX; RIPA, radioimmunoprecipitation assay; s.c., subcutaneous; SUVR, standardized uptake value ratio; TBR, tumor-to-background ratio; UAB, University of Alabama at Birmingham

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