An Optical Probe for Non-invasive Molecular Imaging of Orthotopic Brain Tumors Overexpressing Epidermal Growth Factor Receptor

Richard S. Agnes¹, Ann-Marie Broome¹,², Jing Wang¹, Anjali Verma², Kari Lavik², James P. Basilion¹,²,³

Authors’ Affiliations: Departments of ¹Radiology and ²Biomedical Engineering, ³NFCR Center for Molecular Imaging, Case Western Reserve University.

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Corresponding Author: James P. Basilion, Ph.D., Departments of Radiology, Biomedical Engineering, and Pathology, Case Center for Imaging Research Director, NFCR Center for Molecular Imaging at Case, Case Western Reserve University, Wean Building, Room B-42, 11100 Euclid Avenue, Cleveland, Ohio, 44106; Phone: 216-983-3264; Fax: 216-844-4987; E-mail: jxb206@case.edu.

Notes

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Abstract
We have developed a near-infrared (NIR) probe that targets cells overexpressing the epidermal growth factor receptor (EGFR) for imaging glioblastoma brain tumors in live subjects. A peptide specific for the EGFR was modified with various lengths of monodiscrete polyethylene glycol (PEG) units and a NIR Cy5.5 fluorescence dye. The lead compound, 2, with one unit of PEG displayed good binding (8.9 μM) and cellular uptake in glioblastoma cells overexpressing EGFR in vitro. The in vivo studies demonstrated that the probe was able to selectively label glioblastoma-derived orthotopic brain tumors. In vivo image analyses of peptide binding to the tumors using fluorescence-mediated molecular tomography (FMT) revealed that the compound could distinguish between tumors expressing different levels of EGFR. The data presented here represents the first demonstration of differential quantitation of tumors expressing EGFR in live animals by a targeted NIR fluorescence probe using a molecular imaging device.
INTRODUCTION

Glioblastoma multiforme (GBM) is the most common and most malignant of the glial tumors. In 40-50% of these tumors, mutations resulting in the overexpression or activation of the epidermal growth factor receptor (EGFR) are found (1, 2). EGFR is a tyrosine kinase cell surface receptor that regulates growth and survival including adhesion, migration, differentiation and other cellular processes (3). Thus, the EGFR is considered a validated, molecular biomarker for certain cancers including: non-small cell lung, head and neck, colorectal, and ovarian cancers.

EGF protein and antibodies to the receptor have both been used to probe for EGFR in tumors. An alternate approach to targeting EGFR is to develop small molecular weight molecules that directly bind to the receptor, including peptide-based entities. Numerous studies suggest that small peptides can efficiently bind to surface receptors (4). One way to identify peptide ligands for protein targets is phage display (5, 6). Using this screening method, randomized libraries of peptides can be generated and screened for affinity and selective molecular targeting to cell surface receptor proteins, such as the transferrin receptor (7, 8). Using phage display techniques Li and co-workers have identified a 12-residue linear peptide sequence, GE11, (YHYWGYTPQNV) approaching nanomolar affinity for EGFR (9). We have employed this sequence to develop a fluorescently-labeled tumor-selective agent that can cross the blood-brain-tumor-barrier (BBTB) and non-invasively interrogate the level of EGFR expression in tumors.
Tremendous interest in the development of non-invasive optical imaging technologies exists for diagnosis of cancer and monitoring of the therapeutic response (10, 11). As optical instrumentation advances (e.g. tomographic imaging), the development of optical imaging molecules that are selective for tumors for in vivo studies is fast becoming an important field for cancer research (12). Thus, the ability to differentially image EGFR expression levels might provide non-invasive means to identify tumors that aide in the selection of treatments as well as means of targeted drug delivery.

Specifically, in this work, we synthesized different versions of the imaging agent by varying the length of PEG linker between the peptide and the fluorochrome, Cy5.5. These imaging compounds were then tested in tissue culture cells lines expressing different levels of the EGFR and in orthotopic brain tumors generated from the cell lines. It was determined that the length of the linker critically affected the efficacy of the agent both in tissue culture and in the in vivo setting. Furthermore, these agents were capable of discriminating tumors expressing different levels of EGFR in orthotopic GBM models.

MATERIALS AND METHODS

Probe synthesis and analysis

The peptides were synthesized manually using protocols previously described (13). Peptide was labeled in solution with monoreactive Cy5.5 NHS ester (CyDye, GE Healthcare). Crude fluorophore-labeled peptides were purified by
reversed phase HPLC. The isolated peak was lyophilized and characterized by MALDI mass spectrometry under positive mode. Compounds are characterized by RP-HPLC under two different conditions and by thin layer chromatography with three different solvent conditions. Details are found in Supplementary Information (Table S1 and Table S2). Concentration of stock Cy5.5-labeled peptide solutions in DMSO was determined by UV-Vis spectrometry (Cy5.5 molar extinction coefficient is 250,000 M⁻¹cm⁻¹ at 675 nm) (14).

**Cell culture**

Human glioblastoma-astrocytoma, an epithelial-like cell line U87-MG, and human glioblastoma cell line stably over-expressing the EGFRvIII mutant form of the egfr gene, Gli36Δ5, were used in these studies. U87-MG and A431 cells were recently obtained from American Type Culture Collection (Manassas, VA, USA). Gli36Δ5 cells were obtained from E.A. Chiocca and were authenticated by Research Animal Diagnostic Laboratory at the University of Missouri (Columbia, MO) for interspecies and mycoplasma contamination by PCR analysis. (15). Cell lines were maintained in RPMI or DMEM (Gibco), respectively, and supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were incubated at 37°C in a humidified 5% CO₂ atmosphere. For Gli36Δ5 cells puromycin was also include to maintain the expression of the EGFRvIII plasmid.
Saturation binding assays

To determine binding affinities of the Cy5.5-labeled peptides 1 to 4 to EGFR, a saturation binding assay protocol using labeled fluorescence ligand against cell surface receptors was adapted as previously reported (16). Briefly, Gli36Δ5 cells were plated 20,000 cells per well were plated on a black Costar 96-well plates (cat. no. 3603). Probes were diluted to range of concentration (0 to 25 μM) in growing media with 0.3% bovine serum albumin and added into wells and incubated at 37°C for 90 minutes at 5% CO₂. Cells were then washed with PBS twice and dried. DMSO was added before reading fluorescence (excitation 670 nm, emission 700 nm) with a Tecan Infinite M200 plate reader. Saturation binding assay data were in quadruplicates and analyzed using one site binding classical equation for non-linear regression analysis with the GraphPad Prism version 4.0.

Receptor uptake immunofluorescence

Gli36Δ5 or U87-MG cell lines plated in 96-well culture plates (20,000 cells per well; 3 wells per condition) were incubated over indicated time periods with 1 μM of Cy5.5-labeled EGF peptides 2, 3, and 4 at 37°C and 5% CO₂. Cells were briefly rinsed with HEPES buffer and imaged with a Tecan Infinite M200 fluorescence plate reader (excitation 670 nm, emission 700 nm).

Immunocytochemistry of cells grown on coverslips

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Gli36Δ5 or U87-MG cancer cell lines were plated on coverslips and incubated overnight to promote adherence. The cells were then fixed with 4% paraformaldehyde, rinsed with PBS, and blocked with 1% host serum for 30 min at room temperature. Coverslips were incubated with primary antibody at room temperature for 2 hrs. Antibodies used were mouse anti-human wild type EGFR (1:100 dilution; clone DAK-H1-WT, Dako cat. no. M7289). The coverslips were then rinsed with PBS and counterstained with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) for 10 min at room temperature to visualize the nuclei. After a final rinse with PBS, the coverslips were mounted using Fluor-Mount aqueous media, sealed with nail polish, and observed using epi-fluorescence microscopy.

Orthotopic brain tumor model

NIH athymic nude female mice (5-8 weeks and 20-25g upon arrival, NCI-NIH) were maintained at the Animal Resource Center at Case Western Reserve University according to institutional policies. All procedures were performed aseptically according to Institutional Animal Care and Use Committee (IACUC) approved protocols. Cell for brain implantation were harvested with 1 ml 0.05% trypsin-EDTA (Gibco) and briefly washed with PBS. Trypsin was inactivated by the addition of serum-containing media. The resulting cell suspension was centrifuged at 1000 x g for 3 minutes. The cell suspension was centrifuged and the supernatant was removed after two washes in PBS. Finally, the cells were re-suspended in 2 μl PBS for brain implants per animal (250,000 cells per animal for...
brain implants). Immediately following the cell harvesting procedure, animals were inoculated. For brain tumor implantation, mice were anesthetized by intraperitoneal injection of 50 mgs/kg ketamine/ xylazine and fitted into a stereotaxic rodent frame (David Kopf Instruments). A small incision was made just lateral to midline to expose bregma suture. A small (1.0 mm) burr hole was drilled at AP= +1, ML= -2.5 from the bregma. Glioblastoma cells were slowly deposited at a rate of 1 μl/minute in the right striatum at a depth of 3 mm from dura with a 10 μl syringe (23G needle). The needle was slowly withdrawn and the incision was closed with 2-3 sutures. Brain tumors grew for 10-12 days as per IACUC protocols at which point in vivo imaging studies were conducted. As control for the effects of surgical intervention, animals were also subjected to the implantation procedure but received 2 μL PBS (sham animals). Animals were fed exclusively on a special rodent diet (Harlan Laboratories, Inc.; Tekland 2018S) to reduce autofluorescence.

**In vivo and Ex vivo Fluorescence Imaging**

Mice bearing brain tumors derived from Gli36Δ5 or U87-MG cells were administered with compounds at 1 nmol/ gram via tail vein injection. In vivo competition assays were performed with mixture of compound 2 and 10-fold concentration of non-labeled probe 5. Prior to injection, mice were anesthetized with isoflurane and subjected to tomographic and spectral fluorescence imaging. One hour post injection, animals were re-imaged. Brains were then extracted and imaged. The excised brains were embedded in Tissue-Tek optimum cutting
temperature (OCT) for cryosections for IHC. Fluorescent molecular tomographic images were obtained using FMT2500 (Perkin-Elmer) and three-dimensional reconstructions of fluorescent signals were acquired using the accompanying software TrueQuant. Quantitative fluorescent signals for Cy5.5 of compound 2 were calibrated per manufacturer’s instructions using the 680-channel. Region of interest (ROI) assigned based on the precise placement of cells during implantation at 3-4 mm into the brain. ROI was corroborated with fluorescent signals from ex vivo imaging. Fluorescent multispectral images were obtained using the Maestro™ In-Vivo Imaging System (CRi, Inc.). The yellow filter set appropriate for Cy5.5 was used for emission and excitation light. The tunable filter was automatically stepped in 10 nm increments while the camera captured images at a constant exposure of 200 ms. Fluorescence images were acquired before treatment, immediately post treatment and 1.5 hours after treatment. To compare signal intensities, ROI were selected over the tumor or non-tumor areas and the change in fluorescence signal over baseline was determined. The spectral fluorescent images consisting of autofluorescence spectra and imaging probe were captured and unmixed based on their spectral patterns. The total signal in the ROI is defined in photons measured at the surface of the animal was divided by the area (in pixels). Spectral libraries were generated by assigning spectral peaks to background and fluorescence probe on tissue. The spectral libraries were manually computed using the Maestro™ software, with each tissue used as its own background control.
Immunohistochemistry of cryosection of brain tissue

Sections (2 mm) of whole mouse brains implanted with Gli36Δ5 cells were fixed with 4% paraformaldehyde, cryosectioned onto microscope slides, rinsed with PBS, and blocked with 1% host serum for 30 min at room temperature. Sections were incubated with primary antibody at room temperature for 2 hrs. Antibodies used were mouse anti-human wild type EGFR (1:100 dilution; clone DAK-H1-WT, Dako cat. no. M7289). The coverslips were then rinsed with PBS and counterstained with DAPI for 10 min at room temperature to visualize the nuclei. After a final rinse with PBS, the slides were mounted with coverslips using FluorMount aqueous media, sealed with nail polish, and observed using epi-fluorescence microscopy.

Western blotting

Cell extracts (50μg) were fractionated using SDS-PAGE and transferred onto a nitrocellulose membrane. Immunoblotting was done using a 1:500 dilution of an antibody against wild type EGFR (DAKO, DAK-H1-WT) or a 1:500 dilution of a specific antibody against mutant EGFRvIII (Bioss Inc., cat# bs-2558R). Horseradish peroxidase-conjugated secondary antibodies against mouse IgG (Chemicon) or rabbit IgG (Amersham) were used. Bands were detected using an enhanced chemiluminescence detection system (Pierce).
Statistical analysis

Analyses of data were achieved with GraphPad Prism version 4.00, GraphPad Software, San Diego California USA. Binding affinity was determined with non-linear regression analysis with one site binding hyperbola with an equation: $Y = B_{max} \times \frac{X}{K_d + X}$, where $B_{max}$ is the maximal binding sites and the $K_d$ is the concentration required to reach half-maximal binding. ANOVA analysis at 95% CI was used to compare treatments (*, $P>0.05$; **, $P>0.01$; ***, $P>0.001$). To compare live animal FMT, non-parametric one way ANOVA analyses (Kruskal-Wallis tests) and the median differences were considered significant with $P=0.0220$.

RESULTS

The goal of these studies was to develop a peptide-based NIRF probe that would cross the BBTB and selectively bind to brain tumor cells overexpressing EGFR. For these studies, we employed a peptide discovered through phage display screening against purified human EGFR (9). The peptide was modified to include linkers and a NIRF dye (Fig. 1). To determine the optimal space between the NIRF dye and the peptide, we designed and synthesized a series of peptides to include increasing numbers of discrete ethylene glycol units to serve as linkers between a Cy5.5 and the N-terminal end of the peptide. Cy5.5 and EGFpep were either directly linked or linked via 1, 2, or 3 units of discrete ethylene glycol (AEEA) moieties (Table 1). To determine which of the
compounds optimally interacts with cells expressing EGFR, the apparent binding for each bioconjugate was fluorometrically determined from a saturation binding assay (16) in vitro using a human GBM cell line overexpressing EGFR, Gli36Δ5, (Table 1). Compounds 1, 2, 3, and 4 all bound to the cells with affinities in the micromolar range. Compound 2, which had one linker, had the highest apparent affinity with a Kd at least 2-fold better than compound 1, which had no ethylene linker, 8.9 μM to 18.5 μM respectively. Compounds 3 and 4 have weaker affinities with Kds of 64.4 μM and 123.0 μM, respectively.

We next used immunofluorescence microscopy to determine the fate of the peptide complexes once they bound to glioblastoma cells expressing EGFR. As predicted from the affinity measurements compound 2, which had the highest affinity, also showed the greatest accumulation of fluorescence after incubation with Gli36Δ5 cells (Fig. 2A). Neither compound 1 (no linker) nor either of the molecules with greater linker numbers (compounds 3 and 4) was taken up by the cells to the same extent as compound 2. Interestingly, the peptide with the longest linker and the worst binding affinity, compound 4, was taken up by cells better than compound 3 (Fig. 2B). When tested against U87-MG cells, which express much lower levels of the EGFR, no cellular uptake for any of the compounds was observed (Fig. 2C).

We next tested the ability of these compounds to target EGFR-expressing tumors implanted within the brains of mice. For these studies mice were orthotopically implanted with Gli36Δ5 cells. Approximately 10 days after implantation the animals were administered 1 nmol/gram via tail vein injection
and sacrificed 1 hour later. Brains were harvested and imaged ex vivo for accumulation of the imaging probe. As a control for specificity, we synthesized a Cy5.5-labeled scrambled peptide, compound 6, using the amino acid residues of the parent peptide (compound 5) in random order. Compound 2 targeted the tumor efficiently, accumulating 1.1% of injected dose (Fig. 3A, left graph). In contrast, compound 6 did not target the tumor with delivery of only 0.006% of the injected dose. To further assess specificity, animals that bore Gli36Δ5 brain tumors were administered compound 2 alone or in the presence of a 10-fold excess of unlabeled peptide (compound 5). In animals that received only compound 2, there was significant tumor-associated fluorescence. In contrast, when a 10-fold excess of competitor peptide was co-administered with compound 2 there was approximately a 60% decrease in accumulation of the probe, (Fig. 3A, right graph). Imaging and quantification of identical ROIs taken on the contralateral brain showed little uptake of the probe (data not shown).

To demonstrate that the uptake was associated with human EGFR expression on the Gli36Δ5 cells, the resected brains were fixed and subjected to immunohistochemistry with monoclonal antibodies specific for human EGFR (Fig. 3B). These results demonstrated that only cells that expressed human EGFR were associated with Cy5.5 fluorescence. Notably, no Cy5.5 signal was associated with surrounding mouse brain.

Our final test for compound 2 was to determine if it would be useful for non-invasive in vivo detection and discrimination of tumors differentially
expressing EGFR. For these studies, mice were orthotopically implanted with either Gli36Δ5 cells, which express high EGFR, or U87-MG cells, which express relatively low EGFR levels (Fig. 4A). Following implantation, the tumors were allowed to grow approximately 10-12 days and then mice were administered 1 nmol/gram of compound 2 via tail vein injection. One hour after injection, the mice were anesthetized and the intensity of Cy5.5 fluorescence from the tumor was non-invasively quantified using FMT (Fig. 5A). The tumors formed from Gli36Δ5 cells had approximately 4-fold more fluorescence than either tumors formed with U87-MG cells or control sham surgeries (Fig. 5B). For Gli36Δ5 and U87-MG brain tumors, these fluorescence signals corresponded to injected doses of 1.1% and 0.27% respectively. Statistical analyses showed that the median fluorescence signals between the two groups were significantly different (Fig. 5B). To corroborate these data, tumors were excised and subjected to ex vivo FMT and Maestro fluorescence imaging analyses (Fig. 5C and Fig. 5D). These measurements were in good agreement with those measurements made during the live animal imaging.

We also examined the expression of EGFRvIII in the cell lines. Western blots for the EGFRvIII on both U87-MG and Gli36Δ5 cells showed that the mutant receptor expression is similar for both cell lines (Fig. 4B). In addition, we examined A431 cells, a squamous carcinoma cell line, that expresses high levels of wild type EGFR (Fig. S1A & B). Saturation binding studies indicated that the Kd for binding was similar to that measured with Gli36Δ5 cells (Fig. S1C). Furthermore, incubation of compound 2 with A431 cells displayed increasing
fluorescence in the presence of the EGF ligand, which increases cycling of wild type EGFR in cells (Supplemental Figure S1D).

**Discussion**

The present study has identified a NIRF molecular imaging probe for cells overexpressing EGFR receptor that allows for non-invasive specific detection of tumors expressing the EGFR in live animals. The parent peptide sequenced used to derive compound 5 was initially discovered by Li *et al.* using phage display to identify peptides that bind to purified human EGFR (9). The parent peptide, GE11, has a binding affinity in the nanomolar range as determined from radioligand binding assays and also the additional advantage of being non-mitogenic—not inducing any proliferation of cells in *in vitro* assays (9). The initial applications of the GE11 peptide sequence used in compound 5 were for cancer diagnostics (17) and targeted drug delivery (18) but met with limited *in vivo* success.

Initially Li *et al.* used the GE11 peptide to deliver a gene-polymer complex to tumors (9). Song *et al.* then utilized the peptides conjugated to liposomes to deliver doxorubicin to tumors (18). However, the delivery of the liposomes was not much more effective than the untargeted controls and probably resulted primarily from EPR effects. Further studies by Master *et al.* used bioconjugation via the peptide N-terminal amine of the GE11 sequence to form co-block polymers with high molecular PEG to deliver photodynamic therapy agents to
cells (19). Similarly, these studies did not greatly increase the targeting of PDT agents. For systemic administration of short peptides, it is known that C-terminal amides significantly increases the half-life stability in vivo (4). We, surmised that conjugation of the GE11 peptide via its amine resulted in lower serum stability as well as poor in vivo targeting and drug delivery due in part to the free carboxylate at the C-terminus. We aimed to develop compound 5 into a fluorescence imaging probe for non-invasive detection of tumors overexpressing EGFR and, therefore, incorporated lessons from prior studies into our probe design by conjugating the peptide via its free carboxyl-terminal end.

A concern was that upon conjugation of the dye, the increased hydrophobicity and bulk of the bioconjugate might significantly perturb the interaction between the ligand and receptor. Thus, we decided to introduce spacers comprised of discrete units of ethylene glycol between the peptide and the Cy5.5 dye. Direct conjugation of Cy5.5 to the parent peptide 5 resulted in a significant drop in binding affinity from a nanomolar to micromolar range. This degree of change in binding affinity is not uncommon in the modifications of individual parent peptides, particularly from peptides discovered the rough phage analysis (7, 9). The affinity was improved 2-fold to by inclusion of a single PEG spacer between the peptide and the Cy5.5, but still remained in the low micromolar range. Increased linker lengths did decrease affinity for the cellular EGFR, which may be due to additional bulk introduced by the linkers or to the linkers interference with the binding site in the EGFR. Nevertheless, Cy5.5-labeled peptide compounds 1, 2, and, to a lesser extent, 4 were efficiently
internalized into cells within hours. Despite the significant drop in affinity, the whole set of Cy5.5 compounds with different linker lengths provide a large range of affinities that could play an important role in different \textit{in vivo} applications of the probes when better pharmacokinetics are needed and high affinities are not necessarily required for effective targeting to biomarkers.

The competition assays against non-labeled compound 5 and comparisons with a scrambled sequence 6 suggest specificity for the binding of compound 2 to tumors overexpressing EGFR. To further assess this apparent specificity, we also tested the ability of compound 2 to bind to a brain tumor cell line that does not overexpress EGFR. In \textit{in vitro} assays, we observed that compounds 2, 3, and 4 were not appreciably taken up by U87-MG cells (Fig. 2C), in which we showed that EGFR content was significantly lower than in Gli36Δ5 (Fig. 4A). The low level expression of EGFR in U87-MG is consistent with previous reports (3).

The parent peptide sequence used to derive compound 5 has been used as targeting sequence, but has not been fully utilized for \textit{in vivo} imaging of tumors in live animals, particularly human GBM orthotopic brain tumor models in mice. The \textit{in vitro} results measuring differences in EGFR expression encouraged us to determine if compound 2 could be used to non-invasively differentially detect tumors formed from cells expressing different levels of EGFR expression. Thus, in an orthotopic brain tumor model in live animals we explored the utility of compound 2 to differentially detect glioblastoma brain tumors derived from cell lines with high and low expression of EGFR. The live animal imaging was
achieved with fluorescence-mediated molecular tomography, which provides good resolution and quantitative tomographic images of tumors (20). Analyses of the fluorescent tomographic images showed that tumor accumulation of compound 2 reflects the EGFR content of the tumors with Gli36Δ5 tumors accumulating significantly more fluorescence signal than U87-MG tumors. Along with the corresponding control conditions, which did not generate significant fluorescence, this suggests that compound 2 targets cells overexpressing EGFR. This uptake is significantly more than that measured with the scrambled control or for U87-MG cells treated with compound 2. The modest uptake measured in these latter cases is likely a result of the leaky tumor vasculature and EPR effects (21).

Cancer cells including gliomas have heterogenous expressions of EGFR receptors and its mutant EGFRvIII. To demonstrate if EGFR or its mutant form is responsible for the uptake we are measuring, we used western blots to measure the amount of EGFRvIII present in both U87-MG and Gli36Δ5 cells. These data (Fig. 4B) demonstrated that the level of EGFRvIII expressed in both cell types is approximately equal. In contrast, the wild type EGFR is highly expressed in the Gli36Δ5 cells relative to the U87-MG (Fig 4A). The uptake of compound 2, is markedly higher in Gli36Δ5 cells compared to U87-MG cells for both in vitro (Fig. 2A & B) and in vivo studies (Fig. 5) suggesting that this receptor is responsible for the uptake that we measure.
We also measured the uptake of compound 2 in A431 cells, a cell line which expresses high levels of the wild type EGFR. These studies indicated a similar Kd of binding for compound 2 to the cells and when stimulated with EGF, the cells increased the level of uptake of compound 2. Taken together with our in vitro and in vivo uptake studies, these data suggest that the wild type EGFR is the receptor responsible for the uptake of compound 2. These results are consistent with reports that GE11 based molecules bind to the wild type EGFR in various cell lines including A431 (9,19).

In the brain tumor animal models, compound 2 generated significant fluorescent signals associated with the tumor relative to normal tissues. However, the brain tumor model used in these studies results in a leaky vasculature when assessed by iron oxide nanoparticle imaging (22) and probably explains the ability of this probe to selectively light up EGFR expressing tumors in the brain. There are, however, reports that some peptides cross the intact blood-brain-barrier (BBB), more so for peptides based on phage display sequences. The extent to which these peptides may cross intact BBB still needs to be investigated (8).

EGFR is an attractive drug target for various cancers because the cell surface tyrosine kinase is often associated with its overexpression, which might have a role in the progression of the tumor. Imaging of differential expression of EGFR in various cancers including colorectal and head and neck squamous carcinoma, and non-small cell lung cancer has been explored extensively with PET or PET/CT imaging modalities radiolabeled EGF ligand (23) and EGFR-
targeted antibodies (24, 25), peptides (17), and small molecules (26). The ability to non-invasively observe EGFR levels of tumors in subjects is increasingly important for the purpose of diagnosis of cancer and longitudinal monitoring of drug therapies. Aberrant overexpression of EGFR is also found in 40-50% of high grade gliomas and is ultimately correlated with poor prognosis and drug resistance (1, 2).

There is a significant need to identify tumor margins especially in settings where tissue-sparring resections of tumors is required, such as in brain tumor removal (27-30). Recently, clinical trials in Europe have begun to use fluorescence-guided surgical techniques to achieve more complete brain tumor resections (27-31). Indeed, most recently intraoperative microscopic techniques in combination with molecular imaging probes specific for tumor markers, such as folic acid receptors and transpeptidases, have demonstrated a significant role in future surgical and therapeutic approaches for targeted-fluorescence imaging probes (32, 33). Here, we have developed a fluorescent probe that images tumors overexpressing EGFR and can potentially be utilized to understand the expression of EGFR in the research setting but also has potential applicability to human disease, including receptor detection during surgical interventions.

CONCLUSIONS

We have developed a NIRF probe targeting the EGFR, based on a phage display peptide against the receptor. Various linker lengths between the fluorescence probe and the peptide generated various binding affinities. The lead compound 2 was utilized to non-invasively detect tumors that expressed
different levels of EGFR in live animal imaging models, which was validated using various methods. Thus, we have demonstrated targeting to tumors overexpressing EGFR. We are currently exploring the probe’s selectivity among the EGFR mutants. The probe could prove invaluable for cancer research as a diagnostic tool for longitudinal studies and could translate into utility as a guide for tumor resections in patients in the future.

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References

Table 1.

<table>
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<th>Compound</th>
<th>Peptide (EGF&lt;sub&gt;pep&lt;/sub&gt;)</th>
<th>K&lt;sub&gt;d&lt;/sub&gt; (µM)</th>
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<tr>
<td>1</td>
<td>Cy5.5-YHWYGYPQNV-amide</td>
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<td>2</td>
<td>Cy5.5-(AEEA)&lt;sub&gt;1&lt;/sub&gt;-YHWYGYPQNV-amide</td>
<td>8.9 ± 3.7</td>
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<td>64.4 ± 24.6</td>
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<td>123.0 ± 174.0</td>
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<td>5</td>
<td>YHWYGYPQNV-amide (GE11-amide)</td>
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<td>6</td>
<td>Cy5.5-(AEEA)&lt;sub&gt;1&lt;/sub&gt;,NYQTPVYGWIYH-amide</td>
<td>ND</td>
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Table of the sequences of the peptides studied and the corresponding apparent binding affinities (Kd) from saturation binding assays.
Figure Legends

**Figure 1.** A) EGFR peptide ligands (EGF<sub>pep</sub>) used in this study. EGF<sub>pep</sub> is conjugated to a near-infrared fluorophore, Cy5.5, linked with up to three units of amino-ethoxy-ethoxy-acid (AEEA) at the N-terminal amine of the peptide.

**Figure 2.** *In vitro* studies of the peptides with different linker lengths. A) Gli36Δ5 cells were incubated with 1 μM of compounds 1-4 [Cy5.5-(AEEA)<sub>n</sub>-EGF<sub>pep</sub>] and the cell associated fluorescence was measured using spectrofluorometry at the indicated times. Gli36Δ5 (B) and U87-MG (C) cells were incubated with 1 μM compounds for the indicated times. Uptake of the compounds was assessed using epi-fluorescence microscopy. Representative images are shown. Images were taken at 40X magnification. Scale bar = 50 μm.

**Figure 3.** Near infrared Cy5.5 labeled compound 2 is specific and selective to tumor cells overexpressing EGFR. A) Specificity of the targeting was assessed using a scrambled peptide (6) and via competition against unlabeled parent peptide compound (5). Subjects bearing orthotopic brain tumors derived from Gli36Δ5 cells overexpressing EGFR were treated with (1 nmole/gram mice) of compound 6 (N=3) or compound 2 (N=5). *Ex vivo* analysis of brain tissues showed that compound 2 targets the tumor significantly (p> 0.0001, ***) more efficiently than the scrambled peptide 6. Representative images of the brain tumors are shown above the corresponding bars (left graph). When subjects bearing orthotopic brain tumors derived from Gli36Δ5 cells overexpressing EGFR were co-treated with compound 2 and a ten-fold excess of non-labeled parent compound 5, the signal over the tumor region was significantly reduced. This suggests that the fluorescence labeling of tumor is specific to the peptide sequence of 2. B) The *ex vivo* brain fluorescence images were further validated with immunohistochemistry of the brain tissues. The representative image shows the co-localization of EGFR-targeted probe (red) with cells expressing high levels of EGFR (green). Scale bar = 20 μm.

**Figure 4.** Differential expression levels of EGFR and EGFRvIII in Gli36Δ5 and U87-MG. A) Western blot demonstrating the relative levels of wild type EGFR expressed in Gli36Δ5 and U87-MG glioblastoma cell lysates using an antibody specific to wild type EGFR (DAKO, cat#M7289). B) Western blot comparing the mutant EGFRvIII content in in Gli36Δ5 and U87-MG tumor cells lines using an EGFRvIII specific antibody (Bioss Inc., cat# bs-2558R). β-actin was used a loading control. Densitometry analysis of western blot by Image J comparing the levels of EGFRvIII in Gli36Δ5 and U87-MG cells show similar content of 1800 and 2400 arbitrary units, respectively.

**Figure 5.** Application of near-infrared EGFR probe for *in vivo* differential detection of tumors expressing different EGFR levels. A) Representative image of fluorescence molecular tomography 3D reconstruction showing the targeting of
compound 2 to brain Gli36Δ5 orthotopic brain tumors. Sham, U87-MG, and Gli36Δ5 shows 272 nM, 1608 nM, and 9271 nM amounts of fluorescence in the matching ROIs.  B) Targeting of brain tumor in live animals using FMT was quantified and the graph shows higher detection of tumor with mice bearing the Gli36Δ5 tumor (*, P>0.05). C) Live animal FMT imaging of tumors was further assessed by FMT imaging of explanted brains. With similar trends observed in live animal study, analyses of the reconstructed images of ex vivo brain tissues showed a significant differential detection between mice bearing Gli36Δ5 (high EGFR) brain tumor and mice bearing U87-MG (low EGFR) cells. Compound 2 targeting detects differential levels of EGFR between glioblastoma types. Sham (N=2); U87-MG (N=3); Gli36Δ5 (N=5); ANOVA (P >0.01, **). D) Ex vivo brain samples were also examined using Maestro. The Maestro image analysis reflects the FMT analyses where fluorescence signal is significantly more in mice bearing Gli36Δ5 than in U87-MG glioblastoma cell types, ANOVA (P > 0.001, **).
Figure 1.

A
Figure 2.

A

B

C

Gli36Δ5

U87-MG
Figure 3.

A

![Graph showing average signal over ROI for scrambled, Cy5.5-(AEEA3)-EGF<sub>pep</sub>, 2 alone, and 2 + 10x of 5 conditions.]

B

<table>
<thead>
<tr>
<th>Cy5.5 EGF&lt;sub&gt;pep&lt;/sub&gt;</th>
<th>Anti-EGFR</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Image of Cy5.5 EGF&lt;sub&gt;pep&lt;/sub&gt; staining]</td>
<td>![Image of Anti-EGFR staining]</td>
<td>![Image of Combined staining]</td>
</tr>
</tbody>
</table>
Figure 4.
Figure 5.

A

B

C

D

Ex vivo Brain FMT

Ex vivo Brain Maestro

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