An interleukin 13 receptor α 2–specific peptide homes to human Glioblastoma multiforme xenografts

Hetal Pandya, Denise M. Gibo, Shivank Garg, Steven Kridel, and Waldemar Debinski*  
Departments of Neurosurgery (H.P., D.M.G., S.G., W.D.), Cancer Biology, (S.K., W.D.) and Radiation Oncology (W.D.), Brain Tumor Center of Excellence, Wake Forest University School of Medicine

Comprehensive Cancer Center, Winston Salem, North Carolina

Interleukin 13 receptor α 2 (IL-13Rα2) is a glioblastoma multiforme (GBM)–associated plasma membrane receptor, a brain tumor of dismal prognosis. Here, we isolated peptide ligands for IL-13Rα2 with use of a cyclic disulphide-constrained heptapeptide phages display library and 2 in vitro biopanning schemes with GBM cells that do (G26-H2 and SnB19-pcDNA cells) or do not (G26-V2 and SnB19-αsIL-13Rα2 cells) over-express IL-13Rα2. We identified 3 peptide phages that bind to IL-13Rα2 in cellular and protein assays. One of the 3 peptide phages, termed Pep-1, bound to IL-13Rα2 with the highest specificity, surprisingly, also in a reducing environment. Pep-1 was thus synthesized and further analyzed in both linear and disulphide-constrained forms. The linear peptide bound to IL-13Rα2 more avidly than did the disulphide-constrained form and was efficiently internalized by IL-13Rα2 expressing GBM cells. The native ligand, IL-13, did not bind to IL-13Rα2 when injected by an intravenous route. Therefore, we aimed at identifying ligands for the IL-13Rα2 receptor that can eventually be further developed for various diagnostic, imaging, and therapeutic applications in GBM.

Glioblastoma multiforme (GBM) is one of the most malignant primary brain tumors of astroglial origins. The median survival rate is only 14 months, with 5-year survival rates of <10%. Our focus was to develop novel management strategies for GBM. We previously identified attractive molecular markers and/or targets in GBM tumors that are over-expressed in a vast majority of patients with GBM but not found in normal brain. One such target is interleukin 13 receptor α 2 (IL-13Rα2). This receptor is a monomeric plasma membrane receptor that binds IL-13 but not homologous IL-4, unlike the physiological, normal tissue receptor IL-13Rα1/IL-4Rα, which is shared between the 2 cytokines and is composed of 2 subunits. The latter receptor is moderately elevated in malignancy, whereas IL-13Rα2 is over-expressed in GBM and in head and neck, pancreatic, ovarian, and prostate cancers. Therefore, we aimed at identifying ligands for the IL-13Rα2 receptor that can eventually be further developed for various diagnostic, imaging, and therapeutic applications in GBM.

IL-13Rα2 is a 382 amino acid, type I internalized plasma membrane receptor. It is mostly considered a decoy receptor because it has a very short cytoplasmic tail (17 amino acids in humans), and it is generally not suspected to have signaling activity, with possible exceptions. IL-13Rα2 must be internalized by GBM cells in contact with its ligand because IL-13–directed, Pseudomonas exotoxin A (PE)–containing cytotoxins evoke cell killing. This can take place only when the PE toxin is delivered into the endocytic and then cytosolic cell compartments after ligand-receptor complex internalization. Our laboratory designed various IL-13 ligand mutants that bind specifically to the IL-13Rα2 receptor and do not bind to the physiological receptor.

Keywords: blood-brain tumor barrier, GBM, interleukin-13 receptor α 2, peptides, peptide phages, display library, xenografts.
example, the IL-13.E13K mutant has a glutamic acid at position 13 of the α-helix A substituted by lysine. Also, we previously developed recombinant cytotoxins targeted to IL-13Ra2, which are one of the most potent anti-GBM agents known. These cytotoxins are composed of IL-13, wild type or mutated, fused to various bulky derivatives of PE or diphertheria toxin. The first generation of cytotoxin demonstrated a significant increase in overall survival among patients with recurrent GBM in a phase III clinical trial when patients were treated by physicians more experienced with the method of loco-regional delivery.

In search for more options to attack tumors, peptides offer several potential advantages when used as targeting agents. They are small and relatively easy to synthesize, are usually less or nonimmunogenic, and have attractive pharmacokinetic properties. Their plasma half-life is short, leading to rapid blood clearance and a higher tumor/blood uptake than normal organs/blood uptake. The smaller size of the peptides, compared with proteins and antibodies, is believed to afford better penetration and diffusion into a tumor. Peptides also serve as delivery vehicles to transport chemotherapeutics, labels, and radioisotopes to cancer cells or have been used for diagnostic and imaging purposes.

In the current study, we identified peptide ligands for the IL-13Ra2 receptor for specific targeting of and delivery to GBM tumors. To identify peptides of such characteristics, we used a disulphide-constrained heptapeptide phages display library with a diversity of $1.2 \times 10^9$. We were successful in obtaining peptides that bind IL-13Ra2 and characterized them for their potential use in managing GBM.

**Patients and Methods**

**Cell Culture**

U-251 MG, T98G, and SnB19 GBM cells were obtained from the American Type Culture Collection (Manassas, VA) and grown as recommended. G-26 cells are a murine glioma cell line (gift of Dr. Marzena Wiranowska). The G26-H2 cell line is G-26 cells transfected with a murine glioma cell line (gift of Dr. Marzena Wiranowska). The G26-H2 cell line is G-26 cells transfected with the human IL-13Ra2 receptor gene, and G26-V2 are vector transfected cells. SnB19-asIL-13Ra2 cells are SnB19 GBM cells transfected with an antisense IL-13Ra2 gene, with subsequently diminished expression of the receptor, in contrast to the empty vector-transfected SnB19-pcDNA cells (Supplementary Fig. S1A). The transfected G-26 and SnB19 cell lines were grown in Eagle’s MEM and RPMI 1640 (Hyclone) supplemented with 200 µg/mL of Geneticin. G48a cells were grown and maintained in RPMI 1640 (Lonza) supplemented with glucose, adjusted to 4 g/L of media and 10% FCS. G48a-Lucifearase transfected cells were maintained similar to G48a cells with the addition of blasticidin (200 µg/mL). The use of cell lines is related to their expression of IL-13Ra2 that is checked by Western blotting at least every 2 months.

**Tissues and Reagents**

The Ph.D-C7C phages display library containing $1.2 \times 10^9$ clones was obtained from New England Biolabs. The isolated peptides binding to IL-13Ra2 were synthesized by Anaspec. IL-13Ra2/Fc, IL-13Ra1/Fc, and IgG/Fc recombinant proteins were obtained from R&D Systems. The GBM and normal brain specimens were obtained from the Wake Forest School of Medicine Comprehensive Cancer Center Tumor Bank.

**Panning Procedure**

Phages from the Ph.D-C7C phages display library were panned against GBM cell lines. G26-H2 and SnB19-pcDNA were used as IL-13Ra2 receptor-positive cell lines, whereas G26-V2 and SnB19-asIL-13Ra2 were used as the receptor-negative cell lines. Four biopanning rounds were performed. Each round consisted of 3 negative selections, followed by 1 positive selection. During the negative selection, $1.5 \times 10^{11}$ plaque-forming units (pfu) were added to a 35-mm polystyrene tissue culture dish (Corning) containing $10^7$ receptor-negative cells. The polystyrene tissue culture dishes were blocked with phosphate-buffered saline (PBS) + 1% bovine serum albumin (BSA) to block nonspecific phages binding. The cells were harvested from flasks with use of 0.5 M EDTA/PBS. The dish was incubated for 1 h at room temperature with gentle shaking. The cell/phages mixture was then transferred to a microfuge tube and centrifuged to pellet the cells, and the supernatant with unbound phages was collected. The supernatant was then centrifuged, and the supernatant with unbound phages was collected. The supernatant was then mixed with receptor-negative cells, and the aforementioned procedure was repeated 2 more times. After 3 negative selections, the collected supernatants were mixed with $5 \times 10^6$ receptor-positive cells in a 35-mm polystyrene tissue culture dish. The dishes were incubated for 4 h at 4°C with gentle shaking. The mixture was then transferred to a microfuge tube and centrifuged, and the supernatant with unbound phages was collected. The pellet (cells with the bound phages) was washed $5 \times$ with cold PBS + 1% BSA + 0.1% Tween 20 to remove any unbound phages. The pellet was then resuspended with 100 µL of 0.1 M glycine buffer (pH, 2.2) to elute the IL-13Ra2-binding phages from the cell surfaces and chilled on ice for 10 min. The suspension was then centrifuged, and the supernatant was neutralized with 10 µL of 2 M Tris HCl (pH, 8).

**Phages Amplification and DNA Sequencing**

Phages eluted from receptor-positive cells were amplified in ER2738 Escherichia coli cells with use of the protocol outlined in the NEB Ph.D-C7C phages display manual. The ER2738 E.coli cells have F-factor, which contains a mini-transposon that confers tetracycline resistance and, thus, are suitable for growing the phages on Luria broth agar-tetracycline-5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (LB-Tet-X-gal) plates. The eluted phages were counted by plating various different
dilutions of phages onto LB-Tet-X-gal plates and represented as pfu. For phages DNA isolation, individual phages plaques were picked up, the phages were amplified, and DNA was isolated as directed in the NEB manual. The isolated phages DNAs were sequenced at the Wake Forest University Biomolecular Resource Laboratories.

**Enzyme-Linked Immunoabsorbent Assay**

A total of 100 μL of 10 μg/mL of IL-13Rα2/Fc, IgG/Fc (R&D Biosystems), and BSA control proteins were coated onto a 96-well enzyme-linked immunosorbent assay (ELISA) plate (CoStar) and were incubated for 2 h at room temperature in a humidified container. The proteins were then discarded, and the plate was blocked with 2% milk for another 2 h. Different phages dilutions were prepared with equal volumes of 2% milk and were incubated at room temperature for 15 min. The pre-incubated phages solutions were then coated onto the wells, after discarding the blocking solution. The plate was further incubated at room temperature for 2 h. M13KE, an M13 backbone phages without any surface peptides, was used as a control phages (New England Biolabs). BSA and insulin (Sigma-Aldrich) were used as nonspecific control proteins. For competitive ELISA, the plate was blocked with 1000x of the ligand for 1 h. For the dithiothreitol (DTT)–reducing ELISA experiment, the peptide phages were incubated with 5 mM DTT (Acros Organics) for 20 min and then added onto the wells. After incubation, the solutions were discarded from the wells and the plate was washed 6 times with PBS/0.1% Tween-20 solution. A total of 200 μL of 1:5000 dilution of anti-M13 horseradish peroxidase conjugated antibody (GE Healthcare) was then added onto each well of the plate, and the plate was further incubated for 1 h at room temperature. The plate was washed again 6 times with PBS/tween-20 solution. 2,2'-Azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diaminium salt (Sigma), hydrogen peroxide substrate solution was added, and the plate was incubated for 1 h at room temperature. The color was measured at the absorbance of 405 nm with Spectra max 340 PC (Molecular Devices).

**Cell-Binding Phages Titer Assay**

Equal numbers of G26-H2 and G26-V2 cells and SNB19-asIL-13Rα2 and SnB19-pcDNA cells were incubated with 10^6–10^12 pfu of each phage at 4°C for 4 h. After incubation, the cells were washed 6 times with cold PBS + 1% BSA + 0.1% Tween 20 to remove any unbound phages, and the pellet was resuspended with 100 μL of 0.1 M glycine buffer (pH, 2.2) to elute the IL-13Rα2 binding phages from the cell surfaces. The pellet was placed on ice for 10 min. The suspension was then centrifuged, and the supernatant was neutralized with 10 μL of 2 M Tris HCl (pH, 8). The eluted phages titers were performed as described above.

**Cell Viability Assay**

A total of 1 × 10^3 U-251 MG cells were plated in a 96-well tissue culture plate and incubated at 37°C for 24 h. The cells were plated in quadruplicates for each concentration to be tested. After 24 h, a fixed concentration of the peptides (1 μg/mL) and IL-13.E13K (2.5 μg/mL) in PBS + 0.1% BSA was added with increasing concentrations of the IL13.E13K-PE38QQR cytotoxin. The cytotoxin concentrations ranging from 0.1 to 100 ng/mL were diluted in PBS + 0.1% BSA and added to the plate. The plate was further incubated for 48 h. The cytotoxin concentrations used were 300× and the IL-13.E13K cytokine was 100× molar excess of the cytotoxin. Cells treated with cyclohexamide were used as positive control. After 48 h, cell viability was measured using the MTS/PMS assay (Promega; 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenoxynyl]-2-[4-sulfophenyl]-2H-tetrazolium–phenazine methosulfate) dye, according to the manufacturer’s instructions. The MTS/PMS dye was mixed with the cells and allowed to incubate for 2–4 h, and the absorbance from the assay was measured at 490 nm with use of the Spectra max 340 PC plate reader (Molecular Devices). The viability of the cells was calculated as percentage of untreated control cells.

**Fluorescent Peptide Binding Assay**

GBM cells were seeded on cover slips in a 24-well plate. The cells were allowed to attach for 24 hr. Next, the cells were fixed with 5% paraformaldehyde at 37°C for 15 min. After fixation, the wells were blocked with 10% BSA for 30 min. After blocking, the biotin-labeled peptides (1 μg/mL and 5 μg/mL) were added to the cells in serum-free media and incubated on the shaker for 1.5 h at room temperature. Subsequently, the wells were washed 4 times with PBS and were treated with streptavidin-Alexa fluor 488 (Invitrogen) for 1 h. 4',6-diamidino-2-phenylindole (DAPI) was added to stain the nuclei, and the cover slips were mounted with gel mount (Biomeda). The coverslips were analyzed under a fluorescent microscope (Olympus, IX70) and processed using the Image-Pro plus 5.1 software.

**GBM Tissues**

The tissues were frozen and sliced in 10-micron thick slices and placed on slides. The slides were then frozen at −80°C. Before the experiment, the slides were thawed at room temperature and the tissue slices fixed with acetone at −20°C for 10 min. After fixation, the slides were washed with PBS. Subsequently, the tissue slides were treated with an Endogenous Biotin Blocking kit (Invitrogen) to block endogenous biotin from giving background signal. After this treatment, the tissue slides were blocked with 10% BSA for 1 h. After blocking with BSA, the slides were treated with 10 μg/mL of peptide-biotin in 1% BSA for 1 h. Subsequently, the slides were washed with PBS (4×).
Next, secondary streptavidin-488 was added to the tissue slides, incubated for 1 h, and washed, and the tissue slides were mounted with coverslips with use of gel mount. The slides were then observed under a fluorescent microscope (Olympus, IX70) and processed using the Image-Pro plus 5.1 software.

**Determination of Dissociation Constant (Kd) for the Peptide**

A total of 10 μg/mL of the IL-13Ra2/Fc protein was coated onto an ELISA plate for 2 h at room temperature. Subsequently, the wells were blocked with 5% BSA for 2 h. After the blocking step, 100-fold molar excess of Pep-1-L (unconjugated to biotin) was added to the wells and incubated for 60 min at room temperature. Next, the wells were washed 6 times with PBS/0.1% Tween 20. After washing, various concentrations of the biotin-conjugated Pep-1, ranging from 1 μM to 12 μM, were added, and the plate was incubated for an additional 60 min at room temperature. After incubation, the wells were washed 6 times with PBS/0.1% Tween 20. Streptavidin conjugated to horseradish peroxidase (1:8000) was then added to the wells and incubated for 1 h. Subsequently, the wells were washed 6 times with PBS/0.1% Tween 20. The binding was detected by adding 2,2′-Azino-bis-(3-ethylbenzo-thiazoline-6-sulfonic acid) diammmonium salt (Sigma) and hydrogen peroxide substrate solution, and the plate was incubated at room temperature for 1 h. The color was measured at an absorbance of 405 nm with the Spectra max 340 PC. The calculations were performed as described elsewhere.

**Peptide Internalization Assay**

U-251 MG GBM cells (2.5 × 10⁴) per well were plated in a 24-well plate and allowed to attach for 24 h. After 24 h, 5 μg/mL of peptide was added to live cells and allowed to incubate for 4 h and 15 min. At the end of the incubations, the cells were fixed with 5% paraformaldehyde at 37°C for 15 min. After fixation, each well was washed 4 times with PBS. The cells were then permeabilized using 0.1% Triton-X/0.2% BSA for 10 min at room temperature. The wells were subsequently washed 4 times with PBS, and streptavidin-alexa fluor 488 was added along with Topro-3 nuclear stain (Invitrogen). The cells were allowed to incubate for 1 h at room temperature. The wells were then washed and mounted with gel mount (Biomeda) and observed with an LSM 510 Zeiss Confocal Microscope (Cellular Imaging Core, Comprehensive Cancer Center, Wake Forest University), and the images were processed using the Zeiss LSM Image Browser (version 4.2).

**Peptide-Biotin-Streptavidin-Cy5.5 Conjugate Preparation**

To perform a noninvasive small animal study, a conjugate of the peptide-biotin and streptavidin-Cy5.5 was prepared by incubating 5:1 ratio of peptide-biotin to streptavidin-Cy5.5 overnight at 4°C. The sequence of the Pep-1-L peptide was as follows: H-Ala-Cys-Gly-Glu-Met-Gly-Trp-Val-Arg-Cys-Gly-Gly-Ser-LC-Lys-Lys(biotin)-NH₂.

**GBM Xenograft Tumors**

All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Wake Forest University. For induction of subcutaneous human GBM xenografts, 5–6-week-old female athymic nude mice were injected in the right flank with 5 × 10⁶ G48a cells in 100 μL Hank’s Balanced Salt Solution (HBSS). Mice were regularly monitored for tumor growth, and tumors were measured with a caliper to determine length and width. Tumor volume was calculated using the formula (length × width²)/2. 0.9 nmol of either peptide-biotin-streptavidin-Cy5.5 conjugate, or control scrambled peptide-biotin-streptavidin-Cy5.5 were injected intravenously through the jugular vein when the tumors reached a volume of ~80 mm³. Each treatment group consisted of 3 mice receiving Pep-1/biotin-streptavidin-Cy5.5 conjugate (linear and disulphide), control scrambled linear and disulphide peptides, or just streptavidin-Cy5.5 or saline.

For orthotopic GBM tumor induction, 6-week-old athymic nude mice were anesthetized with a ketamine/xylazine mixture (114/17 mg/kg). With use of a stereotactic instrument, 5 × 10⁵ G48a-Luc cells in HBSS were injected intracranially into the mice. The mice were imaged every week for bioluminescence signals as an indicator for tumor growth. When the mean region of interest of the tumor volume was 2.0514 × 10⁷ photons/s/cm²/ser, the mice were injected with 0.9 nmol of peptide-biotin-streptavidin-Cy5.5 by jugular vein intravenous injections. Each treatment group (n = 5) consisted of mice injected with Pep-1/biotin-streptavidin-Cy5.5 conjugate (linear and disulphide), control scrambled (linear and disulphide) peptides, or just streptavidin-Cy5.5 or saline.

**In Vitro Imaging Using Fluorescent Probes**

The mice induced with either orthotopic or with flank GBM xenograft tumors and injected with peptide-biotin-streptavidin-Cy5.5 conjugates were imaged at different periods, ranging from 30 min to 5 days, for subcutaneous tumors and up to 12 days for orthotopic tumors. The mice were monitored for fluorescent peptide binding to the tumors with use of Xenogen IVIS 100 imaging system (Cellular Imaging Core, Comprehensive Cancer Center, Wake Forest University), and the images were analyzed and processed.
using Living Image software, version 4.0 (Caliper Life Sciences).

Statistical Analysis

Data are represented as means +/− standard error of the mean (SEM). For animal studies, the significant differences between the treatment groups were analyzed using Student’s t test. P < 0.05 was considered to be statistically significant.

Results

Isolation of Phages Binding to the IL-13Rα2

To obtain peptides binding to IL-13Rα2, we adopted biopanning schemes with use of a Ph.D-C7C heptapeptide phages display library25 and GBM cells that either express IL-13Rα2 ectopically (G26-H2) or cells with the receptor knocked down (SnB19-asIL-13Rα2). The levels of the IL-13Rα2 receptor protein in all these and control cells were confirmed by Western blotting (Supplementary Fig. S1A). We applied both negative and positive selections on these cells. The results are summarized in Table 1. Twenty-eight of 30 clones had the same sequence, termed Pep-1 (G26 cells). Five (Pep-2) and 2 (Pep-3) phages clones had the same sequence of 30 that were biopanned on SnB19 cells. Thus, we successfully identified 3 different peptide phages clones from the Ph.D-C7C phages display library with an ability to bind to the IL-13Rα2 receptor.

The Isolated Phages Bind to IL-13Rα2 Recombinant Protein

To confirm that the identified clones from the peptide phages display library bind to the recombinant receptor, we performed ELISA with the IL-13Rα2/Fc chimera protein and IL-13 receptor α 1 (IL-13Rα1/Fc) subunit of the physiological receptor.6 We also used IgG/Fc and insulin as controls (Fig. 1A). The IgG/Fc has the identical Fc fragment that is found in the IL-13Rα2/Fc chimera protein. An M13KE native phages not displaying any foreign peptide served as a phages-binding control. The Pep-1 phages bound specifically to the IL-13Rα2/Fc chimera protein and not to the IL-13Rα1/Fc chimera protein (Fig. 1A). The other 2 peptide phages bound IL-13Rα1/Fc either partially or similarly to IL-13Rα2. The control proteins did not bind the phages (Fig. 1A). Because of the binding activity to IL-13Rα1 of Pep-2 and Pep-3 phages, we focused on Pep-1 clone in our investigations.

The Peptide Phages Clones Bind to the Cell Surface–Expressed IL-13Rα2

Next, to establish that the 3 isolated peptide phages clones would bind specifically to the IL-13Rα2 expressed in its natural setting on cells, we performed a cell-binding phages titer assay. We incubated the same number of IL-13Rα2 receptor over-expressing cells (G26-H2 and SnB19-pcDNA), very low expressors (SnB19-asIL-13Rα2), and IL-13Rα2 receptor–negative cells (G26-V2) with equal pfu of the phages. We found that 200 times more of Pep-1 phages bound to the G26-H2 cells, compared with G26-V2 cells (Fig. 1B), whereas 100 times more of the Pep-1 phages bound to SnB19-pcDNA cells, compared with the SnB19-asIL-13Rα2 cells (Fig. 1B).

IL-13 Does Not Inhibit the Binding of Pep-1 Phages to IL-13Rα2

To determine whether the peptide phages clones interfere with the IL-13 binding site on IL-13Rα2, we performed competitive ELISA with an excess of IL-13.E13K, a mutated form of IL-13.17 We used a 1000-fold excess of IL-13.E13K over the highest concentration of the phages used and pre-incubated the ELISA plate with IL-13.E13K for 1 h. The excess of the ligand did not block binding of Pep-1, Pep-2, or Pep-3 phages (Fig. 1C). The incubation with polyclonal anti-IL-13Rα2 antibody made to the extracellular domain of the receptor did, however, block the binding of the peptide phages, indicating that the antibody might be protecting the site of the phages binding to the receptor (Fig. 1C).

Pep-1 Binds Recombinant IL-13Rα2

We found, unexpectedly, that the Pep-1 phages bound similarly to the receptor protein with and without DTT (Supplementary Fig. S1B). Therefore, we synthesized both disulphide-constrained (DSC) and linear (L) forms of Pep-1. These peptides were synthesized with biotin at their C-terminal ends, and thus, they could be detected using streptavidin probes for fluorescent and chemiluminescent studies. To confirm that the synthesized peptides bound IL-13Rα2, ELISA with IL-13Rα2/Fc recombinant protein was performed. We found that the Pep-1-L bound to IL-13Rα2/Fc at ~1 μg/mL, whereas the DSC and the scrambled control peptides did not bind to the receptor (Fig. 2A). None of the peptides used in this experiment bound to the control IgG/Fc protein (Fig. 2A). Furthermore, Pep-1-L with both the cysteines substituted with the

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Fig. 1. Binding of isolated phages to recombinant proteins. Pep-1, Pep-2, and Pep-3 phage clones in ELISA assay against (A) IL-13Ra2/Fc, IL-13Ra1/Fc chimera proteins, and control proteins IgG/Fc and insulin. (B) Biopanning-selected phages bind to cells over-expressing IL-13Ra2. A cell-binding phages titer assay was done on cells that express the IL-13Ra2 (G26-H2 and SnB19-pcDNA cells) and do not over-express the receptor (G26-V2 and SnB19-asIL-13Ra2). Results are expressed as fold-difference in phages titers obtained between different dilutions of original phages (10^6 to 10^12 pfu). (C) The binding of selected phages was not competed for by the IL-13 ligand, but it was competed for by an antibody against IL-13Ra2. ELISA assay was carried out against the IL-13Ra2/Fc with a 1000-fold excess of the IL-13.E13K ligand over the highest concentration of phages used. M13KE is the control phage without any foreign peptide on its surface. The experiments are representative of three separate experiments in duplicates. The error bars represent SEM.
serine amino acids did not exhibit any binding to IL-13Rα2 protein (Supplementary Fig. S1C).

**Pep-1 Binds to the Receptor at Site(s) Other than the Native Ligand Binding Site**

We demonstrated that the IL-13.E13K ligand did not block the binding of the phages to the receptor protein. We performed a similar experiment with the synthetic peptides. IL-13.E13K ligand did not block the binding of Pep-1-DSC and Pep-1-L. Representative assay of three experiments conducted in duplicates. The error bars represent SEM. (C) Competition of Pep-1-L with IL-13.E13K-PE38QQR cytotoxin for the IL-13Rα2 sites on U-251 MG GBM cells in a cell viability assay. The experiment is representative of three experiments conducted in quadruplicate. (D) Determination of dissociation constant for Pep-1-L. The binding assay was conducted with a 100-fold molar excess of the biotin-unconjugated Pep-1 and increasing concentrations of the biotin-conjugated Pep-1. The peptide binding was detected by streptavidin-HRP. A statistically significant regression analysis value ($R^2 = 0.83$) was obtained.

Peptide concentrations of 0.5, 1, 2.5, and 5 μg/mL correspond to concentrations of 0.25, 0.5, 1.2, and 2.5 μM.

Next, we wanted to verify whether native ligand would affect the binding of the peptide to the receptor on GBM cells. Pep-1-L did not compete for the IL-13.E13K-PE38QQR cytotoxin killing effect on U-251 MG GBM cells, whereas IL-13.E13K neutralized this action of the cytotoxin efficiently, as expected (Fig. 2C).

To determine the binding affinity of the linear Pep-1-L to IL-13Rα2, we performed a competition binding experiment in vitro with the recombinant receptor. A specific binding curve was obtained by blocking the receptor protein with 100-fold molar excess of the linear peptide, unconjugated to biotin. A saturation curve was plotted (Fig. 2D), and the $K_d$ was calculated to be 3.3 μM at a statistically significant regression analysis ($R^2 = 0.83$). This is in the range of affinities of the phages isolated from such libraries.

**Synthetic Pep-1 Binds to IL-13Rα2 on GBM Cells and to GBM Tissues**

We found that both linear and DSC forms of the Pep-1 bound readily to SnB19-pcDNA GBM cells, which overexpress IL-13Rα2, but less intensely to cells with less of the receptor such as SnB19-asIL-13Rα2 (Fig. 3A). However, in accordance with the DTT ELISA results, the linear form of the peptide bound the IL-13Rα2-positive cells with a prominently higher intensity than the disulphide-constrained form (Fig. 3A). Moreover, both linear and DSC control scrambled peptides did not show any ability to bind.
to the tested cells (Fig. 3A). Similar results were obtained with other GBM cell lines because Pep-1-L, but not its scrambled control, bound with higher affinity to U-251 MG cells, which overexpress IL-13Rα2, than T98G cells, which have low levels of IL-13Rα2 (Supplementary Fig. S2).

IL-13Rα2 is internalized in response to the binding by the native ligand, IL-13. When we treated U-251 MG cells with Pep-1-L, we observed that the peptide was internalized at 15 min, with increasing intensity of fluorescence up to 4 h. Pep-1-DSC was also internalized, but less prominently when compared with the linear form (Fig. 3B). Next, we performed peptide binding experiments on GBM tissues and normal brain tissues. We observed that Pep-1-L bound readily to GBM and not to normal brain tissues, whereas the control scrambled peptide did not bind to either of the specimens (Fig. 3C). The GBM tissue used for peptide binding was confirmed to express IL-13Rα2 by immunostaining (Fig. S2D).

**Pep-1 Homes to Human GBM Xenografts**

We performed initial in vivo experiments in nude athymic mice implanted with subcutaneous G48a GBM xenografts. The Pep-1-L/biotin-streptavidin-Cy5.5 conjugate was injected intravenously, and it bound to GBM tumors in a dose-dependent manner (Fig. 4A). Pep-1-L bound to tumors as early as 30 min, with an increasing intensity up to at least 72 h (Fig. 4B). At 2 h, this binding was statistically significant (P < .05; R² = 0.8560), compared with the control peptide (Fig. 4B). As was seen with Pep-1-L, the binding of Pep-1-DSC increased with time and was retained up to 72 h (Supplementary Fig. S3A). Moreover, Pep-1-DSC also homed to the subcutaneous GBM tumors, compared with the control peptide (P < .05; R² = 0.9706); however, one of the experimental mice injected with Pep-1-DSC did not demonstrate any signal and was not included in the analysis (Fig. S3A). The streptavidin-Cy5.5 control was not retained significantly by tumors (Fig. 4 and Supplementary Fig. S3A).

Next, we performed peptide homing studies in GBM orthotopic human xenografts. Although GBM tumors hide behind a blood-brain tumor barrier, we injected the peptide/biotin-streptavidin-Cy5.5 conjugate intravenously. The intracranial tumors were detected readily in all experimental mice (Fig. 5A). We observed that Pep-1-L homed to the intracranial GBM xenografts, compared with the control peptide and the streptavidin-Cy5.5 and saline controls (Fig. 5A). The fluorescence in the intracranial tumors increased with time and was retained up to 288 h, with the largest increase during 24–120 h (Fig. 5A). The retention of Pep-1-L, compared with the control scrambled linear peptide, was statistically significant (P < .01; R² = 0.7718) up to 120 h (Fig. 5A). Similar findings were made for Pep-1-DSC (Supplementary Fig. S3B). However, the difference between the experimental and control disulphide peptide binding were significant only at 2 and 4 h (P < .01; R² = 0.9722) (Supplementary Fig. S3B). The GBM tumor xenograft tumors were harvested, sectioned, and stained for IL-13Rα2 by immunofluorescence and haematoxylin-eosin staining. IL-13Rα2 was abundantly expressed in all of the analyzed tumors (Supplementary Fig. S4). To determine the homing capability of Pep-1 to normal brain, we performed experiments in mice not bearing intracranial tumors. We observed nominal binding for Pep-1-L at 2 h and no binding to the normal brain at subsequent times (Fig. 5B).

**Discussion**

For specific targeting of GBM tumors, we had previously discovered IL-13Rα2, which is over-expressed in approximately three-fourths of patients with GBM. Here, we identified peptide ligands binding to the IL-13Rα2. We used a DSC heptapeptide phages-display library and performed biopanning against GBM tumor cells expressing IL-13Rα2. We isolated 3 peptides and characterized one of them in more detail. This peptide, Pep-1, binds IL-13Rα2 specifically in the context of both recombinant protein and cancer cells. The binding of Pep-1 occurs at a site different from the binding of its natural ligand, IL-13, because the peptide’s binding was not inhibited in the presence of the cytokine. Of interest, the peptide that did not retain disulfide bond-constrained conformation either in the filamentous phages or as a chemically synthesized compound exhibited higher avidity toward the receptor. Moreover, the peptide internalized with the receptor, and its binding can also be readily demonstrated on GBM specimens. Of importance, the peptide homed and bound to both subcutaneous and intracranial GBM tumors expressing IL-13Rα2. These findings open new opportunities for GBM management.

Our biopanning strategy yielded 3 phages against IL-13Rα2, but Pep-1 is the most specific for IL-13Rα2. We found that this peptide binds to the receptor at a...
Fig. 4. Pep-1-L binds to subcutaneous GBM tumor xenografts. (A) Athymic nude mice bearing human GBM G48a subcutaneous tumors were injected i.v. with increasing concentrations of Pep-1-L/biotin-streptavidin-Cy5.5 conjugate (range, 0.1–1.8 nmol). Control mice were injected with either saline or 1.8 nmoles of unconjugated streptavidin-Cy5.5 dye. (B) Athymic nude mice bearing human GBM G48a subcutaneous tumors were injected intravenously with Pep-1-L/biotin-streptavidin-Cy5.5 conjugate or control scrambled linear peptide-biotin-streptavidin-Cy5.5 conjugates and imaged at various times ranging from 30 min to 72 h. The color scale is in units (photon/sec/cm²/steradian)/μW/cm². Control mice were injected with 0.9 nmol of unconjugated streptavidin-Cy5.5 or saline. Representative comparison of tumor binding and retention by Pep-1-L vs. the control scrambled linear peptide (P < .01) is shown at 2 h.
Fig. 5. Pep-1-L binds to orthotopic GBM tumor xenografts. (A) Pep-1-L/biotin streptavidin-Cy5.5 conjugate was injected intravenously in athymic nude mice bearing human GBM G48a intracranial tumors and near infrared fluorescence imaging was carried out for various time points ranging from 30 min to 288 h. Mice were also injected with equal amount of control linear peptide-biotin streptavidin conjugate or unconjugated streptavidin-Cy5.5 or saline. The color scale is in units (photon/sec/cm²/steradian)/μW/cm². Comparison of the tumor binding and retention by Pep-1-L vs. the control scrambled linear peptide (P < .001). (B) The experiment was conducted as in A, but in mice free of intracranial tumors.
site that is not used by the native ligand, because IL-13 did not block the binding of the peptide to IL-13Rα2/Fc chimera protein and the peptide did not neutralize the cytokotoxic effect of IL-13.E13K-PE38QQR. However, the anti-IL-13Rα2 antibody blocked the binding to the receptor, suggesting that the antibody causes steric hindrance for the peptide binding site.

Of interest, Pep-1 recognizes IL-13Rα2 best in a non-disulfide bond–constrained form. Our study may be the first report of this kind when using a phages-display library. However, Pep-1-DSC also showed binding properties but in a context of the receptor expressed on cells. One of the possible reasons for this can be that the Pep-1-DSC is able to recognize the naturally expressed receptor while not being able to bind to the recombinant receptor. Another possibility is that all our selected peptides have Trp and/or Pro residues that may hinder the ability of such peptides to assume a disulfide bond–constrained conformation.

IL-13Rα2 is considered as a decoy receptor for an excess of endogenous IL-13, but it also internalizes. We found that Pep-1-L and DSC peptide were internalized in the cells, although excess of these peptides did not block the binding of the native ligand. This suggests that the Pep-1 might be binding to a region of the receptor that is essential for inducing its internalization. It is plausible that this peptide might reflect yet unknown functions of the receptor that are independent of IL-13.

Of importance, we have demonstrated that Pep-1-L and Pep-1-L-DSC are retained by subcutaneous and intracranial GBM human xenograft tumors in mice. This binding is both dose- and time-dependent. One of the most significant aspects of this study is that Pep-1 homes to intracranial GBM xenografts when injected intravenously. Few targeting agents have been isolated that can cross the blood-brain tumor barrier.27,28 Therefore, the peptides may be used in noninvasive diagnostic examination of GBM. Moreover, our results suggest that Pep-1-L and Pep-1-DSC could be used for different applications (eg, the former for therapeutic targeting and the latter for rapid imaging of GBM). Both peptides can be used in conjunction with IL-13–based targeted imaging and therapy.29–31 In fact, Pep-1 offers a unique opportunity of either double-molecular targeting of IL-13Rα2 or double-labeling of the receptor or specific visualization of the receptor before and during delivery of the receptor-targeted therapy. Thus, it can serve therapeutic delivery or confirmation of eligibility to treatment.32 Furthermore, these peptides could be exploited in image-guided surgical resection of GBM by tumor illumination.33

Supplementary Material

Supplementary material is available online at Neuro-Oncology (http://neuro-oncology.oxfordjournals.org/).

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Conflict of interest statement. Wake Forest University Health Sciences, Office of Technology Asset Management has filed a patent application regarding the use of the peptides in the treatment and diagnosis of tumors expressing IL-13Rα2 (“IL-13 Receptor Binding Peptides”, Provisional Co-operation Treaty Publication No. WO 2010/121125, WD, HP, and DG). All other authors: none reported.

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References


34. Pandya et al.: IL-13Ra2 binding peptides