SELECTION/CONSTRUCTION AND CHARACTERIZATION OF NOVEL PEPTIDE AND RECOMBINANT PROTEIN AGENTS TARGETING INTERLEUKIN 13 RECEPTOR ALPHA 2 IN GLIOBLASTOMA MULTIFORME

BY

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A Dissertation Submitted to the Graduate Faculty of

WAKE FOREST UNIVERSITY GRADUATE SCHOOL OF ARTS AND SCIENCES

in Partial Fulfillment of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

Molecular Genetics and Genomics

May 2011

Winston-Salem, North Carolina

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ACKNOWLEDGEMENTS

First and foremost, I want to thank my advisor, Dr. Waldemar Debinski. Thank you for all your support and encouragement. Thank you for letting me be a part of your laboratory. I have learned a lot from you. Your positive enthusiasm, your strong belief in science, your moral and ethical values are life-long lessons for me. Thank you for providing me your excellent guidance, while allowing me to pursue my scientific ideas. I will take with me a lot of invaluable teachings, including how to critically evaluate ideas and thoughts as well as express scientific viewpoints.

I want to specially thank all my committee members: Dr. Purnima Dubey, Dr. Roy Hantgan, Dr. Steven Kridel and Dr. Mark Lively for all the supportive criticism, encouragement and support. I would also like to acknowledge NCI/NIH RO1 CA74145 (Dr. Waldemar Debinski) grant for financial support.

I want to heartily thank Denise Gibo for her teaching and patience along the way. Thank you for guiding me through those initial years in the laboratory and helping me in those small things that make big things possible. Thank you for always being there and for all the advice and support.

I want to also thank all the past and present Debinski lab members for making my time in the laboratory comfortable and fun. I would like to acknowledge Liz Forbes, Evan Gomes, Kenneth Grant and Ravi Singh for their technical guidance. I would like to express my gratitude for Sherry Raiford and Darlene Cantrell for their care and prompt assistance in situations.
A special thanks to my parents who instilled in me the values of hard work, determination and perseverance, and to my dearest and loveliest sisters, Swati Lodha and Jahnvi Thaker for their exuberant enthusiasm for my every slightest achievement. I want to thank my in-laws, Amma and Appa, for their constant encouragement and their unwavering faith in me. Also, a sincere thanks to my sister-in-law, Hema Subramanian for her unconditional support. I also want to thank my entire family for their understanding and patience, especially, when I have missed engagements, weddings, births and also family gatherings. If it wasn’t for your understanding, my journey wouldn’t have been smooth.

I want to express my gratitude for my friend, Shweta Shankar, for always hearing me out and always being there for me. It is difficult to find people like you. My friends Jennifer Kiger and Michael Blanks for helping me get settled in United States in those initial years. Also, thanks to my friends, Sriram Ramanan, Mayur Choudhary, and Deepak Bharkhada for taking the sting out of being so away from home.

Finally, wholehearted thanks to my dearest and best friend, my husband, Rajesh Subramanian. You have been an inspiration for me. Your encouragement and understanding were paramount in this journey. Thank you for the sacrifices that you have made to help me achieve my goals in life. Thank you for always being there, and for those kind and encouraging words. Your professionalism and hard work is something that I will always aspire to.

This thesis is dedicated to my husband, Rajesh Subramanian and to my sisters - Jahnvi Thaker and Swati Lodha.
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ABBREVIATIONS

GBM - Glioblastoma Multiforme
WHO - World Health Organization
CNS - Central Nervous System
EGFR - Epidermal Growth Factor Receptor
P53 - Protein 53 or Tumor Protein 53
PDGFR - Platelet Derived Growth Factor Receptor
FGFR - Fibroblast Growth Factor Receptor
FGF2 - Fibroblast Growth Factor 2
EGF - Epidermal Growth Factor
LIF - Leukemia Inhibitory Factor
OSM - Oncostatin M
PDGF - Platelet Derived Growth Factor
PTEN - Phosphatase and Tensin Homologue
GATA6 - GATA binding protein 6
RAS - RAt Sarcoma
NF1 - Neurofibromin 1
CDKN2A - Cyclin-Dependent Kinase Inhibitor 2A
TCGA - The Cancer Genome Atlas
BCPC - Brain Cancer Propagating Cells
NSC - Neural Stem Cells
BBB - Blood Brain Barrier
HGA - High Grade Astrocytomas

IL-13Rα2 - Interleukin 13 Receptor Alpha 2

gp100 - Glycoprotein 100

TRP2 - Tyrosine-Related Protein 2

EphA2 - Ephrin type-A Receptor 2

Fra-1 - Fos Related Antigen 1

EGFRvIII - Epidermal Growth Factor Receptor type III

ERK - Extracellular Signal Regulated Kinase Pathway

P13K - Phosphatidylinositol 3-Kinase

IL-13 - Interleukin 13

PE - *Pseudomonas* Exotoxin A

ADP - Adenosine Diphosphate

EF2 - Elongation Factor 2

IL-4Rα - Interleukin 4 Receptor Alpha

IL-13Rα1 - Interleukin 13 Receptor Alpha 1

Th2 - T helper type 2

kDa - Kilodalton

IL-3 - Interleukin 3

IL-5 - Interleukin 5

IL-4 - Interleukin 4

CSF2 - Colony Stimulating Factor 2

IgE - Immunoglobulin chain E

MHC - Major Histocompatibility Complex
Kd – Dissociation Constant

JAK - Janus Kinase

TYK2 - Tyrosine Kinase 2 Deficiency

STAT6 - Signal Transducers and Activators of Transcription 6

STAT3 - Signal Transducers and Activators of Transcription 3

AHR - Airway Hyper Responsiveness

TGF-β1 - Transforming Growth Factor Beta 1

NMR - Nuclear Magnetic Resonance

CT19 - Cancer-Testis Antigen 19

IL-13R - Interleukin 13 Receptor

CT - Cancer-Testis antigen

NFAT1 - Nuclear Factor of Activated T-cells

AP-1 - Activating Protein-1

c-JUN - v-Jun Avian Sarcoma Virus 17 Oncogene Homolog

c-FOS - FBJ Murine Osteosarcoma Viral Oncogene Homolog

AP-2 - Activating Protein-2

GABP - GA Binding Protein

OCT1 - Octamer Transcription Factor 1

GATA3 - Trans-acting T-cell-specific Transcription Factor 3

C-ETS1 - v-ets Erythroblastosis Virus E26 Oncogene Homolog 1

NHBECs - Normal Human Bronchial Epithelial Cells

PAS - Periodic Acid Schiff

MUC5AC - Mucin 5AC
SCCHN - Squamous Cell Carcinoma of the Head and Neck

p38 MAPK - p38 Mitogen-Activated Protein Kinase

JAK1 - Janus Kinase 1

JAK2 - Janus Kinase 2

c/EBPβ - CCAAT/Enhancer-Binding Protein Beta

NF-κB - Nuclear Factor Kappa B

TNFα - Tumor Necrosis Factor Alpha

EMSA - Electrophoretic Mobility Shift Assay

IFNγ - Interferon Gamma

IL-10 - Interleukin 10

siRNA - Short Interfering Ribonucleotide

MMP-8 - Matrix Metalloproteinase-8

HUVEC - Human Umbilical Vein Endothelial Cells

DT - Diphtheria Toxin

CD4TM - Cluster of Differentiation 4 Transmembrane Region

CD3ζ - T-cell Receptor T3 Zeta Chain

CTL - Cytotoxic T Lymphocyte

HLA - Human Leukocyte Antigen

HL - Hodgkin’s Lymphoma

CAR - Coxsackievirus

AA - Anaplastic Astrocytoma

AO - Anaplastic Oligodendrogloma

AOA - Anaplastic Oligoastrocytoma
CB - Cintredekin Besudotox

PRECISE - Phase III Randomized Evaluation of Convection Enhanced Delivery of IL13-PE38QQR with Survival Endpoint

NIRF - Near Infrared Fluorescence

ELISA - Enzyme Linked Immunosorbent Assay

DTT - Dithiothreitol

PFU - Plaque Forming Units

SEM - Standard Error of the Mean

MTS - 3- (4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

PMS - Phenazine Methosulfate

NLS - Nuclear Localization Signal

SV40 – Simian Virus 40

E.coli - Escherichia coli

IPTG - Isopropyl β-D-1-thiogalactopyranoside

FPLC - Fast Protein Liquid Chromatography

EDC - 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride

NHS - N-hydroxysuccinimide

TFP - Tetrafluorophenyl

DOL - Degree of Labeling

BL - Biotin Labeled

nM - Nanomolar

IgG - Immunoglobulin G
HGFR - Hepatocyte Growth Factor Receptor

IL-11 - Interleukin 11

ErbB-2 - Human Epidermal Growth Factor Receptor 2

PSMA - Prostate-Specific Membrane Antigen

TAG-72 - Tumor-Associated Glycoprotein 72

VEGF - Vascular Endothelial Growth Factor

SPECT - Single photon emission computed tomography

PET - Positron emission tomography (PET)

$^{99m}$Tc - Technetium-99m

KeV - Kiloelectron Volt

MMP 2 – Matrix Metalloproteinase 2

MMP 9 – Matrix Metalloproteinase 9

$^{18}$FDG – 2-deoxy-2-fluoro-D-glucose

$^{64}$Cu – Copper-64

uPAR - urokinase-type Plasminogen Activator Receptor

Tie 2 - Tyrosine Kinase with Immunoglobulin and Epidermal Growth Factor Homology domain-2

PRDM14 - proline rich domain proteins

TIP-1 - Tax-Interacting Protein-1

BRP – Bevacizumab-Responsive Peptide

GRP78 - 78 kDa Glucose-Regulated Protein

HSVtk - Herpes Simplex Virus Thymidine Kinase

TAG-72 - Tumor-Associated Glycoprotein 72
MRI - Magnetic Resonance Imaging
5-ALA - 5-Aminolevulinic Acid
PPIX - Protoporphyrin IX
hnRNP - heterogeneous nuclear ribonucleoprotein
CPP - Cell-Penetrating Peptides
PNA - Peptide Nucleic Acids
LET - Linear-Energy Transfer
ABSTRACT

Hetal Pandya

SELECTION/CONSTRUCTION AND CHARACTERIZATION OF NOVEL PEPTIDE AND RECOMBINANT PROTEIN AGENTS TARGETING INTERLEUKIN 13 RECEPTOR ALPHA 2 IN GLIOBLASTOMA MULTIFORME

Dissertation under the direction of
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Professor of Neurosurgery, Radiation Oncology, and Cancer Biology

Glioblastoma multiforme (GBM) is a primary brain tumor of dismal prognosis. Patients with GBM have a median survival rate of 14.5 months inspite of multimodality treatment involving surgery, radio- and chemotherapy. This warrants an intensive search for efficient new therapies. Interleukin 13 receptor alpha 2 (IL-13Rα2) is an internalized, plasma membrane receptor for IL-13. IL-13Rα2 is over-expressed in around 75 percent of GBM patients. One of our goals is to develop specific therapeutic delivery vehicles and imaging agents targeting IL-13Rα2.

Aims: To explore whether small peptide ligands could be identified that bind IL-13Rα2. Peptides may offer better uptake by tumors from circulation or better distribution when used with convection-enhanced delivery (CED), a means of loco-regional delivery to intracranial tumors. Moreover, several anti-cancer therapeutics have defined targets such as proteins, enzymes or DNA, which are localized in distinct intra-cellular compartments like lysosomes, mitochondria and nucleus. Hence, attempt was made to develop recombinant IL-13-based proteinaceous vectors that could deliver therapeutics into specific intra-cellular compartments of IL-13Rα2-positive cells.

Results: To isolate the peptides binding to IL-13Rα2, we screened an M13 cyclic heptapeptide phage-display library, Ph.D-C7C. We identified three different peptide phage clones capable of binding to IL-13Rα2. Amongst these, Pep-1 phage was found to demonstrate a high affinity and specificity to IL-13Rα2, therefore, Pep-1 peptide was synthesized and characterized for binding to IL-13Rα2 using various assays. Pep-1 was found to bind to the receptor at a site distinct from the native ligand. Importantly, when injected by intravenous route, Pep-1 was retained by both subcutaneous and orthotopic GBM xenograft tumors in nude mice. Thus, we were able to select a specific peptide from a phage-display library that homes to IL-13Rα2-positive tumors. In another aim, we constructed a targeted recombinant protein delivery vehicle to the nuclei of cells, using a modified receptor ligand, IL-13.E13K, domain II (D2) of Pseudomonas exotoxin A and SV40 T-antigen nuclear localization signal (NLS); IL-13.E13K-D2-NLS. We also produced control proteins such as IL-13.E13K-D2, IL-13.E13K-NLS and IL-13.E13K. The recombinant
proteins were labeled with fluorescent dyes and their localization was monitored using confocal microscopy. IL-13.E13K-D2-NLS demonstrated a prominent nuclear localization in GBM cells whereas control proteins remained sequestered in the cytosol. Hence, we have demonstrated that all the three components of the recombinant protein are essential for efficient nuclear delivery. Moreover, IL-13.E13K-D2-NLS exhibited precision in recognition as well as intra-cellular localization in IL-13Ra2-overexpressing cells compared to GBM cells with no cell surface expression of the receptor.

**Summary:** Thus, we have selected a specific peptide from a phage-display library that binds to IL-13Ra2. Also, we have generated a non-viral, single-chain recombinant protein vehicle for nuclear delivery to IL-13Ra2-positive cancer cells. Both of these agents have the potential to be further developed for diagnostic, imaging and therapeutic interventions for GBM.

**Clinical Significance:** We were able to demonstrate the imaging applicability of the IL-13Ra2-specific peptide. This peptide ligands can be further conjugated to various imaging beacons and used with different imaging techniques, such as SPECT, PET as well as MRI. Moreover, this GBM-specific peptide can be used as a mean to measure response to therapies delivered by CED. For our nuclear delivery vectors, we are currently assessing conjugation to radioisotopes as a direct application for cancer therapy. The IL-13Ra2-specific peptide, Pep-1, and the nuclear delivery vectors can be conjugated and utilized for delivery of various therapeutics such as radioisotopes ($^{111}$In, $^{125}$I), chemotherapeutics (doxorubicin) and photosensitizers. In addition, the nuclear delivery vectors can also be utilized for gene-, anti-sense gene- or siRNA-mediated therapies. Both the IL-13Ra2 specific peptide and IL-13-based protein vectors can be used for ‘double targeting’ of GBM as they bind independently of each other.
CHAPTER I

INTRODUCTION

**Glioblastoma multiforme**

Glioblastoma multiforme (GBM) is one of the most malignant and treatment-resistant forms of primary brain tumors. GBM tumors are formed by supportive neoplastic astrocytic (glial) cells. The WHO classifies GBM as a grade IV astrocytoma (1;2). GBMs constitute 50-60% of all astrocytic tumors and has an incidence rate of 3 to 4 per 100,000 (3). Primary brain tumors account for 2.4% of all yearly cancer-related deaths. They are also one of the top ten causes of cancer-related deaths in USA for population less than 40 years of age. The median survival rate for patients with GBM is only 14.6 months and most do not survive beyond 2 years of diagnosis. The 2-year survival rate is 26.5% (4).

GBM are heterogenous intraparenchymal tumors with various chromosomal and genetic abnormalities. They display necrosis and subsequent extensive haemorrhage (5). GBM tumors grow rapidly and have a capacity to aggressively invade and infiltrate the surrounding normal brain structures leading to neurological impairment (6). GBM tumors generally do not metastasize outside the central nervous system [CNS]. Histologically, they are made of several different cell types, including the glioma cells, endothelial cells, macrophages and trapped, invaded normal brain tissue (7). They also display mosaic pattern and consist of various histological subtypes, including small cell GBM (27% of all GBM) (8), pleomorphic cell GBM (26%), gemistocytic GBM (25%) (9),
oligodendroglioma (15%), gliosarcoma (2%) (10) and giant cell GBM (1%) (11) and hence the term Glioblastoma multiforme. One of the important characteristics of GBM is the ‘pseudopalisading’ necrosis, in which regions of necrosis are surrounded by densely packed, aligned tumor cell nuclei, which line up next to each other like slats in a picket fence [Figure 1] (12). Also, in GBM, the presence of hypertrophied blood vessels and cells with nuclear pleomorphism are relatively common (13). The signs of neovascularization and pseudopalisading necrosis are pathognomic to GBM [Figure 1].

GBM tumors are classified as primary or secondary tumors. Primary or de novo GBM tumors constitute 60% of all primary brain tumors in patients greater than 50 years of age. Whereas the secondary GBM tumors arising from low grade gliomas, such as WHO grade II and III astrocytomas seem to mostly affect younger population of less than 45 years of age. Also, there seems to exist distinct genetic alteration pathways leading to secondary GBM tumors. The primary GBM tumors exhibit overexpression of epidermal growth factor receptor [EGFR] gene, but mostly have normal p53 status, while the secondary GBM tumors have normal EGFR gene expression but have inactivated p53 (14;15).

One of the other major characteristics of astrocytomas is that the gene/chromosomal deletions and amplifications increase with an increase in the clinical grade of gliomas. The genetic alterations suggested to lead to gliomagenesis are the signaling pathways involved downstream of oncogenic tyrosine kinase receptors like the platelet derived growth factor receptor [PDGFR], the fibroblast growth factor receptor [FGFR] as well as
the EGFR (16), and overexpression of growth factors like fibroblast growth factor 2 [FGF2] (17), epidermal growth factor [EGF] (18), leukemia inhibitory factor [LIF] (19), oncostatin M [OSM] (19) and platelet derived growth factor [PDGF] (20;21). GBM tumors also most commonly exhibit loss of 10q22-25 chromosomal region that harbors tumor suppressors like PTEN (22). They also demonstrate loss of tumor suppressors like EphrinA1 (23;24) and also GATA6 (25). They also have high levels of activated RAS protein (26), however, most GBMs do not exhibit mutations in the RAS gene (27). The activated RAS accumulates due to stimulation of upstream aberrant tyrosine kinase receptor activation (EGFR, PDGFR) and/or also due to loss of RAS-GTPase activator proteins, such as neurofibromin-1 [NF1] (27). The second most frequent genetic alteration observed in gliomas appear to be in the cell-cycle arrest pathways, especially in the INK4A or CDKN2A gene (28). Moreover, recent proteomic analysis of 27 surgical glioma samples and genomic/expression data of GBM from The Cancer Genome Atlas [TCGA] have lead to identification of three main genetic underpinnings for glioma-relevant signal transduction pathways. These consist of either the EGFR or the PDGFR amplification/mutation, or chromosomal loss/mutation of the RAS regulator, NF1 (29-30).

Recent evidence suggests existence of cancer propagating cells, or cancer stem cells in GBM (31). These cells are also known as brain cancer propagating cells [BCPCs] (32). The concept of cancer-stem cell hypothesis defines these cells as a subset of cells present in the tumor with stem-like properties, which have the capacity to initiate new tumors in mice (33). Two major mechanisms have been hypothesized to be responsible for the
generation of BCPCs, including, 1. dedifferentiation of existing brain tumor cells into their progenitors (34) and, 2. generation from neural stem cells [NSC], arising from accumulation of mutations in these cells and their niche microenvironments (35;36). Importantly, these cells have also been touted as the ones responsible for resilience of GBM tumors to therapy (37;38), although this remains to be firmly established (39).

The current standard of care for patients with GBM is tumor debulking by surgical resection, followed by post-operative radiotherapy and concomitant as well as adjuvant chemotherapy with temozolomide (4). Even with the current multimodality treatment strategy, the median survival of patients with GBM is less than 15 months and the 5-year median survival is less than 5% (4). It is apparent from these survival statistics that the current combinatorial treatment approach is inadequate.

There are several factors that act as obstacles for therapeutic efficacy in gliomas. The first and the most important cause for poor prognosis for GBM patients is the issue of tumor recurrence. Surgical debulking is the mainstay treatment for the GBM tumors, however in more than 90% of the cases, the residual glioma cells from the area adjacent to the tumor resection cavity give rise to a recurrent tumor, usually within two-cm radius of the post-resection cavity (40). The second factor is the cellular and molecular heterogeneity across and within these tumors. Alteration in multiple signaling pathways and different phenotypic compositions are the characteristics of GBM tumors. Hence, application of a single treatment modality does not lead to complete eradication of the tumor as it is not pertinent to the whole tumor, both physically and molecularly. The third factor is a
limited delivery across the blood brain barrier [BBB] in the regions of the tumor core and the areas of microscopic tumor infiltration. The fourth obstacle is the non-targeted, non-specific nature of most of the current anti-cancer agents used for treatment. Irradiation and chemotherapy in form of DNA topoisomerases or alkylating agents lead to incomplete elimination of brain tumor cells, and cause damage to normal brain cells.

Considering the above described factors, an ideal therapeutic for GBM would be targeted to tumor-associated antigens, which are abundantly overexpressed in a majority or all of GBM tumor cells. This strategy can lead to lower doses of the therapeutics, with expectations of minimal damage to the normal cells. An efficient approach to make such targeted therapeutics is to take advantage of tumor associated phenotypic changes, such as elevated cell surface receptors or intracellular antigens, like activated cell signaling pathway proteins. In recent times, major efforts have been made to develop molecularly targeted therapeutics against GBM-specific targets that are increasingly favored as drug candidates for GBM treatment.

**Molecular Targeting of GBM**

The basis for molecular targeting of tumors basically can be subdivided into 1. activated intracellular signaling pathway components, and, 2. overexpressed plasma membrane receptors/antigens. Small molecule inhibitors have been identified that modulate the activity of one or more components of the activated signaling pathway molecules, such as EGFR in GBM (41;42). As for the cell-surface tumor-associated plasma membrane receptors, targeted agents including monoclonal antibody-, growth factor- as well as
ligand-based cytotoxins, vaccines, and redirected T cells have been developed for specific targeting of cancer cells.

There are many antigens overexpressed specifically in GBM and are also termed as high grade astrocytoma [HGA]-associated antigens. They include Interleukin 13 receptor alpha 2 [IL-13Rα2], Glycoprotein 100 [gp100], Tyrosine-related protein 2 [TRP2] as their protein expression is found to be elevated in 75%, 13% and 34% of glioma tumors, respectively (43-49). Also, our laboratory has identified a trimolecular phenotypic signature for GBM. This signature consists of three factors/antigens, i.e. IL-13Rα2, Eph type-A receptor 2 [EphA2] and Fos-related antigen 1 [Fra-1] as greater than 95% of tumors express at least two of the three markers, and each tumor has at least one of them (50).

As mentioned earlier, EGFR amplification is also detected in around 40% of GBM tumors (51). Also in approximately 50% of GBM tumors with the EGFR amplification, a mutant of EGFR, called EGFRvIII is found. EGFRvIII has deletion of 267 amino acids from the extracellular domain of the receptor. It does not bind the native ligands efficiently and signals continously due to a constitutive tyrosine phosphorylation, and therefore is highly oncogenic (52). Importantly, EGFR and/or its constitutive active mutant form have been shown to regulate expression of all the three factors discovered in our laboratory which constitute the molecular signature of GBM. We have previously shown that EGF leads to upregulation of IL-13Rα2 levels in GBM cells through EGFR-mediated signaling pathways, such as PI-3K and ERK (53). Also, IL-13Rα2 is a gene
target of the mutant EFGRvIII (54). Similarly, we have observed a large increase in protein levels of Fra-1 in response to EGF stimulation (55). In addition, it has earlier been shown that expression of EGFR and/or EFG FrvIII leads to upregulation of many factors involved in glioma pathogenesis, including EphA2 and Fra-1 (56-58). Thus, the members of the tri-molecular signature identified in our laboratory are at least in part, regulated by either EGFR or the EFG FrvIII mutant or both (54-58).

In spite of overexpression of EGFR and activation of various other signaling pathways, therapeutics targeting to any one component of the heterogenous signaling cascades in GBM have not yet been successful in the clinic (59;60). For this reason, we envision more effective therapeutics would be the ones which take advantage of the relative abundance of tumor-associated cell surface receptors. Our laboratory has been actively involved in developing molecularly targeted therapeutics that specifically recognize tumor cells through their overexpressed plasma membrane receptors, such as IL-13Rα2 and EphA2. These overexpressed tumor associated cell surface receptors are close to ideal candidates for delivery of most of the cancer therapeutics. Our laboratory follows the rational therapeutic approach of conjugating certain cancer chemotherapeutics, biological toxins, radioactive isotopes to receptor ligands, monoclonal antibodies or peptides specific to brain tumor cell surface antigens, in hope of achieving precise recognition and localization in tumor cells in contrast to normal brain cells.

Our laboratory has earlier generated, targeted Interleukin 13 [IL-13]-based bacterial cytotoxins against tumor cells (61). During the course of research, it became apparent
that the high killing activity of this cytotoxin is due to over-expression of IL-13Rα2 on cancer cells (62). These recombinant cytotoxins are composed of IL-13 as a targeting unit, followed by the endosomal translocation (D2) and the enzymatic (protein synthesis inhibitor) protein domains of *Pseudomonas* exotoxin A (PE) (61;63). These cytotoxins were shown to be the most potent anti-glioma agents under both *in vitro* and *in vivo* conditions (62;63).

PE is a 66-kDa pathogenic protein secreted by the bacteria, *Pseudomonas aeruginosa*. The PE protein consists of three major and one minor protein domains (64). Of the three major domains, domain 1a (amino acids 1-252) is the cell-binding domain which recognizes the α₂-macroglobulin receptor on eukaryotic cells (65;66). Domain 2 (amino acids 253-364) is the endosomal translocation domain which transports a part of domain 2 and the entire domain 3 of PE from the endosome into the cytosol (66;67). Once in the cytosol, domain 3 of PE (amino acids 395-613) ADP-ribosylates EF2 factor of the protein synthesis machinery. This arrests the *de novo* protein synthesis and inevitably causes cell death (66;68). Domain 1b (amino acids 365-399) is the minor protein domain whose function is unclear. However, removing domain 1b of the toxin does not alter the function of the PE toxin and thus it may be dispensable (69). Various PE derivatives have been developed like PE40 (deletion of domain 1a) (70), PE38 (deletion of domain 1a and part of 1b) (69) as well as PE38QQR, which is PE38 with substitutions of all the lysines in domain 3 at positions 590, 606 and 613 (71). PE1E and PE4E are full length PE with substitutions of amino acids at positions 57 (PE1E) and at 57, 246, 247 and 249 to
glutamic acid residues. These changes have been shown to diminish the interaction with the $\alpha_2$-macroglobulin receptor on eukaryotic cells (72;73).

Here, I will review the role of IL-13 and IL-13R$\alpha$2 in pathologies such as inflammation and malignant diseases. I will discuss the role and structure of IL-13 and its interactions with IL-13R$\alpha$2 and IL-4R$\alpha$/IL-13R$\alpha$1 receptors. I will also focus on the current pipeline of targeted therapeutics against IL-13R$\alpha$2.

**IL-13 and IL-13 Receptors**

IL-13 is a pleiotropic, immunoregulatory cytokine which has been implicated in inflammatory respiratory diseases and in chronic parasite-induced infection pathologies (74). IL-13 is a type 2 cytokine, produced by activated Th2 helper cells (75). It is a 14-kDa glycosylated protein and consists of a four $\alpha$-helical hydrophobic bundle core (76). The gene for IL-13 is located on chromosome 5q and forms a cytokine gene cluster with IL-3, IL-5, IL-4, and colony stimulating factor 2 [CSF2]. The IL-13 gene is located 12 Kb upstream of IL-4 gene and consists of 4 exons and 3 introns with a 24 amino acid signal peptide and a 146 amino acid open-reading frame (77). There are four postulated glycosylation sites and the protein forms two disulphide bonds (78;79). However, glycosylation does not seem to be necessary for it to bind to its receptor or exert its biologic functions (80). IL-13 receptors in physiology are found on basophils, eosinophils, mast cells, endothelial cells, macrophages, monocytes, fibroblasts, smooth muscle cells and respiratory epithelium, but unlike IL-4, not on T lymphocytes (81). Thus, in summary, IL-13 exerts its effects on many different cell types.
IL-13 is believed to drive isotype switch and secretion of IgE by B lymphocytes (82). It is also responsible for inducing expression of IgE receptors and MHC class II on B lymphocytes, mast cells and basophils, thereby enhancing IgE-mediated responses (83). IL-13 has also been implicated in recruitment, survival and activation of eosinophils (84). Furthermore, it inhibits production of pro-inflammatory mediators, like prostaglandins and reactive oxygen species in monocytes and macrophages (85;86). IL-13 increases epithelial permeability and mucus production in the airways, and induces expression of chemokines such as eotaxin (87;88). IL-13 is implicated mainly in inflammation induced due to asthma (89), allergy (90) and parasitic infections (91-93). IL-13 binds to two different types of receptors: IL-4Rα/IL-13Rα1 and IL-13Rα2 [Figure 2].

**IL-4Rα/IL-13Rα1 receptor**

The IL-4Rα/IL-13Rα1 (IL-4/1L-13R) is a shared receptor between IL-13 and IL-4, and is also known as type II IL-4 receptor. The type II receptor is a heterodimer composed of IL-13Rα1 and IL-4Rα subunits. IL-4Rα also forms a receptor with the γC chain and is known as the IL-4 type I receptor (94). IL-4Rα is the functional subunit of both type I and type II IL-4 receptors [Figure 2]. The IL-4 ligand binds to the IL-4Rα subunit (Kd 20-300 pM) which causes receptor dimerization with IL-13Rα1 subunit, activating the JAK2/TYK2 pathway, and finally recruitment and activation of the STAT6 transcription factor (94-96). IL-13 binds with an intermediate affinity to the IL-13Rα1 subunit, but forms a high affinity complex in presence of the IL-4Rα subunit. Formation of the receptor complex then leads to activation of the JAK2/TYK2 pathway and activation of the STAT6 and also STAT3 transcription factors (97). The IL-4/IL-13R protein is...
ubiquitous and is found at highest levels in the heart, liver, skeletal muscles and the ovaries but is found in low numbers in the brain, lung and kidney [IL-13Rα1, P78552 (I13R1_HUMAN), Protein knowledge database, UniProtKB/Swiss-Prot] and (98). It is also found on bronchial epithelial cells and fibroblasts and is implicated in activation of the airway hyperresponsiveness [AHR] genes (99).

**IL-13Rα2 receptor**

The second type of IL-13 receptor is the monomeric receptor, IL-13Rα2 [Figure 2]. IL-13 binds to this receptor with higher affinity than the IL-13Rα1, and is considered as “decoy” or non-functional receptor in AHR or malignancy (100-103). It does not bind the IL-4 ligand, which is a characteristic feature of this receptor. IL-13Rα2 has a very short cytoplasmic tail (17 amino acids) and no signaling protein binding domains have been identified on it. However, one recent study suggested that the IL-13Rα2 may have some direct signaling function in macrophages, which leads to induction of TGF-β1 mediated fibrosis (104). Importantly, the presence of IL-13Rα2 is frequently detected in malignancy (48;50;105,106).

As IL-13 is the common ligand for the cancer-associated IL-13Rα2 and the IL-4/IL-13R receptor expressed on normal cells, I will next discuss the structure of IL-13, and interaction of IL-4 and IL-13 with the IL-4/IL-13R as well as IL-13Rα2 receptor. I will also emphasize the IL-13 structure/function relationship studies that our laboratory undertook to demarcate the cancer-associated vs. normal organ-expressed IL-13 receptor.
Structure of IL-13 and its interaction with IL-4/IL-13R and IL-13Rα2

NMR solution structure of IL-13

NMR studies carried out by Eisenmesser et al. and Moy et al. (76;79;107) demonstrate that IL-13 assumes a four-helix bundle structure with an up-up-down-down topology with A (residues 10-21), B (residues 43-52), C (residues 61-69) and D (residues 92-110) alpha helices and two β-strands (residues 33-36 and 87-90, respectively). The conserved cysteine residues (Cys29-Cys57 and Cys45-Cys71) form the two disulphide bonds. The A-B and the C-D helices are connected by loops formed by the anti-parallel β-sheet and stabilized via a hydrogen bond between Val34 and Ile90. There are several residues that contribute to the hydrophobic helix bundle core of IL-13, like the conserved Leu17 on helix A as well as Phe95 at the N-terminal of helix D. Thr88 on helix D and Glu12 on helix A are some of the conserved amino acids between IL-13 and other cytokines like IL-2 and IL-4 (79).

Interaction with receptor interfaces

We have identified sites on IL-13 that are responsible for binding/signaling of the ligand to the IL-4/IL-13R versus the HGA-associated IL-13Rα2. We have shown using mutational analysis that changing residues in A, C and D helices of IL-13 altered the binding characteristics of the ligand to the physiological IL-4/IL-13R. Substitution of Glu13 and Glu16 to Lys in helix A impaired signaling though the shared receptor, whereas substitution of Arg at 109 in helix D to Asp abrogated the binding of IL-13 to both IL-13Rα2 and the IL-4/IL-13R (108). IL-13.E13K not only exhibited diminished
signaling through the shared receptor but also had increased binding to the HGA-associated IL-13Rα2 (109). Moreover, IL-13.R66D and -S69D in helix C resulted in reduced signaling through the shared receptor (108).

In alanine-scanning mutagenesis of helix D, K105A, K106A and R109A altered the binding to IL-13Rα2. It was hypothesized that helix A and C of IL-13 might be responsible for binding to the IL-4/IL-13R whereas helix D might be responsible for binding to IL-13Rα2 (110;111). We have therefore proposed that Glu13 is a “hot-spot” for signaling and binding to the IL-4/IL-13R, whereas Lys-105 is the hot spot for interaction with IL-13Rα2 [Figure 3]. The mutational and structural data obtained from the NMR and crystallography studies of the IL-13 mutants (as discussed below) are very much in line with our structure-function relationship studies.

Factors dictating different binding properties of IL-4 and IL-13

IL-4 is also comprised of four-helix bundle with an overall significant structural similarity to IL-13 (112;113). IL-13 has 25% sequence identity with IL-4 (114). Specific amino acids on the helices and overall electrostatic surface potential seem to contribute to the differences in the binding of IL-4 and IL-13 to IL-4R/IL-13R. Glu9 in helix A and Arg88 in helix C contribute significantly to the binding of IL-4 to the IL-4Rα subunit (79). These residues are conserved as Glu12 and Arg65 in IL-13. There also exists a difference in electrostatic surface potential between the IL-13 (Ala9, Glu15 and Glu16) and IL-4 (Thr6, Lys12, Thr13). This difference determines the inability of IL-13 to form intramolecular hydrogen bonds required for binding to IL-4Rα. However, Glu15, Lys104,
and Arg108 residues of IL-13 and Arg121 and Tyr124 of IL-4 create surfaces compatible for binding to the IL-13Rα1 subunit (79).

Analysis of the above structural data of IL-13 for binding to its receptors is in agreement with our mutational analyses that identified Arg108 [corresponding to our IL-13 mutant (R109D)] as well as Glu12 [corresponding to our IL-13 mutant (E13K)] as the most important residues in IL-13 for the binding to its receptors. This can be explained by the fact that Glu12 is one of the residues important for binding to IL-4Rα and Arg108 for binding to IL-13Rα1 (76;79).

**Binding of IL-13 to the IL-13Rα2**

Recently, crystal structure of IL-13 complexed with IL-13Rα2 was studied by Lupardus et al. and compared to the IL-13-IL-13Rα1 complex (115). The study demonstrated that IL-13 bound to IL-13Rα2 in a similar manner as it bound IL-13Rα1, i.e. in “two-site” binding mode, termed as site II and site III.

Site II is region of interaction of A and D helices of IL-13 with the IL-13Rα1 or the IL-13Rα2 receptor. For IL-13-IL-13Rα2 receptor complex, this site consists of four side chains of D-helix of the ligand (K104, K105, F107, and R108), whereas, the site III consists of anti-parallel β sheets of A-B and C-D helix loops of IL-13. As previously determined in our laboratory by alanine scanning mutagenesis, the residues K105 and R108 were found to be the important residues for specific IL-13-IL-13Rα2 interaction compared to IL-13-IL-13Rα1 interaction (111).
**IL-13Ra2 gene, promoter and transcripts**

The gene for IL-13Ra2 is located on chromosome X (Xq13.1-q28) and encodes for a 380-amino acid, 44,176-Da protein. The cDNA for IL-13Ra2 was first isolated from human renal carcinoma cell line, Caki-1 by Caput et al. in 1996 (95). IL-13Ra2 is also known as CT19, CD213A2, as well as IL-13R. IL-13Ra2 is conserved in dog, cow, mouse, rat, chicken, and zebrafish with up to 73, 72, 60, 57, 41 and 38 % amino acid sequence similarity, respectively [ENSEMBL gene tree for IL-13Ra2, genecard]. IL-13Ra2 is categorized as a cancer-testis-like antigen [CT] (98) as its expression is restricted to testis and is not readily expressed in other normal somatic tissues and cells. A large group of CT antigens are encoded by multigene families on chromosome X, especially on the telomeric end (Xq24 to Xq28) and have been classified as CT-X genes (116;117). The IL-13Ra2 undergoes N-glycosylation, which increases the size of the receptor to 60 kDa. It was demonstrated that N-glycosylation is required for optimal IL-13 binding to the receptor, however structural integrity of the receptor was not verified after receptor de-glycosylation studies (118).

Wu et al. (119) have conducted detailed studies on the promoter region of the IL-13Ra2 gene. The promoter region of the IL-13Ra2 contains three TATA boxes and one CCAAT site. Putative sites for several transcription factors such as NFAT1, AP-1 (c-Jun and c-Fos), AP-2, GABP, OCT1, GATA3, PRE and C-ETS1 were present in the promoter region of the gene. However, a 64-bp region identified by deletion analysis was found to contain cis-elements for AP-1, NFAT1 and AP-2. Importantly, AP-1 was identified as a major player along with c-Jun for the promoter activity. This activity of AP-1 was
postulated to be affected by the ERK pathway (119). Recently, the same group also reported the \textit{IL-13Ra2} to have at least two promoters and four transcripts. Promoter N was responsible for expression of IL-13Rα2 transcripts N1, N2 and N3 in normal cell lines whereas Promoter G was implicated in expression of GBM-specific transcript G in GBM cell lines (120). The three transcripts, N1, N2 and N3 consisted of different combinations of initial non-coding exons one to four of the gene, whereas transcript G does not include the non-coding exons three and four. Moreover, both NFAT and AP-1 (c-Fos and c-Jun) transcription factors were found to be essential for expression of transcript G in GBM cells (120). This is in agreement with our studies, wherein we show that AP-1 activators influence the expression levels of IL-13Rα2 (53).

Next, we will look at the role of IL-13Rα2 in airway inflammation and in parasitic infection as well as its expression in various cancers.

\textbf{IL-13Rα2 in airway inflammation and parasitic infection}

IL-13 has been implicated in airway inflammation and also in inflammation associated with parasitic infections. In both scenarios, IL-13Rα2 is up-regulated and plays a significant role in inflammation control, hence preventing further damage to the tissues. It was found that addition of IL-13 to normal human bronchial epithelial cells [NHBEC] increases goblet cell hyperplasia and mucus production. However, treatment with recombinant IL-13Rα2 and anti-IL-13Rα1 antibody reduced the number of periodic acid schiff-positive cells, goblet cells and MUC5AC production (121). Wood et al. have shown increased IL-13 responses in IL-13Rα2 knockout mice (122). These studies
indicate that IL-13Rα2 plays an important role in control and management of airway hyperresponsiveness by inhibiting mucus overproduction by IL-13 through the IL-13Rα1/STAT6 pathway.

Schistosomiasis is a chronic inflammatory disease of the liver and the gut, caused by eggs laid by parasite helminth (*Schistosoma mansoni*). IL-13 and other Th2 cytokines play key role in hepatic fibrosis and granuloma formation to contain the parasite (123;124), but if uncontrolled, the inflammatory response may become disadvantageous to the host. IL-13Rα2 acts as the decoy receptor, capturing excess IL-13, leading to less intense or no granuloma formation and inflammation. The IL-4Rα chain is increased in the early stages of the infection, indicating that it is required for granuloma formation, whereas the IL-13Rα2 is increased in the later stages of the infection to control the excess of IL-13. Also, administration of sIL-13Rα2-Fc was very efficient in blocking liver pathogenesis and an increased hepatic fibrosis was observed in IL-13Rα2 knockout mice (125).

**IL-13Rα2 in Cancer**

IL-13Rα2 is overexpressed in various malignancies notably in GBM, human pediatric brain tumors, brainstem glioma, renal cell carcinoma, squamous cell carcinoma of head and neck, ovarian cell carcinoma and also in pancreatic cancer (48;105;106;126-129). IL-13Rα2 was first found to be over-expressed in a set of 22 of 23 adolescent/adult GBMs that were studied by autoradiography. An excess of hIL-13, and not hIL-4 blocked the binding of IL-13-based cytotoxin to GBM specimens in situ, suggestive of IL-13Rα2 being the most abundant binding moeity in these tissues (48). Notably, study of the
expression of IL-13Rα2 in different brain tumors in situ using $^{125}$I-IL-13, indicated that only few of the low grade brain tumors had IL-13 binding sites, and the number of binding sites increased with the grade of the brain tumor (130). 17/18 GBMs demonstrated the staining for the IL-13Rα2, while 3/11 low grade gliomas, 5/5 high grade gliomas (grade III), 3/5 oligodendrogliomas, and 1/2 gliosarcomas exhibited binding sites for IL-13 (130). Also, analyses of gene expression for IL-13Rα1 and IL-13Rα2 was conducted in 40 human adult tissues, 20 discrete regions of the CNS, 7 fetal tissues, and cultured cell lines. This study demonstrated negligible expression of the IL-13Rα2 in normal CNS, and testis was the only peripheral organ where it was prominently expressed, indicating IL-13Rα2 to be a CT-like antigen. On the other hand, IL-13Rα1 was found to be expressed in low levels in normal CNS as well as in liver, heart, lungs and gastrointestinal tract (98).

Consistent with our previous findings, recent IL-13Rα2 expression analysis of tumor tissue microarrays containing WHO grade II-IV astrocytomas as well as immunohistochemical/western blot analyses of GBM cell lines and tumors, confirmed IL-13Rα2 overexpression in around 75 per cent of GBM patients (50). Moreover, IL-13Rα2 was also found, albeit at lower levels, in patients with anaplastic astrocytomas and some with low grade astrocytomas. Thus, the levels of the receptor protein increased with increasing grade of astrocytomas (50). This study confirmed the presence of high levels of IL-13Rα2 in HGA compared to normal brain, making it an attractive target for molecularly-directed therapy. Furthermore, other laboratories have also documented similar over-expression of IL-13Rα2 in gliomas (43;54;101;131-135).
Using immunohistochemistry and in situ hybridization, over-expression of IL-13Rα2 was also detected in 17 of 28 (61%) brainstem glioma tumor specimens (136) and in 83% of pediatric brain tumor specimens (128) compared to normal brain samples. Of the pediatric brain tumors analyzed, 100% (11 of 11) high grade astrocytoma, 79% (26 of 33) low-grade astrocytomas, 67% (4 of 6) medulloblastoma, and 67% (2 of 3) ependymoma samples were positive for IL-13Rα2. Furthermore, IL-13Rα2 was found to be overexpressed in 83% of the 68 ovarian tumor tissues (106). Obiri et al. reported overexpression of 74 kDa IL-13 binding receptor (IL-13Rα2) in renal cell carcinoma compared to the shared IL-4/IL-13R (126;137;138). Studies conducted in surgical SCCHN specimens and normal head and neck tissues, demonstrated that IL-13Rα2 is overexpressed in 33% of SCCHN (127). Moreover, expression analysis for the receptor was carried out in liver cancer tissues, 27 out of 33 (~81%) of the liver cancer tissues were highly positive for IL-13Rα2 (139). Overexpression of IL-13Rα2 was also shown in pancreatic cancer cell lines and IL-13.PE38QQR inhibited the growth of these cells (129). Thus, various solid tumors over-express IL-13Rα2, while its normal organ presence is low to undetectable.

**Regulation of IL-13Rα2 expression**

As mentioned previously, IL-13 signals through the IL-4/IL-13R complex through the JAK-STAT pathway and the STAT6 transcription factor (140;141). Signaling by the IL-13 through the receptor involves the IL-4Rα subunit, leading to phosphorylation of IL-4Rα, insulin receptor substrate-2, JAK1 and TYK2 (142). IL-13 does not normally signal
through the IL-13Rα2, which is likely a decoy receptor. The major function of IL-13Rα2 seems to be “sequestering” excess of IL-13 and hence protecting the tissues and organs from excessive inflammatory damage. IL-13Rα2 is likely a decoy receptor since it has a short cytoplasmic domain of less than 20 amino acids, with one laboratory data showing tentative signaling function (143). However, dileucine motif (two out of the three leucine amino acids) in the transmembrane region of the receptor is required for efficient internalization of the IL-13Rα2 receptor (144).

Studies carried out by David et al. in human keratinocyte cell line, HaCaT, described for the first time that the expression of IL-13Rα2 mRNA and protein is upregulated when stimulated by IL-13 or IL-4 (145). ERK 1/2 and p38 MAPK together with JAK2 and STAT6 participate in this regulation of IL-13Rα2 expression. Later on, the same group (146) identified three different IL-13Rα2 transcripts (identical to three transcripts found in normal tissues, N1-N3 as discussed previously), most probably regulated by two different promoters for IL-13Rα2 (P1 and P2) in HaCaT cells. Promoter P1 (-1529 to +88) was characterized in detail and was found to contain binding sites for STAT6, C/EBPβ, NF-κB and AP-1 transcription factors. Through further experimentation, it was found that the combinations of TNFα and IL-13, or IL-4, were required for the expression of IL-13Rα2 through P1 promoter. STAT6 transcription factor bound to the proximal STAT6 binding site of the IL-13Rα2 P1 promoter and was responsible for the receptor’s expression. Thus, it seems that IL-13 regulates the expression of IL-13Rα2 in human keratinocytes through IL-4/IL-13R, thereby creating a regulatory feedback loop [Figure 4]. However, on the other hand, it was found that IL-13Rα2 does not induce signal
transduction through the STAT6 pathway as identified by electrophoretic mobility shift assay [EMSA] using STAT6 DNA binding probe and anti-STAT6 antibody (147).

In addition, studies carried out in cultured as well as primary monocytes and respiratory epithelial cells, indicated that the treatment of these cells by IFN-γ resulted in a six-fold increase in surface IL-13Rα2 within hours. However, protein synthesis was not required for this upregulation, suggesting that intracellular stores of IL-13Rα2 are used for this phenomenon (148). Moreover, Zheng et al. hypothesized that the biologic effects of IL-13 are regulated by a number of inflammatory mediators which regulate IL-13 expression at sites of inflammation (149). They demonstrated that IL-4, IL-10 and IFN-γ, in addition to IL-13 upregulate IL-13Rα2 gene expression [Figure 5]. IL-13 leads to IL-13Rα2 protein expression in a blood-leukocyte-independent and IL-4Rα-dependent mechanisms whereas the IL-13Rα1 upregulation is blood leukocyte-dependent and partially IL-4Rα-dependent (149).

**Regulation and potential role of IL-13Rα2 in GBM**

In search of means to increase IL-13Rα2 levels on tumor cells for enhanced delivery of IL-13Rα2-targeted therapeutics, we have shown that in response to EGF there was a 5-fold increase in IL-13Rα2 levels from the background levels in glioma cells. The use of various signaling pathway inhibitors indicate that this increase in the IL-13Rα2 levels was most probably due to increased AP-1 transcription factor, induced by EGF signaling through the PI-3K and ERK pathways (150). Also, an up-regulation of IL-13Rα2 on
GBM cells led to an increased cytotoxic potency of the recombinant IL-13.E13K-PE38QQR cytotoxin (150).

Recent results obtained from co-immunoprecipitation studies of the IL-4Rα subunit and the IL-13Rα2 receptor chains as well as the IL-13Rα2 cytoplasmic chain mutant experiments demonstrated that one of the functions of IL-13Rα2 might be formation of a complex with the IL-4Rα subunit. This event may be thereby inhibiting the formation of the IL-4 (type 1 and type 2) signaling complex and hence eventually inhibiting the activation of STAT6–dependent genes in glioblastoma cells. This is postulated to happen when the short intracellular domain of IL-13Rα2 physically associates with the cytoplasmic region of the IL-4Rα and blocks the binding of JAK1 on IL-4Rα, thus preventing a downstream signal cascade (151). IL-4 has previously been shown to arrest the growth of normal astrocytes and low-grade gliomas (152;153). Moreover, brain infiltrating T lymphocytes are the producers of cytokines, including IL-4 and IL-13. It was hypothesized that IL-13Rα2 leads to inhibition of IL-4 and IL-13 signaling and hence facilitates growth of high-grade astrocytomas (151).

The same group postulated another role for IL-13Rα2, i.e. in IL-4 mediated activation of STAT3, which in turn activates the anti-apoptotic genes in glioblastoma (154). The study suggested that the presence of IL-13Rα2 was required for IL-4 to activate STAT3 and subsequent STAT3 stimulated gene expression, through siRNA mediated down-regulation of IL-13Rα2 and overexpression studies in GBM cell lines. They showed that STAT3 does not physically associate with short cytoplasmic domain of IL-13Rα2.
However, the study failed to reveal a mechanism responsible for IL-13Rα2 in activation of STAT3 and hence the hypothesis that expression of IL-13Rα2 is required for survival of GBM cells needs to be further validated (154). These are only few studies on the role and regulation of IL-13Rα2 in GBM and the results need to be further developed and/or corroborated by additional experimentation.

**Signaling through IL-13Rα2**

Fichtner-Feigl *et al.* (104) showed that IL-13Rα2 has potential signaling activity in the induction of TGFβ1-mediated fibrosis. TGFβ1-mediated fibrosis is observed in various inflammatory states including asthma, ulcerative colitis, pathogen mediated responses and interstitial lung diseases. The paper proposes a two-step signaling model for IL-13Rα2-mediated TGFβ1 induction. In the first step, TNFα and IL-13 co-operatively lead to STAT6 induction resulting in production of membrane bound IL-13Rα2. This IL-13Rα2, in the second step, in response to IL-13 leads to upregulation of AP-1. This AP-1 forms a complex with c-Jun, Jun D and c-Fos which in turn results in transcriptional production of TGFβ1. This is the only study that proposes a signaling function for IL-13Rα2, although the caveats of the study include experiments carried out in a single murine macrophage cell line, an artifical system (the macrophage cell line transfected with the human *IL-13Rα2* gene and a plasmid containing the human *TGFβ1* promoter linked to a luciferase reporter gene to show a correlation between them), with insufficient controls (like lack of a non-specific gene-luciferase reporter gene constructs and scrambled siRNAs) and presented completely opposite results to studies that indicate that IL-13Rα2 might be involved in prevention of fibrosis (155). Moreover, this study does
not propose a mechanism of how signaling would work with the short cytoplasmic domain of the IL-13Rα2 receptor as well as which pathways might possibly be involved in activation of the AP-1 factor. Recently, the same group has also shown similar IL-13-transduced IL-13Rα2-mediated activation of TGFβ1 promoter in pancreatic cancer cell lines (156). Again, some of the shortcomings of the study were same as listed above for the IL-13Rα2 signaling in the macrophage cell line and also no concrete in vivo experimentation was provided.

**Soluble IL-13Rα2**

The first evidence of soluble IL-13Rα2 was reported in 1997 by Zhang et al. who isolated a 45,000-50,000 Mr IL-13 binding protein from the serum and urine of mice. This IL-13 binding protein reportedly had a higher binding affinity for IL-13 and the sequence of this protein was found to be different than that of IL-13Rα1 (157). Later on, Tabata et al. (158) discovered two distinct IL-13Rα2 transcripts expressed in many mouse organs. Further analysis revealed that the two IL-13Rα2 expressed transcripts differed in sizes. The shorter transcript had deletion of exon 10 which led to removal of the transmembrane domain and hence secreted a soluble form of IL-13Rα2 in mice (158). Furthermore, the same group found that humans do not secrete IL-13Rα2 (159). Moreover, O’Toole et al. had previously been unable to detect soluble IL-13Rα2 in healthy human or asthmatic patients (160). In humans, it was found that the low amounts of soluble IL-13Rα2 detected in cell supernatants is due to proteolytic cleavage of the membrane form of the receptor by matrix metalloproteinases, especially MMP-8. The cleaved IL-13Rα2 receptor still retains binding to the IL-13 ligand (161). The above described studies
indicate that soluble IL-13Rα2 is not naturally found in humans, and the small amounts of soluble receptor detected in human are due to the cleavage of the cell surface receptor by MMP-8. However, it remains to be determined whether under malignancy, soluble/cleaved IL-13Rα2 is found in patients and hence can serve as a potential biomarker.

**IL-13Rα2 targeted therapeutics in cancer**

From our discussions on the expression of IL-13Rα2 in various cancers, it is evident that it is a specific marker for tumor cells compared to normal cells and hence can be exploited for targeting various therapeutics as well as imaging agents to these cancers. IL-13Rα2 has been utilized as a cancer-specific antigen to design and develop various targeted anti-tumor therapeutic approaches, including bacterial toxins, viruses, T-cells, vaccines, delivery vectors, and also nano-therapeutics. Following are some of the IL-13Rα2-directed experimental therapeutics that are currently in development as anti-cancer agents.

**IL-13Rα2 targeted cytotoxins**

One of the first therapeutics produced against the receptor were the IL-13 based bacterial cytotoxins, consisting of IL-13 as the targeting ligand fused to a derivative of *Pseudomonas* exotoxin A. Our laboratory designed the first generation of the cytotoxin, hIL-13-PE38QQR, with an intent to target the shared IL-4/IL-13R on adenocarcinoma cells (61). Later on, our laboratory also found that hIL-13-PE38QQR cytotoxin was highly cytotoxic on many of the HGA cell lines, and that excess of hIL-13, but not hIL-4,
inhibited the cytotoxic action of the hIL-13-PE38QQR (63). Although, hIL-13-PE38QQR was very potent against glioma cells, this first generation of IL-13-based cytotoxin was not designed to specifically target the IL-13Rα2 receptor. Hence, we designed second generation of IL-13-cytotoxins. These cytotoxins had a mutation in the IL-13 ligand; IL-13.E13K which showed decreased affinity for the shared receptor and hence were more specific for tumor associated IL-13Rα2. This IL-13.E13K-PE cytotoxin showed diminished killing for normal cells that express the shared IL-4/IL-13R compared to the wild type-based chimera (109).

To further enhance the binding specificity for IL-13-based PE cytotoxins, our laboratory developed third generation of cytotoxins which have multiple mutations in IL-13 targeting unit, such that it had increased binding to IL-13Rα2 and decreased binding to the shared receptor (162). These third generations of cytotoxins include IL-13.E13K.R66D-PE38QQR and PE1E, IL-13.E13K.S69D-PE38QQR and PE1E, IL-13.E13K.R66D.S69D-PE38QQR and PE1E as well as the above cytotoxins with the IL-13.E13Y mutant. All these cytotoxins showed enhanced specificity to IL-13Rα2-expressing GBM cells and decreased cytotoxicity to normal HUVEC cells (162). Subsequently, we have also shown that a recombinant cytotoxin consisting of multiply mutated IL-13 (IL-13.E13Y/R66D/S69D) and a derivative of PE (PE1E), IL-13.E13Y/R66D/S69D-PE1E, eliminated 80% of IL-13Rα2-positive GBM tumors in a murine glioma model (62).
After developing IL-13-based PE cytotoxins, we decided to explore other bacterial toxins and hence we have developed IL-13-based Diptheria toxin-containing cytotoxin [(IL-13.E13K-R66D-S69D/K105R-DT390)- DT-IL-13QM] (163). DT-IL-13QM cytotoxin showed an enhanced, specific cytotoxicity to GBM cells (163). Rustamzadeh et al. have reported the efficacy of DT-IL-13 (Diptheria toxin conjugated to wild type-IL-13) in an orthotopic GBM mice model. The DT-IL-13 cytotoxin when given by local intracranial injections demonstrated significant decrease in tumor growth as well as statistically significant survival (164).

Hypoxia negatively affects the response of many tumors, including GBM, to therapy. We next wanted to know whether the efficacy of IL-13-cytotoxins was affected by the hypoxic microenvironment of GBM tumors. To this end, we screened for the levels of IL-13Rα2, furin endoprotease (a protease that facilitates endosomal translocation of the IL-13-cytotoxins after cell entry by cleaving the PE protein) and the efficacy of the IL-13-cytotoxins themselves (165). We have shown that anoxia and hypoxia decreases the levels of the IL-13Rα2 on GBM cells. However, interestingly, reoxygenation of these cells led to an increase in IL-13Rα2 protein than observed at the pre-hypoxia levels. Furthermore, a similar trend was observed for furin protein and for the efficacy of the DT-IL-13QM. Anoxia/hypoxia decreases the effectiveness of the cytotoxin whereas reoxygenation of the cells increases the potency of the cytotoxin (165).
**IL-13Rα2 targeted viruses**

Another targeting strategy was developed by Zhou *et al.* who engineered a herpes simplex virus 1 (HSV-1) for targeted entry into tumor cells. This virus (R5111) was constructed such that it had deleted viral envelope proteins (gB) that bind to heparin sulfate proteoglycans on host cell surface. IL-13 was inserted into the gC viral envelope protein and an additional IL-13 was added between 24\(^{th}\) and 25\(^{th}\) amino acid of gD, the viral receptor binding unit of the host. These engineered IL-13-based viruses specifically recognized cells expressing IL-13Rα2 and led to rapid viral multiplication in these cells (166). The subsequent second and third phase engineering of the above virus has been carried out to further modify the interaction of gD to the viral receptors on the host cell surface, thereby developing viruses specifically targeted to IL-13Rα2 (167).

**IL-13Rα2 targeted T cells**

Cytolytic T-cells expressing a chimeric-IL-13.E13Y receptor, or zetakine, have been developed that specifically target GBM tumors expressing IL-13Rα2. The IL-13-zetakine is a chimeric immunoreceptor, which was engineered to be expressed on T cells and consisted of IL-13.E13Y, membrane-tethered by extracellular human hinge γ4Fc, huCD4TM, and the intracellular huCD3-ζ cytoplasmic tail (168). Zetakine-expressing CTLs lead to complete eradication of the intracranial GBM tumors in mice after 72 to 96 hr of local delivery into the tumor bed. The CTLs expressing IL-13.E13Y were found to be very specific for glioma cells expressing IL-13Rα2 compared to cell lines expressing the shared IL-4/IL-13R, as earlier established in our laboratory. The advantages of this chimeric-IL-13.E13Y receptor are that it overcomes the requirement of tumor cells to
express HLA Class I molecules as well as the requirement of pre-existence of immune responses to these tumors (168).

**IL-13Ra2 targeted vaccines**

The research by Okada’s group at University of Pittsburgh has led to development of anti-glioma dendritic cell based vaccines based on IL-13Ra2 wild-type and mutated peptide analogues as HLA-A2-restricted CTL epitopes. The peptides utilized were IL-13Ra2\textsubscript{345-353} wild-type (WLPFGFILI) (169) and IL-13Ra2\textsubscript{345-353} mutated peptides [substitutions of the COOH-terminal isoleucine for valine and NH2-terminal tryptophan for alanine (1A9V), glutamic acid (1E9V) and unsubstituted (9V)] (170). Both of the wild type and mutated IL-13Ra2 peptides induced CTLs that displayed lytic activity against IL-13Ra2-expressing glioma cells. However, the mutated IL-13Ra2 peptide CTL epitopes were able to induce higher populations of specific CTLs and protective immunity in IL-13Ra2-expressing syngeneic, intracranial glioma mice model compared to the wild type IL-13Ra2 CTL epitope (170).

Protein and DNA based active immunotherapy attempts utilizing the extracellular protein domains of IL-13Ra2 and gene sequence of IL-13Ra2, were conducted in syngeneic model of IL-13Ra2-positive glioma model (171). Pre-treatment of mice with IL-13Ra2 protein as well as DNA prevented GBM tumor occurrence compared to untreated controls. This IL-13Ra2-mediated immunotherapy might be useful in prevention of tumor recurrence by eradicating microscopic glioma (184). In support of this approach, Nakashima et al. have used IL-13Ra2 as DNA vaccine with the extracellular IL-13Ra2
(ECDα2) protein as prime boost for both prophylactic and therapeutic measures for murine tumor regression in syngeneic sarcoma, breast carcinoma and melanoma models of tumors, expressing IL-13Rα2 (172).

**IL-13Rα2 targeted gene therapy**

Reed-Steinberg cells of Hodgkin’s lymphoma [HL] secrete and proliferate in presence of IL-13 cytokines. Trieu et al. developed recombinant adenovirus mediated gene therapy that secreted soluble IL-13Rα2 and provided it to mice transplanted with xenograft HL tumors. The secreted IL-13Rα2 sequestered IL-13, thereby exhibiting delayed tumor onset and growth (173). An adenovirus gene therapy vehicle (adenovirus serotype 5-Ad5) with the IL-13 targeting unit (LU-13) has also been designed against IL-13Rα2 (218). This is particularly advantageous since glioma cells have low levels of the primary Ad5 and cox-sackievirus [CAR] receptors. This LU-13 was successfully used to transfected luciferase reporter gene in U-87MG subcutaneous glioma tumors in mice. Intratumoral injection of the LU-13 vector in the U87MG tumors led to an increase of at least 300-fold in luciferase expression compared to the wild-type adenovirus control (174). This indicates that the LU-13 engineered adenoviruses are efficient and specific gene therapy delivery vehicles for glioma cells. Recently, Candolfi et al. (175) developed an adenoviral vector (Ad.mhIL-4.mhIL-13-PE), expressing mutated human IL-13.E13K fused PE (mhIL-13-PE) to target IL-13Rα2. The vector was also engineered to express mutated human IL-4, for binding to the physiological IL-13 receptor, IL-4/IL-13R on normal brain cells and hence prevent neurotoxicity. This cytotoxin gene therapy for GBM demonstrated tumor regression and long term survival in >70% of animals with
intracranial GBM human xenografts and syngeneic tumors. Furthermore, it again demonstrated the superiority of the mutant IL-13-based over the wild type IL-13-based cytotoxin (175).

IL-13Rα2 targeted nano-therapy

IL-13Rα2 targeted photothermal ablation agents have been recently developed against HGA. Bernardi et al. have attached anti-IL-13Rα2 antibody to gold-silica nanoshells, which are referred to as immunonanoshells (176). These immunonanoshells when targeted to receptor-positive cells absorb near-infrared light upon exposure to laser light, get activated and lead to photothermal ablation of glioma cells. One of the main advantages of this treatment strategy is the localized activation of the targeted therapeutic, but its efficiency needs to be seen in GBM animal models (176). Moreover, Madhankumar et al. recently developed IL-13Rα2-targeted nano-vesicles, i.e. IL-13 conjugated liposomes, to deliver doxorubicin to glioma cells. This method of delivery led to increased retention and cytotoxicity in the glioma cells and also showed reduced tumor volume in animal studies (177).

Several of the IL-13Rα2-targeted therapeutics mentioned above are in pipeline for further pre-clinical and clinical development. Some of them are already in clinical trials, such as the IL-13Rα2-based peptide or dendritic-cell-based vaccines by the Okado group, which just finished phase I/II trials (178). The efficacy of these vaccines is currently being evaluated against GBM, anaplastic astrocytoma [AA], anaplastic oligodendroglioma [AO], and anaplastic oligoastrocytoma [AOA] tumors. The phase I/II data indicated that
these vaccines were well tolerated and also showed preliminary clinical applicability (178). The native IL-13-based *Pseudomonas* cytotoxins went up to Phase III clinical trials as Cintredekin Besudotox [CB] for recurrent glioblastoma. Cintredekin Besudotox was delivered using convection enhanced delivery [CED] to patients with first recurrent GBM (PRECISE trials). Convection enhanced delivery is a method of loco-regional drug delivery to brain tumors using positive-pressure gradient. Drugs are delivered utilizing catheters placed in either the brain tumor resection cavity or in the tumor mass. In the PRECISE trials, CB showed significant increase in overall survival of patients with recurrent GBM when treated by neurosurgeons experienced in performing CED (179). Inadequate CB delivery to the brain tumor tissue might have been one of the reasons for less than satisfactory results obtained for PRECISE trials than those seen in Phase I/II (179). However, to achieve better drug infusion into the tumor in future, better catheters have been proposed to overcome some of the shortcomings of drug delivery, mainly drug infusate reflux as well as incomplete drug distribution (180).

Almost all of these various IL-13Rα2-directed therapeutics show therapeutic potential, indicating that IL-13Rα2 is one of the attractive targets for molecular, immuno, or nano-therapy, not just in GBM, but also in other IL-13Rα2-positive cancers.

Our goal was to develop novel platforms for imaging and treatment of GBM using IL-13Rα2. Therefore, the specific aims of this dissertation are:

1. **To identify, develop and characterize IL-13Rα2 specific peptides.** For the first platform, we would like to identify, characterize and develop specific small
peptide ligands or peptidomimetics against IL-13Rα2. Small peptides are typically less immunogenic and combine high affinity and selectivity with more desirable pharmacokinetic properties. These IL-13Rα2 peptidomimetics, because of their virtues such as small size, specificity, high tumor/blood ratio could be ideal delivery vectors. One of the major considerations for developing these small peptides is that they may be able cross the blood brain barrier and be delivered through the intravenous route for treatment or imaging of HGA. These peptides can also be conjugated to various cytotoxic peptides, radioisotopes, nanoparticles, chemotherapeutics and also photosensitizers for treatment purposes. The other major application would be that these peptides can be conjugated to various fluorophores, or radioisotopes for imaging purposes.

2. To develop and characterize IL-13Rα2 specific recombinant protein vectors for targeted intra-cellular compartment delivery in GBM cells. For the second platform, we would also like to develop IL-13 based recombinant protein delivery vectors to transport therapeutics into specific intracellular compartments. These ‘double specificity’ vectors will lead to targeting at two levels. First level of specificity will be the recognition of tumor-associated antigen, IL-13Rα2, on GBM tumor cell. After being internalized into the tumor cell through the receptor, it will then exert the next level of specificity by travelling into a specific intracellular compartment. Organelle specific delivery of some of the therapeutics, such as to the nucleus, would lead to an increased efficiency of glioma cell killing, as several isotopes and therapeutics have a small range of
effectiveness or influence inside the cell. Specifically transporting such therapeutics into the nucleus, i.e. to the core of DNA synthesis machinery inside the cell would lead to a rapid and enhanced killing with lower dosage and decreased toxicity to normal cells.
Figure 1. Hallmarks of GBM. 

**A.** necrosis, **B.** microvascular proliferation and hypervascularization, **C.** mitotic figure (*i.*, inset), **D.** pseudopalisading necrosis.
**Figure 2. IL-4 and IL-13 receptors.** Type 1 IL-4 receptor is a heterodimeric complex formed between the γc chain and the IL-4Rα subunit. Type 2 IL-4 receptor is a heterodimer of IL-4Rα and IL-13Rα1 subunits. Type 2 IL-4 receptor binds both IL-4 and IL-13. When it binds IL-13, it is known as the IL-4/IL-13R which is found in various organs. The second IL-13 receptor is a monomeric receptor, IL-13Rα2, and it is overexpressed mainly in pathologic conditions. *(Adapted from Wills-Karp, M.; Finkelman, F.D., Sci. Signal. Vol.1. 2008.)*
Figure 3. Predicted structure of IL-13. The IL-13 ligand consists of four major alpha helices – A, B, C and D. Regions on IL-13 protein that are responsible for binding to its receptors have been categorized as Site-I and Site-II. Site-I consists of alpha helices A and C of IL-13 and is mainly responsible for binding to the IL-4Rα subunit of the physiological IL-4/IL-13R. Mutation of residues Glu13, Arg66, Ser69 disrupts the binding of the ligand to the shared receptor. Site-II comprises of helix D which is responsible for binding to the HGA-associated IL-13Rα2. One of the hotspots for binding to the HGA-associated receptor is residue Lys105 on the IL-13 ligand. Pink– Glu13, Green – Arg66, Brown – Ser69 and Orange – Lys105. (Adapted from Madhankumar AB, Mintz A and Debinski W, Neoplasia, 6, 2004)
Figure 4. Hypothetical IL-13 regulatory feedback loop. IL-13 binds to the IL-13Rα1 subunit of the shared receptor and leads to activation and heterodimerization of the STAT6 transcription factor. STAT6 binds to the promoter of the $IL-13Rα2$ gene, leading to induction and up-regulation of the IL-13Rα2 protein on the cell surface. IL-13Rα2 is considered as a decoy receptor for IL-13 and sequesters away excess of IL-13, thereby maintaining the balance in IL-13 signaling during normal physiology and during inflammation control. Thus, IL-13 may regulate itself by up-regulation of the IL-13Rα2 protein (146).
**Figure 5. Cytokines regulate IL-13Rα1 and IL-13Rα2 expression.** IL-13, IL-4, IL-10 and IFN-γ have been shown to regulate the expression of both IL-13 receptors, including IL-13Rα1 subunit of the shared receptor and IL-13Rα2. Overexpression of IL-13 leads to induction of both IL-13Rα1 and IL-13Rα2 mRNA; IL-10 overexpression leads to induction of IL-13Rα2 mRNA with no change in IL-13Rα1 mRNA and increased amounts of IFN-γ leads to induction and up-regulation of IL-13Rα2 mRNA, whereas it down-regulates the expression of IL-13Rα1 mRNA (149).
References


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CHAPTER II

AN INTERLEUKIN 13 RECEPTOR ALPHA 2-SPECIFIC PEPTIDE HOMES TO HUMAN GLIOBLASTOMA MULTIFORME XENOGRAFTS

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The following manuscript was prepared for submission to Neuro-Oncology and is reprinted with permission. Stylistic variations are due to the requirements of the journal. H. Pandya designed/performed the experiments and analyzed the data. D.M. Gibo and S. Garg assisted in some experiments. S. Kridel acted in an advisory capacity and helped with the manuscript. W. Debinski supervised the project and also acted in an advisory and editorial capacity. H. Pandya and W. Debinski prepared the manuscript.
CHAPTER II

AN INTERLEUKIN 13 RECEPTOR ALPHA 2-SPECIFIC PEPTIDE HOMES TO HUMAN GLIOBLASTOMA MULTIFORME XENOGRAFTS

Abstract

Interleukin 13 receptor alpha 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 using a cyclic disulphide-constrained heptapeptide phage display library and two in-vitro biopanning schemes with GBM cells which do (G26-H2 and SnB19-pcDNA cells) or do not (G26-V2 and SnB19-asIL-13Rα2 cells) over-express IL-13Rα2. We have identified three peptide phages that bind to the IL-13Rα2 in cellular and protein assays. One of the three 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 the disulphide-constrained form and was efficiently internalized by IL-13Rα2-expressing GBM cells. The native ligand, IL-13, did not compete for the Pep-1 binding to the receptor, and vice versa, in any of the assays, indicating that the peptide might be binding to a site on the receptor different from the native ligand. Furthermore, we demonstrated by noninvasive NIRF imaging in nude mice that Pep-1 binds and homes to both
subcutaneous and orthotopic human GBM xenografts-expressing IL-13Rα2 when injected by an intravenous route. Thus, we have identified a linear heptapeptide specific for the IL-13Rα2, which is capable of crossing the blood-brain tumor barrier and homing to tumors. Pep-1 can be further developed for various applications in cancer/inflammatory diseases.

**Keywords:** Peptide phage-display library, Interleukin-13 receptor alpha 2, GBM, blood-brain tumor barrier
Introduction

Glioblastoma multiforme (GBM) is one of the most malignant primary brain tumors of astroglial origins.\(^1\) The median survival rate is only 14 months with 5 year survival rates of less than 10%.\(^2\) Our focus is to develop novel management strategies for GBM. We have previously identified attractive molecular markers/targets in GBM tumors that are over-expressed in a vast majority of GBM patients, but not found in normal brain.\(^3\)\(^-\)\(^5\) One such target is interleukin 13 receptor alpha 2 (IL-13R\(\alpha\)2).\(^3\) This receptor is a monomeric plasma membrane receptor that binds IL-13, but not homologous IL-4, unlike the physiological, normal tissue receptor, IL-13R\(\alpha\)1/IL-4R\(\alpha\) that is shared between the two cytokines and is composed of two subunits.\(^6\) The latter receptor is moderately elevated in malignancy while IL-13R\(\alpha\)2 is over-expressed not only in GBM, but also in head and neck,\(^7\) pancreatic,\(^8\) ovarian,\(^9\) and prostate\(^10\) cancers. Therefore, we aimed at identifying ligands for the IL-13R\(\alpha\)2 receptor, which can eventually be further developed for various diagnostic, imaging and therapeutic applications in GBM.

IL-13R\(\alpha\)2 is a 382 amino acid, type I internalized plasma membrane receptor.\(^11\) It is considered more of a decoy receptor\(^12\), because it has a very short cytoplasmic tail (17 amino acids in humans). The IL-13R\(\alpha\)2 receptor is generally not suspected to have signaling activity with some exceptions.\(^13\) However, the receptor is internalized in GBM cells, because it mediates recombinant cytotoxins’ cell killing and internalization is absolutely required for cell death to happen.\(^3\) Our laboratory has also designed various IL-13 ligand mutants which specifically bind to the IL-13R\(\alpha\)2 receptor and that do not
bind to the physiological receptor.\textsuperscript{14-16} For example, IL-13.E13K mutant has glutamic acid at position 13 of the \( \alpha \)-helix A substituted by lysine.\textsuperscript{14,15} Also, we have previously developed recombinant cytotoxins targeted to the IL-13R\( \alpha \)2, 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 \textit{Pseudomonas} Exotoxin A (PE) or Diphtheria toxin (DT).\textsuperscript{17,18} The first generation of cytotoxin demonstrated a significant increase in overall survival in patients with recurrent GBM in a Phase III clinical trial when patients were treated by physicians experienced with the method of loco-regional delivery.\textsuperscript{19}

In search for more option to attack tumors, peptides offer several potential advantages when used as targeting agents. They are small and relatively easy to synthesize\textsuperscript{20}, usually less or non-immunogenic and have attractive pharmacokinetic properties. Their plasma half-life is short, leading to rapid blood clearance and a higher tumor/blood vs. normal organs/blood uptake. The smaller size of the peptides when compared to proteins/antibodies is believed to afford better penetration and diffusion into a tumor. Peptides also serve as delivery vehicles to transport chemotherapeutics\textsuperscript{21}, labels and radioisotopes to cancer cells\textsuperscript{22,23} or they have been used for diagnostic and imaging purposes.\textsuperscript{23}

In the current work, we have identified peptide ligands for the IL-13R\( \alpha \)2 receptor for specific targeting of and delivery to GBM tumors. To identify peptides of such characteristics, we employed a disulphide-constrained heptapeptide phage display library
with a diversity of $1.2 \times 10^9$. We were successful in obtaining peptides that bind IL-13Rα2 and characterized them for their potential utility in managing GBM.
Materials 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 murine glioma cell lines (gift of Dr. Marzena Wiranowska). G26-H2 cell line is G-26 cells transfected with human IL-13Rα2 gene and G26-V2 are vector transfected cells. SnB19-asIL-13Rα2 are SnB19 GBM cells transfected with anti-sense IL-13Rα2 gene with subsequently diminished expression of the receptor, in contrast to the empty vector-transfected SnB19-pcDNA cells (Supplementary Fig. S1A) [Appendix I]. The transfected G-26 and SnB19 cell lines were grown in Eagle’s MEM and RPMI 1640 (Hyclone, Logan, UT) substituted with 200 µg/ml of Geneticin. G48a cells were grown and maintained in RPMI 1640 (Lonza, Walkersville, MD) supplemented with glucose, adjusted to 4 gm/liter 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-13Rα2 that is checked by Western blotting, at least every two months.

Tissues and Reagents

The Ph.D-C7C phage display library containing 1.2 x 10^9 clones obtained from New England Biolabs (Ipswich, MA). The isolated peptides binding to IL-13Rα2 were synthesized by Anaspec (San Jose, CA). IL-13Rα2/Fc, IL-13Rα1/Fc and IgG/Fc recombinant proteins were obtained from R&D Systems (Minneapolis, MN). 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 phage library were panned against GBM cell lines. G26-H2 and SnB19-pcDNA were used as IL-13Rα2-positive cell lines while G26-V2 and SnB19-*as*IL-13Rα2 were used as the receptor-negative cell lines. Four biopanning rounds were carried out. Each round consisted of three negative selections followed by one positive selection. During the negative selection, 1.5 x 10^{11} plaque forming units (pfu) were added to a 35-mm polystyrene tissue culture dish (Corning Inc., Corning, NY) 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 non-specific phage binding. The cells were harvested from flasks using 0.5 M EDTA/PBS. The dish was incubated for 1 hr at room temperature (RT) with gentle shaking. The cell/phage mixture was then transferred to microfuge tube, centrifuged to pellet cells and the supernatant with unbound phages was collected. The supernatant was again mixed with receptor-negative cells and the above procedure was repeated two more times. After three negative selections, the collected supernatants were mixed with 5x10^6 receptor-positive cells in 35-mm polystyrene tissue culture dish. The dishes were incubated for 4 hrs at 4 °C with gentle shaking. The mixture was then transferred to a microfuge tube, centrifuged and supernatant was discarded. The pellet (cells with the bound phages) was washed 5x 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-13Rα2 binding phages from the cell surfaces and chilled on ice for 10 min. The suspension was then centrifuged and the supernatant neutralized with 10 μl of 2 M Tris HCl, pH 8.

**Phage amplification and DNA sequencing**

Phages eluted from receptor positive cells were amplified in ER2738 *E.coli* cells using the protocol outlined in the NEB Ph.D-C7C manual. The ER2738 *E.coli* cells have F-factor which contains a mini-transposon which confers tetracycline resistance and hence 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 plaque forming units (pfu). For phage DNA isolation, individual phage plaques were picked up, phage amplified and DNA isolated as directed in the NEB manual. The isolated phage DNAs were sequenced at the Wake Forest University Biomolecular Resource Laboratory Services.

**Enzyme-linked Immunoabsorbent assay**

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 ELISA plate (CoStar, Bethesda, MD) and incubated for 2 hr at RT in a humidified container. The proteins were then discarded and the plate blocked with 2% milk for another 2 hr. Different phage dilutions were prepared with equal volumes of 2% milk and incubated at RT for 15 min. The pre-incubated phage solutions were then coated onto the wells, after discarding the blocking solution. The plate was
further incubated at RT for 2 hr. M13KE; a M13 backbone phage without any surface peptides was used as a control phage (New England Biolabs). BSA and Insulin (Sigma-Aldrich, St. Louis, MO) were used as non-specific control proteins. For competitive ELISA, the plate was blocked with 1000x of the ligand for 1 hr. For the Dithiothreitol (DTT) reducing ELISA experiment, the peptide phages were incubated with 5 mM DTT (Acros Organics, Geel, Belgium) for 20 min and then added onto the wells. After incubation, the solutions were discarded from the wells and the plate washed 6x with PBS/0.1% Tween-20 solution. 200 μl of 1:5000 dilution of anti-M13 horseradish peroxidase conjugated antibody (GE Healthcare, Piscataway, NJ) was then added onto each well of the plate and the plate was further incubated for 1 hr at RT. The plate was washed again six times with PBS/tween-20 solution. 2,2’-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Sigma, St. Louis, MO) and H₂O₂ substrate solution was added and the plate was incubated for 1 hr at RT. The color was measured at the absorbance of 405 nm with Spectra max 340 PC (Molecular Devices, Sunnyvale, CA).

Cell-binding phage titer assay

Equal numbers of G26-H2 and G26-V2 cells as well as SNB19-α3IL-13Rα2 and SnB19-pcDNA cells were incubated with 10⁶ to 10¹² pfu of each phage at 4°C for 4 hr. After incubation, the cells were washed 6 times with cold PBS+1% BSA+0.1% Tween 20 to remove any unbound phages, 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
neutralized with 10 µl of 2M Tris HCl, pH 8. The eluted phage titers were performed as described above.

**Cell viability assay**

$1 \times 10^3$ U-251 MG cells were plated in a 96 well tissue culture plate and incubated at 37 ºC for 24 hr. The cells were plated in quadruplicates for each concentration to be tested. After 24 hr, a fixed concentration of the peptides (1 µg/ml) and IL-13.E13K (2.5 µg/ml) in PBS + 0.1 % BSA were 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 hr. The peptide concentrations used were 300x and the IL-13.E13K cytokine was 100x molar excess of the cytotoxin. Cells treated with cyclohexamide were used as positive control. After 48 hr, cell viability was measured using the MTS/PMS assay (Promega, Madison, WI) \[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] / [phenazine methosulfate] dye as per the manufacturer’s instructions. The MTS/PMS dye was mixed with the cells and allowed to incubate from 2 to 4 hr and the absorbance from the assay was measured at 490 nm using the plate reader Spectra max 340 PC (Molecular Devices, Sunnyvale, CA). 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 ºC 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 hr at RT. Subsequently, the wells were washed 4x with PBS and treated with streptavidin-Alexa fluor 488 (Invitrogen, Carlsbad, CA) for 1 hr. DAPI (4', 6-diamidino-2-phenylindole) was added to stain the nuclei and the cover slips mounted with gel mount (Biomed, Foster City, CA). The coverslips were analyzed under the fluorescent microscope (Olympus, IX70, Center Valley, PA) 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 ºC. Before the experiment, the slides were thawed at RT and the tissue slices fixed with acetone at -20 ºC for 10 min. After fixation, the slides were washed with PBS. Subsequently, the tissue slices were treated with Endogenous Biotin Blocking kit (Invitrogen, Carlsbad, CA) to block endogenous biotin from giving background signal. After this treatment, the tissue slices were blocked with 10% BSA for 1 hr. After blocking with BSA, the slides were treated with 10 µg/ml of peptide-biotin in 1% BSA for 1 hr. Subsequently, the slides were washed with PBS (4x). Next, secondary streptavidin-488 was added to the tissue slides, incubated for 1 hr, washed and the tissue slides mounted with coverslips using gel mount. The slides were then observed under the fluorescent microscope (Olympus, IX70, Center Valley, PA) and processed using the Image-Pro plus 5.1 software.
Determination of dissociation constant (Kd) for the peptide

10 µg/ml of the IL-13Rα2/Fc protein was coated onto ELISA plate for 2 hr at RT. Subsequently, the wells were blocked with 5% BSA for 2 hr. 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 RT. Next, the wells were washed 6x 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 incubated for an additional 60 min at RT. After incubation, the wells were washed 6x with PBS/0.1% Tween 20. Streptavidin conjugated to horseradish peroxidase (1:8,000) was then added to the wells and allowed to bind to the biotin-conjugated peptide for 1 hr. Subsequently, the wells were washed 6x with PBS/0.1% Tween 20. The binding was detected by adding 2,2’-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diaminonitromethane salt (Sigma, St. Louis, MO) and H2O2 substrate solution and the plate was incubated at RT for 1 hr. The color was measured at the absorbance of 405 nm with Spectra max 340 PC (Molecular Devices, Sunnyvale, CA).

Peptide Internalization Assay

U-251 MG GBM cells (2.5 X 10⁴) per well were plated in a 24 well plate and allowed to attach for 24 hr. After 24 hr, 5 µg/ml of peptide was added to live cells and allowed to incubate for 4 hr 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 4x with PBS. The cells were next permeabilized using 0.1% Triton-X/0.2% BSA for 10 min at RT. The wells were subsequently washed 4x with PBS and streptavidin-alexa fluor 488 was added along with Topro-3 nuclear stain (Invitrogen, Carlsbad, CA). The cells were
allowed to incubate for 1 hr at RT. The wells were next washed and mounted with gel mount (Biomed, Foster City, CA) and observed with LSM 510 Zeiss Confocal Microscope (Cellular Imaging Core, Comprehensive Cancer Center, Wake Forest University) and the images processed using Zeiss LSM Image Browser (version 4.2).

**Peptide-Biotin-Streptavidin-Cy5.5 conjugate preparation**

To carry out small animal non-invasive 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.

**GBM xenograft tumors**

All animal experiments were carried out in accordance with the guidelines of Institutional Animal Care and Use Committee (IACUC) 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 \(5 \times 10^6\) 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: \((\text{length} \times \text{width}^2)/2\). 0.9 nmole of either peptide-biotin-streptavidin-Cy5.5 conjugate or control scrambled peptide-biotin-streptavidin-Cy5.5 were injected by \(i.v\) through the jugular vein, when the tumors reached a volume of \(~80\ \text{mm}^3\). Each treatment group consisted of 3 mice receiving Pep-1/biotin-streptavidin-Cy5.5 conjugate (linear and disulphide), or control scrambled linear and disulphide peptides, or just streptavidin-Cy5.5 or saline.
For orthotopic GBM tumor induction, 6-week athymic nude mice were anesthetized with the ketamine/xylazine mixture (114/17 mg/kg) respectively. Using a stereotactic instrument, 5 X 10^5 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 average ROI of the tumor volume was 2.0514 x 10^7 photons/sec/cm^2/sec, the mice were injected with 0.9 nmole of the 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), or control scrambled (linear and disulphide) peptides, or just streptavidin-Cy5.5 or saline.

**IVIS 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 time intervals 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 using Xenogen IVIS 100 imaging system (Cellular Imaging Core, Comprehensive Cancer Center, Wake Forest University) and the images were analyzed/processed using Living Image Version 4.0 software (Caliper Life-sciences).

**Statistical Analysis**

Data are represented as means +/- SEM. For animal studies, the significant differences between the treatment groups were analyzed by Student’s t test. P value < 0.05 was
considered significant.
Results

Isolation of phages binding to the IL-13Rα2

In order to obtain peptides binding to IL-13Rα2, we adopted biopanning schemes using a Ph.D-C7C heptapeptide phage display library 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) [Appendix 1]. We alternated negative and positive selections on these cells. The results are summarized in Table 1. 28 out of 30 clones had the same sequence, termed Pep-1 (G26 cells). 5 (Pep-2), and 2 (Pep-3) phage clones had the same sequence out of 30 that were biopanned on SnB19 cells. Thus, we have successfully identified three different peptide phage clones from the Ph.D-C7C phage 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 phage display library bind to the recombinant receptor, we carried out ELISA with the IL-13Rα2/Fc chimera protein and IL-13 receptor alpha 1 (IL-13Rα1/Fc) subunit of the physiological receptor. We also used the IgG/Fc and Insulin as controls (Fig. 1A). The IgG/Fc has the identical Fc fragment, which is found in the IL-13Rα2/Fc chimera protein. An M13KE native phage not displaying any foreign peptide served as phage binding control. The Pep-1 phage bound specifically to the IL-13Rα2/Fc chimera protein and not to the IL-13Rα1/Fc chimera protein (Fig. 1A). The other two peptide phages bound IL-13Rα1/Fc either
partially or similarly to IL-13Rα2. The control proteins did not bind the phages (Fig. 1A).

Due to the binding activity to IL-13Rα1 of Pep-2 and Pep-3 phages, we focused on Pep-1 clone in our investigations.

The peptide phage clones bind to the cell surface-expressed IL-13Rα2

Next, to establish that the three isolated peptide phage clones would bind specifically to the IL-13Rα2 expressed in its natural setting on cells, we carried out a cell-binding phage 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) as well as IL-13Rα2 receptor-negative cells (G26-V2) with equal pfu of the phages. We found that 200 times more of Pep-1 phage bound to the G26-H2 cells as compared to G26-V2 cells (Fig. 1B), whereas 100 times more of the Pep-1 phage bound to SnB19-pcDNA cells as compared to the SnB19-asIL-13Rα2 cells (Fig. 1B).

IL-13 does not inhibit the binding of Pep-1 phage to IL-13Rα2

To determine whether or not the peptide phage clones interfere with the IL-13 binding site on IL-13Rα2, we carried out 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 60 min. The excess of the ligand did not block binding of Pep-1, Pep-2 or Pep-3 phage (Fig. 1C). The incubation with polyclonal anti-IL-13Rα2 antibody (Ab) made to the extracellular domain of the receptor did however block the binding of the peptide phage, indicating the antibody might be protecting the site of the phage binding to the
receptor (Fig. 1C).

**Pep-1 binds recombinant IL-13Rα2**

We found unexpectedly that the Pep-1 phage bound similarly to the receptor protein with and without DTT (Supplementary Fig. S1B) [Appendix I]. 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 hence they can be detected using streptavidin probes for fluorescent as well as 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 around 1 µg/ml whereas the DSC and the scrambled control peptides did not bind to the receptor at all (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 serine amino acids did not exhibit any binding to IL-13Rα2 protein (Supplementary Fig. S1C) [Appendix II].

**Pep-1 binds to the receptor at the site/s other than the native ligand binding site**

We demonstrated that the IL-13.E13K ligand did not block the binding of the phage to the receptor protein. We carried out a similar experiment with the synthetic peptides. IL-13.E13K ligand did not block the binding of Pep-1-L to the IL-13Rα2/Fc protein (Fig. 2B). The Pep-1-DSC and the scrambled peptide again did not bind to the receptor at all (Fig. 2B). Similar to what we had observed in the experiments with the phage clones, the Ab against IL-13Rα2 blocked the access of Pep-1-L to the recombinant receptor (Fig.
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 IL-13.E13K-PE38QQR cytotoxin killing effect on U-251 MG GBM cells\textsuperscript{16} while 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-13R\(\alpha\)2, we performed a competition binding experiment \textit{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 3.292 \(\mu\)M at a statistically significant regression analysis (\(R^2 = 0.83\)).

**Synthetic Pep-1 binds to IL-13R\(\alpha\)2 on GBM cells and to GBM tissues**

We found that both linear and disulphide-constrained forms of the Pep-1 bound readily to SnB19-pcDNA GBM cells, which over-express IL-13R\(\alpha\)2, but less intensely to cells with less of the receptor like SnB19-\textit{as}IL-13R\(\alpha\)2 (Fig. 3A). However, in accordance with the DTT ELISA results, the linear form of the peptide bound the IL-13R\(\alpha\)2-positive cells with a prominently higher intensity than the disulphide-constrained form (Fig. 3A). Moreover, both linear and disulphide-constrained 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 over-express IL-13Rα2 compared to T98G cells which have low levels of IL-13Rα2 (Supplementary Fig. S2) [Appendix I].

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 hr. Pep-1-DSC was also internalized, but less prominently when compared to the linear form (Fig. 3B). Next, we carried out 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 had been confirmed to express IL-13Rα2 by immunohistochemistry.

**Pep-1 homes to human GBM xenografts**

We carried out initial *in vivo* experiments in nude athymic mice, implanted with subcutaneous (s.c.) 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 hr (Fig. 4B). At 2 hr, this binding was statistically significant (*p*<0.05, $R^2 = 0.8560$) compared to the control peptide (Fig. 4B). As it was seen with Pep-1-L, the binding of Pep-1-DSC increased with time and was retained up to 72 hr (Supplementary Fig. S3A) [Appendix I]. Moreover, Pep-1-DSC also homed to the s.c. GBM tumors compared to the control peptide (*p*<0.05, $R^2 = 0.9706$), however one of the
experimental mice injected with Pep-1-DSC did not demonstrate any signal and was not included in the analysis (Fig. 4B). The streptavidin-Cy5.5 control was not retained significantly by tumors (Fig. 4 and Supplementary Fig. S3A) [Appendix I].

Next, we carried out peptide homing studies in GBM orthotopic human xenografts. Even though GBM tumors hide behind a blood-brain tumor barrier, we injected the peptide/iodoacetamide-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 to the control peptide, and the streptavidin-Cy5.5 as well as saline controls (Fig. 5A). The fluorescence within the intracranial tumors increased with time and was retained up to 288 hr with the largest increase between 24 and 120 hr (Fig. 5A). The retention of Pep-1-L compared to the control scrambled linear peptide was statistically significant ($p<0.01$, $R^2 = 0.7718$) up to 120 hr (Fig. 5A). Similar findings were made for Pep-1-DSC (Supplementary Fig. S3B) [Appendix I]. However, the difference between the experimental and control disulphide peptide binding were significant only at 2 and 4 hr time-points ($p<0.01$, $R^2 = 0.9722$) (Supplementary Fig. S3B) [Appendix I]. The GBM tumor xenograft tumors were harvested, sectioned and stained for IL-13Ra2 by immunofluorescence and haematoxylin/eosin staining. IL-13Ra2 was abundantly expressed in all of the analyzed tumors (Supplementary Fig. S4) [Appendix I]. To determine the homing capability of Pep-1 to normal brain, we have carried out experiments in mice not bearing intracranial tumors. We observed nominal binding for Pep-1-L at 2 hr and no binding to the normal brain whatsoever at following time points (Fig. 5B).
Discussion

For specific targeting of GBM tumors, we had earlier uncovered IL-13Rα2, which is over-expressed in around three quarters of GBM patients. Here, we identified peptide ligands binding to the IL-13Rα2. We employed a disulphide-constrained heptapeptide phage-display library and carried out biopanning against GBM tumor cells expressing IL-13Rα2. We isolated three 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 takes place 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. Interestingly, the peptide that did not retain disulfide bond-constrained conformation in either the filamentous phage or as a chemically synthesized compound exhibits higher avidity towards the receptor. Moreover, the peptide internalizes with the receptor and its binding can also be readily demonstrated on GBM specimens. Importantly, the peptide homes and binds to both subcutaneous and intracranial GBM tumors expressing IL-13Rα2. These findings open new opportunities for GBM management.

Our biopanning strategy yielded three phages against the IL-13Rα2, but Pep-1 is the most specific for IL-13Rα2. We found that this peptide binds to the receptor at a 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 cytotoxic 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.

One of the interesting findings is that Pep-1 recognizes IL-13Rα2 best in a non-disulphide bond constrained form. Our study may be the first report of this kind when using a phage-display library. However, Pep-1-DSC showed binding property as well, but more 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 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 as well as DSC peptide did get internalized into the cells, even though 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.

Importantly, we have demonstrated that Pep-1-L, and -DSC are retained by the subcutaneous as well as the 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.\textsuperscript{25,26}
Hence, the peptides may be used in non-invasive diagnostic examination of GBM. Moreover, our results suggest that Pep-1-L and Pep-1-DSC could be used for different applications, e.g., 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/therapy. 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. Furthermore, these peptides could be exploited in image-guided surgical resection of GBM by “tumor illumination”.
Acknowledgements: We would like to thank Dr. Evan Gomes for assistance with the some of the jugular vein injections.
Figure 1. Binding of isolated phages to recombinant proteins.

Pep-1, Pep-2, and Pep-3 phage clones in ELISA assay against (A) IL-13Rα2/Fc, IL-13Rα1/Fc chimera proteins, and control proteins: IgG/Fc and insulin. (B) Biopanning-selected phages bind to cells over-expressing IL-13Rα2. A cell-binding phage titer assay was done on cells which do express the IL-13Rα2 (G26-H2 and SnB19-pcDNA cells) and which do not over-express the receptor (G26-V2 and SnB19-asIL-13Rα2). Results expressed as fold-difference in phage titers obtained between different dilutions of original phage (10^6 to 10^{12} pfu). (C) The binding of selected phages is not competed for by the IL-13 ligand, but it is competed for by an antibody against IL-13Rα2. ELISA assay was carried out against the IL-13Rα2/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.
**Figure 2.** Pep-1 binds to the receptor at site other than its ligand binding site.

(A) ELISA of the synthetic Pep-1 binding to IL-13Rα2/Fc in both linear and disulphide-constraint forms. IgG/Fc served as a control. (B) A 1000-fold excess of IL13.E13K was utilized to 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 quadruplicates. (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 values ($R^2 = 0.83$) was obtained.
Figure 3. Synthetic Pep-1 binds to IL-13Rα2.

(A) Fluorescent peptide binding experiment on cells over-expressing IL-13Rα2 (SnB19-pcDNA) and cells with receptor knock down using anti-sense-IL-13Rα2 (SnB19-asIL-13Rα2). Pep-1-L and Pep-1-DSC as well as control linear and disulphide-constrained scrambled peptides (1 µg/ml and 5 µg/ml) were incubated with fixed cells for 1 hr and detected using streptavidin-Alexa fluor 488. (B) Synthetic Pep-1 is internalized into GBM cells. Biotinylated peptides (5 µg/ml) were incubated with live U-251 MG GBM cells for 15 min and 4 hr. The internalized peptides were detected using streptavidin-Alexa fluor 488. The cell nucleus was labeled with Topro-3 DNA dye. The pictures were taken using the LSM 510 confocal microscope. (C) Fluorescent peptide binding experiment on GBM tumor tissue and normal brain tissue. Biotinylated Pep-1-L and control scrambled peptide (10 µg/ml) were incubated with the cells and detected using streptavidin-Alexa fluor 488. The pictures were taken using the Olympus IX70 fluorescence microscope. The pictures are representative of three independent experiments.
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Linear | Disulphide-constrained
Figure 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 (ranging from 0.1 to 1.8 nmoles). 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 i.v. with Pep-1-L/biotin-streptavidin-Cy5.5 conjugate or control scrambled linear peptide-biotin-streptavidin-Cy5.5 conjugates and imaged at various time points ranging from 30 min to 72 hr. The color scale is in units (photon/sec/cm²/steradian)/µW/cm². Control mice were injected with 0.9 nmole 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 < 0.01) is shown at 2 hr.
Figure 5.  Pep-1-L binds to orthotopic GBM tumor xenografts.

(A) Pep-1-L/biotin-streptavidin-Cy5.5 conjugate was injected i.v. in athymic nude mice bearing human GBM G48a intracranial tumors and NIRF imaging was carried out for various time points ranging from 30 min to 288 hr. Mice were also injected with equal amount of control linear peptide-biotin-streptavidin conjugate and 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 ($p<0.001$). (B) The experiment was conducted as in A, but in mice free of intracranial tumors.
B

Luciferin

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Pep-1-L (nmole)
Table 1. Sequences of peptides isolated by biopanning

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References


CHAPTER III

MOLECULAR TARGETING OF INTRACELLULAR COMPARTMENTS SPECIFICALLY IN CANCER CELLS

Hetal Pandya, Denise M. Gibo and Waldemar Debinski

The final, definitive version of the following manuscript has been published in Genes & Cancer. 2010; 1 (5): 421-433. This manuscript is reprinted with permission from SAGE Publications Ltd; Copyright © 2010 SAGE Publications, Inc, All rights reserved. Stylistic variations are due to the requirement of the journal. H. Pandya performed the experiments, analyzed the data and wrote the manuscript. W. Debinski helped prepare the manuscript and acted in an advisory and editorial capacity.
CHAPTER III

MOLECULAR TARGETING OF INTRACELLULAR COMPARTMENTS SPECIFICALLY IN CANCER CELLS

Abstract

We have implemented a strategy in which a genetically engineered, single-chain protein specifically recognizes cancer cell and is trafficked to a targeted subcellular compartment, such as the nucleus. The recombinant protein termed IL-13.E13K-D2-NLS has a triple functional property: (i) it binds a cancer-associated receptor, interleukin 13 receptor alpha 2 (IL-13Rα2), using modified IL-13 ligand, IL-13.E13K, (ii) exports its C-terminal portion out of the endosomal compartment using Pseudomonas aeruginosa exotoxin A (PE) translocation domain (D2), and (iii) travels to and accumulates in the nucleus guided by the nuclear localization signal (NLS). Here, we have demonstrated that this protein is transported into the brain tumor cells’ nucleus, using three different methods of protein conjugation to dyes for the purpose of direct visualization of the protein’s intracellular trafficking. IL-13.E13K-D2-NLS, and not the controls like IL-13.E13K-D2, IL-13.E13K-NLS, or IL-13.E13K accumulated in nuclei very efficiently, which increased with the time the cells were exposed to the protein. Also, IL-13.E13K-D2-NLS did not exhibit nuclear transport in cells with low expression levels of IL-13Rα2. Thus, it is possible to recognize cancer cells through their specific receptors and deliver a conjugated protein that travels specifically to the nucleus. Hence, our molecular targeting strategy succeeded in generating a single-chain proteinaceous agent capable of
delivering drugs/labels needed to be localized to the cells’ nuclei or potentially any other subcellular compartment, for their optimal efficacy or ability to exert their specific action.
**Introduction**

Molecular targeting of cancer cells is achieved (a) specifically through the use of ligands/antibodies against tumor-associated or tumor-specific receptors, and (b) non-specifically using plasma membrane permeable agents targeting activated/over-expressed intracellular elements, such as the oncogenes. In the field of non-viral gene therapy of cancer that employs recombinant proteins, we have pioneered the use of proteinaceous vectors for the targeted intracellular transport of proteins/non-proteinaceous compounds (1-3). Some bacterial toxins, such as *Pseudomonas aeruginosa* exotoxin A (PE) or Diphtheria toxin (DT), possess an ability to exit the endocytic compartment after being internalized in the process of receptor-mediated internalization and being proteolytically activated by a calcium-dependent serine endoprotease, furin (4-7). This “get cleaved and exit endocytic compartment” ability is possible due to the presence of a specialized domain of PE, domain II (abbreviated here as D2) (8;9).

Previously, we have exploited PE translocation ability to traffic other, non-PE, or repeated PE peptide sequences into the cell cytosol (10). This was achieved by incorporating non-PE peptides or an additional catalytic domain III of PE within dispensable domain Ib of the toxin. This domain Ib is downstream of both the furin cleavage site and the N-terminal sequence important for initiation/conduct of exiting of the C-terminal portion of the toxin from the endocytic vesicles (includes portion of domain II and domain III of PE toxin) (11). We demonstrated for the first time that in this manner, PE can serve as a vector for intra-cytosolic delivery of various proteins (12). This approach served as a basis for further developments of, e.g., intracellular vaccines.
or prompted the use of DT protein, having very similar properties to PE toxin, for the same purpose of intra-cytosolic delivery (14).

Most anti-cancer therapeutics have defined targets such as oncogenes, enzymes or DNA, all of which are localized to distinct intracellular compartments like plasma membrane, cytosol, mitochondria or nuclei. We reasoned that having direct delivery vectors for therapeutics/labels to these subcellular compartments will lead to an increased specificity, efficacy and less toxicity. To this end, we have designed a true multiple-specificity delivery vehicle targeting the Interleukin 13 Receptor alpha 2 (IL-13Rα2) for efficient transport to the nuclei of Glioblastoma multiforme (GBM) cells. GBM is a high-grade astrocytoma representing the most common form of primary brain tumors. The treatment of patients with GBM is still a major challenge and the median survival rate is 14.5 months after diagnosis (15). Several factors specific to GBM have been uncovered in recent years (16-19). For example, a tri-molecular signature of GBM has been documented that includes IL-13Rα2, EphA2 receptor and a Fos-related antigen 1 (Fra-1) (20). All three factors belonging to the signature are suitable for therapeutic targeting of GBM (21). For example, IL-13Rα2 is expressed in >75% of GBM tumor specimens (22;23). Our laboratory has characterized IL-13Rα2 receptor as a cancer/testis-like antigen (24). IL-13Rα2 is believed to act as a decoy receptor (25). However, it has been shown that IL-13 ligand binds to IL-13Rα2 receptor and is internalized through receptor-mediated endocytosis (26;27). Hence, drugs attached to the IL-13 ligand can be internalized and delivered specifically inside the glioma cells. Our laboratory has designed and produced IL-13-based cytotoxins, which have shown anti-GBM tumor
activity under both in vitro and in vivo conditions (28). Our laboratory has also designed various IL-13 ligand mutants which specifically bind to the IL-13Rα2 receptor and which do not bind to the physiological receptor that is shared with IL-4 (29;30). IL-13 mutant, such as IL-13.E13K, which has an amino acid residue at position 13 substituted for lysine, demonstrated specific binding to IL-13Rα2, but not to the physiological receptor for IL-13 (31).

The nuclear localization signals (NLS) are employed by a large number of important proteins, which travel to and from the nucleus. One well-characterized sequence from Simian Virus 40 (SV40) large T-antigen is a proven efficient NLS (32-35). This particular NLS is a classical monopartite NLS and comprises of short stretch of basic positively charged amino acids, containing several arginine and lysine residues (36). In the nucleus import pathway, the NLS sequence binds to cytosolic proteins, known as importins/karyopherins which recognize and transport NLS-containing proteins to the nuclear pore complex (37).

For our nuclear targeting delivery vector, we combined the above described recognition and transport signals into a single-chain recombinant protein. Thus, our vector consists of IL-13 or the IL-13 mutant, IL-13.E13K that specifically recognizes IL-13Rα2 followed by Domain II (D2) of PE for endosomal translocation, and finally, the SV40 T antigen NLS to guide the remaining portion of PE to the nucleus. We demonstrate here, in a direct way, that molecularly targeted, genetically engineered proteins specifically recognize GBM cancer cells, travel to and accumulate in these cells’ nuclei.
Results


We aim at developing effective drug/radioactive isotope delivery vehicles to specific intracellular compartments of cancer cell, based preferentially on recombinant proteins. Hence, we have developed here a recombinant protein delivery vehicle to the nuclei of GBM cells. This delivery vehicle recognizes the IL-13Rα2, which is overexpressed on GBM cells. IL-13.E13K-D2-NLS and its control proteins, IL-13.E13K-NLS, IL-13.E13K-D2 as well as IL-13.E13K, which are not expected to either leave the endosomal compartment or reach the nucleus (Fig. 1A) were produced in *E.coli* and purified using FPLC system. IL-13.E13K-D2-NLS was induced in BL21 *E.coli* cells using IPTG (Fig. 1B). The induced protein was isolated and further processed using a disulphide-shuffling method and purified using FPLC column, as described previously (38;39). The separated protein from the column was >90% pure (Fig. 1C, inset). The controls IL-13.E13K-D2, IL-13.E13K-NLS and IL-13.E13K recombinant proteins were expressed, processed and purified in a similar manner (e.g., Fig. S1 and not shown) [Appendix II].


We next wished to confirm that all the purified recombinant proteins bind to the IL-13Rα2 receptor on GBM cells as intended. To this end, we carried out a cell viability assay in which these recombinant proteins bound to the IL-13Rα2 receptor and protected
against cytotoxic action of IL-13.E13K-PE38QQR. IL-13.E13K-PE38QQR, as mentioned above, is a recombinant cytotoxin that binds to IL-13Rα2, is internalized and leads to cell killing through the cleaved active portion of PE, enzymatic domain III. As expected, all recombinant proteins of interest blocked the action of the cytotoxin, resulting in no cell killing: IL13.E13K-D2-NLS (Fig. 1D); IL-13.E13K-D2 (Fig. S1C) [Appendix II]; IL-13.E13K-NLS and IL-13.E13K (not shown). These results confirm that all the recombinant proteins retain IL-13.E13K ligand binding properties and compete specifically for the IL-13Rα2.

**IL-13.E13K-D2-NLS localizes to the nuclei of U-251 MG GBM cells**

Next, we wished to monitor the intracellular journey as well as the subcellular localization of our targeted proteins. To this end, we fluorescently labeled these proteins using three different approaches/methods. For the first approach, we labeled the carboxyl amino acids of the proteins, so as not to modify the primary amines (lysines) present in the NLS domain of the protein. Thus, we utilized the EDC-Sulfo-NHS and Alexa fluor 488 labeling techniques. IL-13-D2-NLS and IL-13-D2 were labeled at their carboxylate groups on amino acids with alexa fluor 488-hydrazide via EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) and Sulfo-NHS (N-hydroxysuccinimide) (see Materials and Methods). In the second approach, we directly labeled the primary amines of the proteins, which are present also in the lysines, with the Alexa Fluor 488-TFP reactive dyes. And for the third approach, we carried out an indirect labeling method. Thus, we initially conjugated the proteins at the primary
amines with biotin. The biotinylated-proteins were detected using HRP-streptavidin and the signals amplified using Tyramide signal amplification method.

After labeling the proteins using the first conjugation method, i.e. EDC-Sulfo-NHS Alexa fluor 488 labeling, we performed cell localization experiments in U-251 MG GBM cells. We observed that the IL-13-D2-NLS effectively localized to the nucleus at 1 hr (Fig. 2A-C) preceded by membrane and cytosolic localization seen at 15 min (Fig. 2A). We also performed Z-stack analysis to confirm the localization of the protein inside the nucleus. The Z-stack analysis for the experiment shown in Fig. 2B demonstrated unequivocally the nuclear localization of IL-13-D2-NLS (Fig. 2C). On the other hand, IL-13-D2 did get internalized into the U-251 MG cells and was found to be primarily in the peri-nuclear region, but it did not travel into the nucleus at either 15 min or 1 hr. of the experiment (Fig. 2D and E).

The above experiments strongly suggested an ability of IL-13-D2-NLS, but not IL-13-D2, to localize to U-251 GBM cells’ nuclei. However, in order to obtain even higher resolution pictures and confirm the nuclear localization using a different conjugation method, we carried out a direct labeling of IL-13.E13K-D2-NLS and IL-13.E13K-D2 with the Alexa-fluor 488 dye. In this approach, Alexa fluor 488 tetrafluorophenyl (TFP) reactive dye molecules attach directly to the primary amines of the amino acids of the proteins forming stable protein-dye conjugates. The conjugate of the proteins were visualized using either SDS-PAGE (Fig. 3A) or fluorescence signals (Fig. 3B). The Typhoon scan showed that only protein-dye conjugates emitted fluorescence signals, but
not the unconjugated proteins (Fig. 3B). The labeled IL-13.E13K-D2-NLS was observed as a doublet on the gel, this can be attributed to differential labeling of the protein and hence different molecular weight bands. We next repeated the localization experiments in U-251 MG cells and confirmed what we had observed earlier: IL-13.E13K-D2-NLS localized to the nuclei at 1 hr (Fig. 3C and D) whereas IL-13.E13K-D2 protein never trafficked into the nucleus (Fig. 3E).

In order to examine whether yet another visualization method would document the same nuclei-localization ability of our recombinant constructs; we decided to use a signal amplification method via tyramide molecules. We initially labeled our proteins using biotin-XX sulfosuccinimidyl ester (biotin-XX, SSE); which reacts very efficiently with the primary amines of the proteins forming stable protein-biotin conjugates. The biotinylated proteins were analyzed using SDS-PAGE/Western blot and the protein-biotin conjugates were detected using streptavidin-HRP. The Western blot indicated that both the proteins had been biotinylated (Fig. 4A). The number of biotins on each of these proteins was quantified by performing FluoReporter Biotin Quantitation assay based on the standard curve in Fig. 4B. Using a quadratic fit equation, the IL-13.E13K-D2-NLS and IL-13.E13K-D2 had similar levels of labeling (DOL) of 13.87 and 14.45 when labeled at a protein to dye molar ratio of 1:4 and 1:8, respectively. Next, these biotinylated proteins were tested in the neutralization of cell killing in a cytotoxicity assay, as shown in Fig. 4C. Both biotinylated IL-13.E13K-D2-NLS and IL-13.E13K-D2 (BL) proteins blocked the action of IL-13Rα2-specific cytotoxin-mediated U-251 MG cell killing (Fig. 4C). The cell localization experiment was then conducted and the
proteins were detected using HRP-streptavidin tyramide signal amplification procedure. We found that in the case of IL-13.E13K-D2-NLS, the protein was bound to the cell membrane with some cytosolic localization at 5 min (Fig. 5A). At 4 hr, cells nuclei started to demonstrate IL-13.E13K-D2-NLS localization, whereas almost all the cells had a significant portion of the protein inside their nuclei at 8 and 24 hr (Fig. 5A). The Z-stack analyses of the 24 hr experimental time point shown in Fig. 5B clearly demonstrated that the IL-13.E13K-D2-NLS traveled to and accumulated in the nucleus. In the case of IL-13.E13K-D2, the protein was mostly bound to the cell membrane with some molecules undergoing internalization at 5 min, while at 8 and 24 hr, the protein was predominantly internalized and localized in the perinuclear region of cells (Fig. 5C). The Z-stack analyses of the 24 hr experimental time point however failed to demonstrate any significant migration of IL-13.E13K-D2 protein to the nucleus (Fig. 5D).

We have also carried out experiments wherein we have labeled the proteins with different molar ratios of the biotin. The protein:dye ratios used were 1:12, 1:8 and 1:4. When both IL-13.E13K-D2-NLS and IL-13.E13K-D2 proteins were labeled at a 1:12 ratio, we observed similar localization patterns for these proteins as described above, except we did not observe any nuclear localization at 4 hr (not shown). This is suggestive of higher occupancy of lysine sites in the NLS by the dye, thus influencing the signal’s functional capability.

We also carried out cell localization experiment with another control protein, IL-13.E13K-NLS, which is devoid of Domain 2 of PE and should not be able to undergo
endosomal translocation and subsequent nuclear transport; it should behave like the IL-13.E13K ligand alone. We thus labeled IL-13.E13K-NLS and IL-13.E13K proteins with the biotin. The SDS PAGE/Western blot of the IL-13.E13K-D2-NLS, IL-13.E13K-D2, IL-13.E13K-NLS and the IL-13.E13K proteins conjugated with biotin and probed with streptavidin-HRP indicated that all the proteins were similarly labeled (Fig. S2A) and also that all the biotin-conjugated proteins bound to the IL-13Rα2 on GBM cells (Fig. S2B) [Appendix II]. We then used all the conjugated proteins concomitantly in an experiment using U-251 MG cells (Fig. 5E). As expected from the previous experiments, IL-13.E13K-D2-NLS traveled to the cells’ nuclei, while IL-13.E13K-D2 did not (Fig. 3H). Both IL-13.E13K-NLS and IL-13.E13K ligand alone behaved like IL-13.E13K-D2, since they did demonstrate perinuclear localization, but no nuclear transport whatsoever at 24 hr of experiment in GBM cells (Fig. 5E). Comparatively the same results were obtained at 5 min, 4 and 8 hr time points to that observed at 24 hr.

**IL-13.E13K-D2-NLS localizes to the nucleus of G48a GBM cells**

We repeated the nuclear trafficking of IL-13.E13K-D2-NLS experiments in another GBM cell line, G48a (40), which over-expresses IL-13Rα2. We obtained corresponding results to the U-251 MG cells. Again, almost all the cells had the IL-13.E13K-D2-NLS protein inside their nuclei not only at 8 hr and 24 hr, but already at 4 hr of the experiment (Fig. 6A). Again, at 5 min, we observed mainly plasma membrane binding with partial internalization of the protein (Fig. 6A). The Z-stack analysis for the 24 hr experiment directly documented the nuclear localization of IL-13.E13K-D2-NLS (Fig. 6B). Also similar results were observed for the IL-13.E13K-D2, IL-13.E13K-NLS and IL-13.E13K
proteins in G48 cells as in U-251 MG cells (Fig. 6C). IL-13.E13K-D2 as well as IL-13.E13K-NLS and IL-13.E13K were not found to have ability to localize to nuclei at any of the time-points and exhibited mainly membrane/cytosolic/perinuclear localization (Fig. 6C, and data not shown).

**IL-13.E13K-D2-NLS does not localize to the nucleus of LN229 GBM cells**

We carried out identical experiments with biotin-labeled IL-13.E13K-D2-NLS in LN229 cells, very low expressors of IL-13Rα2 (41). We observed that the protein displayed weak binding to the cell surface with some internalization, but we did not observe any nuclear localization for the IL-13.E13K-D2-NLS protein at any of the experimental time points (Fig. 7A) similarly to the three control proteins used in our assays (Fig. 7C). The Z-stack analysis for the IL-13.E13K-D2-NLS protein localization in the LN229 cells at 24 hr depicted a low internalization activity and no nuclear localization for the protein in these cells (Fig. 7B).
Discussion

We have attempted to construct a universal proteinaceous module that would recognize cancer cells specifically and be able to travel via intracellular routes specifically to the cells’ nuclei. We have achieved this goal by targeting IL-13Rα2 in GBM cells with a single chain protein composed of a modified receptor ligand, modified specialized cytosol translocation bacterial toxin domain D2 of PE, and a nuclear localization signal from the SV40 T-antigen to form the IL-13.E13K-D2-NLS delivery vector. We have shown in a direct tracking/visualization experiments that this vector binds to plasma membranes of GBM cells, enters endocytic compartment, concentrates in the perinuclear region and then enters nuclei in a time-dependent manner. IL-13.E13K-D2-NLS persistently remains and accumulates in the nuclei up to at least 24 hr. Control proteins without either the NLS or the D2 of PE, and the IL-13.E13K ligand, could follow all of these steps with the exception of the last one, i.e. the efficient entry into the nucleus. Thus, we have directly demonstrated the journey of a designer protein-based vector from the cell surface to the nucleus of GBM cells.

To ascertain the direct demonstration of the phenomenon of IL-13.E13K-D2-NLS traveling from the membrane of cells to their nuclei, we have used three different methods of protein labeling with tractable dyes. First, we conjugated the proteins at the carboxyl groups of amino acids with Alexa fluor 488 using EDC-Sulfo-NHS labeling technique and could demonstrate that NLS-guided vector traveled readily to the GBM cells nuclei, but the signals obtained were relatively weak and the procedure cumbersome as well as not readily reproducible. Therefore, we moved to the approach of directly
conjugating proteins at primary amines with Alexa-fluor 488 and again observed analogous nuclear localization of the protein. We observed similar cell localization for recombinant proteins containing both wild-type IL-13 and mutant IL-13.E13K domains. The signals improved, but we strived to work out a system in which intracellular journey of IL-13.E13K-D2-NLS could be even more easily demonstrated. We then tried attaching proteins at primary amines with biotin and subsequently used HRP-streptavidin and tyramide-alexa fluor 488 signal amplification method as a potentially more optimal technique. We repeated the intracellular localization experiments and found readily detectable nuclear localization of IL-13.E13K-D2-NLS and cytoplasmic/perinuclear localization of IL-13.E13K-D2. Also, the control proteins like IL-13.E13K-NLS and IL-13.E13K demonstrated internalization but no nuclear localization. Very few cells had IL-13.E13K-NLS and IL-13.E13K sporadically in the nucleus. This could be due to large number of these protein molecules in the vicinity of these cells’ nuclei. Another reason can be attributed to the cancer cell metabolism and increased number of nuclear import factors like importin/karyopherins in malignant cells (42). With the last conjugation method, wherein we biotinylated the proteins and then used tyramide amplification method for detection, we also learned indirectly the importance of the lysine amino acids used for the conjugation and contained within the NLS, since lowering the number of attached biotins had favorable effect on IL-13.E13K-D2-NLS accumulation to nuclei. These experiments strongly suggest a possibility of attaching various labels/drugs/dyes to a protein like IL-13.E13K-D2-NLS in expectation of efficient delivery to the nuclei.
We have originally found that it is possible to target cancer cells and deliver peptides/proteins into the cell cytosol using modified PE (43). This finding was exploited in trying to accomplish the same goal with DT derivatives, which proved to be successful too (44). In a recent report, a MAb was linked to the NLS peptide conjugated to Auger electron emitter (45). The construct was able to recognize cells and kill them although no direct demonstration of nuclear delivery was provided. It is also difficult to explain why the NLS peptides leave the antibody and the endocytotic compartment in order to travel to nuclei. Another group has engineered a recombinant protein similarly to our current design (37). This protein targets a non-specifically over-expressed EGFR and when conjugated to a $\alpha$-emitter, kills EGFR-positive GBM cells. The construct was shown to internalize, but no direct evidence for the journey to the nucleus was provided. Besides, $\alpha$-emitters are not necessarily needed to be in the nucleus in order to kill cells, since their cytotoxic radiation has a range of several layers of cells. Therefore, it cannot be excluded that only internalization might be required for such conjugates to work.

IL-13.E13K-D2-NLS might be best suited for conjugation with radiopharmaceuticals like auger electrons, chemotherapeutics and photosensitizers. Auger electrons are low energy electrons, similar to $\beta$-decay, that are emitted when another electron from a high-energy transition state moves to a low-energy inner-vacancy shell, transferring the differential energy to these electrons (38). Therefore, auger electron therapy is very useful for specific tumor cell killing due to very low bystander effects (39). Chemotherapeutics like standard doxorubicin was used successfully in delivering to brain tumors by liposomes (40) but directing it specifically to nuclei of the targeted cells could promise both higher
efficacy and safety through a novel dual-targeted approach. Also, delivering Topotecan, a DNA topoisomerase 1 inhibitor to the cells’ nuclei will be more effective. Photosensitizers, used in photodynamic therapy, would also be an ideal conjugating therapeutic. Since, photosensitizers generate singlet oxygen species which are active only around 40 nm radius of action and have to been shown to be more efficacious if localized to the nucleus (41;42).

In summary, we have generated multiple-specificity vector for nuclear delivery targeting the IL-13Rα2 positive cancer cells, which can be further used for radio labeling and/or conjugation to various labels/chemotherapeutics/genetic elements offering more effective and safer treatment options for GBM and other cancers.
Materials and Methods

**Cell culture.** Human GBM cell lines U-251 MG and LN229 were obtained from American Type Culture Collection (Manassas, VA). U-251 MG cells were maintained in DMEM (Lonza, Walkersville, MD) supplemented with 1x non-essential amino acids (Invitrogen, Carlsbad, CA) and 10% FCS (Hyclone, Logan, UT). LN229 cells were grown in DMEM supplemented with 10% FCS. G48a cells were grown and maintained in RPMI 1640 (Lonza, Walkersville, MD) supplemented with glucose, adjusted to 4 gm/liter of media and 10% FCS (51).

**Cloning, production and purification of targeted proteins.** A duplex primer cloning strategy was employed wherein SV40 T-antigen NLS 5’ and 3’ sequence primers were synthesized (Invitrogen) and made into duplex DNA (containing Xho1/BamH1 ends) by incubating the primers in favorable annealing conditions. The annealed duplex was then subcloned into the IL-13-D2 containing plasmid using Xho1/BamH1 at the 3’ end to produce IL-13-D2-NLS. The IL-13-D2 plasmid was engineered by sub-cloning it from a previously generated IL-13-D2-PE38QQR plasmid (25). The IL-13 mutant recombinant constructs were made by replacing the wild type IL-13 sequence from the parent plasmid with the mutant IL-13.E13K sequence (43). The NH2-terminal end of NLS domain was joined to the COOH terminal of IL-13.E13K domain using the HindIII site to form the IL-13.E13K-NLS plasmid. Also, all of these recombinant constructs were transformed in DH5α *E.coli* cells for amplification. All the constructs were sequenced at DNA sequencing Laboratory of the Comprehensive Cancer Center of Wake Forest University.
and analyzed for their in-frame DNA sequence using an automated sequence analyzer prior to protein expression.

Also, the IL-13/IL-13.E13K-D2-NLS and other control DNA constructs have been created in a manner such that it enables the expression of these proteins under the IPTG inducible T7 promoter in BL21 (λDE3) E.coli protein expression system as previously described (54). In brief, the recombinant constructs were transformed in BL21 cells and the cells were grown in Luria-broth media supplemented with 100 µg/ml of ampicillin at 37°C shaker. When the A600 of the bacterial culture media reached around 1.4, the recombinant protein expression in the cells was induced by addition of 1 nmol/L of IPTG and allowed to incubate for further 90 min. The expressed proteins in the inclusion bodies were then denatured using 7 M Guanidine (MP Biomedicals, Salon, OH) and 1,4-Dithiothreitol (Sigma, St. Louis, MO). The reduced protein was then renatured in a buffer containing arginine/L-glutathione oxidase (Sigma, St. Louis, MO). The protein was further dialyzed and purified by SP Sepharose ion-exchange liquid chromatography (GE Healthcare, Piscataway, NJ) using Fast Protein Liquid Chromatography system (GE Healthcare, Piscataway, NJ). The purified proteins were subsequently run on SDS-PAGE gels to identify the purity of the isolated proteins. All the proteins obtained were >90% pure.

**Colorimetric MTS/PMS cell viability assay.** 1 X 10³ U-251 MG cells were plated per well in quadruplicates for each concentration to be tested. IL-13.E13K-PE38QQR is an IL-13Rα2 based cytotoxin against GBM (55). After 24 hours incubation at 37°C for the
cells to attach, a fixed concentration (i.e. 1 µM) of the IL13.E13K-D2-NLS and other purified proteins as well as biotin-labeled (BL) proteins were added and incubated at 37°C for 1 hr. After 1 hr. incubation, increasing concentrations of the IL-13.E13K-PE38QQR ranging from 0.1 to 100 ng/ml was added and the plate was incubated for 48 hr. Cells treated with cyclohexamide and just the cytotoxin were used as controls. After 48 hr., cell viability was measured using the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]/ PMS [phenazine methosulfate] dye (Promega, Madison, WI) as per the manufacturer’s instructions. The absorbance from the assay was measured at 490 nm using the plate reader Spectra max 340 PC (Molecular Devices, Sunnyvale, CA) and data was plotted as percentage of control versus concentration of the toxin used.

**IL-13-D2-NLS and IL-13-D2 labeling with EDC-Sulpho-NHS and Alexa fluor 488 labels.** Purified IL-13-D2-NLS and the IL-13-D2 proteins were labeled at their carboxylate amino acids via EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride). EDC reacts with a carboxyl group on an amino acid of the protein and forms an amine reactive O-acylisourea intermediate that swiftly reacts with an amino group to form an amide bond and release the isourea by-product. The intermediate is unstable in aqueous solutions and therefore, two-step conjugation procedures require N-hydroxysuccinimide (Sulfo-NHS) stabilization. Sulfo-NHS reacts with the O-acylisourea intermediate and stabilizes it. Next, Alexa fluor 488-hydrizide was added, which replaced the Sulfo-NHS and formed a stable amide bond on the carboxyl groups of the protein to form labeled protein conjugates.
The proteins were initially dissolved in the activation buffer (0.05 M MES, 0.5 M NaCl, pH 6) at the concentration of 1 mg/ml using buffer-exchange columns. Later, 2 mM EDC (Thermo Scientific, Waltham, MA) and 5 mM Sulfo-NHS (Thermo Scientific, Waltham, MA) were added to the proteins and allowed to react for 15 min at RT. Subsequently, 0.14 µl of 2-mercaptoethanol was added to quench the unreacted EDC. The protein-EDC-Sulfo-NHS conjugates were then dissolved in the coupling buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5) using buffer exchange columns (Pierce, Rockford, IL). Next, alexa fluor 488 hydrazide (dissolved in the coupling buffer) (Invitrogen, Carlsbad, CA) was added at 25 molar concentration excess to the proteins and incubated in the dark at RT for 30 minutes. After incubation, 10 mM hydroxylamine (Thermo Scientific, Rockford, IL) was added to quench the excess fluor. The excess unreacted hydrazide fluor was removed using Pierce protein desalting columns.

Localization studies on IL-13Ra2 positive U-251 MG cells using Alexa fluor 488 EDC-Sulfo-NHS labeled proteins. 25,000 U-251 MG GBM cells were plated on coverslip/per well in a 24-well plate. The wells were plated in duplicates for each time point. The cells were allowed to adhere to the coverslips for 24 hr., after which 500 nM each of the Alexa fluor 488 EDC-Sulpho-NHS labeled proteins were added to the U-251 MG cells for 15 min and 4 hr. After the incubations, the cells were fixed with acetone (pre-chilled at -20°F) for 10 min and washed with PBS 4x times. The coverslips were then mounted on the slides using the gel mount (Biomeda, Foster City, CA) and observed with LSM 510 Zeiss Confocal Microscope (Cellular Imaging Core, Comprehensive Cancer Center,
Wake Forest University) and the images processed using Zeiss LSM Image Browser (version 4.2).

The proteins were directly labeled with alexa fluor 488 dye using the Alexa fluor 488 microscale protein labeling kit from Invitrogen (Carlsbad, CA) as per the manufacturer’s instructions. A molar ratio of 25 of the dye to the protein was used to label both the proteins. The proteins were run on 12% SDS-PAGE. The gel was scanned using Typhoon 9210 (Amersham Pharmacia Biotech) for fluorescence signals and later stained using Coomassie blue dye.

**Localization studies on IL-13Ra2 positive U-251 MG cells for Alexa fluor 488 directly labeled proteins.** 25,000 U-251 MG GBM cells were plated on coverslips per well in a 24-well plate. The wells were plated in duplicates for each time point. After 24 hr. for allowing the cells to adhere and attach to the plate, 1 µM/well of alexa fluor directly labeled proteins were added to the U-251 MG cells for 15 min and 4 hr. After the incubations, the cells were fixed with 5% paraformaldehyde (Ted Pella, Redding, CA) for 15 mins at 37°C and washed with 1X PBS (3 times). The cells were then permeabilized with 0.1% Triton-X-100/0.2% BSA-PBS for 10 min at RT. After permeabilization, the cells were washed 3 times with 1X PBS. Subsequently, Topro-3 iodide (Invitrogen, Carlsbad, CA) was added at a concentration of 1:1000 dilution of the 1 mM stock to stain the cell nuclei. The coverslips were then mounted on the slides using the gel mount (Biomedica, Foster City, CA) and observed with LSM 510 Zeiss Confocal Microscope.
Direct labeling of IL-13.E13K-D2-NLS and IL-13.E13K-D2 with biotin and tyramide signal amplification system. Biotin-XX microscale protein labeling kit (Invitrogen, Carlsbad, CA) was used to label the proteins as per the manufacturer’s instructions. A different molar ratio of 12, 8 or 4 biotin-dye to the proteins was used. The biotin-labeled proteins were run on a gel and a Western blot carried out using streptavidin-HRP (Pierce, Rockford, IL) to detect for biotin-labeled proteins. The number of biotin molecules attached to the proteins was determined by the FluoReporter Biotin Quantitation assay kit (Invitrogen) as per the manufacturer’s guidelines. The fluorescent signals were measured using the plate reader Spectra max 340 PC (Molecular Devices, Sunnyvale, CA) and data was plotted as concentration of the standard Biocytin in pmoles versus relative fluorescence units.

Localization studies on IL-13Ra2 positive U-251 MG, G48a and IL-13Ra2 low expressors-LN229 cell with biotin-conjugated proteins. 12,500 U-251 MG or G48a or LN229 GBM cells were plated on coverslips per well in a 24-well plate. The wells were plated in duplicates for each time point. After 24 hr., 1 μM/well of biotin-labeled proteins was added onto the cells for 15 min, 4, 8 and 24 hr. After the incubations, the cells were fixed with 4% paraformaldehyde (Ted Pella, Redding, CA) for 15 mins at 37°C and washed with PBS 4x times. The cells were then permeabilized with 0.1% Triton-X-100/0.2% BSA-PBS for 10 min at RT. After permeabilization, the cells were washed 3
times with 1X PBS. After washings, Tyramide signal amplification kit (Invitrogen, Carlsbad, CA) using Alexa fluor 488 dyes and HRP-streptavidin was carried out as per manufacturer’s instructions. ToPro-3 iodide (Invitrogen, Carlsbad, CA) was added at a concentration of 1:1000 dilution of the 1 mM stock to stain the cell nuclei. After the tyramide staining, wells were washed and mounted with gel mount (Biomeda, Foster City, CA) and observed with LSM 510 Zeiss Confocal Microscope (Cellular Imaging Core, Comprehensive Cancer Center, Wake Forest University) and the images processed using Zeiss LSM Image Browser (version 4.2).

**Immunoblotting.** 500 ng/well of each of the recombinant biotin conjugated proteins were loaded onto a 12% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Pierce, Rockford, IL). Blots were blocked with 5% milk-phosphate buffered saline (PBS) for 1 hr. at room temperature (RT). Biotin-proteins were detected using streptavidin conjugated with horseradish peroxidase (Thermo Fisher Scientific, Rockford, IL) diluted 1:16000 in blocking buffer. Detection was performed using the ECL plus Western Blotting Detection System (Amersham Biosciences, UK). Membranes were exposed to autoradiographic film Kodak Biomax XR. Films were scanned at 600x dpi and images compiled using Jasc Paint Shop Pro v 6.0.
Figure 1. IL-13.E13K-D2-NLS is a nucleus-targeting vector in GBM cells.

A. Schema of single-chain targeted recombinant proteins. (I) recombinant proteins: IL-13.E13K-D2-NLS composed of building blocks of targeting ligand, endosomal translocation PE domain and the SV40 T antigen nuclear localization signal sequence; (II) IL-13.E13K-D2 composed of the targeting ligand and the endosomal translocation domain; (III) IL-13.E13K-NLS consisting of the targeting unit and NLS; (IV) IL-13.E13K targeting unit alone. B. Purification and functional activity of IL-13.E13K-D2-NLS. E.coli BL21 cells were transformed with the recombinant IL-13.E13K-D2-NLS cDNA construct and protein expressed by induction with IPTG. The 12% SDS PAGE gel shows the pre- and post-IPTG induced cell protein extracts. C. The expressed protein was further processed and purified on an SP Sepharose column using the FPLC system (inset). D. The purified protein was subjected to competition assay for the IL-13Rα2 against the IL-13.E13K-PE38QQR toxin. 1 µg/ml of the purified IL-13.E13K-D2-NLS was used to compete against 0.01 to 100 ng/ml of the cytotoxin and the cytotoxicity measured using the MTS/PMS assay.
Figure 2. EDC-Sulfo-NHS-Alexa fluor 488 labeled IL-13-D2-NLS localizes to the nucleus of U-251 MG GBM cells.

A. U-251 MG cells were treated with 500 nM of the EDC-Sulfo-NHS-Alexa fluor 488-labeled IL-13-D2-NLS and the subcellular localization monitored using Zeiss 510 LSM confocal microscope. B. As in A, U-251 MG cell at 1 hr. DIC panel refers to Differential Image Contrast image for depicting the cell morphology. C. Confocal Z-stack analysis of a U-251 MG cell treated with the labeled IL-13-D2-NLS protein at 1 hr. D and E. U-251 MG cells were treated with 500 nM of the EDC-Sulfo-NHS-Alexa fluor 488-labeled IL-13-D2 and the intracellular localization monitored using the Zeiss 510 LSM confocal microscope.
Figure 3. IL-13.E13K-D2-NLS travels to and very efficiently accumulates in the nuclei of GBM cells, IL-13Rα2 over-expressors.

A. Conjugation of IL-13.E13K-D2-NLS and IL-13.E13K-D2 to Alexa fluor 488. The primary amines in the IL-13.E13K-D2-NLS and the IL-13.E13K-D2 were conjugated directly to the tetrafluorophenyl (TFP)-reactive Alexa fluor 488 dye. The labeled proteins were run on 12 % SDS-PAGE gel and stained with Coomassie blue. B. As in A, but the blot scanned using Typhoon 9210 to detect fluorescent proteins. C and D. Alexa fluor 488 labeled IL-13.E13K-D2-NLS localizes to the nucleus of U-251 MG GBM cells. The intracellular localization of IL-13.E13K-D2-NLS labeled with Alexa-fluor 488 in U-251 MG cells was examined using confocal microscope. E. As in C and D, but the protein used was control IL-13.E13K-D2. The data is representative of three independent experiments.
Figure 4. Biotin-labeled IL-13.E13K-D2-NLS retains binding to IL-13Rα2 on GBM cells.

A. Western blot for the biotin-labeled IL-13.E13K-D2-NLS and IL-13.E13K-D2 probed with streptavidin-HRP. B. Standard curve for the Bio-fluoreporter assay for quantification of the biotin molecules on the labeled proteins. Biocytin was used as the standard and the fluorescence was measured using the Spectra max 340 plate reader. C. Neutralization of the cytotoxicity assay for the biotin-labeled (BL) IL-13.E13K-D2-NLS and the IL-13.E13K-D2. 1 µM each of the biotin-labeled proteins was competed against the IL-13.E13K-PE38QQR cytotoxin.
**Figure 5.** Biotin-labeled IL-13.E13K-D2-NLS retains nuclear localization ability in GBM cells.

A. Biotin-labeled IL-13.E13K-D2-NLS (1 µM) was added to U-251 MG cells for different time points and the biotin signal was amplified by tyramide-alexa fluor 488 labeling technique. Subcellular localization of the proteins was monitored using confocal microscope. B. The Z-stack confocal analysis of IL-13.E13K-D2-NLS nuclear accumulation. C and D. The biotin-labeled control proteins do not localize to the nuclei of U-251 MG GBM cells. The localization experiment was carried out as for IL-13.E13K-D2-NLS. Cells not exposed to the labeled protein were used as controls. The Z-stack analysis is that of the experiment at 24 hr. The pictures are representative of three independent experiments. E. The biotin-labeled IL-13.E13K-NLS and the IL-13.E13K do not localize to the nuclei of U-251 MG GBM cells. Localization experiment was carried out for the IL-13.E13K-D2-NLS, IL-13.E13K-D2, IL-13.E13K-NLS and the IL-13.E13K proteins in U-251 MG cells and their localization patterns monitored using Zeiss 510 LSM confocal microscope. At 24 hr., only the IL-13.E13K-D2-NLS was prominently localized in the nucleus whereas the other three proteins had major perinuclear, but no nuclear localization. The data is representative of two independent experiments.
Figure 6. The biotin-labeled IL-13.E13K-D2-NLS localizes to the nuclei of G48a GBM, but not of LN229 cells.

A. Cellular localization experiments carried out in another high IL-13Rα2 expressor G48a cells. Wells having no labeled proteins were used as controls. B. Z-stack analysis of G48a cells treated with IL-13.E13K-D2-NLS at 24 hr. C. Cellular localization experiments for the IL-13.E13K-D2-NLS, IL-13.E13K-D2, IL-13.E13K-NLS and the IL-13.E13K were carried out in G48a cells concomitantly and their trafficking documented by confocal microscope. Cells with no labeled proteins were used as controls. The data is representative of two independent experiments.
Figure 7. The biotin-labeled IL-13.E13K-D2-NLS does not localize to the nuclei of LN229 cells.

References


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CHAPTER IV

DISCUSSION

Recent approaches in molecular targeting of GBM include directing treatment strategies to overexpressed GBM-associated antigens like activated intracellular signaling pathways as well as their components and also to cell membrane receptors. The latter treatment strategies include vaccines, virus-directed therapies, re-directed cytolytic T cells, as well as bacterial toxins conjugated/fused to a targeting moiety such as monoclonal antibodies, peptides or native ligands. Our laboratory has been on the forefront in the development of GBM-specific therapeutics, including IL-13-based cytotoxins consisting of IL-13 as the targeting ligand and the translocation and cytotoxic protein domains of PE and DT (1;2).

In continuation with our search for improved and more effective treatment and imaging modalities for GBM, the main aims of this dissertation were:

1. **To identify, develop and characterize IL-13Ra2 specific peptides**

2. **To develop and characterize IL-13Ra2 specific recombinant protein vectors for targeted intra-cellular compartment delivery in GBM cells**

We have successfully achieved our aim of developing the above mentioned IL-13Ra2 targeted therapeutic technologies as follows:

1. **To identify, develop and characterize IL-13Ra2 specific peptides**

Peptides are one of the desirable delivery vectors as they present attractive properties of
small size, high affinity and selectivity in a single package. Our aim was to develop IL-13Rα2-peptidomimetics which could cross the blood-brain tumor barrier and be delivered through the intravenous route for treatment or imaging of HGA. Furthermore, smaller compounds may offer better distribution when used with convection-enhanced delivery, which is a form of loco-regional delivery to brain.

By means of a cyclic M13 heptapeptide phage display library, we have identified three different peptide phage clones binding to IL-13Rα2. Among the three peptide phages, Pep-1 phage exhibited the highest affinity and specificity towards IL-13Rα2. Synthetic peptides based on the sequence displayed on the Pep-1 phage (both linear and disulphide constrained forms- Pep-1-L and Pep-1-DSC) also demonstrated binding to IL-13Rα2. Moreover, using competition binding experiments, we identified that Pep-1 might be binding to a site on the receptor other than the IL-13 binding site. Confocal microscopy experiments indicated that Pep-1 is internalized by GBM cells. Experiments carried out in subcutaneous human GBM xenografts using NIRF imaging for Pep-1-L and Pep-1-DSC demonstrated readily detectable binding and retention by the tumors compared to the control scrambled peptides. Importantly, similar experiments performed in orthotopic human GBM xenografts indicated that Pep-1 homes to intracranial tumors when injected into circulation by crossing the blood-brain tumor barrier.

Thus, we have selected a peptide from a cyclic phage-display library that binds to IL-13Rα2. This peptide binds to the receptor at a site other than that of the native ligand. Pep-1 shows an ability to home to tumors and hence it has a potential to be further
developed for diagnostic, imaging and therapeutic interventions for GBM. This IL-13Rα2-peptide may also be utilized for double targeting of the receptor, as it binds independently of the ligand. In addition, it can be efficiently used for monitoring IL-13-based therapy or vice versa.

2. To develop and characterize IL-13Rα2 specific recombinant protein vectors for targeted intra-cellular compartment delivery in GBM cells

Most anti-cancer therapeutics have defined targets such as proteins, enzymes or DNA, which are localized in distinct intra-cellular compartments like cytosol, mitochondria or nucleus. We reasoned that direct delivery of therapeutics to intracellular compartments can lead to an increased specificity, efficacy and less toxicity. To this end, we have designed a multiple-specificity delivery vehicle targeting the nucleus of GBM cells as a final stop and IL-13Rα2 for cell recognition and as an entry site.

The targeted delivery vehicle to the nucleus was constructed using a modified receptor ligand, IL-13.E13K, domain II (D2) of Pseudomonas exotoxin A [PE] and SV40 T-antigen nuclear localization signal (NLS); IL-13.E13K-D2-NLS. Domain II of PE contains a site of proteolytic cleavage and is responsible for the translocation of the toxin, and any other protein fused to it, from the endosome to the cytosol. We also produced control proteins that lack essential components of the designer protein, such as IL-13.E13K-D2, IL-13.E13K-NLS and IL-13.E13K. The preservation of the binding of these proteins to IL-13Rα2 receptor on GBM cells was confirmed by neutralization of cell killing mediated by the IL-13 based cytotoxin. Next, the proteins were labeled with
fluorescent dyes and their localization in GBM cells was monitored using confocal microscopy by three different labeling techniques. IL-13.E13K-D2-NLS demonstrated a prominent nuclear localization in GBM cells, under all conditions whereas control IL-13.E13K-D2, IL-13.E13K-NLS and the IL-13.E13K remained sequestered in the cytosol. Thus, we have successfully generated a non-viral, mutliple-specificity, single-chain recombinant protein vehicle for nuclear delivery of IL-13Rα2-positive cancer cells.

After developing IL-13Rα2 specific small peptides, and recombinant single-chain proteins for intracellular nuclear delivery, our next step will be conjugation of different therapeutic and imaging modalities to these delivery vectors. For our IL-13Rα2 specific peptides, we have shown their application as optical imaging agents for GBM tumors. For our nuclear delivery vectors, we are in the process of conjugating the radioisotope, $^{111}\text{In}$ for therapeutic purposes. In future, these peptides and recombinant proteins can be conjugated to various different imaging beacons and/or therapeutics, and hence can be efficiently applied in different diagnostic and therapeutic arenas. In the following sections, I will discuss some of the major applications for both the IL-13Rα2-specific peptides and single-chain nuclear delivery protein vectors.
**Peptide phage display library**

Phage display libraries constitute collections of billions of different peptides, antibodies, receptors expressed on the surface of bacteriophages (3;4). These proteins or peptides are engineered to be expressed on the tips of some of the coat proteins of the phages. The genetic information of the molecules displayed on the phage surfaces is encoded within the phage genome and hence it is easy to identify the sequence of the molecule of interest.

Peptide phage display libraries have been extensively used to isolate and characterize peptides for various diseases like atherosclerosis, arthritis, inflammation, cancer and have been utilized for various functions, including cancer imaging and treatment. Peptide phage display library have also been employed in the field of material sciences, such as the peptides identifying indium nitride. These peptides can further be utilized for various biomimetic applications as well as for development of various affinity-based optical and electrical biosensors (5). Furthermore, peptide phages recognizing markers on embryonic (6) and neural (7) stem cells have been identified. These findings have great implications in viral vector-mediated gene therapy and in regenerative medicine.

**Peptide Phage display libraries in cancer research**

Peptide phage display libraries have been employed to select peptides for treatment of various tumors and cancers (8;9). Recently, they are increasingly being utilized for identification of specific peptides against tumor-associated antigens, including receptors, carbohydrates/lectins as well as tumor vasculature (10-12). Furthermore, peptide phage
display libraries have been successfully used to identify peptides binding to various tumor-associated plasma membrane receptors like EphA2 (10), HGFR (13), IL-11 receptor (14), ErbB-2 (15), EGFR (16), PSMA (17) and TAG-72 (18). Peptides isolated from phage display libraries against several angiogenesis-associated proteins and receptors like integrins (19;20), VEGF (21) and FGF (22) have also been reported. But, these studies constitute only a handful of papers wherein peptide phage display libraries have been employed to find peptidomimetics for specific tumor-associated receptors. Most of the studies constitute selection of these peptides on cancer cells by in vitro biopanning or in tumor-bearing mice by in vivo biopanning, giving rise to tumor-specific peptide phages with unknown targets.

These tumor specific peptides and peptide phages selected from phage display libraries have been widely used for various applications ranging from delivery agents for therapeutics, gene therapy, monitoring response to treatment as well as for imaging.

**Phage display-derived peptides as imaging agents**

Peptides isolated against specific tumors have been extensively employed for various imaging platforms, like SPECT, PET and optical imaging. Some of the common radionuclides routinely employed for SPECT imaging are $^{99m}$Tc (6 hr half-life, 140 KeV gamma emission) and $^{111}$In (2.8 day half-life and 173 and 247 KeV gamma photon emission) due to their short lives and easy conjugation chemistries. Peptides successfully utilized to image tumors using SPECT imaging include, $\alpha$$\beta_3$ integrin specific $^{99m}$Tc labeled RGD peptides (23), $^{111}$In-DOTA-galectin-3 peptide in prostate carcinoma (24),
MMP-2/9 binding peptides (25) and also $^{111}$In-DOTA-GSG-KCCYSL binding to ErbB-2 (15).

$^{18}$FDG [2-deoxy-2-fluoro-D-glucose] is usually employed to image cancer cell metabolism during PET imaging, but not all cancer cells efficiently utilize $^{18}$FDG. Hence, tumor targeting agents have been employed to increase uptake of PET tracers like $^{18}$FDG and $^{64}$Cu by cancer cells. Many tumor specific peptides identified from the phage display libraries have been used for PET imaging, like the $^{18}$FDG-RGD peptide for $\alpha_v\beta_3$ integrin (26), and $^{64}$Cu-FSRYLWS peptide for targeted imaging of uPAR [urokinase-type plasminogen activator] receptor (27).

Near-infrared optical imaging using fluorophores like Cy5.5, Cy7 and near infrared quantum dots is being increasingly used for optical imaging of tumors. Near infrared fluorescence with emission wavelengths between 650 and 900 nm is ideally suited to image deeper tissues. At NIRF wavelengths, absorption of light by hemoglobin, water, lipids, and other molecules is reduced and also tissue autofluorescence is minimal. This allows for potential imaging up to 7-14 cm deep with increased sensitivity (28). Several peptides isolated from the phage display library have been employed for optical imaging using near infrared fluorophores, including the RGD peptide against integrins (29), HVGGSSV peptide for identification of tumors responding to a combined therapy of radiation and SU11248, a VEGF receptor tyrosine kinase inhibitor (30), as well as various other studies.
Peptides as gene therapy vehicles

Efforts have been carried out to deliver p53 gene-therapy to Tie2 [Tyrosine kinase with immunoglobulin and epidermal growth factor homology domain-2] overexpressing solid tumors via a vector consisting of a 12-mer peptide isolated from a phage display library and polyethylenimine (31). Moreover, siRNA against PRDM14 gene (important in breast cancer carcinogenesis) has been delivered via liposomes coated with MCF-7 breast cancer cell-specific targeting peptide DMPGTVP, isolated from a f8/8 phage display library (32).

Peptides for monitoring efficacy of a therapeutic regimen

Peptides that bind to tumors responding to anti-angiogenesis therapy can be identified based on the hypothesis that distinct biomarkers are upregulated when the endothelial cells undergo changes under a therapeutic regimen. Peptide phage display libraries have been utilized to identify early biomarkers in tumors responding to a combined radiation and chemotherapeutic regimen. In 2008, Han et al. identified a peptide from a T7 peptide phage display library which can noninvasively identify tumors responding to a combined therapy of radiation and SU11248 (VEGF receptor tyrosine kinase inhibitor) compared to the tumors that do not respond to the therapy (30). The authors have also shown that this peptide, HVGGSSV, can bind to tumors that are responding to treatment with various other tyrosine kinase inhibitors like PTK787 (inhibitor of all known VEGF receptors), AEE788 (inhibits both VEGF and EGFR receptors) and SU5416 (VEGF specific inhibitor) (30). The major advantage of such peptides is that they can identify patients early on during the treatment who do not respond to a particular therapy, and hence
provide ample time to modify the treatment, thereby minimizing toxicity and reducing expenses.

Furthermore, the same group demonstrated that the HVGGSSV peptide can also bind to TIP-1 [Tax-interacting protein-1], a radiation-induced receptor. The HVGGSSV peptide was used to target TIP-1 and deliver nanoparticle albumin-bound paclitaxel (HVGGSSV-nab-paclitaxel) to irradiated mouse model of lung cancers (33). Treatment with HVGGSSV-nab-paclitaxel led to a significant growth regression of both the Lewis lung carcinomas and H460 lung carcinomas compared to the controls (33). Therefore, it seems like that the HVGGSSV peptide binds to TIP-1 protein on tumor microvasculature, which is induced due to the treatment with either tyrosine kinase inhibitor or radiation or both.

Recently, a LLADTTHHRPWT peptide from 12-mer M13 peptide phage display library has been isolated. This peptide binds to tumors that exhibit early response to treatment with bevacizumab, a humanized monoclonal antibody against human VEGF in murine model of colorectal cancer (34). This peptide, termed as bevacizumab-responsive peptide (BRP), exhibited a higher uptake in tumors treated with bevacizumab than with phosphate-buffered saline treated controls as observed by NIRF optical imaging (34). The next question that naturally arises is whether this technique can be successfully used to identify pharmacological changes during treatment with tumor cell-associated small molecule signaling pathway inhibitors (i.e. not associated with the vasculature) or with other cytotoxic therapeutics.
**Phage display-derived peptide as therapeutic delivery agents**

Peptides isolated from the phage display library have been used as specific, targeted delivery vehicles to transport various therapeutics to tumors and tumor-associated vasculature. Pro-apoptotic, antimicrobial peptides like (KLAKLAK)$_2$ have been delivered through alpha v-integrin-specific RGD peptides to the tumor-vasculature in nude mice bearing breast carcinoma xenografts (35). Tumor-bearing mice treated with the (RGD-4C)-GG-(KLAKLAK)$_2$ induced tumor regression and showed increased survival compared to mice treated with unconjugated mixture of RGD-4C peptide and (KLAKLAK)$_2$ (35). Similarly, conjugates made from the RGD peptides and an anti-cancer chemotherapeutic, doxorubicin, demonstrated decreased tumor growth and toxicity than free doxorubicin (19).

As discussed earlier, HVGGSSV peptide isolated from the T7 peptide phage display library was used to deliver nanoparticle albumin-bound paclitaxel to irradiated mouse model of lung cancers through TIP, a radiation induced receptor (33). Recently, Passarella RJ and co-workers isolated a peptide, GIRLRG, which can bind to tumors treated with ionizing radiation. GIRLRG peptide binds to a radiation-induced, 78 kDa, glucose-regulated protein [GRP78] receptor. Furthermore, paclitaxel was delivered to these tumors by a polyester nanoparticle drug delivery system conjugated to the GIRLRG peptide (36). In irradiated murine breast and glioma tumor xenografts, the GIRLRG-delivered paclitaxel significantly delayed tumor tripling time by 55 and 12 days, respectively (36). Thus, targeted peptides can be effectively used to deliver therapeutics specifically to the site of tumor, and thereby increase efficacy and decrease side effects.
**Phage display-derived peptide as virus delivery agents**

For efficient gene therapy, novel chimeras of eukaryotic viruses with prokaryotic bacteriophages have been created. In this chimeric virus system known as AAV/P, a eukaryotic gene cassette from an adeno-associated virus (AAV) was inserted in an intergenomic region of M13 phage (P) expressing RGD-4C peptides. The RGD-4C peptide targets the αv integrins expressed on endothelial and tumor cells. This AAVP chimeric virus was engineered to carry the herpes simplex virus thymidine kinase (HSVtk) gene for molecular imaging and tumor targeting of prostate carcinoma tumors in nude mice (37). HSVtk acts as a suicide gene when combined with ganciclovir treatment. It can also be used for effective PET imaging, when used with HSVtk-specific radiolabeled nucleoside analogs. Tumor regression was observed in mice injected with RGD-4C-AAVP-HSVtk compared to non-targeted AAVP-HSVtk after ganciclovir treatment (37). Also, increased PET signals were observed in tumors of mice injected with the targeted AAVP-HSVtk compared to the non-targeted controls (37). In addition, similar system has also been shown to have effectiveness in a human sarcoma xenograft model in mice (38).

**Applications of Peptide phages**

Phages with their respective surface-displayed peptides have been utilized for targeting tumors, and for induction of immune responses to the tumors. Eriksson et al. have tested a M13-based peptide phage against mouse melanoma for recruitment of inflammatory cells to the tumor. This treatment lead to induction of Th1 cytokines and regression of tumors in 50% of the mice (39). Moreover, prostate carcinoma specific phages have been
labeled with Alexa Fluor 680 and used for optical imaging of prostate tumors in mice (40). Peptide phages have also been radiolabeled for imaging of tumors. For example, tumor-associated glycoprotein 72 [TAG-72] specific phage clones from a f88-4/Cys6 phage display library were conjugated to technetium-99m for imaging purposes (41).

Recently, modified filamentous M13 phages have been demonstrated to form efficient phage-liposome nano-webs to deliver a photosensitizer, zinc naphthalocyanine to SKBR-3 breast cancer cells. These phages were engineered to express eight glutamic acids on the major coat protein of the phages. Under *in vitro* conditions, these M13 phage-liposomes led to accumulation of the photosensitizer in the cells and mediated their destruction when irradiated with an infrared light. This application can be extended to M13 phages displaying specific cancer-targeting or tumor-homing peptides on phage coat protein pIII, while the liposome binding anionic peptides can be displayed on coat protein VIII. These liposomes can carry any anti-cancer drug/s. Hence, this phage-liposome apparatus can lead to specific delivery of drugs to tumors and ultimately their eventual destruction (42).

**Potential applications for the IL-13Rα2-specific peptides and peptide phages**

We have shown that our IL-13Rα2-specific peptides can be successfully employed for non-invasive, optical imaging of GBM tumors. These peptides will further be explored for various other imaging and therapeutic applications. As discussed above, our peptides against IL-13Rα2 can similarly be conjugated to various therapeutics like the anti-apoptotic peptides tachyplesin and/or (KLAKLAK)$_2$ (43), chemotherapeutics like
doxorubicin (19;44), liposomes (45), radioisotopes like technetium-99m (99mTc) (41), and 111In for nuclear imaging (24;46) as well as treatment, quantum dots (47;48), imaging and radiotherapy with 125I (13) and many other therapeutic/imaging modalities. Besides the IL-13Rα2-specific peptides, the peptide phages can also be similarly applied for efficient drug/radiotracer delivery to tumors.

In addition, we plan to develop IL-13Rα2 peptide-based intra-cellular compartment delivery vectors utilizing the endosomal translocation domain and the nuclear localization signal. Again, the logic here is to achieve double targeting at the cellular and subcellular levels, thereby increasing specificity, efficacy and limiting toxicity. The other advantage of a small peptide-intracellular compartment delivery construct would be increased diffusion and penetration into the tumor. In addition, these intracellular delivery peptides might possess the ability to cross the blood-brain tumor barrier, and hence therapeutics conjugated to these constructs may be delivered non-invasively to GBM patients.

**Image-guided surgical resection**

There are some instances wherein imaging has been employed for tumor detection during image-guided surgical resection. Retrospective pre-operative MRI-based analysis in a set of GBM patients indicate that maximal surgical resection (>98%) led to increased survival (49). However, in some cases, the evidence is weak due to patient selection bias in terms of age and prognosis (50). Nevertheless, studies have been done where tumor resection was carried out by pre-operatively planning the resection co-ordinates based on MRI as well as PET tumor contours. It was observed that PET-based guidance led to
improvement in patient survival while MRI based resection was not significantly correlated to better survival (51).

Furthermore, clinical trials have been carried to determine survival using 5-aminolevulinic [5-ALA]-based fluorescence-guided tumor resection. 5-ALA leads to a high accumulation of fluorescent protoporphyrin IX [PPIX] in epithelia as well as malignant glioma and “lights up” the tumor. This is also known as ‘tumor painting’ or ‘tumor illumination’. The major advantage of this technique is that the surgeons can visualize the fluorescent tumor at the time of resection using a fluorescent microscope and hence can effectively determine tumor boundaries. Intra-operative surgical guidance with 5-ALA fluorescence showed a decreased residual tumor and an improved 6-month progression-free survival (52). The shortcoming of this technique is that 5-ALA is not specific for any tumor associated marker but is selectively uptaken by cancer cells. It will be interesting to employ our IL-13Rα2-specific peptide conjugated to a fluorophore for fluorescence-image guided resection of gliomas. Because these peptides are specific for a glioma-associated antigen, they might be able to better delineate between the cells in the tumor core as well as the infiltrating glioma cells vs normal brain cells, and therefore lead to maximal tumor resection as well as possibly increased survival than 5-ALA-based fluorescence-guided resection.

As discussed earlier, the bacteriophage backbone (with the respective specific peptides on the coat proteins) has been used as efficient platform for imaging purposes as well as for some therapeutic purposes. It would be worthwhile to employ our IL-13Rα2-specific
peptide phages for the purpose of GBM tumor imaging/diagnosis, or as delivery agents for gene therapy or nano-therapy.

In summary, the IL-13Rα2-specific peptides/peptide phages are potentially versatile agents for delivery and treatment purposes of GBM as well as other IL-13Rα2-expressing tumors. These receptor-directed peptide/peptide phages can be employed for numerous applications, including treatment as well as diagnosis of tumor type, size, and location.

In the following section, I will discuss the merits of our non-viral, recombinant protein delivery vector and its various applications.
**IL-13Ra2-directed nuclear delivery recombinant single-chain protein**

For IL-13Ra2-directed intracellular compartment delivery vehicles, we have utilized a mutant IL-13 ligand specific for IL-13Ra2 (IL-13.E13K), an endosomal compartment exit-facilitating protein domain of PE (D2) and nuclear localization signal sequence of SV40 T-antigen. We have shown, mainly through confocal microscopy, that these protein delivery vehicles are not only specific for GBM cells expressing IL-13Ra2, but also efficiently localize in the nucleus of these cells. Moreover, we have also demonstrated that domain 2 of PE is required for efficient travel to the nucleus.

We chose to develop intra-cellular localization constructs to the nucleus, since it houses the genetic information as well as the all important transcriptional machinery of the cells, and therefore making it one the most desirable target for anti-cancer drug delivery/development.

Our design strategy for the intracellular delivery vector is very versatile. The IL-13 targeting ligand can be exchanged for any targeting ligand (including monoclonal antibodies, peptides, and natural receptor ligands) against tumor-associated cell surface receptors. In addition, other endosomal translocating domains such as GALA peptide, electroneutral lipids like DOPE, as well as the translocation protein domain from Diphtheria toxin can also be used. Moreover, localization signal sequence for many intra-cellular organelles like the lysosomes (53), peroxisomes (54), mitochondria (55) as well as other nuclear localization signals (56) can be exchanged with the current SV40 T-antigen signal sequence for targeted drug delivery to one or more of the intracellular
compartments of the cell.

**Nuclear localization signal sequence**

The transport to the nucleus occurs through the nuclear pore complexes located on the nuclear membrane of the cell. The nuclear pore complex regulates transport of molecules in and out of the nucleus (57). Molecules < 9 nm can diffuse freely through the nuclear pore, but large molecules up to 25 nm in diameter or around 1000 kDa require active transport to pass through the pore (58). This active transport is mediated by karyophilic molecules bearing targeting signals like the nuclear localization signals. Nuclear localization signal sequences are classified as either classical or non-classical sequences. Classical NLS sequences are either monopartite or bipartite signal sequences and comprised of basic amino acids. Monopartite NLS sequences have one cluster of basic amino acids, whereas bipartite sequences have two basic clusters separated by 10-12 neutral amino acids (59; 60). Importin α/β are molecules of the nuclear import-export pathway which recognize the classical NLS sequences on proteins, and transport them inside the nucleus through the nuclear pore complex by an energy-dependent mechanism (61). Non-classical NLS sequences, like M9-sequences found on heterogeneous nuclear ribonucleoprotein [hnRNP] A1 protein do not have basic amino acids and bind to transportins instead of importins (62).

**SV40 T-antigen nuclear localization signal**

The SV40 T-antigen NLS is a classical, monopartite signal sequence with a basic amino acid sequence of $^{126}$PKKKRKV$^{132}$. It was the first NLS sequence to be identified (59).
SV40 T–antigen NLS sequence can assist in nuclear transport of proteins up to 465 kDa (63). Mutations studies have indicated the importance of basic amino acids, especially Lys-128 in nuclear import (59). Detailed studies on SV40 T-antigen NLS by Jans et al. have established that amino acids (T-antigen 111-135) flanking the NLS monopartite sequence (T-antigen 126-132) facilitate faster nuclear accumulation than the basic NLS sequence (64). These flanking amino acids do not contain another NLS sequence but possess several phosphorylation sites. Subsequently, it was found that phosphorylation of $S^{111/112}$ by casein kinase II (65;66) and inhibition of phosphorylation at site $T^{124}$ (67) on the T-antigen increases the nuclear transport efficiency. Moreover, it was later discovered that phosphorylation at site $S^{120}$ of T-antigen (DNA-dependent protein kinase) also increased nuclear transport kinetics (68). Considering this, we have designed and utilized the following NLS sequence in our constructs – SSDDEATADS$^{120}$QHAA$^{124}$PPKKRKV$^{132}$EDP. We have observed very efficient translocation of our designer protein into the nucleus. Besides, it is known that under certain pathological conditions, especially cancer, the levels of importins are increased (69), which may also explain the efficient localization of our constructs into the nuclei of GBM cells.

**Non-viral delivery vector for gene therapy**

Most of the current nuclear delivery vehicles have been developed and characterized for use in non-viral vector mediated gene therapy. These non-viral nuclear delivery vehicles have been designed by utilizing non-specific, cell-penetrating peptides [CPP] like penetratin, tat, transportan, and other CPP peptides conjugated to the NLS peptide for
transportation of DNA, peptide nucleic acids [PNA] and antisense oligodeoxynucleotides to the nucleus of a cell (70).

When DNA is injected directly into the cell cytoplasm, it is very unstable and is susceptible to degradation by proteases. Besides, free DNA is not very efficient in travelling to the nucleus. DNA microinjected into the cytoplasm reached the nucleus in 6-8 hr, whereas, cells which had their nuclear membrane permeabilized, the DNA transport to the nucleus occurred in 90 min (71). The basic amino acid NLS sequences can easily be conjugated to negatively charged DNA through electrostatic interactions (72;73). DNA can also be conjugated to NLS peptides through covalent modification of cationic polymers like polyethyleneimine (74).

**Delivery of auger electrons to the nuclei of a cell**

Auger electrons like Indium-111 ($^{111}\text{In}$) have a very short path length ranging from nanometers to micrometers (mostly $<1 \mu m$) in tissues. They also have high-linear-energy transfer [LET] values ranging from 4-26 keV/µM (75). For $^{111}\text{In}$, the dose of radiation absorbed to the nucleus is 2 to 35-fold higher when it decays in the nucleus compared to when $^{111}\text{In}$ decays on the cell surface or in the cytoplasm (76;77), indicating that $^{111}\text{In}$ is very cytotoxic when delivered directly to the DNA of the cell. Moreover, when targeted to a particular tumor-associated antigen, it can be highly specific in destruction of cancer cells while sparing the surrounding normal cells. These short-range emitters can be advantageous when targeting individual cells, small clusters of tumor cells or micrometastases. Efforts have been carried out to target HER2 positive human breast...
cancer with $^{111}$In-NLS-Trastuzumab (78;79). The indium-conjugated trastuzumab significantly slowed the growth of HER2-positive breast cancer xenografts in mice compared to the unconjugated trastuzumab (80). In these studies, the NLS peptides were chemically conjugated to the trastuzumab antibody. Other studies, including the one in which anti-CD33 antibody (HuM195) has been used to target $^{111}$In to the nucleus of human leukemia cells, have utilized NLS peptides which were conjugated to the antibody through a linker, however no concrete evidence of nuclear localization was provided (81).

**IL-13Ra2-directed nuclear and cytoplasmic delivery of drugs/labels**

In addition to developing the nuclear delivery constructs for IL-13Ra2-expressing tumor cells, we have also established that an endosomal translocation facilitating protein was required for efficient transport into the nucleus. This is in line with previous findings, when Tkachenko et al. injected gold nanoparticles-conjugated to SV40 T-antigen NLS directly into the cytoplasm of the HepG2 cells; they entered the nucleus of the cells. However, when these constructs were provided in the HepG2 growth media, the constructs surprisingly entered the cells through receptor-mediated endocytosis and accumulated in the cytoplasm, but could not localize to the nucleus (82;83). This study suggests that constructs with the NLS sequences cannot enter the nucleus, unless they are capable of endosomal escape.

A limited amount of work performed in the field of nuclear delivery vectors has either been through 1.) chemical conjugation of NLS peptide/s to monoclonal antibodies or other targeting ligands, which are subsequently conjugated to various therapeutics like
radioisotopes (double conjugation); or 2.) use of non-specific plasma membrane penetrating peptides [CPP] to enter the cell; and/or 3.) most of these vectors have been created without any endosomal translocation facilitating protein domains/peptides. Our nuclear delivery vector is one of the foremost designer protein-targeted delivery vehicles, combining the specific cell targeting, endosomal and nuclear localization units into a single-chain protein. Besides, recombinant chimeric proteins can easily be mass produced in bacterial system and hence may have a higher potential to be efficiently translated into clinic compared to other mAbs-NLS peptide vectors.

Furthermore, we have utilized a mutant form of native IL-13, IL-13.E13K, in construction of our delivery vectors. In this variant of IL-13, the negatively charged glutamic acid at position 13 of the α-helix A of the native ligand has been replaced by a positively charged lysine. We have previously shown that this modification in amino acid of the ligand alters the binding of IL-13 to the IL-4/IL-13 shared receptor, but exhibits increased binding to the IL-13Rα2 receptor (84). Therefore, our IL-13.E13K-based nuclear delivery vectors are more specific for cancer-associated IL-13Rα2 receptor than for the shared IL-13/IL-4 receptor that is expressed on normal brain cells. This was further confirmed by studies in other laboratories which have utilized IL-13.E13K/Y in their IL-13Rα2–directed therapies (85;86). For example, Kahlon et al. have utilized IL-13.E13Y-based redirected CTLs in a murine glioma model (85). In their studies, they found that IL-13.E13Y directed CTLs were specific for cells expressing IL-13Rα2, but did not mediate any CTL activation for cell lysis of cell lines with IL-13Rα1+/IL-13Rα2− expression (85). Furthermore, Candolfi et al. observed severe neurotoxicity when native
IL-13-PE38QQR cytotoxin (Cintredekin Besudotox) was injected into naïve mouse brain (86). This is because native IL-13 can also bind the IL-4/IL-13R expressed on normal brain cells. Cintredekin Besudotox when injected at both low and high doses was very toxic and lead to several neurological deficits. However, injection of mutant IL-13.E13K–PE38QQR cytotoxin displayed lesser neurotoxicity in a dose-dependent manner, and exhibited no neuropathological abnormalities at the lower dose (86). This indicates that mutant IL-13-based constructs are more specific for GBM in situ than the native ligand constructs.

The IL-13.E13K-D2-NLS nuclear delivery vectors can be conjugated (single conjugation) to various radioisotopes like auger electrons, chemotherapeutics and several other therapeutics to specifically deliver them to their site/s of action. These delivery vectors can also be used to transport DNA into the nuclei and hence be used as effective gene therapy delivery agents. Furthermore, they can be applied for genetically-mediated therapy like siRNA or anti-sense gene therapy. These IL-13Rα2-specific nuclear delivery vectors can also be utilized for delivery of photosensitizers as their optimal site of action resides in the nucleus.

One of our immediate goals is conjugation of the IL-13.E13K-D2-NLS vector to $^{111}$In to for delivery into the nuclei of GBM cells. Furthermore, after exploiting the SV40 T-antigen NLS, we next plan to use a human NLS sequence from NF-κB p50 (EEKVQRKRQKLM) protein to make our nuclear localization delivery proteins, as it is one of the well-studied and well-characterized human NLS (87;88).
The IL-13.E13K-D2 protein is a cell cytoplasmic delivery vector. We have previously used IL-13.E13K-D2 to specifically and successfully deliver bacterial toxins (1;89) to IL-13Rα2-expressing glioma cells. IL-13.E13K-D2 can be further conjugated to many different cytotoxins which exert their action in the cytoplasmic compartment of a cell. It can be used to deliver various cellular death inducing proteins like chlorotoxin, plant toxin ricin, or chemotherapeutics and also radioisotopes like Iodine-131.

**Combinatorial therapy with the IL-13Rα2-specific peptides and IL-13-based vectors**

Combinatorial therapy by mixing both our IL-13Rα2-specific peptides and the IL-13-based delivery vectors would be another efficient approach for GBM tumor targeting. Our IL-13Rα2-specific peptides are exceptionally suited for the task as they bind to the receptor at a site other than the IL-13 ligand binding site. Hence, they can be used for double-targeting of GBM tumors. The fluorescent-probe conjugated IL-13Rα2-specific peptide can be used for ‘tumor illumination’, while an IL-13-based therapeutic, like ¹¹¹In-IL-13.E13K-D2-NLS, can be utilized for therapy through convection-enhanced delivery. This strategy can help us in identifying the efficacy of the therapy. Even though the IL-13Rα2 peptide binds at a site other than the ligand binding site, it is internalized into GBM cells. Therefore, the peptide can also be used in a reverse fashion, wherein the peptide-based therapeutic can be used for treatment and an IL-13 fluorescent probe can be utilized for tumor illumination. Secondly, a bacterial cytotoxin and a radioisotope can be attached to the peptides or the IL-13-based vectors (or vice versa) to deliver them to glioma cells. In addition to the above mentioned therapeutic and diagnostic applications for our targeting/delivery vectors, they can be efficiently utilized for combinatorial
therapy for GBM with other HGA-associated antigen-targeted therapeutics, including EphA2 and EGFR-directed therapies or in other cancers wherein IL-13Rα2 is overexpressed (90-93).

In conclusion, we have developed two different delivery vectors targeting IL-13Rα2 with different characteristics. The IL-13Rα2-specific peptides are small, can possibly cross the blood-brain tumor barrier and hence may have better uptake from circulation or better diffusion in the tumor when delivered through convection-enhanced delivery. On the other hand, the single-chain recombinant protein IL-13-based vectors are intra-cellular nuclear delivery vehicles and hence can be efficiently used to transport therapeutics which have their site of action in the nuclei of a cell. They also make excellent non-viral gene delivery vehicles. Moreover, our genetically engineered intracellular delivery vectors are very specific for cancer cells due to their ability for ‘double-targeting’ and hence may cause no harm to normal brain cells when used for therapy. Both of these vectors can be further conjugated to myriad of therapeutics/imaging beacons for precise delivery to cancer cells.


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APPENDIX I

AN INTERLEUKIN 13 RECEPTOR ALPHA 2-SPECIFIC PEPTIDE HOMES TO HUMAN GLIOBLASTOMA MULTIFORME XENOGRAFTS

Hetal Pandya, Denise M. Gibo, Shivank Garg, Steven Kridel and Waldemar Debinski

SUPPLEMENTARY FIGURES

The following manuscript was prepared for submission to Neuro-Oncology and is reprinted with permission. Stylistic variations are due to the requirements of the journal. H. Pandya designed/ performed the experiments and analyzed the data. D.M. Gibo and S. Garg assisted in some experiments. S. Kridel acted in an advisory capacity and helped prepare the manuscript. W. Debinski supervised the project and also acted in an advisory and editorial capacity. H. Pandya and W. Debinski prepared the manuscript.
**Figure S1.** GBM cells used for biopanning, isolated phages that bind IL-13Rα2 independent of disulphide bonds formation and control synthetic peptide binding to the receptor.

(A) IL-13Rα2 expression by Western blot in malignant glioma cell lines used for biopanning with Ph.D.-C7C Phage-display library. G26-H2 and SnB19-pcDNA cells were used for positive selection whereas the G26-V2 and SnB19-asIL-13Rα2 cells were used for negative selection. (B) A DTT ELISA was performed with Pep-1, Pep-2, and Pep-3 phages. M13KE is the control M13 phage. The results are representative of three separate assays in duplicates. The error bars correspond to SEM. (C) ELISA binding assay to recombinant proteins was carried out with Pep-1-L and Pep-1-DSC, and their controls: scrambled peptides or a serine linear peptide in which cysteines in the sequence of Pep-1-L were substituted with serines.
C

O.D. - A405 nm

Concentration (μg/ml)

IL-13Rα2-Fc
IgG-Fc
BSA

Pep-1-L
Pep-1-DSC
Pep-1-L (C/S)
Control Pep-1-L
Control Pep-1-DSC
Figure S2. Pep-1 binds to IL-13Rα2-expressing GBM cells.

Fluorescence of peptide binding to cells over-expressing IL-13Rα2, U-251 MG (A and C) and T98G (B) cells that express the receptor to a much smaller degree. Biotinylated linear, disulphide-constrained Pep-1 and control scrambled peptide (1 µg/ml and 5 µg/ml) were incubated with cells and detected using streptavidin-Alexa fluor 488. The pictures were taken using the Olympus IX70 fluorescence microscope. The pictures are representative of three independent experiments.
C

U-251 MG

1 µg/ml

5 µg/ml

Scrambled Pep-1-L

No peptide
Figure S3. Pep-1-DSC homes to GBM xenografts in mice.

(A) Athymic nude mice bearing human GBM G48a s.c. tumors were injected i.v. with Pep-1-DSC/biotin-streptavidin-Cy5.5 conjugate or control scrambled streptavidin-Cy5.5 conjugates, and imaged at various time points ranging from 30 min to 72 hr. The color scale is in units (photon/sec/cm²/steradian)/µW/cm². Control mice were injected with 0.9 nmole of unconjugated streptavidin-Cy5.5 or saline. Representative image (24 hr) of comparison of tumor binding and retention by Pep-1-DSC vs. the control scrambled disulphide peptide (*p* < 0.05). (B) Equal amounts of Pep-1-DSC/biotin-streptavidin-Cy5.5 conjugate or the control scrambled peptide-biotin-streptavidin conjugate were injected i.v. into athymic nude mice bearing human GBM G48a orthotopic tumors and NIRF imaging was carried out for various time points ranging from 30 min to 288 hr. Control mice were injected with unconjugated streptavidin-Cy5.5 or saline. The color scale is in units (photon/sec/cm²/steradian)/µW/cm². Comparison of tumor binding and retention by Pep-1-DSC vs. the control scrambled peptide (*p* < 0.0001).
Figure S4. Expression of IL-13Ra2 in G48a GBM xenografts.

(A) Tumors were analyzed for the expression of IL-13Ra2 by immunofluorescence. IL-13Ra2 (red) and nucleus stained with DAPI (blue). (B) H & E staining of the G48a xenografts. The sections were obtained from mice treated with Pep-1-L (a), control scrambled Pep-1-L (b), and saline (c).
APPENDIX II

MOLECULAR TARGETING OF INTRACELLULAR COMPARTMENTS SPECIFICALLY IN CANCER CELLS

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SUPPLEMENTARY FIGURES

The final, definitive version of the following manuscript has been published in Genes & Cancer. 2010; 1 (5): 421-433. This manuscript is reprinted with permission from SAGE Publications Ltd; Copyright © 2010 SAGE Publications, Inc, All rights reserved. Stylistic variations are due to the requirement of the journal. H. Pandya performed the experiments, analyzed the data and wrote the manuscript. W. Debinski helped prepare the manuscript and acted in an advisory and editorial capacity.
**Figure S1.** Purification and functional activity of IL-13.E13K-D2.

A. Recombinant IL-13.E13K-D2 protein was expressed in *E.coli* BL21 cells by induction with IPTG. The 12% SDS PAGE shows the pre- and post-IPTG induced cell protein extracts. B. The expressed protein in the IPTG induced cells was further processed and purified using the SP Sepharose FPLC system. C. The purified IL-13.E13K-D2 was used in a competition assay for the IL-13Rα2 against the IL-13.E13K-PE38QQR cytotoxin. The cell viability was measured using an MTS/PMS assay. Data was plotted as % of control versus the cytotoxin concentration. The IL-13.E13K-D2 neutralized efficiently the effect of cytotoxin.

A. All recombinant proteins were conjugated to the biotin-dye and a Western blot assay was carried out to confirm biotinylation of these proteins. Streptavidin-HRP was used as secondary for detection. B. Biotinylated proteins were used in a competition assay for the IL-13Rα2 against the IL-13.E13K-PE38QQR cytotoxin. All the biotinylated proteins neutralized the effects of the cytotoxin establishing that they all still effectively bind to IL-13Rα2.
APPENDIX III

IL-13Rα2 EXPRESSION STUDIES IN RESPONSE TO INCREASING CONCENTRATIONS OF IL-13 AND IL-13.E13K LIGANDS

We examined the levels of IL-13Rα2 on GBM cells’ surface in response to IL-13 and IL-13.E13K, as we wanted to know whether the receptor was being degraded and/or down-regulated in response to these ligands.

U-251 MG GBM cells were treated with different concentrations of IL-13 and IL-13.E13K (1, 10 and 100 nM respectively) and cell lysates were collected at different timepoints ranging from 0 to 24 hr. Western blotting experiments were carried out with the cell lysates and IL-13Rα2-specific antibody was used for detection. We found that the total levels of the IL-13Rα2 remained either unchanged or increased with the time, but not with increasing concentrations of the ligand (Figure. 1 A-F). At no timepoint and ligand concentration was the receptor down-regulated.

Since Western blots give a measure of total cellular protein, we next evaluated the cell-surface levels of IL-13Rα2. To this end, flow-cytometry experiments were carried out using U-251 MG cells. Cells were treated with 10 nM of the IL-13 or the IL-13.E13K ligand and collected at various timepoints (0 to 24 hr). IL-13Rα2-specific primary antibody and anti-IgG-Alexa fluor 488 secondary antibody were used for detection. An IgG isotype was used as a control. The results of the flow cytometry experiments were similar to that obtained by the Western blots. In presence of the IL-13 and the IL-
13.E13K mutant ligands, the cell-surface levels of the IL-13Rα2 receptor remained constant at increasing timepoints (Figure 2 A-E).

Thus, our studies indicate that the total IL-13Rα2 protein levels remain relatively steady in presence of high concentrations of the ligands, and in fact, the receptor levels may even increase with time. Moreover, IL-13Rα2 remains constant on GBM cell surface when exposed to both wild-type and mutant IL-13. This observation is particularly important since we are targeting the IL-13Rα2 receptor to deliver various IL-13 and IL-13-mutant based therapeutics and delivery vectors. This observation implies that these IL-13-based therapeutics and delivery vectors can be continuously delivered to IL-13Rα2-expressing GBM cells without down-regulating the receptor.

Moreover, our experiments may indicate that the expression of *IL-13Rα2* gene is either constitutive or there are intracellular pools of the receptor protein, or both. Some studies point towards the presence of intracellular stores of IL-13Rα2. Studies carried out in cultured as well as primary monocytes, and respiratory epithelial cells demonstrated that the treatment of these cells by IFN-γ resulted in a six-fold increase in surface IL-13Rα2 within hours (1). Protein synthesis was not required for this upregulation as determined by cyclohexamide inhibition experiments, suggesting that intracellular stores of IL-13Rα2 may be present (1).

Furthermore, studies carried out by Daines *et al.* indicate that the distribution of the IL-13Rα2 receptor in the cytoplasmic (intracellular), cell-membrane and the soluble
compartments does not depend on the overall levels of expression of the receptor. The IL-13Rα2 receptor was found to majorly exist as intracellular pools. Surface IL-13Rα2 was released as a soluble form of the receptor, but the levels of the receptor always remained constant indicating that the receptor from the intracellular pool is continuously trafficked onto the cell surface (2).

Thus, our studies may point towards intracellular stores of the receptor in GBM cells which are continuously cycled to the cell surface to maintain receptor levels constant on the plasma membrane. Again from a drug delivery viewpoint, the steady presence of the receptor, following a continuous exposure to the ligand signifies the attractiveness of targeting this receptor.
**Figure 1.** IL-13Ra2 receptor is not down-regulated or degraded in response to higher concentrations and prolonged exposure to the native/mutant IL-13 ligand: Western Blot analyses of the IL-13Ra2 receptor at

A., 1 nM B., 10 nM and C., 100 nM of native IL-13 ligand;

D., 1 nM E., 10 nM and F., 100 nM mutant IL-13.E13K ligand at various timepoints ranging from 0 to 24 hr.
**Figure 2.** IL-13Rα2 receptor remains constant on GBM cell surface: Flow cytometry analysis of the IL-13Rα2 receptor on intact U-251 MG GBM cells.

A., I., Analysis of the U-251 MG cell population, II., U-251 MG cells, no primary antibody, III., U-251 MG cells, IgG isotype control.

B., IL-13Rα2 on U-251 MG GBM cells in response to 10 nM of the native IL-13 ligand at various timepoints (0 to 24 hr). Anti-IL-13Rα2 used as primary.

C., Comparison of the IgG isotype and the wild-type IL-13 treated U-251 MG GBM cells at 24 hr detected using the anti-IL-13Rα2 antibody.

D., IL-13Rα2 on U-251 MG GBM cells in response to 10 nM of the mutant IL-13.E13K ligand at various timepoints. Anti-IL-13Rα2 used as primary.

E., Comparison of the IgG isotype and the mutant IL-13.E13K treated U-251 MG GBM cells at 24 hr detected using the anti-IL-13Rα2 antibody.
References


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**Abstracts**


2. **Pandya H**, Garg S, Gibo DM, Kridel S and Debinski W. Selection of Heptapeptide that Binds Interleukin 13 Receptor Alpha 2 At a Site Distinct than the Native Ligand Binding Site. 18th Annual Residents’ and Fellows' Research day, Division of Surgical Sciences, Wake Forest University (Nov. 2010)
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5. **Pandya H**, Garg S, Gibo DM, Kridel S and Debinski W. Selection of a Linear Heptapeptide that Binds Interleukin 13 Receptor Alpha 2 at a Site Distinct from the Native Ligand. 17th Annual Residents’ and Fellows’ Research day, Division of Surgical Sciences Surgical Science Research day (Oct. 2010), Wake Forest University.

the Native Ligand. 2009 Joint Meeting of the Society for Neuro-Oncology and the AANS/CNS Section on Tumors (Oct. 22-24 2009), New Orleans, USA


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**Invited Oral Presentations**


2. **Pandya H**, Garg S, Gibo DM, Kridel S and Debinski W. Isolation and Characterization of Interleukin 13 Receptor Alpha 2-Specific Heptapeptides. The Third Quadrennial Meeting of the World Federation of Neuro-Oncology (WFNO) and the Sixth Meeting of Asian Society for Neuro-Oncology (ASNO) – (May 11-14, 2009), Yokohama, Japan
Academic Achievements and Awards

• Gold Medal Award (1st place) for best basic science poster presentation at 18th Annual Residents’ and Fellows’ Research day, Division of Surgical Sciences, Wake Forest University (Nov. 2010)
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• Alumni Student Travel Award, Wake Forest University Graduate School of Arts and Sciences (Apr. 2009)
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