A novel ligand delivery system to non-invasively visualize and therapeutically exploit the IL13Rα2 tumor-restricted biomarker

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Our objective was to exploit a novel ligand-based delivery system for targeting diagnostic and therapeutic agents to cancers that express interleukin 13 receptor alpha 2 (IL13Rα2), a tumor-restricted plasma membrane receptor overexpressed in glioblastoma multiforme (GBM), meningiomas, peripheral nerve sheath tumors, and other peripheral tumors. On the basis of our prior work, we designed a novel IL13Rα2-targeted quadruple mutant of IL13 (TQM13) to selectively bind the tumor-restricted IL13Rα2 with high affinity but not significantly interact with the physiologically abundant IL13Rα1/IL4Rα heterodimer that is also expressed in normal brain. We then assessed the in vitro binding profile of TQM13 and its potential to deliver diagnostic and therapeutic radioactivity in vivo. Surface plasmon resonance (SPR; Biacore) binding experiments demonstrated that TQM13 bound strongly to recombinant IL13Rα2 (Kd ~5 nM). In addition, radiolabeled TQM13 specifically bound IL13Rα2-expressing GBM cells and specimens but not normal brain. Of importance, TQM13 did not functionally activate IL13Rα1/IL4Rα in cells or bind to it in SPR binding assays, in contrast to wtIL13. Furthermore, in vivo targeting of systemically delivered radiolabeled TQM13 to IL13Rα2-expressing subcutaneous tumors was demonstrated and confirmed non-invasively for the first time with 124I-TQM13 positron emission tomography imaging. In addition, 131I-TQM13 demonstrated in vivo efficacy against subcutaneous IL13Rα2-expressing GBM tumors and in an orthotopic synergeic IL13Rα2-positive murine glioma model, as evidenced by statistically significant survival advantage. Our results demonstrate that we have successfully generated an optimized biomarker-targeted scaffolding that exhibited specific binding activity toward the tumor-associated IL13Rα2 in vitro and potential to deliver diagnostic and therapeutic payloads in vivo.

Keywords: glioblastoma multiforme, interleukin-13 receptor, PET, radioimmunotherapy.

Glioblastoma multiforme (GBM) is an invariably fatal malignancy with a mean survival time of approximately 14 months despite the availability of multimodality treatments, including surgery, radiation, and chemotherapy.1 The residual and infiltrating cells left after therapies, such as surgery and radiation, lead to recurrent disease in the resection cavity and beyond. Molecular-targeted therapies that target tumor-associated biomarkers have emerged as innovative approaches to eradicate these highly infiltrative tumors but not harm normal brain. Similarly, molecular targeted therapies offer promise in systemic malignancies that also express tumor-restricted biomarkers.

Interleukin 13 receptor alpha 2 (IL13Rα2) is an attractive GBM-associated biomarker that we and others have previously identified to be overexpressed in approximately 75% of GBMs but not in normal brain tissue or significantly on other organ sites except the testes.2–5 In addition to GBMs, IL13Rα2 has been reported to be overexpressed in peripheral nerve sheath tumors, medulloblastomas, and meningiomas.6–9 This receptor is
thought to serve as a protective decoy receptor that has recently been shown to sequester IL13 and prevent it from inducing apoptosis through the physiologically abundant IL13Ra1/IL4Ra heterodimer in GBM.\(^\text{10}\) IL13Ra1 is a distinct physiologically abundant receptor that weakly binds IL13 and needs to recruit IL4Ra for a high-affinity IL13 interaction requisite to mediate the common physiological functions of IL13.\(^\text{11}\) The differential expression of IL13Ra2 in cancer has led many groups to develop means of targeting this attractive biomarker with diagnostic and therapeutic agents. One approach to targeting IL13Ra2 has been to use derivatives of IL13, the high-affinity natural ligand for IL13Ra2.\(^\text{12,13}\) However, because IL13 also interacts with the physiologically abundant IL13Ra1/IL4Ra heterodimer expressed in normal brain,\(^\text{3}\) we rationally designed our derivative ligands to overcome this interaction with the non-cancer associated receptor. This selectivity is possibly attributable to our prior work in which we extensively used site-directed mutagenesis to identify specific amino acids on IL13 that are responsible for its binding toward the physiologically abundant IL13Ra1/IL4Ra heterodimer and the cancer-restricted IL13Ra2.\(^\text{14,15}\) We found that specific mutations introduced to amino acids at the 13th, 66th, and 69th positions on IL13 decreased the binding/activation of the physiologically abundant IL13Ra1/IL4Ra receptor (Fig. 1).\(^\text{14,15}\) Of importance, these mutations that impair binding to the physiologically abundant IL13 receptor have no adverse effect on the strong binding to the tumor-associated IL13Ra2.\(^\text{14,15}\) In addition, we found that a mutation in a separate region of IL13, a lysine-to-arginine mutation at the 105th position in the D-helix, enhanced IL13 affinity toward the cancer-restricted receptor IL13Ra2.\(^\text{16,17}\) Therefore, to further improve targeting the tumor-associated IL13Ra2, we for the first time combined all 4 IL13Ra2-targeting mutations to produce an optimized targeted quadruple mutant of IL13 (TQM13; IL13.E13K/R66D/S69D/K105R), a multiply mutated IL13 derivative that we hypothesized would demonstrate enhanced binding to the tumor-associated IL13Ra2 but not to the physiologically abundant IL13Ra1/IL4Ra heterodimer (Fig. 1). In addition, on the basis of our prior work, we produced a matched negative control (IL13-binding deficient mutant of IL13, BDef13; IL13.E13K/R66D/S69D/K105A) that only differs from TQM13 by a single amino acid substitution, but lacks appreciable binding to both IL13 receptors (Fig. 1).\(^\text{16,17}\)

In addition to demonstrating in vitro and in vivo tumor targeting of our optimized ligand, we used a mutant derivative of IL13 (TQM13) to non-invasively image real-time biomarker IL13Ra2 expression via positron emission tomography (PET) and to selectively deliver toxic β-emitting radioactivity to IL13Ra2-expressing malignancy. This, for the first time, manifests the potential of using noninvasive imaging to assess real-time IL13Ra2 expression for the purpose of personalizing our targeted therapy against malignancies that express this attractive tumor-associated biomarker.

Materials and Methods

Tissue culture equipment was from Corning Glass (Corning, NY). The G48a MG cell line was developed in the laboratory of Dr. Waldemar Debinski.\(^\text{18,19}\) G26-H2 cells were generated in-house, as previously reported.\(^\text{20}\) MTS/PHS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium), inner salt/phenazine methosulfate was purchased from Promega (Madison, WI). SDS-PAGE and Western blot transferring equipment were from Bio-Rad (Hercules, CA) and Invitrogen (Carlsbad, CA). Antibodies were obtained from R&D Systems (Minneapolis, MN) and Santa Cruz Biotechnology (Santa Cruz, CA). IODOGEN reagents for \(^{125}\)I, \(^{124}\)I, and \(^{131}\)I labeling were purchased from Pierce (Rockford, IL).

Construction of Plasmids and Expression/Purification of Recombinant Proteins

For TQM13 and BDef13 plasmid construction, the plasmid for IL13.E13K/R66D/S69D (obtained from W.D.\(^\text{21}\)) was further mutagenized at the 105th position as we previously described.\(^\text{16,17}\) TQM13 cDNA was amplified and inserted into the directional pET100 TOPO vector as instructed by the manufacturer (Invitrogen), which contained an N-terminus polyhistidine tag. All plasmids carrying the gene constructs of interest were under a T7 promoter-based expression system and

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**Fig. 1. Schematic of IL13, TQM13, and BDef13 and their interaction with the physiologically abundant IL13Ra1 and the tumor-restricted IL13Ra2.** Although IL13 binds to both receptors, TQM13 contains hotspot mutations that prevent binding to the physiologically abundant IL13Ra1/IL4Ra but not the cancer-restricted IL13Ra2.\(^\text{15}\) BDef13 is a negative control that differs from TQM13 only in the 105th amino acid position, which we have shown dramatically decreases affinity towards both IL13 receptors.\(^\text{16}\)
constructed as described.\textsuperscript{14} Plasmids were isolated using Qiagen kits and sequenced in-house to verify the correct modifications prior to use.

Protein synthesis in \textit{Escherichia coli} and purification were performed as previously described.\textsuperscript{15,17} The inclusion body fraction of the bacterial cells was isolated using the detergent-based BugBuster protein extraction reagents according to the manufacturer’s recommendations (EMD Chemicals). Denatured recombinant proteins were purified via nickel-based affinity chromatography (Qiagen), then renatured using the disulfide-shuffling method as described elsewhere.\textsuperscript{22} wtIL13 and IL13.E13K recombinant proteins were obtained from W.D. and were expressed/purified in a prokaryotic system as previously described.\textsuperscript{14,15}

\textbf{Western Blot}

Western blotting was done as described previously.\textsuperscript{15} Membranes were incubated with primary antibody overnight at 4°C and with secondary antibody conjugated with HRP for 1 h at room temperature. Detection was done using the enhanced chemiluminescence plus Western Blotting Detection System (GE Healthcare) with the LAS-3000 imaging system (Fujifilm), and images were compiled using Adobe Photoshop Elements, version 5.0 (Adobe Systems).

\textbf{Circular Dichroism Spectroscopy}

Circular dichroism (CD) measurements were made with a Jasco-720 spectropolarimeter. All measurements were performed at 23°C, using a .05 cm path-length quartz cuvette. Proteins (0.2 mg/mL) were resuspended in phosphate-buffered saline (PBS) and then analyzed. Reported spectra were the average of 3 consecutive runs for each sample. Spectra from PBS alone were subtracted from each sample so that the resulting spectra reflected only the CD contribution of the proteins.

\textbf{Cytotoxicity Assay}

Cytotoxicity assays were performed as described previously. In brief, $1 \times 10^3$ G26-hIL13Ra2(+) cells were plated on each well of a 96-well plate in 150 g/mL of media and allowed to adhere overnight. The following day, 25 mL of blocker (8 g/mL; e.g., IL13 derivatives in 0.1% BSA/PBS buffer or buffer alone) was added. After an hour of incubation at 37°C, 25 mL of varying concentrations of IL13-based bacterial cytotoxin was added in quadruplicate and incubated at 37°C in 5% CO$_2$/95% O$_2$ for 48 h. After 48 h, the MTS/PMS cell proliferation assay was performed as instructed by the manufacturer (Promega). Ten microliters of dye was added to each well and incubated for 2–4 h. Plates were then read using a microplate reader at an absorbance of 490 nm. Cyclohexamide-treated, cell-containing wells served as the background for the assay. Background was subtracted from each data point, which was divided by the value from the wells that were not treated with cytotoxin to obtain the fraction of cells remaining. The fraction of cells remaining was multiplied by 100 to obtain the percent of control.

\textbf{IL13Ra2-Binding Biacore Experiment}

Measurements were performed in a Biacore T100 instrument by monitoring the changes in response units (RU) at the biosensor surface.\textsuperscript{23} Recombinant hIL13Ra2-Fc (R&D Systems) in sodium acetate (pH 4; Biacore AB) was coupled to the carboxymethyl dextran layer of a CM5 research-grade chip using standard amine coupling procedures, expecting to achieve a surface density of 10 000–12 000 RU in the sample channel; excess ethanolamine then blocked any remaining reactive groups in both the sample and reference channels.\textsuperscript{24} The running buffer used in this study was HBS-EP 1X (Biacore AB). Purified TQM13 and BDef13 were separately injected in series (0, 100, 300, and 1000 nM) over the immobilized receptor, followed by a regeneration cycle with 0.1% SDS. The biosensor was equilibrated for 300 s with buffer prior to the next cycle. Binding was quantified as the increase in RUs (sample minus reference channel signals) at 60 s after the end of injection, compared with a baseline established at 20 s prior to injection. Kinetic data were analyzed with a bivalent analyte model to determine the forward and reverse rate constants for complex formation between TQM13 (or BDef13) and immobilized IL13Ra2-Fc. This approach yielded a 20-fold reduction in $\chi^2$, compared with a single-site kinetic model. Therefore, the dissociation constant $K_d$ was calculated from the ratio $k_{on}/k_{off}$ for the initial binding step, as previously described.\textsuperscript{23}

\textbf{IL13Ra1-Dependent TF-1 Cell Proliferation Assay}

TF-1 cells (pre-leukemic human B cells, which express the shared IL13/4 receptor)\textsuperscript{25} were grown in the presence of different concentrations of wtIL13 or its mutants in 96-well culture plates. After 72 h of incubation at 37°C, the rate of proliferation of the TF-1 cells was determined by a colorimetric MTS/PMS cell proliferation assay as instructed by the manufacturer (Promega). Cyclohexamide-treated, cell-containing wells served as the background for the assay. Background was subtracted from each data point, which was divided by the value from the wells that were not treated with cytokines to obtain the proliferation rate, expressed as percentage of controls.\textsuperscript{16} In the competitive assay, TF-1 cells were pre-treated with TQM13 for 2 h prior to the addition of wtIL13 for growth.

\textbf{IL13Ra1/IL4Ra-Binding Biacore Experiment}

This study was performed similarly to the IL13Ra2-binding Biacore experiment. Recombinant hIL4Ra-Fc (R&D Systems) was immobilized instead. wtIL13, IL13.E13K, and TQM13 were pre-incubated with or without recombinant human IL13Ra1-Fc (R&D Systems) in equal molar concentrations for 1 h at 37°C.
to reconstitute the binding complex prior to delivery to the immobilized IL4Rα binding partner. Binding signal for each experimental ligand complex was expressed as a percentage of the positive control (wtIL13 + IL13Ra1).

**Iodination and Instant Thin-Layer Chromatography**

Recombinant IL13 derivatives were labeled with $^{125}$I (or $^{124}$I, $^{131}$I) using the IODO-GEN method as recommended by the manufacturer (Pierce). Instant thin-layer chromatography was performed using 0.9% saline as the mobile phase to ensure labeling efficiency.

**Electrophoretic Mobility Shift Assay**

Radiolabeled $^{125}$I-TQM13 and $^{125}$I-BDef13 were incubated with excess molar amounts of soluble recombinant hIL13Ra2.Fc (R&D Systems) at 37°C for 1 h with constant gentle mixing. Sample preparation and electrophoretic mobility shift assay (EMSA) were then performed using the NativePAGE gel system (Invitrogen) according to the manufacturer’s recommendations. Digital detection was done using a storage phosphor screen with the Typhoon Trio (GE Healthcare), and images were compiled using Adobe Photoshop Elements, version 5.0 (Adobe Systems).

**Immunohistochemistry**

Immunohistochemistry detecting IL13Ra2 expression was performed as previously described.26 Photomicrographs were taken with a 40x magnification lens with a Retiga 4000 camera using ImagePro Plus, version 5.1. Images were processed with Adobe Photoshop Elements, version 5.0 (Adobe Systems).

**Autoradiography**

 Autoradiography was performed as previously described4 on snap-frozen G48a subcutaneous tumor sections or clinical GBM specimens obtained from the Wake Forest University Brain Tumor Center of Excellencepository. Digital detection was done using a storage phosphor screen with the Typhoon Trio (GE Healthcare), and images were compiled using Adobe Photoshop Elements, version 5.0 (Adobe Systems). All clinical tissue sections were scored independently by 2 reviewers on the basis of the overall specific signal intensity throughout the section and assigned a score (0, none; 1, low; 2, moderate; or 3, strong).

**Receptor Binding Study on Tumor Cells**

G48a cells were plated at $8 \times 10^4$ cells per well in 24-well plates and allowed to adhere overnight. For saturation curve binding analysis, cells were incubated in 300 µL of growth medium in duplicates containing 0.1–20 nM concentrations of $^{125}$I-TQM13 (specific activity 233 cpm/fmol) for 2 h at 4°C. Cells were then washed gently 3 times with ice-cold PBS and solubilized in 500 µL of 0.1% NaOH. Lysate was harvested into scintillation vials; scintillation cocktail was added, and its activity was counted using a Beckman Coulter LS6000SC scintillation counter. Non-specific binding was determined at each concentration of labeled TQM13 by adding 500-fold molar excess of the non-labeled TQM13. Non-specific binding was subtracted from all the data points to produce specific binding values. The results represent an average of duplicate determinations in 1–3 independent assays with variations being <20% from the mean. Saturation binding studies were performed similarly with U-251 and B16 tumor cells. In competition studies, $8 \times 10^4$ G48a cells were incubated with serial dilutions of TQM13 at 4°C for 1 h. Next, labeled TQM13 was added at a final concentration of 0.5 nM and incubated with cells for an additional 2 h. Then, the cell-bound radioactivity was determined as above.16 Binding data were analyzed with GraphPad Prism software and receptor number estimated by GraphPad radioactivity calculator (GraphPad Software).

**In Vivo Biodistribution Experiment**

Actively growing G48a cells were implanted subcutaneously into male athymic nude (nu/nu) mice at $1 \times 10^7$ cells per mouse in 200 µL of PBS/Matrigel (BD Biosciences), as recommended by the manufacturer. When tumors reached 500 mm$^3$, mice were injected intravenously with 100 µCi $^{125}$I-TQM13 ($n=6$) or BDef13 ($n=2$) and killed 24 h after injection. Muscle and tumor tissues were harvested, weighed, snap-frozen, and counted in a γ-counter. Counts per wet weight were calculated for each tissue sample, and the tumor-to-muscle ratio was obtained.

**PET Experiment**

Luciferase-expressing G48a GBM subcutaneous tumors were initiated in the front and hind flanks of male athymic nude (nu/nu) mice as described in the biodistribution study (2 tumors per mouse). When tumors reached 500 mm$^3$, mice were injected intraperitoneally with luciferin-D (50 mg/kg) and imaged using the IVIS-100 Imaging System (Caliper Life Sciences) for tumor confirmation prior to PET imaging study. Mice were then injected intravenously with 100 µCi $^{124}$I-TQM13 ($n=2$) or BDef13 ($n=2$) and imaged at 2, 4, 24, and 48 h after injection with use of a microPET P4 scanner (Siemens/CTI Concorde). Before and during the 20-min scans, all mice were anesthetized and maintained by 1.5–2.5% isoflurane. PET images were analyzed by defined region of interest (ROI) using AMIDE software (Stanford School of Medicine).
In Vivo Antitumor Experiment

G48a GBM cells were implanted subcutaneously into male athymic nude (nu/nu) as described in the biodistribution experiment. The treatment started when established tumors were formed. Radiolabeled \(^{131}\)I-TQM13 (200 μCi/mouse) or controls (unlabeled TQM13, \(^{131}\)I-labeled control or saline mock injection) were injected intratumorally and repeated 7 days after the initial treatment (n = 5 per group). Tumor measurements were obtained weekly using a digital caliper. When tumors reached a volume exceeding 1000 mm\(^3\), mice were killed, and their tumors were removed then snap frozen.

Orthotopic IL13Ra2-expressing GBM tumors were established by stereotaxic implantation of \(2 \times 10^5\) actively growing G26-H2 murine glioma cells in C57BL/6 mice. In brief, mice were anesthetized with a ketamine/xylazine mixture (114/17 mg/kg), and a 0.45 mm burr hole was made 2 mm lateral and 0.5 posterior to the bregma in the right cerebral hemisphere through a scalp incision. Stereotaxic injection was performed on a Just For Mice stereotaxic apparatus (Harvard Apparatus) using a 10-μL syringe (Hamilton) with a 30-gauge, 1-inch flat needle, inserted through the burr hole to a depth of 3.2 mm, and constant infusion at a rate of 0.5 μL/min to a total volume of 5 μL was delivered by a Nanomine Programmable Syringe Pump (Harvard Apparatus).27,28

Five days after implantation, mice were imaged using the IVIS-100 Imaging System (Caliper Life Sciences) as described in the PET experiment to confirm tumor establishment. Animals with confirmed intracranial tumors via luminescent signal were randomly stratified to treatment and control groups. They were then administered 2 treatments of radiolabeled \(^{131}\)I-TQM13 (n = 8), radioactivity control (n = 5) or mock injection with saline (n = 7) at day 10 (90 μCi/mouse) and day 20 (142 μCi/mouse) after tumor implantation. During these treatments, mice were again anesthetized with ketamine-xylazine, and therapeutic agents or controls were administered via the same burr hole made for tumor implantation. To mimic convection-enhanced delivery, drugs were infused at a constant rate of 0.7 μL/min by positive pressure from a Nanomine Programmable Syringe Pump (Harvard Apparatus). Animals losing ≥20% of their body weight or having trouble ambulating or feeding were euthanized. Mice with tumors growing outside of their intracranial space were eliminated from the study.

Statistical Analyses

Statistical significance was determined using Student’s t test. Error bars represented mean ± standard deviation. To evaluate the anti-tumor efficacy, Kaplan-Meier survival curves of the control and radiolabeled construct treatment groups were compared via the log-rank test. All data were analyzed using GraphPad Prism software (GraphPad Software).

Results

Recombinant Protein Expression and Purification

To express recombinant proteins, we used a prokaryotic expression system under T7-promoter control. Pre- and post-induction cultures demonstrated tight regulation of expression, evidenced by a prominent 16-kDa protein band both on stained gel and Western blot analyses (Fig. 2A and B). Proteins were expressed in the insoluble inclusion bodies (Fig. 2C). They were therefore subsequently solubilized, denatured, then refolded and purified as we previously described,15 yielding 0.5–1.5 mg of purified recombinant protein per liter of culture (Fig. 2D).

To determine whether TQM13 had refolded correctly and that the multiple mutations did not alter the characteristic α-helix rich secondary structure of IL13, CD analysis was performed. We found that our recombinant TQM13 had a spectrum with 2 minima at approximately 208 and 222 nm, similar to wild-type IL13 (Fig. 2E). Similar results were obtained for the binding-deficient control, BDef13 (not shown). This suggests that the novel targeted multiply-mutated IL13 derivative maintained a similar secondary structure to IL13.

TQM13 Demonstrates Affinity Toward the Cancer-Associated IL13Ra2

IL13Ra2-expressing GBM cell lines and tumors are highly sensitive to chimeric cytotoxins consisting of human IL13 and derivatives of bacterial toxins, such as Diphtheria toxin or Pseudomonas exotoxin A.14 Of importance, we have demonstrated that this potent killing effect by these cytotoxins is mediated through specific targeting of the cancer-restricted IL13Ra2.14 The G26 murine glioma cell line G26-H2 expresses human IL13Ra2 and is highly susceptible to IL13-based cytotoxins, in contrast to the G26 parental cell line, which does not express any IL13 receptors and is not susceptible to IL13-based cytotoxins (Fig. 3A).16,20 To determine whether TQM13 had affinity toward IL13Ra2, we pre-incubated G26-H2 murine glioma cells with excess TQM13 to saturate and block the IL13Ra2 binding sites prior to applying a potent IL13Ra2-targeted bacterial cytotoxin. In the absence of competing TQM13, G26-H2 cells were effectively killed by the IL13Ra2-targeted IL13-based cytotoxin (Fig. 3A). In contrast, when the cells were pre-incubated with excess TQM13, the cytotoxin no longer killed cells, indicating binding and saturation of IL13Ra2 sites by TQM13 (Fig. 3A). As expected, such competition was not observed with BDef13 (Fig. 3A) because it did not effectively bind and saturate IL13Ra2 to prevent cell killing by the cytotoxin. These data indicate that TQM13, but not the BDef13 control, binds to the tumor-associated IL13Ra2.

We next quantified the IL13Ra2 binding of TQM13 or BDef13 using surface plasmon resonance in which we separately delivered the ligands over immobilized
recombinant hIL13Rα2-Fc. TQM13 exhibited rapid, concentration-dependent binding to IL13Rα2 and little dissociation during buffer delivery (Fig. 3B and C). These kinetic studies demonstrated that, at each concentration tested, TQM13 bound rapidly and strongly to the cancer-restricted IL13Rα2 with a $K_d$ of $\approx 5$ nM for the initial binding step. In contrast, BDef13 bound $\approx 25$-fold more slowly and dissociated $\approx 10$-fold more quickly, yielding a 250-fold weaker $K_d$ of $\approx 1.2$ mM (Fig. 3B and D). The concentration dependence of the maximum binding RU signals yielded a similar pattern of tight binding with TQM13 and significantly decreased binding of BDef13 toward the tumor-associated IL13Rα2 (Fig. 3B).

**TQM13 Does Not Bind or Activate the Physiologically Abundant IL13Rα1/IL4Rα**

To assess activity of TQM13 toward the physiologically abundant IL13Rα1/IL4Rα receptor, we performed a functional cell proliferation assay on TF-1 cells, which are pre-leukemic human B cells that express the physiologically abundant IL13Rα1/IL4Rα heterodimer and only proliferate in the presence of cytokines, such as GM-CSF, IL13, or IL4.25,29,30 As expected, the cells proliferated in a dose-dependent manner in response to wtIL13. In contrast, TQM13 did not induce TF-1 cell proliferation even when present at very high concentrations, such as 1000 ng/mL (Fig. 4A). To examine whether the lack of TF-1 cell proliferation was attributable to abrogated function versus disrupted binding toward the physiologically abundant IL13Rα1/IL4Rα heterodimer, we performed a competition assay in which we pretreated TF-1 cells with 100x excess of TQM13 prior to adding wtIL13. However, excess TQM13 did not compete with wtIL13 for the IL13Rα1/IL4Rα binding sites and, thus, did not prevent wtIL13-induced proliferation (Fig. 4B), indicating a lack of effective binding.

To further investigate potential interactions between TQM13 and IL13Rα1/IL4Rα in a direct manner, we reconstituted the physiologically relevant binding complex in a Biacore experiment. We immobilized soluble recombinant human IL4Rα onto a flow cell and confirmed its

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**Fig. 2.** Expression and purification of TQM13 and BDef13. (A) SDS-PAGE analysis of TQM13 protein expression in *E. coli*. (B) Western blot probing for IL13/derivatives of TQM13 pre- and post-IPTG induction samples. (C) SDS-PAGE analysis of TQM13 purification process via BugBuster and Nickel-based affinity chromatography. (D) Western blot for IL13/derivatives of purified TQM13 and BDef13. (E) Circular dichroism spectroscopy measures helical structures of TQM13 in comparison to that of wtIL13, demonstrating two spectral minima at approximately 208 and 222 nm for both, which indicates similar α-helical enriched secondary structures. Similar findings were observed for BDef13 (not shown).
functionality by injecting IL4, which bound fast and tight (not shown). In contrast, as expected, wtIL13 did not bind the immobilized IL4R

receptor (Fig. 4C), because wtIL13 must first bind (weakly) to IL13R

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to recruit the IL4R

receptor into the heterodimer.15 Similarly, IL13.E13K and TQM13 did not bind alone to IL4R (not shown). However, when injected as a preformed wtIL13/IL13Rα1 complex, there was significant binding to the immobilized IL4Rα, reconstituting the wtIL13/IL13Rα1/IL4Rα complex (Fig. 4C). In contrast, mixtures of IL13.E13K and IL13Rα1 showed significantly decreased binding (~70%) to the immobilized IL4Rα, demonstrating directly for the first time the adverse effects of even a single mutation on the physiological receptor binding. Furthermore, mixtures of the multiply mutated TQM13 and IL13Rα1 demonstrated a 90% overall binding reduction to IL4Rα, compared with the wtIL13 mixture, a >60% additional decrease, compared with the single mutant. Of importance, there was no statistically significant difference between premixed TQM13/IL13Rα1 binding to immobilized IL4Rα and background (IL13Rα1 alone) binding to IL4Rα. These data for the first time directly demonstrate a significant additive effect of combining multiple mutations on redirecting IL13 away from its physiologically abundant receptor complex (Fig. 4C).

125I-TQM13 Binds Tumor Samples Expressing the Cancer-Associated IL13Rα2

To demonstrate the potential of using TQM13 to deliver diagnostic and therapeutic radioisotopes to IL13Rα2-expressing GBMs, we radioiodinated TQM13 with 125I and confirmed the radiochemical purity at ≥98% using ITLC (Supplemental Figure 1). Furthermore, we directly confirmed binding of radiolabeled TQM13 to IL13Rα2 using an electrophoretic mobility shift assay (EMSA). We only observed a shift in the molecular weight (mw) of radiolabeled TQM13 mixed with soluble IL13Rα2 but not in BDef13 mixed with the receptor, indicating that only TQM13 bound IL13Rα2 (Fig. 5A). In addition, we found that 125I-TQM13 bound IL13Rα2 expressed on tumor samples by performing digital autoradiography on snap-frozen IL13Rα2-expressing G48a subcutaneous tumor sections (Fig. 5B), because these tumors express high amounts of IL13Rα2, confirmed here by IHC (Fig. 5B, Supplemental Figure 2). Of importance, G48a tumor specimens bound 125I-TQM13, but not the radiolabeled binding-deficient control (Fig. 5B). The binding of TQM13 was highly specific because it was displaced by excess unlabeled TQM13 but not excess unlabeled BDef13. Furthermore, we found a direct relationship between

Fig. 3. Binding activities of TQM13 and BDef13 to the cancer-restricted IL13Rα2. (A) Cytotoxicity assay on G26-hIL13Rα2(+) cells (G26-H2) with IL13-based bacterial cytotoxin in the presence or absence of TQM13 and BDef13 as competitive blockers, demonstrating that TQM13 but not BDef13 successfully saturated IL13 binding sites and protected cells from being targeted by cytotoxin. IL13-based cytotoxin did not affect G26-hIL13Rα2(−) cells. (B) Biacore binding concentration-dependent signal changes for TQM13 and BDef13 binding to the cancer-restricted IL13Rα2. (C) SPR kinetic profiles for TQM13 binding to IL13Rα2. The solid lines were obtained by fitting these data, and those in a duplicate experiment, to a bivalent analyte model to obtain the forward and reverse rate constants which yielded a 4.9 nM Kd for the initial binding step. (D) SPR kinetic profiles for BDef13; solid lines as in (C), 1.2 µM Kd.
IL13Rα2 expression and 125I-TQM13 binding to cells and tumor specimens (Supplemental Figure 3). Thus, radioiodinated TQM13 retained its affinity toward IL13Rα2 expressed on GBM tumors.

**Radiolabeled TQM13 Binds to Clinical GBM Specimens**

The cancer-restricted IL13Rα2 has been shown to be overexpressed in the vast majority of GBMs but is not present in normal brain tissue.4 Previously reported autoradiographic and immunohistochemical analysis indicated that the prevalence of IL13Rα2 expression in patients with GBM is approximately 75%.3,31,32 To determine whether the binding activity by TQM13 matched the previously reported prevalence of the receptor, we performed autoradiography with 125I-TQM13 on 10 GBM specimens. All tissue sections were scored independently by 2 reviewers on the basis of the overall specific signal intensity throughout the section and assigned a general qualitative score (0, none; 1, low; 2, moderate; or 3, strong). Our results indicated that 125I-TQM13 bound to 8/10 samples tested with 3 samples having low binding, 4 moderate binding, and 1 strong binding (Fig. 5C). This was similar to the expected number of IL13Rα2-positive samples.

**TQM13 Specifically Binds to IL13Rα2-Expressing Tumors In Vivo**

In vivo tumor targeting studies were performed on mice bearing subcutaneous IL13Rα2-expressing G48a tumors at 24 h after intravenous injection of 125I-TQM13 or 125I-BDef13. 125I-TQM13 preferentially bound to IL13Rα2-expressing tumors at a 7-to-1 tumor-to-muscle ratio 24 h after intravenous delivery of radiolabeled ligands. In contrast, 125I-BDef13 did not specifically bind to IL13Rα2-expressing tumors because its activity remained similar to normal muscle activity at 24 h after injection (Fig. 6A). In addition, we tested the tumor targeting of 125I-TQM13 in B16 tumors that do not express IL13Rα2 and found no significant tumor uptake over background muscle but a significant difference between uptake in IL13Rα2-expressing G48a tumors (Supplemental Figure 4; P ≤ .05 between G48a-IL13Rα2(+) tumors and B16-IL13Rα2(−) tumors).

Fig. 4. Activation and binding of IL13 and mutants towards the physiologically abundant IL13Rα1/IL4Rα heterodimer. (A) TF-1 proliferation assay in the presence of TQM13 or wtIL13 demonstrating only wtIL13 but not TQM13 results in IL13Rα1/IL4Rα induced proliferation (**P ≤ .01, ***P ≤ .001). (B) TF-1 proliferation assay with 10 ng/mL wtIL13 in the presence or absence of 100-fold excess TQM13. (C) SPR study on binding activity of IL13 and mutants to the reconstituted IL13Rα1/IL4Rα complex. wtIL13 or IL13Rα1 alone did not demonstrate significant binding towards immobilized IL4Rα. However, when wtIL13 and IL13Rα1 were premixed, the complex bound to immobilized IL4Rα, reconstituting the physiologically abundant receptor. Conversely, premixing IL13.E13K and IL13Rα1 resulted in a 70% decrease in binding. Premixing TQM13 and IL13Rα1 resulted in a ~90% decrease in binding, which was not significantly different from background binding (IL13Rα1 alone). **P ≤ .01 comparing [wtIL13 + IL13Rα1] complex and [TQM13 + IL13Rα1] complex, *P ≤ .05 comparing (IL13.E13K + IL13Rα1) complex with [TQM13 + IL13Rα1] complex.
Encouraged by these promising in vivo results, we evaluated the feasibility of using TQM13 as a PET probe to non-invasively image real-time expression of the attractive tumor-associated biomarker IL13Rα2 in the G48a GBM subcutaneous model. PET scans at 24 and 48 h after intravenous radioligand delivery demonstrated significant localization to the IL13Rα2-expressing tumors by 124I-TQM13 but not 124I-BDef13 (Fig. 6B). ROI analysis quantified the tumor-to-muscle ratio by 124I-TQM13 be 7-to-1 at 48 h after injection, whereas 124I-BDef13 remained similar to background activity (Fig. 6C). In addition, radioactivity was transiently localized in the bladder and excreted in the urine (not shown).

Radiolabeled 131I-TQM13 Demonstrates Safety and Antitumor Activity Against Subcutaneous IL13Rα2-Expressing Tumor Xenografts In Vivo

After confirming that TQM13 targets IL13Rα2-expressing tumors in vivo, we tested the potential of TQM13 as a delivery vehicle for molecular-targeted radiotherapy by performing antitumor experiments in mice bearing the IL13Rα2-expressing G48a GBM xenografts. GBM cells were implanted subcutaneously in the flank of immunocompromised mice, and IL13Rα2-targeted radiotherapy was initiated after tumors reached a measurable size of about 100 mm3 (Fig. 7A). For this pilot experiment, we administered 2 intratumoral injections of 200 μCi 131I-TQM13 (7 days apart), which resulted in tumor growth inhibition and increased overall survival (Fig. 7A and B). All mice treated with 131I-TQM13 survived until at least day 35 after treatment initiation, whereas none of the control animals remained at this time (Fig. 7B). Of importance, no adverse effects or toxicities were observed in the treated mice, compared with the controls. Thus, this indicates for the first time to our knowledge the suitability of targeting IL13Rα2 with molecular-targeted radiotherapy in vivo using our novel ligand.

Radiolabeled 131I-TQM13 Demonstrates Safety and Antitumor Activity Against Orthotopic Synergic IL13Rα2-Expressing Tumors In Vivo

To further demonstrate the therapeutic potential of 131I-TQM13, we tested its in vivo safety and efficacy...
in an orthotropic GBM model after loco-regional delivery to mimic the potential translational scenario. For this orthotopic study, we used the G26-H2 synergeic murine glioma model that we and others have developed to express human IL13Rα2. In addition to the intracranial location of the tumors, using this model allowed us to evaluate the potential safety and efficacy of 131I-TQM13 in animals with an intact immune system. Tumor cells were stereotactically implanted intracranially, and tumor establishment was verified in all mice via bioluminescent imaging prior to treatment. Expression of IL13Rα2 in implanted tumors was also confirmed via postmortem autoradiography (Supplemental Figure 5) in selected animals. To test the safety and efficacy of 131I-TQM13, tumor-bearing mice were administered loco-regionally with 94 μCi and 142 μCi of 131I-TQM13 on days 10 and 20, respectively, after tumor implantation. Loco-regional administration was performed after stereotactically targeting the tumor with a needle and administering the therapy over 20–30 min under constant pressure from a syringe pump, similar to convection-enhanced delivery. Our results demonstrated that 131I-TQM13 significantly increased survival in mice bearing orthotopic tumors, compared with radioactive control and saline mock injection (Fig. 8). Furthermore, 2 (25%) of 8 treated intracranial tumor-bearing mice from the 131I-TQM13 group were cured and demonstrated no evidence of tumor burden at the conclusion of the study (day 80). Of importance, no adverse effects or toxicities were observed in the treated mice, compared with the controls. These data confirm and strengthen our finding that the novel ligand TQM13 is a potential platform to target the tumor-associated IL13Rα2 with molecular-targeted radiotherapy.
mutants at high doses. Furthermore, the disruption of TQM13 binding to the physiologically abundant IL13Rα2 receptor was further demonstrated by its inability to block wtIL13-mediated IL13Rα2/IL4Rα receptor activation even at 100-fold excess concentration, in contrast to what has been reported for the single mutant IL13.E13K. The additive effects of multiple mutations in abrogating the interaction between TQM13 and IL13Rα1/IL4Rα were further confirmed by a significant (~60%) binding reduction in our SPR (Biacore) reconstitution experiment, compared with a single mutant (IL13.E13K), and a 90% overall reduction, compared with wtIL13 (Fig. 4). This broken interaction between TQM13 and IL13Rα1/IL4Rα promises to increase the therapeutic window and strengthen the probability of success for IL13Rα2-targeted agents.

In addition to in vitro binding studies, we for the first time demonstrated the feasibility of using TQM13 to target IL13Rα2-expressing tumors after systemic injection using non-invasive PET imaging (Fig. 6). This is of great significance to other IL13Rα2-expressing tumors beside GBM, such as peripheral nerve sheath tumors, medulloblastomas, and meningiomas, because TQM13 can be used to non-invasively assess IL13Rα2 expression using a PET scan. Of importance, this information can be used to personalize biomarker-targeted therapies only to patients expressing the targeted receptor. This approach of personalized anti-cancer therapy has been shown to predict response to biomarker-targeted therapy a priori when used to target other tumor-restricted biomarkers.37-40

A significant challenge to delivering molecular-targeted therapeutics to GBM is exclusion from the brain by the blood-brain barrier (BBB) after systemic injection. As expected for a macromolecule, we have found that IL13 and TQM13 do not cross the intact BBB (unpublished), although we are currently studying the ability to target GBM in the presence of a tumor-induced leaky BBB. However, despite the limitations caused by the BBB, investigators are working on various mechanisms of bypassing the BBB to deliver molecular therapeutics to GBM, including using molecular targeted nanoparticles capable of crossing the BBB or altering the BBB with drugs and/or radiation to make it more permeable to macromolecules.41,42 Therefore, by demonstrating significant tumor targeting of TQM13
in vivo after systemic injection (Fig. 6), we validated its potential as a tumor-targeting ligand capable of delivering diagnostic and therapeutic entities selectively to biomarker-expressing tumors.

A promising alternative to systemic delivery of anti-GBM agents is direct loco-regional injection of therapeutics into tumors or resection cavities. In addition to bolus injections, convection enhanced delivery (CED) has been used as a clever approach of bypassing the BBB by directly perfusing therapeutic agents straight into the brain parenchyma or intratumorally. Previous clinical trials using CED for targeted cytotoxins demonstrated that large areas of human brain can be infused by this technique without producing tissue injury, increased intracranial pressure, or systemic toxicity resulting from drug leaking into the circulation. Furthermore, recent innovations in delivering other therapeutic agents to GBM via CED demonstrated that incorporating imaging to confirm proper catheter placement significantly increased the efficacy of this approach. Thus, direct intraparenchymal delivery is an innovative approach of safely delivering sufficiently higher doses of macromolecular anti-cancer therapeutics to GBM, compared with systemic delivery. The development of local delivery methods of bypassing the BBB opens the door to use of molecular-delivered radioactivity as a viable therapeutic option against GBM. Therefore, we conjugated the cytotoxic β-emitting radioisotope $^{131}$I to TQM13 and demonstrated specific inhibition of tumor growth after direct injection into IL13Ro2-expressing GBMs. We used subcutaneous human tumor xenografts in this pilot in vivo experiment to directly demonstrate the targeting specificity of TQM13, compared with the untargeted, binding deficient BDef13. Furthermore, considering the unique clinical challenges posed by brain tumors and the critical role played by the immune system in cancer radio-immunotherapy, we proceeded to evaluate safety and therapeutic efficacy of $^{131}$I-TQM13 in mice bearing synergetic intracranial gliomas. We once again confirmed the antitumor effect of $^{131}$I-TQM13 evidenced by significantly prolonged survival in the treatment group compared with that of controls. These data indicate that TQM13, our novel IL13Ra2-targeting ligand, is suitable for loco-regional convection enhanced delivery, which makes it a promising delivery vehicle for GBM management.

Our work demonstrated for the first time the potential of IL13Ra2-targeted radiotherapy approaches. Of importance, molecular delivery of cytotoxic radioactivity to GBMs has recently shown promising therapeutic potential. Specifically, investigators used an $^{131}$I-labeled antibody that targeted Tenascin-C (TN-C), an extracellular matrix brain glycoprotein shown by some groups to be overexpressed in GBM. Early phase clinical trials indicated incremental benefit of $^{131}$I-labeled antibody in combination with surgery, radiation therapy, and chemotherapy with temozolomide. Although we also used $^{131}$I labeling in our pilot in vivo experiments because of its ready availability and ease of radiolabeling, β-emitting radioisotopes may be suboptimal for local delivery in brain cancer, as evidenced by radiotoxicity that occurred to the normal surrounding brain tissue in the pioneering clinical trial that used $^{131}$I-labeled anti-TN-C antibodies. Because $^{131}$I emits β-rays that travel 0.08–2.3 mm (10–230 cells), it will inevitably kill untargeted surrounding normal brain tissue. However, α-emitting radionuclides, such as $^{211}$Bi or $^{212}$Bi, are up to 1000-fold more potent than β-particles emitted by $^{131}$I and have a significantly shorter range (6–8 cells). This is of great significance in GBM, in which tumor cells infiltrate normal brain parenchyma that may be damaged if the range of radio-toxic emissions is too long. Furthermore, α-particles efficaciously elicit irreparable double-stranded DNA breaks, in contrast to β-emitters. These advantages are supported by the only clinical study to date that delivered an α-emitter in GBM via loco-regional delivery, in which $^{211}$Bi was conjugated to 81C6, an anti-TN-C antibody. This study concluded that there was significantly less radionecrosis, compared with patients treated with $^{131}$I-81C6 anti-TN-C antibody. This and other clinical studies in different malignancies confirm that the strength and short distance traveled by α-particles may make them more suitable than β-emitters for some molecular radiotherapy applications. Of importance, IL13Ra2 is a receptor membrane protein that is internalized after engaging IL13 or mutants, which is highly advantageous when using short-range α-emitting radioisotopes, in contrast to TN-C, which is an extracellular matrix protein. Thus, this proof-of-concept work validating our novel ligand as a carrier for IL13Ra2-targeted radioimmunotherapy manifests the therapeutic potential of our approach, which can be further optimized with other choices of radioisotopes in future studies.

Antibodies have played a key role in the development of molecular targeting systems that are capable of delivering cytotoxic radioisotopes specifically to biomarker-expressing tumor cells. However, whole antibodies are suboptimal for molecular-targeted radiotherapy approaches because of their long plasma half-life that results in prolonged exposure of normal tissue to radiation. Although smaller-sized antibody fragments and peptides have shown promise in improving these shortcomings, our approach exploits IL13, a natural high-affinity ligand that we successfully retargeted to bind only the tumor-associated IL13Ra2 but not the physiologically abundant IL13Ra1/IL4Ra. We used rational site-directed mutagenesis to identify the structurally significant parts of IL13 and used this knowledge to engineer TQM13 to optimize its potential as an anti-tumor ligand. In our system, this approach may be advantageous over using antibodies because of the strong binding and very poor dissociation after ligand is bound to IL13Ra2 (Fig. 3). These favorable binding characteristics are not typically seen by antibody derivatives and are more characteristic of the irreversible biotin/avidin binding. Thus, the data presented here demonstrate for the first time the power of this approach in our receptor system and potentially for...
targeting other tumor-associated receptors with cytotoxic radionuclides.

Conclusion

On the basis of these promising studies that demonstrated a novel approach of targeting IL13Rα2 using rational site-directed mutagenesis, we anticipate that our optimized ligand will aid in targeting the GBM-associated IL13Rα2 biomarker with diagnostic and therapeutic entities, including radioisotopes.

Supplementary Material

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

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