Published in final edited form as:

Mol Pharm. 2016 July 5; 13(7): 2563–2570. doi:10.1021/acs.molpharmaceut.6b00372.

ImmunoPET imaging of CD146 expression in malignant brain tumors

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Abstract

Recently, the overexpression of CD146 and its potential as a therapeutic target in high-grade gliomas, the most lethal type of brain cancer, was uncovered. In this study, we describe the generation of ⁸⁹Zr-Df-YY146, a novel ⁸⁹Zr-labeled monoclonal antibody (mAb) for the targeting and quantification of CD146 expression in a mouse model of glioblastoma, using noninvasive immunoPET imaging. YY146, a high affinity anti-CD146 mAb, was conjugated to deferoxamine (Df) for labeling with the long lived positron emitter ⁸⁹Zr ($t_{1/2}$: 78.4 h). *In vitro* assays, including flow cytometry, immunofluorescence microscopy, and Western blot were performed with two glioblastoma cell lines, U87MG and U251 to determine their CD146 expression levels. Also, YY146 and Df-YY146's CD146-binding affinities were compared using flow cytometry. *In vivo* CD146-targeting of ⁸⁹Zr-Df-YY146 was evaluated by sequential PET imaging, in athymic nude mice bearing subcutaneously implanted U87MG or U251 tumors. CD146 blocking, *ex vivo* biodistribution, and histological studies were carried out to confirm ⁸⁹Zr-Df-YY146 specificity, as well as the accuracy of PET data. *In vitro* studies exposed elevated CD146 expression levels in U87MG cells, but negligible levels in U251 cells. Flow cytometry revealed no differences in affinity between YY146 and Df-YY146. ⁸⁹Zr-labeling of Df-YY146 proceeded with excellent yield (~80%), radiochemical purity (> 95%), and specific activity (~44 GBq/μmol). Longitudinal PET revealed prominent and persistent ⁸⁹Zr-Df-YY146 uptake in mice bearing U87MG tumors that peaked at 14.00 ± 3.28 %ID/g (n=4), 48 h post injection of the tracer. Conversely, uptake was significantly lower in CD146-negative U251 tumors (5.15 ± 0.99 %ID/g, at 48 h p.i.; n=4; $P < 0.05$). Uptake in U87MG tumors was effectively blocked in a competitive inhibition experiment, corroborating the CD146-specificity of ⁸⁹Zr-Df-YY146. Finally, *ex vivo* biodistribution validated the accuracy of PET data and histological examination successfully correlated tracer uptake with *in situ* CD146 expression. Prominent, persistent, and specific uptake of ⁸⁹Zr-Df-YY146 was observed in brain tumors, demonstrating the potential of this radiotracer for noninvasive PET

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Financial Disclosure

Authors declare no conflict of interest.

Supporting Information

Additional figures and tables are provided as supporting information

imaging of CD146 expression. In a future clinical scenario, ^{89}Zr -Df-YY146 may serve as a tool to guide intervention and assess response to CD146-targeted therapies.

Graphical Abstract

Keywords

CD146; ^{89}Zr ; immunoPET; brain tumor; monoclonal antibody (mAb)

Introduction

To date, glioblastoma – the most common brain malignancy - remains a devastating disease for which current management strategies often fail to prolong patient median survival beyond a few months¹. In spite of recent advancements in the field of neurooncology, an urgent need remains for the development of novel, highly effective therapeutics. The highly heterogeneous character of glioblastoma phenotypes hinders the implementation of across-the-board therapeutic strategies that serves the majority of patients^{2, 3}. A promising approach is personalized medicine, which heavily relies on identifying patient-specific molecular signatures that allow for tailored targeted therapies. Within this context, the necessity to scrutinize the dysregulation of a receptor or molecular pathway becomes evident, as only certain patient populations will benefit from a particular targeted treatment. Hence, it is necessary to have precise tools, not only for diagnosis but to monitor the early efficacy of therapy, especially given the fast progression of glioblastomas. In this regard, positron emission tomography (PET) is now recognized as one of the most valuable molecular imaging technique to scrutinized tumor phenotypes *in vivo*⁴. ImmunoPET, a PET imaging modality that uses antibodies as targeting probes, have been proven effective in noninvasively determining the expression levels of several cancer-associated antigens *in vivo*^{5–7}. Additionally, immunoPET has been shown to outperform magnetic resonance imaging (MRI) and ^{18}F -FDG PET in terms of sensitivity and specificity in several clinical scenarios^{8, 9}.

CD146 has emerged as a promising target in a variety of diseases, including melanoma, breast cancer, and prostate cancer. CD146, also known as MCAM or MUC18, plays an important role in several processes involved in tumor angiogenesis, progression, and metastasis¹⁰. In melanoma, CD146 has been shown to mediate homotypic and heterotypic cell adhesion and its overexpression linked to upregulation of matrix metalloproteinases (MMPs) and the acquisition of a complex metastatic behavior¹¹. Moreover, CD146 is highly

upregulated in more than 80% of metastatic melanomas, but not observed in the benign lesions. Tissue array studies performed in primary melanomas revealed CD146 as an independent, highly specific negative prognostic marker of patient survival¹². Similar results have been observed in other cancer types where CD146 expression invariably correlated with poor survival, including breast cancer, lung carcinoma, ovarian cancer, and gastric cancer^{13–16}. Furthermore, the low expression of CD146 in normal tissues makes CD146 an attractive candidate for targeted therapies. In fact, targeting CD146 with monoclonal antibodies has not only demonstrated value for the determination of CD146 expression *in vitro*, but also has proven efficacious at inhibiting tumor growth and metastasis *in vivo*¹⁷. Recently, the expression of CD146 was described and correlated with aggressiveness in high-grade gliomas¹⁸.

In anticipation of the implementation of novel CD146-targeted therapies for brain cancer, we developed an immunoPET imaging agent based on YY146, an anti-CD146 monoclonal antibody (mAb). YY146 was labeled with ⁸⁹Zr (T_{1/2}: 78.4 h), and the expression of CD146 was monitored in a mouse model of glioblastoma. Owing to the excellent targeting properties of ⁸⁹Zr-Df-YY146 *in vivo*, we accurately determined both expression levels and tumor burden noninvasively in mice bearing CD146-positive tumor xenografts, in a specific manner. In a future clinical scenario, PET imaging with ⁸⁹Zr-Df-YY146 will be of great utility to identify patient subpopulations with increased likelihood of responding to CD146-targeted therapies, and to monitor early response to such management strategies.

Materials and Methods

Reagents

YY146 was generated and purified as described previously¹⁸. Chelex 100 resin and p-SCN-Bn-deferoxamine were purchased from Sigma Aldrich and Macrocyclics, Inc., respectively. Unless otherwise noted, all other materials and reagents were obtained from Thermo Fisher Scientific. Mili-Q (resistivity > 18.2 MΩ·cm) was employed in the preparation of buffers and solution. In addition, buffers were treated with Chelex 100 resin to remove heavy metal contaminants.

Cancer cell lines and Animal models

Human glioblastoma cell lines, U87MG and U251, were purchased from the American Type Culture Collection (ATCC) and cultured following provider's recommendations. Briefly, U87MG cells were culture in Dulbecco's modified Eagle medium (Invitrogen) supplemented with penicillin (100 U/mL; Invitrogen), streptomycin (100 µg/mL; Invitrogen), and FBS [10% (wt/vol); Sigma-Aldrich] and incubated in a 5% CO₂ atmosphere at 37°C. U251 cells were culture under similar condition using RPMI 1640 medium (Invitrogen). Cells were employed for *in vitro* and *in vivo* studies after they reached 80–90% confluency.

Animal studies were performed under the approval of the University of Wisconsin Institutional Animal Care and Use Committee. U87MG and U251 tumor xenografts were generated by subcutaneous injection of 100 µL of a 1:1 Matrigel (BD Biosciences, San Jose,

CA) and cell culture media suspension containing 1×10^6 cancer cells, in the lower flank of athymic female nude mice. Tumor size was regularly monitored and in vivo studies were carried out when the tumor reached 5–10 mm in diameter, approximately 3 weeks after implantation.

Isotope production and radiochemistry

^{89}Zr was produced in a GE PETTrace biomedical cyclotron via irradiation of natural Yttrium targets with 16 MeV protons. Isotope purification was accomplished by trapping ^{89}Zr in a hydroxamate-functionalized column, from which it was eluted in 1 M oxalic acid with and specific activity of $\sim 111 \text{ GBq}/\mu\text{mol}$. YY146 was conjugated via amine-isothiocyanate reaction to deferoxamine (Df) chelator for posterior radiolabeling with ^{89}Zr . For conjugation, $\sim 3 \text{ mg}$ of YY146 in $500 \mu\text{L}$ of PBS were adjusted to pH 8.5–9.0 by adding aliquots of Na_2CO_3 (0.1 M), and a solution of p-SCN-Bn-deferoxamine in DMSO was added to the antibody mixture. The pH was readjusted to 8.5–9.0, and the reaction was left to proceed for 2 h, after which the conjugated mAb was purified by size exclusion chromatography using PD-10 columns. A 5:1 chelator to mAb ratio was employed to ensure minimal disruption in YY146 binding affinity. During radiolabeling, $\sim 121 \text{ MBq}$ ($\sim 3 \text{ mCi}$) of ^{89}Zr were buffered with HEPES (0.5 M; pH = 7.0), 75–100 μg of Df-YY146 added to the radioactive solution, and the reaction was left to proceed for 1 h at 37°C . ^{89}Zr -Df-YY146 was purified by PD-10 columns with PBS as the mobile phase. In order to determine labeling yields and radiochemical purity, $2 \mu\text{L}$ samples of the radiolabeling reaction and the purified fractions were spotted into radio instant thin-layer chromatography (iTLC) plates, run with 50 mM EDTA (pH = 4.5), and read in a phosphor-plate reader (Perkin Elmer). Labeled antibody stayed at the origin ($R_f = 0$) whereas free ^{89}Zr moved with the solvent front ($R_f = 1$).

The number of chelator per antibody was determined via isotopic dilution using a slightly modified reported method¹⁹. Briefly, 3.7 MBq ($100 \mu\text{Ci}$) of $^{89}\text{ZrC}_2\text{O}_4$ were dispensed into 1.5 mL eppendorf vial containing $200 \mu\text{L}$ of HEPES buffer (0.5 M, pH = 7.0) and 0.1, 0.2, 0.5, or 1.0 nmol of non-radioactive ZrCl_4 was added to the vials for a final $250 \mu\text{L}$ volume. Following, 0.1 nmol of Df-YY146 was added to the mixture and the reaction was incubated at 37°C for 1 h. The reaction was quenched with EDTA (1nM), and $2 \mu\text{L}$ samples were taken and the ^{89}Zr labeling yield of each reaction determined by iTLC. Radiolabeling yield was plotted against moles of ZrCl_4 , and a linear regression analysis was performed to determine the amount of ZrCl_4 resulting in a 50% labeling of the protein (N_{50}). N_{50} was divided by twice the moles of the mAb to obtain the chelator to mAb ratio.

Flow Cytometry and Western blot

YY146 and Df-YY146 immunoreactivity towards U87MG and U251 cells was assessed and compared using flow cytometry. Cells were harvested and suspended in PBS containing 2% BSA at a final concentration of 1×10^6 cells/mL. Cells were then incubated with different concentrations of YY146 and Df-YY146 for 30 min at room temperature (RT), rinsed with PBS thrice, and centrifuged at $150 \times g$ for 5 min. Cell pellets were resuspended in PBS (2% BSA) containing AlexaFluor488-labeled goat anti-mouse IgG ($5 \mu\text{g}/\text{mL}$) and incubated for

30 min at RT. Samples were analyzed on a BD FACS Calibur cytometer (Becton-Dickinson) and fluorescence intensity histograms generated using FlowJo software (Tree Star, Inc.).

For western blot assays, cells were lysed with RIPA buffer (Boston BioProducts) and collected cell lysates centrifuged at 12000rpm for 20 minutes. Total protein concentration was determined with an enhanced bicinchoninic acid (BCA) protein assay kit (Beyotime). Cell lysates were mixed with gel-loading buffer, and an equal amount of protein was loaded and separated on 10% SDS/PAGE. Following electrophoretic transfer onto nitrocellulose membranes (iBlot 2 Dry Blotting system), membranes were blotted using a iBind Western System overnight at 4°C, with anti-Mel-CAM (CD146) mouse mAb (Santa Cruz Biotechic) and anti-β-actin rabbit mAb (LI-COR) as primary antibodies, and IRDye-680L-labeled donkey anti-mouse and IRDye-800CW-labeled donkey anti-rabbit conjugated as secondary mAb (LI-COR Biosciences). Membranes were visualized in an Odyssey® CLx Infrared Imaging System (LI-COR Biosciences) and images analyzed using Image Studio Lite (LI-COR Biosciences).

Saturation binding assay

CD146 receptor saturation assay was carried out to determine the YY146 binding affinity (Kd) and the number of CD146 molecules per U87MG cell. To the wells of 96-well filter plates (Corning®, Sigma-Aldrich, St. Louis, MO), $\sim 1.0 \times 10^5$ U87MG cells were seeded, and varying concentrations of ^{89}Zr -DF-YY146 (range 0.03 nM – 100 nM; SA: 48.9 GBq/μmol) were added. Non-specific binding was determined for each concentration by adding 1 μM of unlabeled YY146. The plate was incubated at room temperature with gentle agitation for 2 h, then rinse three times with PBS containing 0.1% BSA, blow-dried, and filters collected and counted in an automated γ-counter. Saturation binding isotherms were plotted using GraphPad Prism software (GraphPad Software, San Diego, CA), and the affinity constant (Kd) and maximum specific binding (Bmax) determined.

PET imaging and Biodistribution

For noninvasive in vivo PET imaging, mice bearing U87MG or U251 tumor xenografts (n = 3–4) were injected intravenously with 200 μL of a PBS solution containing 3.7–5.5 MBq (12.6–18.7 μg) of ^{89}Zr -Df-YY146. Longitudinal studies were designed considering the long circulation of mAb and ^{89}Zr physical half-life and carried out by acquiring PET images at 4, 24, 48, 72, 120, and 168 h after injection of the radiotracer. Before each PET scan, mice were anesthetized with isoflurane (4% induction; 2% maintenance) and place in the scanner in a prone position. List mode scans (time window: 3.432 ns; energy window: 350–650 keV) of 40 million coincidence events each, were acquired in an Inveon microPET/microCT scanner (Siemens Medical Solution). A blocking study was performed in which mice were injected a large dose (50mg/kg) of unlabeled YY146, 24 h prior the administration of ^{89}Zr -Df-YY146 (n=4). PET images were reconstructed using a three-dimensional Ordered Subset Expectation Maximization (OSEM3D) algorithm. Quantification of PET images was accomplished in an Inveon Research Workplace (Siemens Medical Solution) workstation via region of interest (ROIs) analysis; tissue uptake values were reported as percent injected dose per gram of tissue (%ID/g).

In order to corroborate the accuracy of PET data and to obtain a more detailed biodistribution of the tracer in each group, an *ex vivo* biodistribution study was performed. Immediately after the last imaging time point at 168 h p.i., mice were euthanized by CO₂ asphyxiation, and all major tissues/organs harvested, weighed, and counted in an automatic gamma counter (Perkin Elmer). Uptake was also expressed as %ID/g (mean $\pm$ SD).

Confocal microscopy

Immunofluorescence staining of both cancer cells and tumor tissue was performed to assess CD146 expression following a previously reported method¹⁸. In brief, tumors were harvested from mice, embedded in optimal cutting temperature (OCT) compound, frozen, and sectioned into 5 μ m thick slices. Slices were fixed with cold acetone for ~10 min and then blocked with donkey serum for 30 min. Following, tissue sections were incubated overnight at 4 °C with a mixture of YY146 (10 μ g/mL) and rat anti-mouse CD31 mAb (BD Biosciences). Sections were rinsed with PBS, and bound primary mAb was visualized using AlexaFluor488-labeled goat anti-mouse IgG (Invitrogen) and Cy3-labeled donkey anti-rat IgG (The Jackson Laboratory) antibodies. Fluorescent images were recorded using a Nikon Digital Eclipse C1 plus microscope, equipped with 488 nm, 546 nm, and 633 nm excitation lasers.

Statistics analysis

A minimum sample number of 3 were employed to assure statistical power. Differences between groups were analyzed for statistical significance using the unpaired, two-tailed Student's t-test. A confidence interval of 95% was selected and $P < 0.05$ was considered to be statistically significant.

Results

In vitro assays

Flow cytometry experiments were carried out to determine CD146 expression levels in U87MG and U251 cancer cells. U87MG cells displayed markedly high fluorescence indicative of enhanced YY146 binding and elevated CD146 expression, whereas U251 cell showed notably lower or background fluorescence readings (Figure 1A). No appreciable differences were observed when binding affinities were compared between YY146 and Df-YY146, in both U87MG and U251 cells. These results indicate that the presence of the chelator did not alter YY146 immunoreactivity. CD146 immunofluorescence staining also revealed strong YY146 binding to the membrane of U87MG cells, while only background signal in U251 cell (Figure 1B). Finally, Western blot corroborated the results from flow cytometry and immunofluorescence experiments. As seen in Figure 1C, the lane corresponding to U87MG cell lysates showed a very intense band around 110 kDa (MW: 113 kDa) consistent with the presence of high levels of CD146 protein expression. On the contrary, U251 cells showed very low, undetectable CD146 levels. Finally, the number of CD146 receptors per cell and YY146 binding constant (K_d) were determined in U87MG cells through a receptor saturation binding assay. A CD146-affinity constant of 11.29 nM and an average density of ~101,000 CD146 molecules per U87MG cells were determined (Figure S1).

Radiotracer development

In order to facilitate radiolabeling with ^{89}Zr and subsequent PET imaging, deferoxamine chelator was conjugated to YY146 via primary amine-isothiocyanate conjugation reaction. Isotopic dilution experiments using non-radioactive zirconium showed an average of 2.9 chelators per antibody (Figure S2). Df-YY146 was successfully radiolabeled with ^{89}Zr with excellent yields ($> 80\%$; non-decay corrected) and radiochemical purity ($> 95\%$). High specific activities of ^{89}Zr -Df-YY146 (SA; $\sim 44\text{ GBq}/\mu\text{mol}$) were achieved owing to the high radiochemical purity of ^{89}Zr -oxalate.

PET Imaging

Thanks to the long physical half-life of ^{89}Zr ($T_{1/2}$: 78.4 h), the *in vivo* biodistribution of ^{89}Zr -Df-YY146 was monitored up to 7 days (168 h) after intravenous administration into tumor bearing mice. Maximum intensity projection (MIP) PET images revealed a prominent accumulation of ^{89}Zr -Df-YY146 that allowed for accurate tumor delineation in mice bearing CD146-overexpressing U87MG xenograft (Figure 2). In these animals, tumor-to-muscle ratios were high 4 h post injection (p.i.) of the tracer and continued to rise to values well over 10, at the 48 h p.i. imaging time point.

ROI quantitative analysis of PET images (Figure 3, Table S1) unveiled initially high ^{89}Zr -Df-YY146 activity in blood pool and liver, which declined over time evidencing the slow clearing kinetics of the tracer (Figure 3, Table S2). In mice bearing CD146-negative U251 xenografts, liver uptake went from $10.68 \pm 0.94\%$ ID/g at 4 h p.i. to $4.37 \pm 0.38\%$ ID/g at 168 h p.i. ($n=4$), while blood radioactivity showed a more pronounced decline from 12.73 ± 0.71 to $1.47 \pm 0.15\%$ ID/g, in the same period of time. Liver and blood uptake in U87MG-bearing mice were similar in magnitude and trend to those observed in U251 tumor bearing mice: 12.58 ± 2.12 vs. $5.65 \pm 1.44\%$ ID/g for liver, and 14.23 ± 1.22 vs. $1.25 \pm 0.21\%$ ID/g for the heart at 4 h and 168 h p.i., respectively. Simple pharmacokinetic analysis of the time-activity curves derived from the heart revealed similar ^{89}Zr -Df-YY146 circulation half-lives of 20.3 ± 1.9 h and 23.8 ± 2.1 h for U87MG and U251 groups, respectively (Figure S3). A prominent tracer accretion in CD146-positive U87MG tumors was readily noticeable at early time points ($8.19 \pm 1.67\%$ ID/g, at 4 h p.i.), and continue to increase reaching a peak value of $14.00 \pm 3.28\%$ ID/g at 48 h p.i. ($n=4$). U87MG tumor uptake remained high throughout the course of the longitudinal study. In accordance with low CD146 expression levels in U251 tumors, ^{89}Zr -Df-YY146 uptake in U251 tumors peaked at a significantly lower ($P < 0.0001$) value ($5.45 \pm 0.15\%$ ID/g; $n=4$), 24 h after administration of the tracer. In both groups, non-target tissues, such as muscle, displayed similar accumulation of the tracer, which was negligible compared to uptakes observed for target tissues and the organs involved in the hepatobiliary excretion of ^{89}Zr -Df-YY146. Bone uptake became appreciable in late PET images (after 72 h p.i.), being most obvious in mice bearing U251 tumors. The nature of such uptake is attributed to the limited stability of ^{89}Zr -deferoxamine complexes *in vivo*, together with the bone seeking properties of zirconium. However, bone uptake was not quantified due to inherent inaccuracies in PET quantification resulting from partial volume effects.

PET images showed a clear reduction in U87MG uptake when mice were intravenously administered a large dose of YY146 (50 mg/kg), 24 h prior to infusing ^{89}Zr -Df-YY146. Blocking of CD146 resulted in a ~45% reduction in U87MG peak tumor uptake (14.0 ± 3.28 vs. 8.23 ± 1.83 %ID/g at 48 h p.i.; $n=3$) and in a statistically significant decrease ($P < 0.05$) at 4, 24, and 72 h p.i. imaging time points. Blocking effects disappeared at longer (120 and 168 h p.i.) time intervals, plausibly due to a slower clearance of ^{89}Zr -Df-YY146 (half-life: 27.0 ± 1.0 h; Figure S3) from the mouse circulation. ^{89}Zr -Df-YY146 uptake in non-target tissues remained comparable between the blocked and CD-146 positive groups. Altogether, these results attested to the CD146-specific character of ^{89}Zr -Df-YY146 uptake *in vivo*.

Biodistribution

After the final *in vivo* PET scan at 168 h p.i., mice were euthanized and major tissues collected for *ex vivo* gamma counting. These studies served to corroborate the accuracy of the data obtained in PET and provided a more detailed profile of ^{89}Zr -Df-YY146 biodistribution (Figure 4; Table S2). An excellent concordance was observed in 168 h p.i. PET and biodistribution data, corroborating a significant difference in uptake between CD146-positive and CD146-negative tumors (8.59 ± 2.62 vs. 2.79 ± 0.33 %ID/g; $P < 0.05$; $n=4$). No major differences ($P > 0.05$) in ^{89}Zr -Df-YY146 accumulation in U87MG tumors were appreciated among blocked and non-blocked groups, demonstrating the transitory nature of the blocking effect. Besides tumor, other major tissues including liver, kidneys, and spleen revealed significant tracer uptake (Fig 4, Table S2); these organs are known to mediate the excretion of monoclonal antibodies and their degradation products. Bone uptake was determined by harvesting and counting the epiphysis of the femur –bony part that appeared brightest in PET images. Bone activities were highest in mice bearing U251 (12.30 ± 6.62 %ID/g), followed by blocking and U87MG groups with 9.67 ± 1.39 and 6.13 ± 2.81 %ID/g, respectively ($n=3-4$). Overall, *ex vivo* biodistribution certified the ability of image-derived quantification to represent accurately ^{89}Zr -Df-YY146 biodistribution *in vivo*, and shed light on the fate of the radiolabeled tracer at a later time after its administration.

Histology

CD146/CD31 immunofluorescence co-staining unveiled prominent fluorescence signal (green) in U87MG tumor tissue, corresponding with a high *in situ* CD146 expression, but only marginal signal in CD146-negative U251 tumor tissue (Figure 5). Other organs showing marked ^{89}Zr -Df-YY146 accumulation were stained. Poor fluorescence intensities, indicative of low CD146 expression levels were detected in liver, kidney, and spleen, proving the nonspecific character of tracer uptake in these organs. In general, *in situ* CD146 expression, as determined by immunofluorescence staining, was in line with the observation made in PET imaging and biodistribution studies.

Discussion

Glioblastoma remains one of the most lethal types of cancer. Despite intense research efforts, current management strategies fail to improve patient survival, with only 5% of glioblastoma patient surviving five years after initial diagnosis¹. Several factors contribute to the poor prognosis of glioblastoma, including its extreme genotypic and phenotypic

heterogeneity, the inability to perform complete tumor resection, and its high radio/chemo-resistance²⁰. In addition, multiple studies have linked aggressiveness and treatment resistance to the presence of tumor cell subpopulation with cancer stem cell (CSC) properties^{21, 22}. CSC fractions overexpressing CD133, a specific marker of stemness, can range from 1% up to 50% in highly aggressive glioblastomas. Such fractions are likely to increase after first line treatments, owing to the enhanced sensitivity of 'normal' glioblastoma cell resulting in an enrichment on CSC phenotypes within the tumor²³. CSCs, which are responsible for disease dissemination and acquisition of *de novo* resistance to treatment, have been suggested to act as true cancer reservoirs. Though significant efforts have been devoted to finding susceptible molecular targets to treat normal and CSC glioblastoma cell populations, finding new effective biomarkers that would translate into future successful therapies remains a priority. Combination therapies targeting multiple pathways driving tumor proliferation and dissemination have better chances at succeeding; however, due to inter-patient variability, this paradigm would require the implementation of noninvasive companion diagnostics in order to identify and select the right treatment for the right patient.

We have recently reported the overexpression of CD146 in high-grade gliomas and its correlation with WHO tumor grade in humans¹⁸. Additionally, its value as a therapeutic molecule to treat not only normal glioblastoma cells but to selectively target CSC was proposed. In light of the potential employment of CD146 as a therapeutic target in glioblastoma, we developed a molecular imaging agent that facilitates the noninvasive PET imaging of CD146 expression *in vivo*. Well suited for the task was YY146, an anti-CD146 monoclonal antibody produced in our laboratory that shows excellent affinity against the CD146 antigen *in vivo*. Consequently, we employed YY146 as a targeting agent to create a PET imaging radiotracer. Given its suitable physical half-life (78.4 h), which matches the pharmacokinetic time-scale of most antibodies *in vivo*, and amenable coordination chemistry, ⁸⁹Zr was employed as radioactive synthon. Owing to the efficient incorporation of ⁸⁹Zr to YY146, we generated ⁸⁹Zr-Df-YY146 with a radiochemical purity and specific activity suitable for *in vivo* PET imaging studies.

⁸⁹Zr-Df-YY146 displayed excellent targeting properties shortly after its administration into mice bearing CD146-positive tumor xenografts. The excellent tumor-to-background contrasts seen in PET images of mice bearing U87MG tumors were contrasted by a significant lower radioactivity in U251 tumors, demonstrating that ⁸⁹Zr-Df-YY146 tumor accretion is sensitive to variation in CD146 expression levels. We also corroborated that ⁸⁹Zr-Df-YY146 targeting was specific. Such specificity was evidenced during CD146 blocking experiments where administering a high dose of YY146 (50 mg/kg) to U87MG-bearing mice resulted in significant decline in tumor uptake values. ⁸⁹Zr-Df-YY146 peak uptake in U87MG tumors (14.00 ± 3.28 %ID/g at 48 h p.i.) was twice as high as in the liver (7.17 ± 1.12 %ID/g), organ displaying the second highest uptake. Overall, ⁸⁹Zr-Df-YY146 showed markedly lower accumulation in non-target tissues, as well as faster clearance rate compared to U87MG tumors, which revealed longer tracer residency times. These results attest to the feasibility of our tracer to study CD146-expressing malignancies outside the central nervous system (CNS), particularly those located in the abdominal cavity.

Given a strong affinity for phosphate, ^{89}Zr is readily incorporated into nascent hydroxyapatite formations in bone, particularly at the epiphysis where most active bone formation takes place²⁴. As seen by PET, ^{89}Zr bone depositions were observed at the epiphyses of tibia and femur bones, as early as 48 h p.i. and increased over time indicating a gradual transchelation of ^{89}Zr *in vivo*. Such accumulation was consistent with the presence of transchelated ^{89}Zr in the circulation. The U87MG group displayed delayed and markedly lower bone accumulation plausibly due to the sequestration and tumor internalization of ^{89}Zr -Df-YY146, which resulted in decreased bioavailability of ^{89}Zr in the circulation. Numerous research efforts have been devoted to finding ^{89}Zr chelators with enhanced *in vivo* stability; however, to date deferoxamine remains the most suitable choice of chelator for the implementation of ^{89}Zr -based radiopharmaceuticals. Nonetheless, bone mineralization of ^{89}Zr is likely to be of less impact in human patients due to slower bone turn over (0.7% vs. 0.1% for cancellous bone in mice vs. human) and lower radiotracer doses employed in human clinical applications²⁵.

A conceivable limitation of our study was the utilization of subcutaneous models of brain carcinogenesis. Despite its usefulness for evaluating tumor targeting *in vivo*, it is evident that such artificial xenograft models do not resemble the real environment in which brain tumors present in humans. A major difference is the absence of the hardly penetrable blood-brain barrier (BBB), which renders the infiltration of most therapeutic agent negligible. The presence of the BBB has ignited a debate on the validity of antibody targeting within the brain. To our advantage, glioblastoma characterizes by an extensive disruption in BBB's integrity, facilitating the extravasation of macromolecules into tumor's interstitium. Additionally, several strategies for transient disruption or facilitated transport through intact BBB have been explored with measurable success²⁶.

All this mounting body of evidence, together with our recently reported results on the targeting of intracranial brain malignancies using a homologous radiotracer, indicates that ^{89}Zr -Df-YY146 holds objective potential for imaging *in situ* brain malignancies¹⁸. In a clinical scenario, imaging of specific tumor biomarkers provides several advantages as patient screening tool and in evaluating the progression or response to treatment. In fact, the detection specificity achieved with antibody-based imaging approaches is unparalleled by other anatomical imaging modalities, such as CT or MRI, which are often unable to unequivocally discern tumor burden from other processes such as pseudoprogression and inflammation^{20, 27}. Finally, CD146-targeted molecular imaging may offer benefits not only for patient identification, staging, and segregation but also to expose intra-tumoral heterogeneities in CD146 expression.

Conclusion

This study demonstrates the feasibility of immunoPET to scrutinize CD146 expression *in vivo* using ^{89}Zr -labeled antibody. ^{89}Zr -Df-YY146 showed excellent properties as an imaging radiotracer including elevated and specific uptake in brain tumors expressing high levels of CD146. Our encouraging results warrant further exploration toward implementing CD146-based antibody therapies, not only for brain cancer but to treat a myriad of diseases having CD146 dysregulation as the cornerstone. In a clinical practice that increasingly relies on

precision medicine, having an ample arsenal of targeting agents is of vital importance to determine the best disease management paradigm. This work is just a small contribution to creating that armory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the University of Wisconsin-Madison, National Science Foundation (DGE-1256259), National Institutes of Health (NIBIB/NCI 1R01CA169365, P30CA014520, T32CA009206, and 5T32GM08349), American Cancer Society (125246-RSG-13-099-01-CCE), and Strategic Priority Research Program of the Chinese Academy of Sciences (H1808-81201140).

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Figure 1.

In vitro characterization of CD146 expression in two glioblastoma cell lines. **A)** Flow cytometry analysis of U87MG and U251 cells incubated with different concentrations of YY146 and Df-YY146. **B)** Immunofluorescent staining of U87MG and U251 cells. Scale bars: 50 µm. Green color represents CD146 expression. **C)** Western blot looking at CD146 and β-actin protein expression in U87MG (2) and U251 (3) cell lysates. Lane (1) is the molecular weight marker.

Figure 2.

Maximum intensity projection (MIPs) of sequential PET images acquired in mice bearing glioblastoma tumors after ^{89}Zr -Df-YY146 administration. Mice bearing U87MG (top row) or U251 (middle row) tumor xenografts were intravenously injected 3.7–5.5 MBq of ^{89}Zr -Df-YY146 and serial PET scans recorded at 4, 24, 48, 72, 120, 168 h p.i. (n=4). A third group of mice bearing U87MG tumors (bottom row) was administered a blocking dose of YY146 (50mg/kg) prior to injection of the radiotracer (n=3).

Figure 3. Time-activity curves, derived from ROI analysis of PET images, describing ^{89}Zr -Df-YY146 uptake in the tumor, heart/blood, liver, and muscle tissues.

Figure 4.
Ex vivo biodistribution studies of ^{89}Zr -Df-YY146 in mice bearing U87MG or U251 tumors. Mice were sacrificed at 168 h post-injection and radiotracer distribution profile in all major tissues determined by gamma counting.

Figure 5.

Immunofluorescence CD146/CD31 co-staining of two glioblastoma tumor tissues, liver, kidney, and spleen. CD146-bound YY146 was stained with AlexaFluor488-labeled goat anti-mouse secondary antibody (green). Endothelial cells were stained for CD31 with a rat anti-mouse CD31 antibody and Cy3-labeled donkey anti-rat IgG (red). Cell nuclei were stained with DAPI (blue). All images were acquired on a confocal microscope using similar settings. Scale bars: 50 μm.