

Glypican-3 targeted thorium-227 alpha therapy reduces tumor burden in an orthotopic xenograft murine model of hepatocellular carcinoma

Kevin P. Labadie¹, Donald K. Hamlin², Aimee Kenoyer³, Sara K. Daniel¹, Alan F. Utria¹, Andrew D. Ludwig¹, Heidi L. Kenerson¹, Lily Li⁴, Jonathan G. Sham¹, Delphine L. Chen⁵, Johnnie J. Orozco³, Raymond S. Yeung¹, Chris Orvig⁶, Yawen Li², D. Scott Wilbur², James O. Park¹

¹ Department of Surgery, University of Washington, Seattle, WA, United States.

² Department of Radiation Oncology, University of Washington, Seattle, WA, United States.

³ Clinical Research Division, Fred Hutch Cancer Research Center, Seattle, WA, United States.

⁴ Life Sciences Division, TRIUMF, Vancouver BC, V6T 2A3, Canada and Medicinal Inorganic Chemistry Group, Department of Chemistry, University of British Columbia, Vancouver BC, Canada

⁵ Department of Radiology, University of Washington, Seattle, WA, United States.

⁶ Medicinal Inorganic Chemistry Group, Department of Chemistry, University of British Columbia, Vancouver BC, Canada

Conflict of interest disclosure statement: No potential conflicts of interest relevant to this article exist.

Corresponding author

James O. Park

Professor, Hepatobiliary Surgical Oncology

Department of Surgery

University of Washington Medical Center

1959 NE Pacific Street, Box 356410, Health Sciences Bldg. Room BB-442

Seattle, WA 98195-6410

Office: 206-685-4672, Facsimile: 206-598-1984; jopark@uw.edu

ORCID ID 0000-0002-4239-5392

First Author

Kevin P. Labadie

Resident Physician

Department of Surgery

University of Washington Medical Center

1959 NE Pacific Street, Box 356410, Health Sciences Bldg. Room BB-442

Seattle, WA 98195-6410

Office: 206-685-4672, Facsimile: 206-598-1984; labadiek@uw.edu

ORCID ID: 0000-0002-5729-3524

Word count: 4,992

Figures/Tables: 9

Running title: GPC3 targeted ²²⁷Th therapy for HCC

ABSTRACT

Hepatocellular carcinoma (HCC) is a significant cause of morbidity and mortality worldwide with limited therapeutic options for advanced disease. Targeted alpha therapy (TAT) is an emerging class of targeted cancer therapy in which alpha-particle-emitting radionuclides, such as thorium-²²⁷Th, are specifically delivered to cancer tissue. Glypican-3 (GPC3) is a cell surface glycoprotein highly expressed on HCC. In this study, we describe the development and *in vivo* efficacy of a ²²⁷Th-labeled GPC3 targeting antibody conjugate (²²⁷Th-octapa- α GPC3) for treatment of HCC in an orthotopic murine model.

METHODS

The chelator *p*-SCN-Bn-H₄octapa-NCS (octapa) was conjugated to a GPC3 targeting antibody (α GPC3) for subsequent ²²⁷Th radiolabeling (octapa- α GPC3). Conditions were varied to optimize radiolabeling of ²²⁷Th. *In vitro* stability was evaluated by measuring percentage of protein-bound ²²⁷Th by gamma-ray spectroscopy. An orthotopic athymic Nu/J murine model using HepG2 cells was developed. Biodistribution and blood clearance of ²²⁷Th-octapa- α GPC3 was evaluated in tumor bearing mice. Efficacy of ²²⁷Th-octapa- α GPC3 was assessed in tumor bearing animals with serial measurement of serum alpha-fetoprotein at 23 days after radionuclide injection.

RESULTS

Octapa-conjugated α GPC3 provided up to 70% ²²⁷Th labeling yield in 2 h at room temperature. In the presence of ascorbate, $\geq 97.8\%$ of ²²⁷Th was bound to α GPC3-octapa after 14 d in phosphate buffered saline. In HepG2 tumor-bearing mice, highly specific GPC3 targeting was observed, with significant ²²⁷Th-octapa- α GPC3 accumulation in the tumor over time and minimal accumulation in normal tissue. 23 days after treatment, significant reduction in tumor burden was observed in

mice receiving 500 kBq/kg ^{227}Th -octapa- α GPC3 by tail vein injection. No acute off-target toxicity was observed and no animals died prior to termination of the study.

CONCLUSION

^{227}Th -octapa- α GPC3 was observed to be stable *in vitro*, maintain high specificity for GPC3 with favorable biodistribution *in vivo*, and result in significant antitumor activity without significant acute off-target toxicity in an orthotopic murine model of HCC.

Keywords: Hepatocellular carcinoma (HCC), glypican-3 (GPC3), targeted alpha therapy (TAT), radioimmunotherapy

INTRODUCTION

Hepatocellular carcinoma (HCC) is a significant cause of morbidity and mortality worldwide, particularly in advanced stages (1-3). Although novel combination therapies are being developed (e.g. immune checkpoint and tyrosine kinase inhibitors), these regimens are limited by modest efficacy and significant adverse effects (4-7). New therapies for treatment of advanced HCC are needed.

Targeted alpha-particle therapy (TAT) is a promising new class of cancer therapies which create double-stranded DNA breaks via a high linear energy transfer (LET) and induce cytotoxic T-lymphocyte driven antitumor immune response (8-12). Thorium-227 (^{227}Th , $t_{1/2}$ 18.7 d) is an alpha particle emitting radionuclide that has been attached to monoclonal antibody conjugates using bifunctional octadentate ligands, such as isothiocyanato-benzyl-DOTA and 3,2-hydroxypyridinone (3,2-HOPO) derivatives (13). The decay scheme of ^{227}Th is shown in Figure 1. While alpha-emitters are generally appealing because of the higher radiation payload deposited over shorter distances (high LET), minimizing the risk of off-target toxicity, the long half-life of ^{227}Th makes it advantageous for the treatment of solid tumors. ^{227}Th -labeled antibody conjugates are currently being evaluated in the treatment of several malignancies including acute myeloid leukemia, multiple myeloma, renal cell carcinoma, non-Hodgkin's lymphoma, mesothelin-positive mesothelioma and ovarian cancer (14-17). Glypican-3 (GPC3) is a cell surface glycoprotein highly expressed in hepatocellular carcinomas (HCC) making it a promising target for novel therapeutic and diagnostic applications (18-23). To our knowledge, no report of ^{227}Th TAT directed at GPC3 for the treatment of HCC has been published to date.

The objective of this study was to develop a ^{227}Th -labeled GPC3 antibody conjugate (^{227}Th -octapa- α GPC3) and evaluate the radiolabeling properties, *in vivo* biodistribution and efficacy in a GPC3-positive hepatic orthotopic xenograft murine model of HCC.

MATERIALS AND METHODS

Conjugation of α GPC3 and α BHV1 With Octapa

$^{227}\text{Th}(\text{NO}_3)_4$ was purchased from Oak Ridge National Laboratory and was purified from its decay daughters prior to use (24). The bifunctional chelator *p*-SCN-Bn-H₄octapa (octapa) was synthesized at the University of British Columbia (chemical structure shown in supplemental Figure S1) (25,26). The anti-GPC3 antibody (α GPC3) was generated and produced at Fred Hutchinson Research Center antibody core facility as previously described (22,27). The isotype control (IgG₁) antibody against bovine herpes virus, α BHV1 was a generous gift from the Orozco laboratory(28). The conjugation reaction was previously described (25,26). Detailed information regarding conjugation and radiolabeling is included in the *Supplemental Methods*.

^{227}Th radiolabeling of α GPC3-octapa

The α GPC3-octapa conjugate with the highest octapa-to- α GPC3 ratio and highest antigen binding was used in ^{227}Th labeling reactions. Optimization of the radiolabeling yield was accomplished by varying the reaction time, pH and temperature. Appropriate amounts of unlabeled mAb conjugates were added to the purified product to provide 0.5 μCi (18.5 kBq) and 70 μg antibody per dose. The remaining ^{227}Th labeled α GPC3-octapa was used for *in vitro* stability studies.

***In vitro* stability of ^{227}Th -octapa- α GPC3**

Solutions of ^{227}Th -labeled mAbs were incubated at room temperature (RT) and pH 7.0 for 4 h, followed by refrigeration at 4°C, in the presence or absence of ascorbate acid. At 4 h, 24 h, 3 d, 7 d and 14 d time points, the percentage of protein-bound ^{227}Th was determined by gamma-ray spectroscopy, monitoring the 236 keV (12.9%) gamma peak of ^{227}Th . Stability in serum and in the presence of an excess of metal ions such as Ca^{2+} , Fe^{3+} , Mg^{2+} , Cu^{2+} and Zn^{2+} will be evaluated in future studies.

Development of orthotopic xenograft model

GPC3-positive HepG2-Red-FLuc (HepG2) cells expressing *Luciferase* were purchased from PerkinElmer (Bioware, cat. no. BW134280, RRID:CVCL_5I98) and were maintained in a monolayer at 37°C in Dulbecco's modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) in a humidified chamber with 5% CO_2 .

This study was performed in accordance with the University of Washington Office of Animal Welfare guidelines for the humane use of animals, and all procedures were reviewed and approved by the Institutional Animal Care and Use Committee (Protocol #4304-02). Experiments were carried out in compliance with the ARRIVE guidelines (29). A hepatic subcapsular orthotopic xenograft model was generated and described in supplemental methods (23). To monitor orthotopic tumor growth, whole blood was obtained from animals by submandibular bleed and serum concentration of alpha-fetoprotein (AFP) was determined (30).

Biodistribution and blood clearance studies

Tumor-bearing mice were injected with ^{227}Th -octapa- α GPC3 or ^{227}Th -octapa- α BHV1 (70 μg , 500kBq/kg). Tumors and normal organs were harvested, weighed and ^{227}Th activity was measured by gamma counter; measured activity was a net sum of activity from all

daughters. The percent injected dose of radioisotope per gram (% ID/g) of blood, tumor or organ was calculated after correcting for radioactive decay using an aliquot of the injectate, as were the tumor-to-normal organ ratios of absorbed radioactivity.

After tumor-bearing mice were injected with ^{227}Th -octapa- α GPC3 or ^{227}Th -octapa- α BHV1 (70 μg , 500 kBq/kg), serial retro-orbital blood sampling was performed at 5, 15, 30, 60, 120, 240 min, and then collected on necropsy at 24 h, 7 d and 23 d after injection. Blood samples were measured by gamma counter and corrected for ^{227}Th activity.

^{227}Th radioimmunotherapy

After animals were confirmed to have tumors by IVIS imaging, they were included in the study and serum AFP was measured. Tumor-bearing animals were assigned to one of four experimental groups based on serum AFP measurements to ensure comparable tumor burden among cohorts. In unblinded fashion, animals either received no treatment, α BHV1-octapa radiolabeled with 500kBq/kg ^{227}Th , α GPC3-octapa radiolabeled with 250 or 500 kBq/kg of ^{227}Th via tail vein injection without anesthesia. Twenty-three days after injection, animals were euthanized to evaluate for early anti-tumor effect of ^{227}Th -octapa α - α GPC3. Serum was obtained for AFP measurement and randomly selected livers were harvested and placed in 10% (w/v) neutral-buffered formalin.

Statistical analysis

Statistical analysis was performed with GraphPad Prism (version 8.0.0, GraphPad Software, San Diego, California USA, RRID:SCR_002798). D'Agostino & Pearson normality test was performed to determine if Gaussian distribution. Continuous variables were expressed as medians and means and compared by Student's t-test or Mann-Whitney test. One way-ANOVA

or Kruskal-Wallis with Dunn's multiple comparison test was performed. In all cases, a *P* value of ≤ 0.05 was considered statistically significant.

RESULTS

GPC3 Conjugation with Octapa is Highly Efficient

Octapa was conjugated to α GPC3 via reactions conducted using 5, 10 and 15 eq of octapa to determine the optimal conjugation ratio. The isoelectric points of the α GPC3-octapa shifted toward the acidic pI as the number of eq offered increased (Figure 2). α GPC3-octapa 5eq demonstrated maximum shift of binding to GPC3⁺ cells by flow cytometry (Supplemental Figure S2), however, it resulted in lower chelates per antibody compared to the 10 eq, therefore 10 eq conjugation ratio was used for subsequent experiments. The protein recovery from the conjugation process was >85% and the final concentrations were 4.6 mg/mL and 4.75 mg/mL for α GPC3-octapa and α BHV1-octapa, respectively. Mass spectral analysis of α GPC3-octapa and α BHV1-octapa conjugates produced by reaction of 10 eq of octapa indicated that there was an average of 3.3 octapa moieties on α GPC3 and 5 octapa moieties on α BHV1.

²²⁷Th-Labeled α GPC3-octapa is Stable *in vitro*

Labeling conditions were optimized using α GPC3-octapa and small quantities of ²²⁷Th in 0.1 M HNO₃ (85.1-314.5 kBq, or 2.3-8.5 μ Ci). Labeling yield increased from 16%, 25% to 37% as the reaction time increased from 30 min, 1 h to 2 h, respectively, but did not significantly increase after 2 h. Among the different reaction solutions and pH tested, the highest consistent yields were obtained when 200 μ L of 0.05 M sodium citrate with 1 mM EDTA (pH 5.5) was combined with 5-10 μ L ²²⁷Th in 0.1 M HNO₃ with the pH adjusted to 5-5.5 using 1 M sodium citrate. To this, 200 μ g of α GPC3-octapa (4.0 mg/mL) was added and allowed to react for 2 h at

37°C (43-70% radiochemical yield). Reactions conducted at the elevated temperature only accounted for about 4% higher yields, therefore subsequent reactions were conducted at RT.

In vitro stability of the ^{227}Th -labeled mAb-octapa conjugates was evaluated with and without ascorbic acid. Because the gamma counter cannot distinguish ^{227}Th activity from its radioactive progeny, gamma spectroscopy was used to analyze radio-iTLC strips from and determine the percentage of antibody-bound ^{227}Th . After 14 d, $\geq 97.8\%$ of ^{227}Th was observed to be bound to αGPC3 -octapa in the presence of ascorbic acid (Figure 3).

αGPC3 -octapa Maintains Ligand Binding *in vitro* and *in vivo*

The affinity for GPC3 by αGPC3 -octapa was assessed with flow cytometry using HepG2 cells. αGPC3 -octapa maintained high affinity for GPC3 with only modest reduction in binding affinity compared to unconjugated αGPC3 (Figure 4). In tumor-bearing mice 24 h, 7 d and 23 d after tail vein injection of ^{227}Th -octapa- αGPC3 , elevated levels of radioactivity were detected in the tumor tissue compared to surrounding liver and other organs (Figure 5A, Supplemental Table S1). %ID/g of ^{227}Th -octapa- αGPC3 remained high in tumor tissue over 23 d (Figure 5B). The ratio of %ID/g of ^{227}Th -octapa- αGPC3 in tumor tissue compared to adjacent normal liver parenchyma steadily increased over time (Figure 5C), ^{227}Th -octapa- αBHV1 , an irrelevant isotype antibody conjugate, did not significantly bind to the tumor tissue as expected (supplemental Figure S3).

^{227}Th -octapa- αGPC3 did not significantly accumulate in normal tissues at 1, 7 and 23 d after injection (Figure 5A). %ID/g of ^{227}Th -octapa- αGPC3 was $<5\%$ in all tested normal tissues by day 23 after injection. The primary mode of decay for ^{227}Th is alpha decay, resulting in daughter radium-223 particles, a radionuclide which preferentially accumulates in bone. Measurement of radioactivity of the femur demonstrated low gamma counts after injection with ^{227}Th -octapa- αGPC3 compared to ^{227}Th -octapa- αBHV1 , where radioactivity was observed to accumulate in

bone over time. This is presumably due to the highly specific targeting and preferential accumulation of ^{227}Th -octapa- α GPC3 in the tumor, resulting in lower circulating radioactivity for bone deposition. High-resolution gamma-ray spectroscopy was performed on select tissues demonstrating radium accumulation in bone (Supplemental Table S2), but further evaluation will be part of future study.

Blood radioactivity was more rapidly cleared after ^{227}Th -octapa- α GPC3 injection compared to ^{227}Th -octapa- α BHV1 with serum half-life of 14 and 17 hours, respectively. This more rapid clearance may be secondary to increased accumulation of the radioimmunoconjugate in the tumor over time (Figure 6).

^{227}Th -octapa- α GPC3 Reduces Tumor Burden in Murine Model

To assess efficacy of ^{227}Th -octapa- α GPC3 TAT, tumor-bearing mice received either no treatment, 500 kBq/kg ^{227}Th -octapa- α BHV1, 250 or 500 kBq/kg ^{227}Th -octapa- α GPC3 by tail vein injection. Serum AFP was significantly lower in mice treated with ^{227}Th -octapa- α GPC3 compared to control groups 23 d after therapy administration (Figure 7). The treatment effect was most pronounced after therapy with 500 kBq/kg ^{227}Th -octapa- α GPC3, although a modest effect was observed after therapy with 250kBq/kg. AFP increased significantly after therapy with 500 kBq/kg ^{227}Th -octapa- α BHV1, indicating that GPC3 targeted thorium delivery induced tumor cell killing rather than the presence of systemically circulating antibody-bound ^{227}Th .

To assess for organ-specific toxicity after administration of the ^{227}Th radioimmunoconjugates, serum markers of end organ dysfunction were collected 23 d after injection, and no significant aberrations were identified in comparison to control (Supplemental Figure S4). No animals died prior to termination of study.

DISCUSSION

In this report, we describe the development of a thorium-227 radioimmunoconjugate targeting GPC3 and demonstrate its *in vivo* efficacy in the treatment of HCC in an orthotopic murine xenograft model. The ^{227}Th -octapa- α GPC3 radioimmunoconjugate was observed to be stable *in vitro*, maintain its specificity for GPC3 with a favorable biodistribution, and result in tumor reduction without undesired significant acute toxicity. These findings add to previous studies establishing the basis for a GPC3 targeted theranostic platform whereby different radioimmunoconjugates can be used for diagnostic/surveillance imaging and treatment (22,23,31,32). Such a platform could improve current treatments of HCC by enabling earlier identification of disease or recurrence, increase the accuracy of staging, and allow for more targeted treatment with less systemic toxicities.

To radiolabel our antibody, we utilized a picolinic acid (pa)-containing chelate, octapa, an octadentate acyclic ligand that enables ^{227}Th radiolabeling of antibodies at RT, helping to maintain the three-dimensional conformation and immunoreactivity of the conjugated targeting antibody (25,26). Optimization of the reaction conditions resulted in efficient ^{227}Th -labeling (up to 70% radiolabeling yield) and product with high radiochemical purity. The high *in vivo* stability of the ^{227}Th -octapa complex is demonstrated by the low bone uptake throughout the 23-day study period. In its initial description, H₄octapa was used to label trastuzumab with indium-111 and lutetium-177 for imaging and therapy, respectively, of mice bearing ovarian cancer xenografts (25,26). Previously ^{227}Th has been radiolabeled to antibodies using an octadentate hydroxypyridino (HOPO) for the treatment of CD33⁺ myeloid leukemia, CD70⁺ renal cell carcinoma, mesothelin-positive malignancies (13). This study is the first to describe pa ligands as a new class of ligand for ^{227}Th radiolabeling of antibody conjugates.

We have previously described conjugating GPC3 with DOTA chelate for yttrium-90 (^{90}Y - αGPC3) radioimmunotherapy(22,23,33). Yttrium-90 produces beta ionizing radiation, with lower energy and longer path length compared to ^{227}Th , which produces alpha ionizing radiation. These differences in properties are important as higher energy transfer results in a lower LD₅₀, and a shorter path length decreases the radius of tissues affected by the radiation (10). Alpha therapies might be desirable over beta therapies if highly specific targeting is possible. While research into TAT for hematologic malignancies has focused on ^{211}At ($t_{1/2}$ 7.21 h), and a recent study for HCC described using actinium-225 (^{225}Ac , $t_{1/2}$ 9.92 d), we elected to use ^{227}Th ($t_{1/2}$ 18.7 d) for its longer half-life which we hypothesize is advantageous in the treatment of solid tumors (31,34,35).

Administration of ^{227}Th -octapa- αGPC3 led to highly specific tumor uptake, rapid blood clearance and robust antitumor activity without significant acute toxicity. Within one day of injection of ^{227}Th -octapa- αGPC3 , significant intra-tumoral accumulation was observed compared to control. Tumor to liver ratio of ^{227}Th -octapa- αGPC3 steadily increased over time and minimal off-target uptake was observed, indicating highly specific targeting and clearance. We observed modest bone uptake over time in the irrelevant isotype antibody control group, which is expected given ^{227}Th daughter molecule ^{223}Ra delivers radiation to sites of increased osteoblastic metabolism (36).

The observed therapeutic effect of ^{227}Th -octapa- αGPC3 was dependent on antibody targeted delivery of radiation, as evidenced by the lack of therapeutic effect observed in the non-targeting ^{227}Th -octapa- αBHV1 control group. Using an established marker of tumor burden in our model, serum AFP, we observed the therapeutic effect after treatment with 500 kBg/kg ^{227}Th - αGPC3 to be consistent, with a reduction in serum AFP in all animals except one (23).

Interestingly, we observed marked increase in tumor burden in the α BHV1 control group. The mechanism of this finding is not understood but could be related to bone marrow toxicity and suppression of alloreactive immune cells, which are present in athymic mice (37,38).

No significant acute off-target toxicity was observed in our study with all animals surviving until study completion. There was moderate amount of radioactivity identified in the bone, particularly in our control group. One of the challenges of using alpha-particle emitters for therapy is the presence of multiple radioactive daughter products that may dislocate from target site. Although this may lead to cytopenias and marrow toxicity, data from human trials with similar radioisotopes are reassuring. In the phase 3 ALSYMPACA trial, difference in cytopenia rates were seen in patients with prior docetaxel dosing, suggesting differences in cumulative marrow damage being more implicated over direct radioactive effects. A recently published manuscript describing α GPC3 TAT using ^{225}Ac conjugated to the humanized monoclonal antibody GC33 in a heterotopic murine xenograft model demonstrated modest anti-tumor activity while observing significant bone marrow suppression and toxicity (31). Although experimental models tested were different, we postulate that the difference in toxicity between our studies is due to improved specificity of our antibody, highlighting the importance of effective targeting for delivery of the alpha therapy.

There are several limitations to our study. GPC3 expression in HCC is variable and differs based on the degree of differentiation (39). Human HepG2 cells demonstrate high expression of GPC3 (40), and may not recapitulate low or intermediate grade HCC. The studies were performed in athymic mice that lack mature T-cells. Although competent leukocytes are present in this model, it does not represent the complex tumor microenvironment of human HCC. We elected to omit a non-radiolabeled antibody conjugate control group due to extensive prior work by our group demonstrating that α GPC3 antibody alone does not lead to robust antitumor response (23). Tumor

size was measured indirectly in our model by serum AFP. Direct tumor size measurements via ultrasonography or bioluminescent imaging were not performed due to environmental health and safety constraints at our core facilities with radioactive animals. Some untreated animals observed spontaneous reduction in AFP and tumor size without treatment, possibly secondary to an alloreactive response from native immune cells. While these animals existed in all groups, this requires further investigation. More studies are warranted to evaluate the potential toxicity of ^{227}Th -octapa- α GPC3. One of the studies would be to perform dosimetry analysis to understand the radiation dose from ^{227}Th and its alpha-emitting decay progenies, especially ^{223}Ra . Additionally, it is worth noting that radiation nephropathy and other toxicities were not able to be fully assessed with only 23 days of monitoring(41). A longer period of observation in addition to hematologic analysis, which was not performed due lack of appropriate experimental equipment, is planned for future investigations.

CONCLUSION

In conclusion, we report the development of a GPC3-targeted ^{227}Th conjugate using octapa and demonstrate it to be stable *in vitro*, maintain high specificity for GPC3 with favorable biodistribution *in vivo*, and result in significant antitumor activity without undesirable acute toxicity. To our knowledge, this is the first description of a thorium-227 radiopharmaceutical targeting GPC3 and is a promising addition to the theranostic approach to treating HCC.

Financial support:

NIH/NCI Cancer Center Support Grant P30 CA015704. The isotope(s) used in this research was supplied by the U.S. Department of Energy Isotope Program, managed by the Office of Isotope

R&D and Production NSERC CREATE IsoSiM at TRIUMF for a PhD research stipend (LL) and NSERC Discovery Grant (RGPIN-42394-13) as well as NSERC (CHRP 493725-16) and the Canadian Institutes of Health Research (CIHR, CPG-146482) as a Collaborative Health Research Project (CO). TRIUMF receives federal funding via a contribution agreement with the National Research Council of Canada.

Key Points

QUESTION: Does glypican-3 targeted thorium radionuclide therapy localize intratumorally and reduce tumor burden in a mouse model?

PERTINENT FINDINGS: Thorium was reliably and efficiently labeled to a glypican-3 targeting antibody via octapa chelator. This radioimmunoconjugate maintained affinity for the target antigen in vitro and in vivo. Significant levels of thorium accumulated intratumorally. Orthotopic mice treated with glypican-3 directed thorium therapy had significant reductions in their tumor burden compared to control animals.

IMPLICATIONS FOR PATIENT CARE: This study identifies a new approach to treating HCC using a personalized and targeted approach against a highly expressed antigen in HCC.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global Cancer Statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2018;68:394-424.
2. Kim D, Li AA, Perumpail BJ, et al. Changing trends in etiology-based and ethnicity-based annual mortality rates of cirrhosis and hepatocellular carcinoma in the United States. *Hepatology.* 2019;69:1064-1074.
3. Wong MC, Jiang JY, Goggins WB, et al. International incidence and mortality trends of liver cancer: a global profile. *Sci Rep.* 2017;7:45846.
4. Finn RS, Qin S, Ikeda M, et al. Atezolizumab plus bevacizumab in unresectable hepatocellular carcinoma. *N Engl J Med.* 2020;382:1894-1905.
5. Finn RS, Ryoo BY, Merle P, et al. Pembrolizumab as second-line therapy in patients with advanced hepatocellular carcinoma in KEYNOTE-240: A randomized, double-blind, phase III trial. *J Clin Oncol.* 2020;38:193-202.
6. Llovet JM, Kudo M, Cheng A-L, et al. Lenvatinib (len) plus pembrolizumab (pembro) for the first-line treatment of patients (pts) with advanced hepatocellular carcinoma (HCC): Phase 3 LEAP-002 study. *J Clin Oncol.* 2019;37:TPS4152-TPS4152.

7. Yau T, Kang Y-K, Kim T-Y, et al. Nivolumab (NIVO) + ipilimumab (IPI) combination therapy in patients (pts) with advanced hepatocellular carcinoma (aHCC): Results from CheckMate 040. *J Clin Oncol*. 2019;37:4012-4012.
8. Targeted Alpha Therapy Working G, Parker C, Lewington V, et al. Targeted Alpha Therapy, an Emerging Class of Cancer Agents: A Review. *JAMA Oncol*. 2018;4:1765-1772.
9. Sgouros G, Roeske JC, McDevitt MR, et al. MIRDO Pamphlet No. 22 (abridged): radiobiology and dosimetry of alpha-particle emitters for targeted radionuclide therapy. *J Nucl Med*. 2010;51:311-328.
10. Kassis AI. Therapeutic radionuclides: biophysical and radiobiologic principles. *Semin Nucl Med*. 2008;38:358-366.
11. Poty S, Francesconi LC, McDevitt MR, Morris MJ, Lewis JS. alpha-Emitters for Radiotherapy: From Basic Radiochemistry to Clinical Studies-Part 1. *J Nucl Med*. 2018;59:878-884.
12. Malamas AS, Gameiro SR, Knudson KM, Hodge JW. Sublethal exposure to alpha radiation (²²³Ra dichloride) enhances various carcinomas' sensitivity to lysis by antigen-specific cytotoxic T lymphocytes through calreticulin-mediated immunogenic modulation. *Oncotarget*. 2016;7:86937-86947.

13. Ramdahl T, Bonge-Hansen HT, Ryan OB, et al. An efficient chelator for complexation of thorium-227. *Bioorg Med Chem Lett*. 2016;26:4318-4321.
14. Hagemann UB, Ellingsen C, Schuhmacher J, et al. Mesothelin-targeted thorium-227 conjugate (MSLN-TTC): Preclinical evaluation of a new targeted alpha therapy for mesothelin-positive cancers. *Clin Cancer Res*. 2019;25:4723-4734.
15. Hagemann UB, Mihaylova D, Uran SR, et al. Targeted alpha therapy using a novel CD70 targeted thorium-227 conjugate in in vitro and in vivo models of renal cell carcinoma. *Oncotarget*. 2014;5:56311-56326.
16. Hagemann UB, Wickstroem K, Wang E, et al. In vitro and in vivo efficacy of a novel CD33-targeted thorium-227 conjugate for the treatment of acute myeloid leukemia. *Mol Cancer Ther*. 2016;15:2422-2431.
17. Wickstroem K, Hagemann UB, Cruciani V, et al. Synergistic effect of a mesothelin-targeted (²²⁷Th) conjugate in combination with DNA damage response inhibitors in ovarian cancer xenograft models. *J Nucl Med*. 2019;60:1293-1300.
18. Nakatsura T, Yoshitake Y, Senju S, et al. Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. *Biochem Biophys Res Commun*. 2003;306:16-25.

19. Moek KL, Fehrmann RSN, van der Vegt B, de Vries EGE, de Groot DJA. Glypican 3 overexpression across a broad spectrum of tumor types discovered with functional genomic mRNA profiling of a large cancer database. *Am J Pathol.* 2018;188:1973-1981.
20. Nishida T, Kataoka H. Glypican 3-Targeted Therapy in Hepatocellular Carcinoma. *Cancers (Basel).* 2019;11.
21. Sham JG, Kievit FM, Grierson JR, et al. Glypican-3-targeting F(ab')₂ for ⁸⁹Zr PET of hepatocellular carcinoma. *J Nucl Med.* 2014;55:2032-2037.
22. Sham JG, Kievit FM, Grierson JR, et al. Glypican-3-targeted ⁸⁹Zr PET imaging of hepatocellular carcinoma. *J Nucl Med.* 2014;55:799-804.
23. Ludwig AD, Labadie KP, Seo YD, et al. Yttrium-90-Labeled Anti-Glypican 3 Radioimmunotherapy Halts Tumor Growth in an Orthotopic Xenograft Model of Hepatocellular Carcinoma. *J Oncol.* 2019;2019:4564707.
24. Ferrier MG, Li Y, Chyan MK, et al. Thorium chelators for targeted alpha therapy: Rapid chelation of thorium-226. *J Labelled Comp Radiopharm.* 2020;63:502-516.
25. Price EW, Cawthray JF, Bailey GA, et al. H₄octapa: an acyclic chelator for ¹¹¹In radiopharmaceuticals. *J Am Chem Soc.* 2012;134:8670-8683.

26. Price EW, Zeglis BM, Cawthray JF, et al. H(4)octapa-trastuzumab: versatile acyclic chelate system for ¹¹¹In and ¹⁷⁷Lu imaging and therapy. *J Am Chem Soc.* 2013;135:12707-12721.
27. Wayner EA, Hoffstrom BG. Development of monoclonal antibodies to integrin receptors. *Methods Enzymol.* 2007;426:117-153.
28. Pagel JM, Matthews DC, Kenoyer A, et al. Pretargeted radioimmunotherapy using anti-CD45 monoclonal antibodies to deliver radiation to murine hematolymphoid tissues and human myeloid leukemia. *Cancer Res.* 2009;69:185-192.
29. Percie du Sert N, Hurst V, Ahluwalia A, et al. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *Exp Physiol.* 2020;105:1459-1466.
30. Golde WT, Gollobin P, Rodriguez LL. A rapid, simple, and humane method for submandibular bleeding of mice using a lancet. *Lab Anim (NY).* 2005;34:39-43.
31. Bell MM, Gutsche NT, King AP, et al. Glypican-3-targeted alpha particle therapy for hepatocellular carcinoma. *Molecules.* 2020;26.
32. Kelada OJ, Gutsche NT, Bell M, et al. ImmunoPET as Stoichiometric Sensor for Glypican-3 in Models of Hepatocellular Carcinoma. *bioRxiv.* 2020:2020.2001.2031.926972.

- 33.** Labadie KP, Ludwig AD, Lehnert AL, et al. Glypican-3 targeted delivery of (89)Zr and (90)Y as a theranostic radionuclide platform for hepatocellular carcinoma. *Sci Rep.* 2021;11:3731.
- 34.** O'Steen S, Comstock ML, Orozco JJ, et al. The alpha-emitter astatine-211 targeted to CD38 can eradicate multiple myeloma in a disseminated disease model. *Blood.* 2019;134:1247-1256.
- 35.** Orozco JJ, Back T, Kenoyer A, et al. Anti-CD45 radioimmunotherapy using (211)At with bone marrow transplantation prolongs survival in a disseminated murine leukemia model. *Blood.* 2013;121:3759-3767.
- 36.** Suominen MI, Fagerlund KM, Rissanen JP, et al. Radium-223 inhibits osseous prostate cancer growth by dual targeting of cancer cells and bone microenvironment in mouse models. *Clin Cancer Res.* 2017;23:4335-4346.
- 37.** Festing MF, May D, Connors TA, Lovell D, Sparrow S. An athymic nude mutation in the rat. *Nature.* 1978;274:365-366.
- 38.** Benestad HB, Hersleth IB, Rolstad B. Thymus and T cells are not essential for rat leucopoiesis. *Eur J Haematol.* 1989;43:150-157.

- 39.** Suzuki M, Sugimoto K, Tanaka J, et al. Up-regulation of glypican-3 in human hepatocellular carcinoma. *Anticancer Res.* 2010;30:5055-5061.
- 40.** Gao W, Tang Z, Zhang YF, et al. Immunotoxin targeting glypican-3 regresses liver cancer via dual inhibition of Wnt signalling and protein synthesis. *Nat Commun.* 2015;6:6536.
- 41.** Kiess AP, Minn I, Vaidyanathan G, et al. (2S)-2-(3-(1-Carboxy-5-(4-211At-Astatobenzamido)Pentyl)Ureido)-Pentanedioic Acid for PSMA-Targeted alpha-Particle Radiopharmaceutical Therapy. *J Nucl Med.* 2016;57:1569-1575.

TABLES/FIGURES

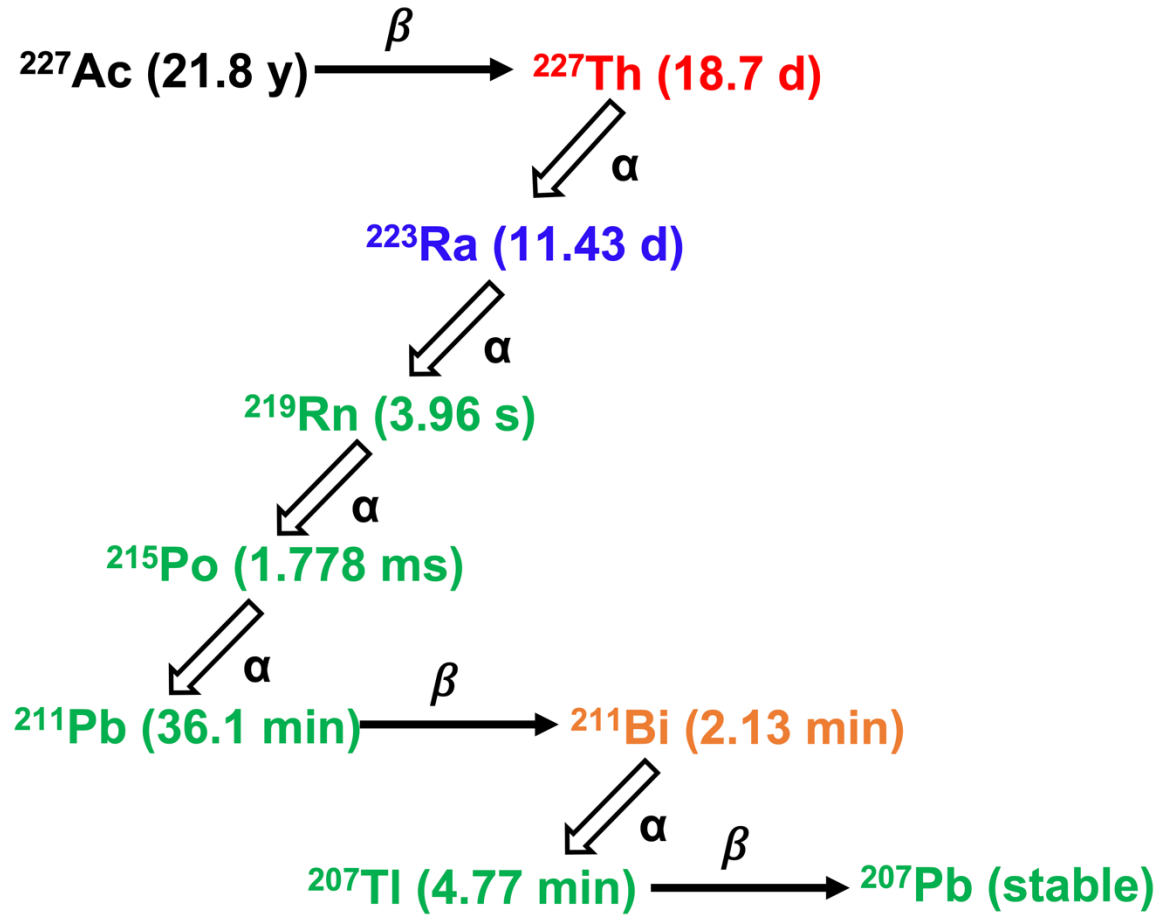


Figure 1. Decay scheme of ^{227}Th .

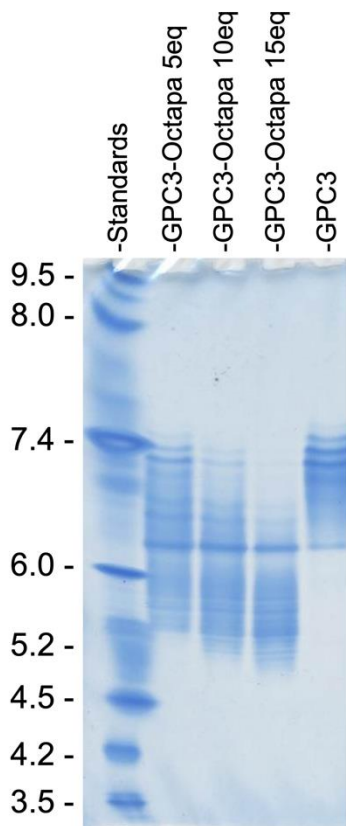


Figure 2. Conjugation with octapa to α GPC3. Stained isoelectric focusing gel showing the pI changes in the unconjugated GPC3 mAb (right lane), when 5, 10 and 15 Eq of the octapa-NCS reagent are reacted with α GPC3 mAb.

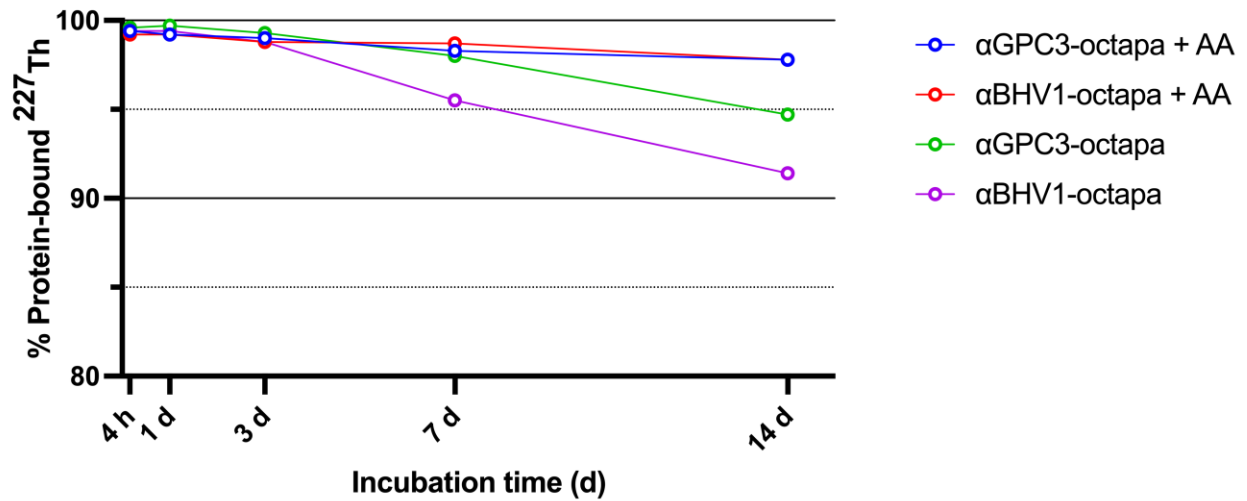


Figure 3. ²²⁷Th-labeled mAbs are stable *in vitro*. Shown is the percentage bound thorium of αBHV1-octapa and αGPC3-octapa incubated in PBS over 14 d with and without ascorbic acid (AA).

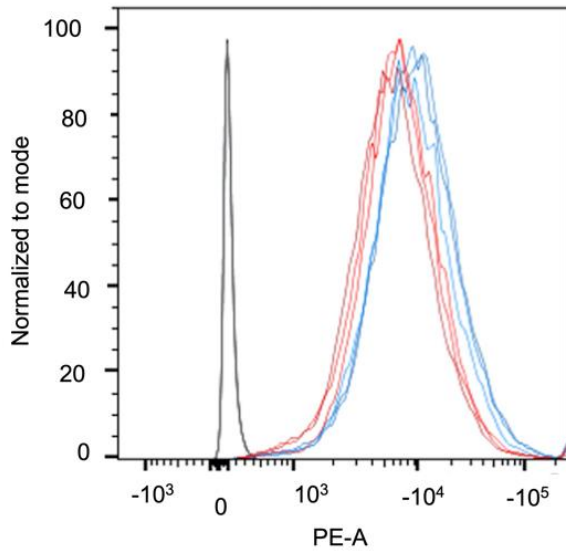


Figure 4. α GPC3-octapa maintains binding affinity for GPC3 *in vitro*

In vitro GPC3 binding assessed by flow cytometry on human HepG2 cells with unconjugated (blue), and α GPC3-octapa (red) compared to unstained control (black). Three biologic replicates samples shown.

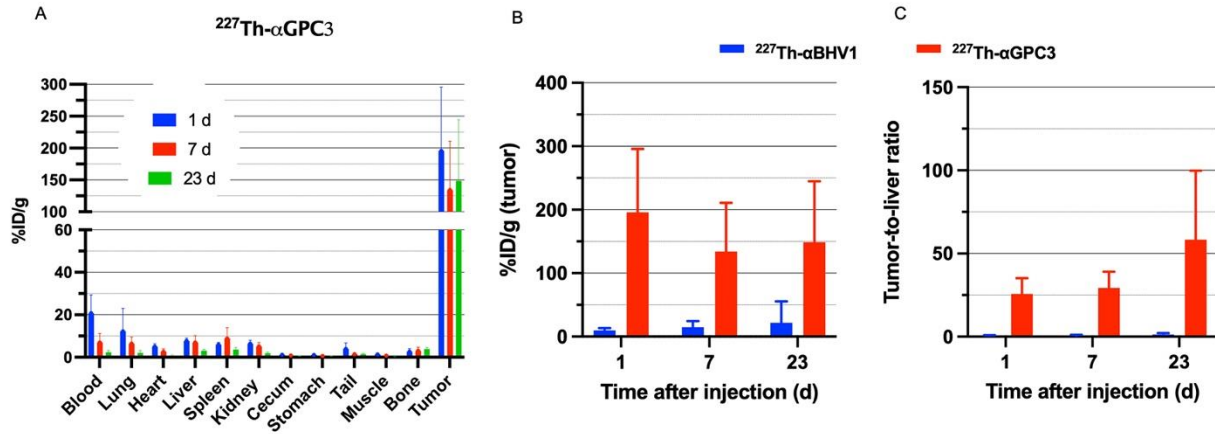


Figure 5. Comparative biodistribution of $^{227}\text{Th-}\alpha\text{GPC3}$ and $^{227}\text{Th-}\alpha\text{BHV1}$. A) Tissue biodistribution of $^{227}\text{Th-}\alpha\text{GPC3}$ in tumor bearing mice 1 d (n=7), 7 d (n=7) and 23 d (n=6) after injection. B) ^{227}Th accumulation in tumor tissue and C) the tumor to liver ratio 1 d (n=7), 7 d (n=5 αBHV1 , n=3 αGPC3) and 23 d (n=4) after injection with $^{227}\text{Th-}\alpha\text{BHV1}$ or $^{227}\text{Th-}\alpha\text{GPC3}$. Bar denotes mean, error bar denotes standard deviation.

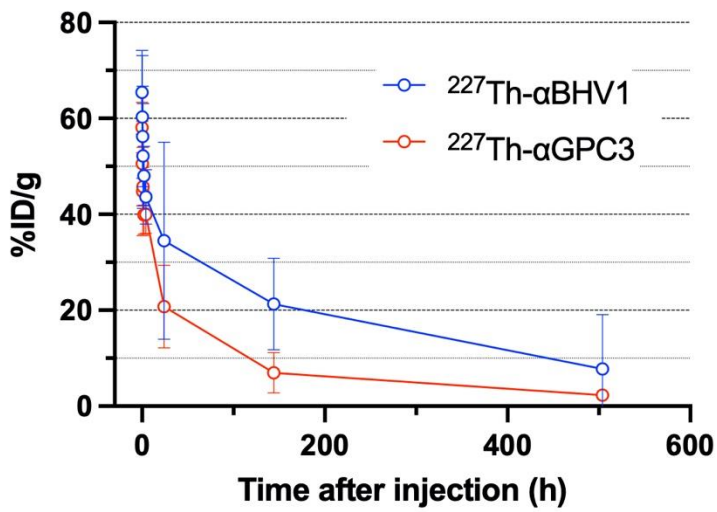


Figure 6. Comparative blood clearance of ^{227}Th radiolabeled αGPC3 -octapa and of αBHV1 -octapa. Comparative blood clearance profiles in tumor bearing mice at 5, 15, 30, 60, 120, 240 min, 24 h, 7 d and 23 d after injection with ^{227}Th radiolabeled αGPC3 -octapa and αBHV1 -octapa (n=4/time point). Symbol denotes mean, error bar denotes standard deviation.

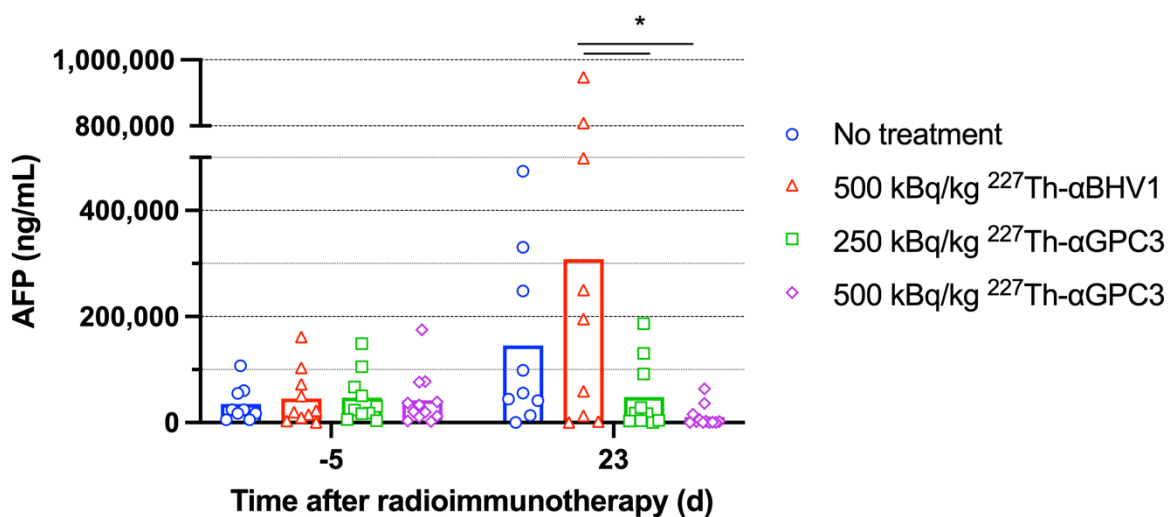
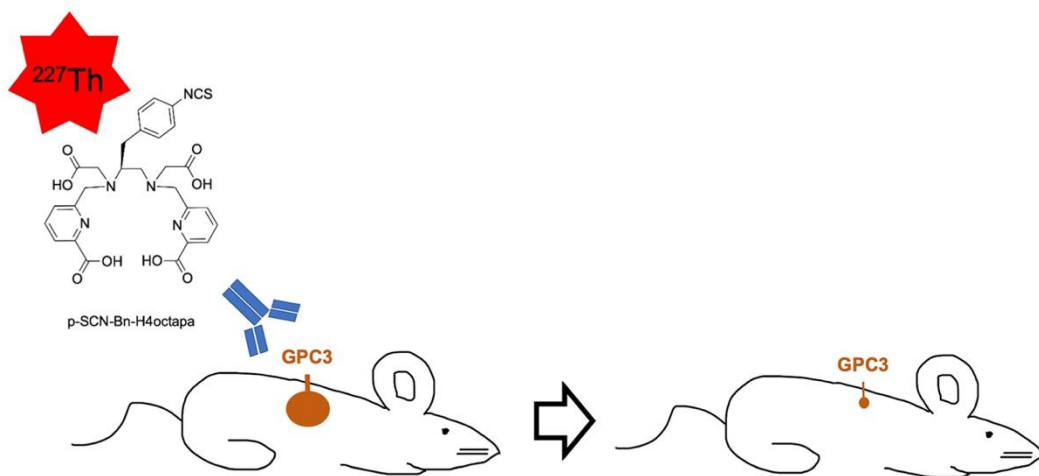


Figure 7. $^{227}\text{Th-}\alpha\text{GPC3}$ reduces tumor burden in murine model

Serum AFP before (day -5) and 23 days after receiving no treatment (n=9), or tail vein injection of 500 kBq/kg $^{227}\text{Th-}\alpha\text{BHV1}$ (n=9), 250 kBq/kg (n=10) or 500 kBq/kg $^{227}\text{Th-}\alpha\text{GPC3}$ (n=12). Bar represents mean. Symbols denote individual mice. *denotes $p < 0.05$ after unpaired two-way ANOVA with Sidak multiple comparison test.



Graphical Abstract

SUPPLEMENTAL METHODS

Conjugation of α GPC3 and α BHV1 With Octapa

Reagents and solvents purchased from commercial sources were analytical grade or higher and were used without further purification unless noted. High purity nitric acid was obtained from Honeywell Fluka as TraceSELECT™ Ultra grade. Antibodies were demetallated prior to and after octapa-NCS conjugation as previously described(1). Pipette tips, syringes and plastic vials used for monoclonal antibody (mAb) conjugation and ^{227}Th labeling were acid-washed to rid the surface of trace metals.

Size-exclusion high-performance liquid chromatography (HPLC) and radio-HPLC analyses were performed using a system that employs a Hewlett-Packard quaternary 1050 gradient pump, a Protein-Pak glass 300SW column (7.5 mm x 300 mm, 10 μm ; Waters Corp., Milford, MA), a variable wavelength UV detector and a Beckman 170 radiation detector. Gamma-ray spectroscopy was performed on a GEM18180-P HPGe detector coupled to a PC-based multichannel analyzer (AMETEK, Oak Ridge, TN). The spectra were analyzed using the Maestro-32 software (ORTEC, Oak Ridge, TN). Calibration setting numbers 72 and 92 on a Capintec CRC-55tR dose calibrator were used for freshly purified ^{227}Th solutions and solutions 1 day after purification, respectively(2). A Wallac Wizard 1470 gamma counter was also used to determine radiochemical purity of labeled products and count animal tissues.

The conjugation reaction followed a protocol similar to previously described(3,4). Briefly, the conjugation reaction was conducted in HEPES buffer (50 mM HEPES, 150 mM NaCl, pH 8.5). The HEPES buffer was passed over a Chelex 100 (BioRad, USA) column to remove trace metals prior to use. Metal-free mAb (α GPC3 or α BHV1) was reacted with 5, 10 or 15 eq of octapa-NCS at room temperature (RT) with gentle mixing for about 16 h. Then the

mixture was transferred to a Slide-A-Lyzer™ dialysis cassette and dialyzed against 4 L metal-free citrate buffer (50 mM citrate, 15 mM NaCl, pH 5.5) three times to quench the reaction. This was followed by dialysis against 3 L of metal-free saline changing twice per day for another 3 days. The octapa conjugates were then removed from the Slide-A-Lyzer™ dialysis cassette and stored under 4 °C.

Isoelectric focusing (IEF) analyses were conducted on a XCell SureLock Mini-Cell Electrophoresis system using pH 3-10 IEF gels (Thermo Fisher Scientific, Waltham, MA) to determine how the isoelectric point (pI) of the antibodies changed because of the conjugation of octapa-NCS. SERVA IEF standards used included: Cytochrome C; Pig heart origin; 10.7 pI, Ribonuclease A; Bovine pancreas origin; 9.5 pI, a Lectin; Lens culinaris origin; 8.3, 8.0, 7.8 pI, a Myoglobin; Horse muscle origin; 7.4, 6.9 pI, Carbonic anhydrase; Bovine erythrocytes origin; 6.0 pI, a B. Lactoglobulin; Bovine milk origin; 5.3, 5.2 pI. Trypsin inhibitor; Soybean origin; 4.5 pI. Glucose oxidase; Aspergillus niger origin; 4.2 pI, Amyloglucosidase; Aspergillus niger origin; 3.5 pI. Size exclusion (SE)-HPLC analyses were performed to assess the purity of the antibody conjugates. Mass spectral analyses of octapa-NCS conjugated mAbs were performed in duplicate on an Applied Biosciences SciEX 4800 MALDI TOF/TOF analyzer (Wellborn, TX) to estimate the average number of conjugates per α GPC3 or α BHV1 molecule.

Optimizing ^{227}Th Radiolabeling of α GPC3-octapa

Before being used for radiolabeling reactions, ^{227}Th was purified to remove its radioactive daughter, ^{223}Ra . Briefly, ^{227}Th was dissolved in 3M HNO_3 and H_2O_2 , followed by heating to dryness. The residue was dissolved in 3M HNO_3 and the solution was loaded on a TEVA resin (Eichrom Tehnologies, Lisle, IL, USA). The column was rinsed with 3M, 1M and 0.5 M HNO_3 , and the purified ^{227}Th was eluted using 0.1 M HNO_3 . Any purified ^{227}Th that was not used within

two days was purified again before use(2). Antibody labeling conditions were optimized using small quantities of ^{227}Th . In a typical reaction 200 μL of 0.05 M sodium citrate and 1 mM EDTA (pH 5.5) or 0.5 M ammonium acetate (NH_4OAc) (pH 5.5) was combined with 5-10 μL ^{227}Th in 0.1 M HNO_3 (85.1-314.5 kBq, or 2.3-8.5 μCi). Metal-free sodium citrate buffer (1 M) was used to adjust the pH to 5-6.5, and subsequently 200-500 μg of αGPC3 -octapa (4.0-5.7 mg/mL) was added and allowed to react for 30-120 min at RT or 37 $^\circ\text{C}$. The radiolabeling yield is also affected by the concentration of the ligand. The concentration dependence was not evaluated in this study, but is of interest in future studies.

^{227}Th Labeling of Antibodies for Animal Studies

To 200 μL of sodium citrate solution (0.05 M with 1 mM EDTA, pH 5.5), was added 60 μL of ^{227}Th in 0.1 M HNO_3 (180-210 μCi , 6,660-7,770 kBq). The pH of the solution was adjusted to 5.5 by adding an appropriate amount of 1 M sodium citrate. It was allowed to react for 2 h at RT after 1 mg of αGPC3 -octapa (0.217 mL of 4.6 mg/mL) or αBHV1 -octapa (0.21 mL of 4.75 mg/mL) was added to the reaction mixture. The ^{227}Th labeled mAbs were purified using Econo-Pac[®] 10DG columns (BIO-RAD, Hercules, CA).

***In vitro* Stability of ^{227}Th -octapa- αGPC3**

Free ^{227}Th and antibody associated ^{227}Th were separated by eluting 7-cm iTLC SG strips (Agilent, Santa Clara, CA) using 0.05 M sodium citrate with 1 mM EDTA (pH 5.5). The iTLC strips were cut into 3 sections. One 3-cm section that includes the origin to show activity bound to (denatured) protein, one 3-cm section that includes the solvent front to show free radioactivity, and a 1-cm section between the other two sections to determine if the radioactivity at the origin and at the solvent front were completely separated. The regions of the radio-iTLC strips that

correspond to ^{227}Th labeled αGPC3 -octapa and free ^{227}Th were measured on the HPGe detector using the same counting geometry. Although the percentage of protein-bound ^{227}Th determined by gamma-ray spectroscopy was similar to that determined by automated gamma counter at 4 h, significant differences were observed for all later time points as the daughter ^{223}Ra and other radioactive progenies could not be distinguished from ^{227}Th by the automated gamma counter.

Cell Lines and Tissue Culture

Cells were grown to 70-80% confluency, detached with 0.25% trypsin and passaged into new cell culture flasks per manufacturer instructions (PerkinElmer). Cells were passaged between 4-10 times between thawing and use in described experiments. *Mycoplasma* testing was not performed.

Flow Cytometry

HepG2 cells were resuspended in cold phosphate-buffered saline (PBS) at a concentration of 1×10^6 cells/mL. One microgram of unconjugated αGPC3 , αGPC3 -octapa or αBHV1 -octapa was added to the cell suspension and incubated for 45 min at 4°C . After incubation, samples were washed in cold PBS, and incubated with $1\mu\text{g}$ of FITC-labeled goat- α -mouse IgG_1 secondary antibody (Southern Biotech, cat. no. 1070-02) on ice for 30 min in the dark. Cells were then washed in cold PBS and were analyzed with a LSRII (BD Biosciences, San Jose, CA) using the FACS Diva software. A minimum of 10,000 cells were analyzed for each sample in triplicate. Data analysis was performed on the FlowJo software, version 8.8.6 (TreeStar, Ashland, OR; RRID:SCR_008520).

Development of Orthotopic Xenograft Model

The orthotopic xenograft model was generated and described previously⁽¹⁾. Briefly, after a week of acclimatizing, 8-week-old female athymic Nu/J animals (20-25g) (Jackson Laboratories)

were anesthetized using 1.5% inhaled isoflurane and the left lobe of the liver was exposed through an upper midline laparotomy. Approximately 2×10^6 Luciferase-expressing GPC3-positive HepG2 cells in 25 μ L of Dulbecco's modified Eagle medium containing 50% Matrigel (BD Biosciences) were injected into the subcapsular space of the anterior left hepatic lobe. Four weeks after injection, a 75 mg/kg intraperitoneal injection of VivoGlo luciferin (Promega) was administered and bioluminescent imaging was performed using an IVIS Lumina II system (PerkinElmer) to verify tumor establishment.

Serum AFP Measurement

To monitor orthotopic tumor growth, whole blood was obtained from animals by submandibular bleed and collected in Eppendorf tubes(5). Serum was extracted from the fresh whole blood and the serum concentration of alpha-fetoprotein (AFP) was determined on the UniCel Dxl 800 Access Immunoassay System (Beckman Coulter) using an Access AFP Alpha-fetoprotein pack (Quest Diagnostics; RRID:SCR_005210) and reported in nanograms/milliliter (ng/mL). Serum AFP has been extensively validated to correlate with tumor size in our model (1,6).

SUPPLEMENTAL TABLES

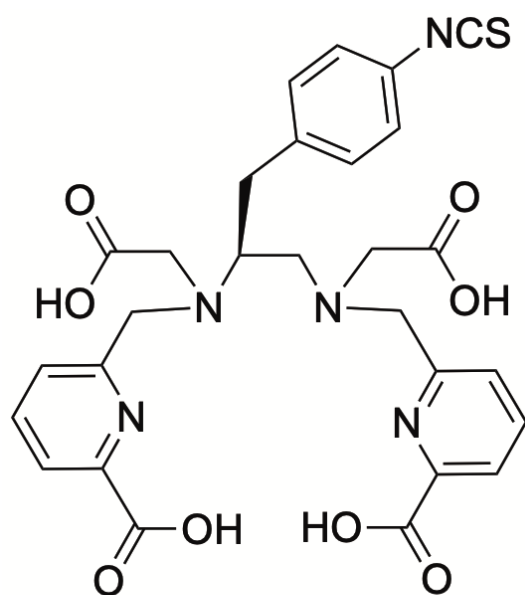
Tumor: Organ Ratio	DAY 1		DAY 7		DAY 23	
	Avg	Stdev	Avg	Stdev	Avg	Stdev
Blood	12.0	6.8	60.9	58.9	90.8	70.0
Lung	23.4	18.6	26.9	5.6	118.4	80.7
Heart	46.4	27.4	80.2	34.3	301.1	191.5
Liver	25.7	9.5	29.3	9.7	58.3	41.5
Spleen	35.7	19.2	18.7	10.5	49.1	31.6
Kidney	29.1	7.2	31.1	8.5	101.5	70.5
Cecum	163.5	62.8	356.9	312.7	480.0	306.3
Stomach	166.6	77.6	360.9	216.8	794.2	590.6
Tail	52.3	33.3	115.8	47.2	115.8	77.3
Muscle	133.7	61.8	776.9	872.1	829.5	734.2
Bone	96.2	50.5	76.6	40.2	46.9	40.8

Supplemental Table S1 – Tumor to organ ratios after injection of 500 kBq/kg ²²⁷Th-αGPC3 at 1, 7 and 23 days after injection.

	$^{223}\text{Ra}/^{227}\text{Th}$ ratio	Normalized $^{223}\text{Ra}/^{227}\text{Th}$
Standard	0.78	1.00
Bone	2.86	3.67
Kidneys	0.29	0.37
Liver	0.37	0.48
Spleen	0.56	0.71
Tumor	0.30	0.38

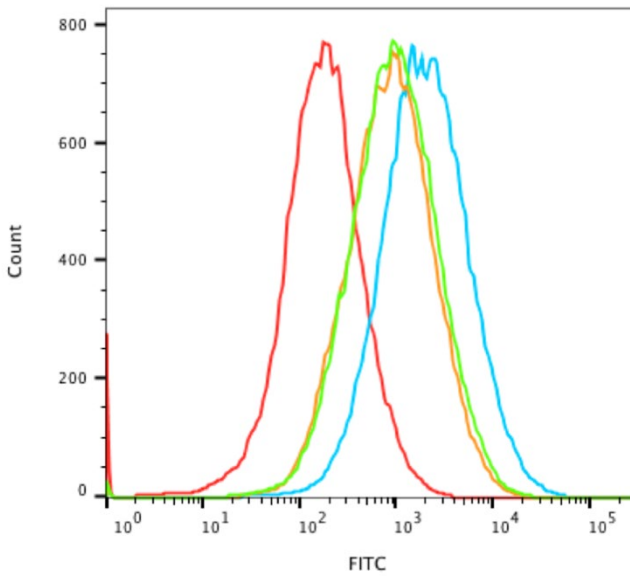
Supplemental Table S2. Distribution of ^{223}Ra and ^{227}Th in tissues. $^{223}\text{Ra}/^{227}\text{Th}$ ratios and normalized $^{223}\text{Ra}/^{227}\text{Th}$ ratios in selected tissues 21 days after injection of of 500 kBq/kg ^{227}Th - αGPC3 determined by high-resolution gamma-ray spectroscopy. The standard is an aliquot of the injectate.

SUPPLEMENTAL FIGURES



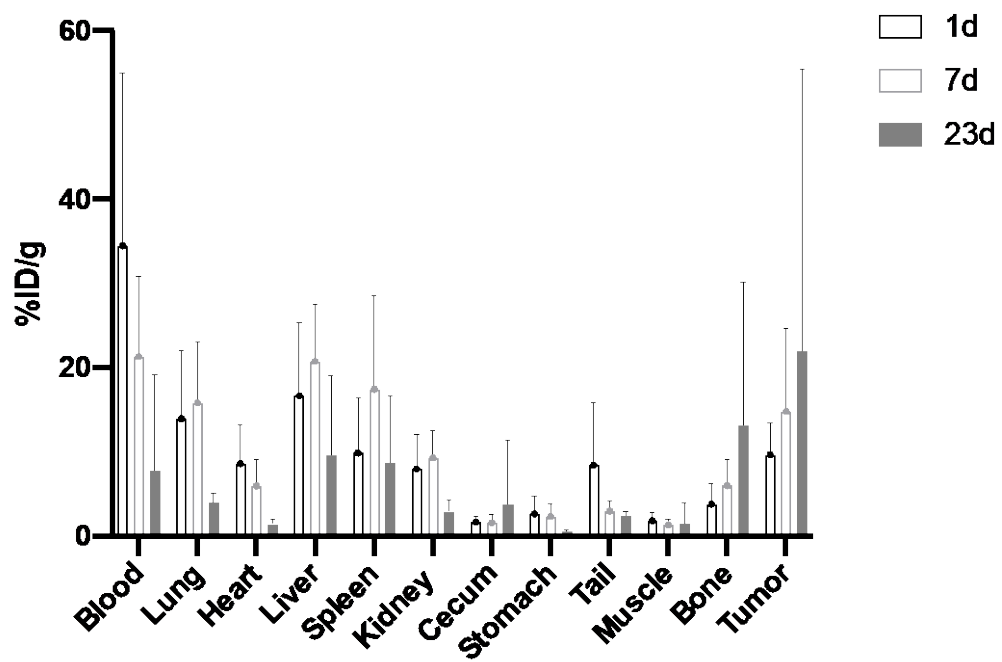
p-SCN-Bn-H4octapa

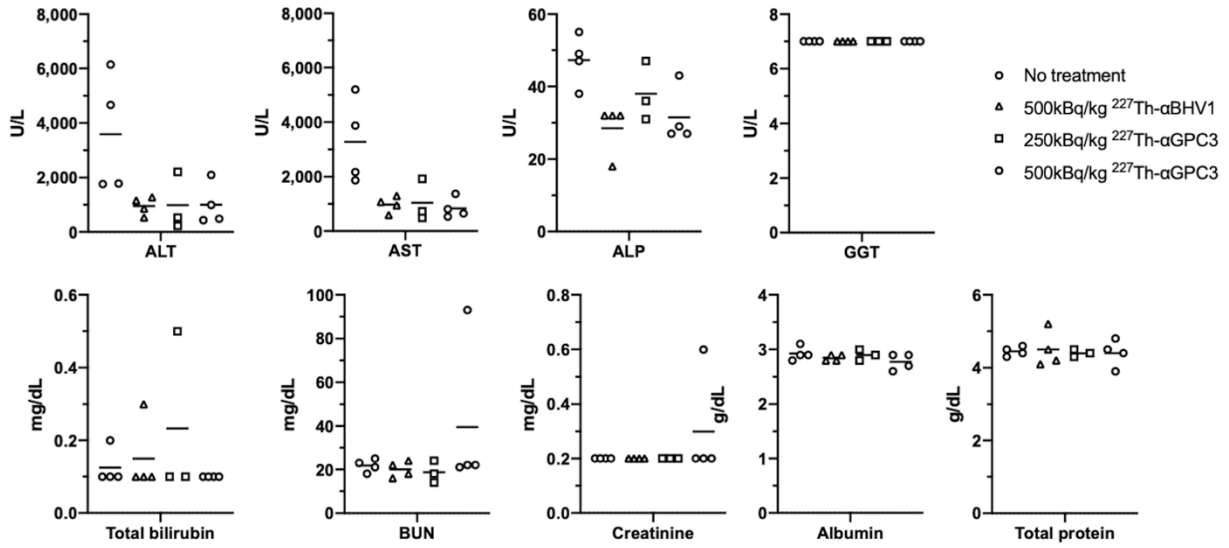
Supplemental Figure S1. Chemical structure of octapa



Supplemental Figure S2. *In vitro* GPC3 binding assessed by flow cytometry on human HepG2 cells with unconjugated (red), and α GPC3-octapa 5eq (blue), α GPC3-octapa 10eq (green), α GPC3-octapa 15eq (orange).

^{227}Th - αBHV1





Supplemental Fig S4. Serum chemistry profiles of animals 23 d after receiving no treatment, 500 kBq/kg $^{227}\text{Th-}\alpha\text{BHV1}$, 250 kBq/kg $^{227}\text{Th-}\alpha\text{GPC3}$ or 500 kBq/kg $^{227}\text{Th-}\alpha\text{GPC3}$. Line represents mean. Symbols denote individual mice.

Supplemental References

1. Ludwig AD, Labadie KP, Seo YD, et al. Yttrium-90-Labeled Anti-Glypican 3 Radioimmunotherapy Halts Tumor Growth in an Orthotopic Xenograft Model of Hepatocellular Carcinoma. *J Oncol*. 2019;2019:4564707.
2. Ferrier MG, Li Y, Chyan MK, et al. Thorium chelators for targeted alpha therapy: Rapid chelation of thorium-226. *J Labelled Comp Radiopharm*. 2020;63:502-516.
3. Price EW, Cawthray JF, Bailey GA, et al. H4octapa: an acyclic chelator for 111In radiopharmaceuticals. *J Am Chem Soc*. 2012;134:8670-8683.
4. Price EW, Zeglis BM, Cawthray JF, et al. H(4)octapa-trastuzumab: versatile acyclic chelate system for 111In and 177Lu imaging and therapy. *J Am Chem Soc*. 2013;135:12707-12721.
5. Golde WT, Gollobin P, Rodriguez LL. A rapid, simple, and humane method for submandibular bleeding of mice using a lancet. *Lab Anim (NY)*. 2005;34:39-43.
6. Labadie KP, Ludwig AD, Lehnert AL, et al. Glypican-3 targeted delivery of (89)Zr and (90)Y as a theranostic radionuclide platform for hepatocellular carcinoma. *Sci Rep*. 2021;11:3731.