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Bifunctional Phage-Based Pretargeted Imaging of Human Prostate Carcinoma Bifunctional Phage Based Pretargeted Imaging

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Abstract

Introduction—Two-step and three-step pretargeting systems utilizing biotinylated prostate tumor-homing bacteriophage (phage) and ¹¹¹In-radiolabeled- streptavidin or biotin were developed for use in cancer radioimaging. The *in vivo* selected prostate carcinoma-specific phage (G1) displaying up to five copies of the peptide IAGLATPGWSHWLAL, was the focus of the present study.

Methods—The ability of G1 phage to extravasate and target prostate tumor cells was investigated using immunohistochemistry. G1 phage were biotinylated, streptavidin was conjugated to diethylenetriaminepentaacetic acid (DTPA), and biotin was conjugated to 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). Biodistribution studies and single photon emission computed tomography (SPECT)/CT imaging of xenografted PC-3 tumors via two-step pretargeted ¹¹¹In-labeled streptavidin and three-step pretargeted ¹¹¹In-labeled biotin were performed in SCID mice to determine the optimal pretargeting method.

Results—The ability of G1 phage to extravasate the vasculature and bind directly to human PC-3 prostate carcinoma tumor cells *in vivo* was demonstrated via immunocytochemical analysis. Comparative biodistribution studies of the two-step and three-step pretargeting strategies indicated increased PC-3 human prostate carcinoma tumor uptake in SCID mice of 4.34 ±0.26 %ID/g at 0.5 hours post-injection of ¹¹¹In radiolabeled biotin (utilized in a three-step protocol) compared to that of 0.67 ±0.06 %ID/g at twenty four hour postinjection of ¹¹¹In radiolabeled streptavidin (employed in a two-step protocol). *In vivo* SPECT/CT imaging of xenografted PC-3 tumors in SCID mice with the three-step pretargeting method was superior to that of the two-step pretargeting method, and, importantly, blocking studies demonstrated specificity of tumor uptake of ¹¹¹In-labeled biotin in the three-step pretargeting scheme.

Conclusion—This study demonstrates the use of multivalent bifunctional phage in a three-step pretargeting system for prostate cancer radioimaging.

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Keywords

Bacteriophage; Bifunctional Phage; Two-step pretargeting; Three-step pretargeting; Radioimaging; Prostate Cancer

1. Introduction

Investigations into the use of radioimmunoimaging (RII) and radioimmunotherapy (RIT) have been ongoing for more than two decades. Radiolabeled monoclonal antibodies against CD20 have been successfully utilized in patients with non-Hodgkin's B-cell lymphoma [1,2]. Another antibody showing clinical promise is Trastuzumab (Herceptin), a humanized monoclonal antibody that has been utilized in both RII and RIT studies for many types of solid human tumors expressing the HER2/neu tumor antigen [3–9]. However, the unfavorable pharmacokinetic properties of whole antibodies and antibody fragments, and the resulting toxicity towards normal tissue, are major drawbacks to the use of directly labeled antibodies for the delivery of radionuclides to solid tumors [10,11]. An alternative strategy to the use of radiolabeled antibodies is to partition the delivery of the antibody and the radionuclide using pretargeting techniques [12,13]. One form of pretargeting has focused on the use of the streptavidin (SA)/avidin-biotin system because of the extraordinarily high affinity ($K_D = 10^{15} \text{ M}^{-1}$) of SA for biotin, as well as, the potential for signal amplification due to the tetrameric architecture of SA. Fritzberg and co-workers employed a monoclonal antibody (NR-LU-10)/SA conjugate to pretarget epithelial cellular adhesion molecule (Ep-CAM), expressed on human colon carcinoma xenografts for the efficient delivery of β -emitting ^{90}Y [14] and α -emitting ^{212}Bi [15]. Other pretargeting methods for the diagnosis and therapy of cancer have been developed [16–23]. One such approach focuses on the use of phage to pretarget tumor antigens [23]. Phage are multivalent and thus should have an increased avidity for the intended target compared to that of bivalent antibodies or antibody fragments. Phage are also easily derivatized via their coat proteins, which allows multiple reporter tags to be coupled to each virion. Thus, the development of pretargeted, multivalent and bifunctional phage may have benefits over implementation of antibodies for the RII or RIT of cancer.

However, the successful implementation of phage as imaging agents would require a better understanding of phage pharmacokinetics and biodistribution *in vivo*. Our laboratory previously analyzed the biodistribution properties of commonly employed phage display libraries in mice and demonstrated that phage were primarily cleared through the organs of the reticuloendothelial system [24]. Based on these studies, we devised a new *in vivo* selection scheme to obtain tumor-homing phage able to extravasate the vasculature and bind directly to the tumor tissue in comparison to the commonly utilized selection procedures that result in vasculature binding phage clones [25,26]. One phage clone that was obtained in our laboratory, G1, displaying the foreign peptide sequence IAGLATPGWSHWLAL on coat protein III, was developed into a multivalent, bifunctional, biological nanoparticle for the *in vivo* targeting and optical imaging of prostate cancer [27]. Phage have been employed by other groups to image a variety of disease pathologies. Kelly et al. [28] *in vivo* imaged Lewis lung carcinoma and inflammation through the use of fluorescently labeled phage targeting osteonectin and vascular cell adhesion molecule-1, respectively. Segers et al. [29] exploited phage displaying a peptide with affinity for phosphatidylserine, by labeling with ultrasmall iron oxide particles for use as a contrast agent for the magnetic resonance imaging of apoptosis. Phage have also been directly radiolabeled with $^{99\text{m}}\text{Tc}$ for the *in vivo* imaging of infection [30,31]. However, because of the previously described unfavorable pharmacokinetics of phage particles, the use of phage directly labeled with radioisotopes would lead to extended exposure of non-target tissues (such as the liver and spleen) and potential harmful radioactive-induced damage. Nevertheless, we

reasoned that phage can be effective radioimaging agents; however, the delivery of the targeting phage and the radiolabel should be segregated as part of a pretargeting strategy.

Formerly a pretargeting strategy was employed by our laboratory for the development of phage-based biological nanoparticles displaying multiple copies of tumor-homing peptides to be used in cancer imaging [23]. The first pretargeting technique we attempted centered on phage displaying an engineered analog of the natural peptide hormone, α -melanocyte stimulating hormone (α -MSH), that binds the melanocortin-1 receptor overexpressed on malignant melanoma [23]. The engineered phage (MSH2.0) were examined for their ability to target malignant melanoma in a C57 mouse *in vivo* using a two-step pretargeting scheme. It was theorized that the two-step pretargeting system would allow the clearance of the majority of unbound phage before the injection of the imaging label. However, even at twenty four hours postinjection of the ^{111}In -radiolabeled SA there were significant levels of activity within non-targeted tissues. Therefore, we decided to examine the potential use of biotinylated, tumor homing phage for use in a three-step pretargeting protocol.

It was decided to examine the PC-3 human prostate tumor-targeting phage, G1, to determine if they could function in a pretargeting approach as *in vivo* SPECT radioimaging agents for the detection of prostate carcinoma. G1 phage were previously selected *in vivo* in SCID mice bearing human PC-3 prostate carcinomas and were subsequently examined both *in vitro* and *in vivo* for use as a biological nanoparticle for the targeting of prostate carcinoma [27]. The less than optimal biodistribution and retention of ^{111}In -radiolabeled SA in C57 mice bearing B16-F1 mouse melanoma tumors observed by our laboratory as well as others [32–34], prompted us to explore whether a three-step pretargeting scheme may make the best use of phage for the radioimaging of tumors. The three-step pretargeting system involved first, administration of biotinylated G1 phage, followed by injection of avidin, and finally, ^{111}In -labeled DOTA-biotin injection. It was hypothesized that the small molecular weight of biotin would favor fast clearance of excess radiolabel as well as reduce non-target tissue uptake. Biotinylated G1 phage were able to localize to PC-3 carcinomas in SCID within four hours and were used in both two-step and three-step pretargeting protocols. Analysis of *in vivo* biodistribution data revealed increased tumor uptake and retention of ^{111}In -labeled biotin used in the three-step method over that of ^{111}In -labeled SA used in the two-step method within xenografted PC-3 tumors in SCID mice. The liver uptake of ^{111}In -labeled biotin was distinctly improved over the two-step pretargeting method even after twenty four hours post injection of ^{111}In -labeled SA. Kidney uptake and retention of ^{111}In -labeled SA and ^{111}In -labeled biotin were found to be similar for all time points investigated. SPECT/CT imaging studies revealed the three-step pretargeting method a more efficient technique for the visualization of the pretargeted tumor than that of the two-step pretargeting method and blocking studies verified the specificity of ^{111}In -biotin tumor uptake. These data provide proof of the utility of bifunctional, multivalent phage as a biological nanoparticle to facilitate the three-step pretargeted radioimaging of prostate cancer.

2. Materials and Methods

2.1. Materials

Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO), unless otherwise stated.

2.2. Cell lines

The human prostate carcinoma cell line PC-3, [35] was grown in Ham's F12K media, 7% fetal bovine serum (FBS), 2 mM L-glutamine, and 48 $\mu\text{g}/\text{ml}$ gentamicin at 37° C in 5% CO_2 . The

cell line was tested for pathogens before injection into mice by the Cell and Immunobiology Core Facility at the University of Missouri.

2.3. Generation of clonal phage populations

The G1 phage clone was previously isolated [27] and was amplified in *Escherichia coli* (E. coli) K91 Blue Kan. Purification of the amplified phage included polyethylene glycol (PEG) precipitation and cesium chloride ultracentrifugation [35]. Following dialysis in 1X Dulbecco's PBS, Ca²⁺ and Mg²⁺-free (PBS) the phage particle concentration in virions (V) was determined spectrophotometrically and the amount of infectious units (transducing units, TU) were determined by titring on E. coli K91 Blue Kan [35]. Proper DNA sequence was verified by DNA sequencing at the University of Missouri DNA Core Facility.

2.4. Biotinylation of phage

Biotinylation of G1 phage was performed as previously described [23]. Briefly, a suspension of phage in 100 mM NaH₂PO₄ (pH 7.4 with NaOH) was mixed with a 1000-fold molar excess of NHS-PEO₄-biotin (Pierce Biotechnology, Rockford, IL) dissolved in DMSO. This solution was allowed to incubate on a rotator at room temperature for 2 hours. A final concentration of 400 mM ethanolamine (pH 9.0) was added to the solution to quench the reaction followed by incubation for 1 hour on a rotator at room temperature. The biotinylated phage preparation was then extensively dialyzed against TBS to remove excess free biotin. Determination of the extent of biotinylation was performed as described previously [36,37]. In short, the biotinylated phage were digested with proteinase K and the resulting biotin and biotinylated peptide fragments were analyzed in an inhibition ELISA.

2.5. Covalent attachment of DTPA to SA

2-(4-isothiocyanatobenzyl)-diethylenetriaminepentaacetic acid (DTPA) (Macrocyclics, Dallas, TX) was dissolved in carbonate buffer (0.5 M sodium carbonate, pH 9.5) and SA was suspended in 1X PBS. DTPA was added to the SA to yield a mixture containing 50 fold more DTPA than SA. This solution was rotated overnight at 4°C, followed by removal of excess DTPA from the mixture of DTPA, SA, and DTPA-SA (DTPA-SA) complex with Zeba Desalt Spin Columns (Pierce Biotechnology, Rockford, IL).

The number of DTPA molecules per SA was then investigated. A solution of 0.1 M SA-DTPA conjugate was incubated with 0.5 M europium (Eu) in an ammonium acetate (0.1 M, pH 5.5) buffer for 1.5 hours at 37°C. Excess Eu was removed with Zeba Desalt Spin Columns (Pierce Biotechnology, Rockford, IL). The absorbance at 465nm [38] of the purified Eu-DTPA-SA complex was then measured and the Lambert – Beer law applied to determine the concentration of Eu/DTPA.

2.6. ¹¹¹In-radiolabeling of DOTA-biotin and DTPA-SA

100 µg of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-biotin (Macrocyclics, Dallas, TX) was dissolved in ammonium acetate (0.1 M, pH 5.5) and added to a solution of 5.6 MBq of ¹¹¹InCl₃ (specific activity = 370 MBq/mL) (Mallinckrodt Inc., St. Louis, Missouri) for a total reaction volume of 100 µL and was incubated at 85°C for 45 minutes. Free ¹¹¹In was removed from the reaction by the addition of EDTA (0.05 mM final concentration). The resulting radiolabeled DOTA-biotin was then purified by reverse phase-high pressure liquid chromatography (RP-HPLC) using a linear gradient of water/0.1% TFA and acetonitrile/0.1% TFA. Purified sample was flushed with N₂ gas for 30 minutes to remove the acetonitrile. The pH of the final solution was adjusted to 6.0 with 0.1 N NaOH and saline (0.9% w/v of NaCl) for animal studies. The resulting specific activity of the radiolabeled DOTA-biotin was, on average, 504 kBq/µg. DTPA-SA was radiolabeled with ¹¹¹In by the

addition of 9.3 MBq to 300 µg DTPA-SA in 10 mM HEPES (pH 7.0) followed by incubation at 37°C for 1.5 hours. Excess ^{111}In was removed with Zeba Desalt Spin Columns (Pierce Biotechnology, Rockford, IL). The resulting specific activity of the radiolabeled DTPA-SA was, on average, 24.43 kBq/µg.

2.7. Animal studies

All animal studies were conducted in accordance to the NIH guide for Care and Use of Laboratory Animals and the Policy and Procedures for Animal Research at the Harry S. Truman Memorial Veterans Hospital. Four- to 6-week-old (approximately 20 grams) ICRSC-M SCID (SCID) outbred mice were obtained from Taconic (Germantown, New York). The animals were provided with water *ad libitum* and fed biotin free rodent chow for 5 days prior to injection. SCID mice receiving 5×10^6 PC-3 human prostate carcinoma cells were subcutaneously inoculated in the right flank or intrascapular, region. Four weeks post-inoculation, tumors were approximately 1 cm in diameter.

SCID mice with xenografted PC-3 human prostate carcinoma tumors received a tail vein injection containing 1×10^{11} virions of G1 phage. Previous work [23,27] revealed the optimal tumor uptake and tumor to non-tumor ratios of phage uptake to be four hours postinjection. Thus, the phage were allowed to circulate within the mouse for four hours before the mice were then sacrificed and tissues and organs harvested. Liquid nitrogen was mixed with enough methylbutane to attain a temperature of -60°C and the tissues were then frozen by immersion for 20 to 30 seconds. Ten µm thick slices were obtained from the frozen tissues, immediately fixed in 95% EtOH, and air dried overnight. Immunohistology was performed using anti-phage (gifted to us by Dr. George Smith), anti-platelet endothelial cell adhesion molecule-1 (PECAM-1) (Santa Cruz Biotechnology, Santa Cruz, California), and anti-ErbB-2 antibodies (USBiological, Swampscott, Massachusetts). Briefly, the slides containing normal or cancerous tissue were blocked overnight in 5% BSA in TBS. The presence of phage within the tissues was probed using an anti-phage antibody, followed by an anti-rabbit secondary antibody conjugated to Alexa Fluor 488 or Alexa Fluor 647 (Invitrogen, Carlsbad, CA). Endothelial cells were visualized using anti-PECAM and anti-goat antibodies conjugated to Alexa Fluor 488. Prostate tumor cells were visualized with anti-ErbB-2 antibody and anti-sheep secondary antibody conjugated to Alexa Fluor 546. The presence of fluorophore was detected using laser scanning confocal microscopy performed on a Bio-Rad MRC 600 confocal microscope (University of Missouri Molecular Cytology Core Facility).

Two-step tumor targeting studies included groups of 3 mice receiving tail vein injections of 1×10^{11} virions of biotinylated phage. Phage were allowed to circulate for four hours followed by another tail vein injection of 1.85 MBq of ^{111}In -labeled DTPA-SA. Mice were then sacrificed by cervical dislocation at 0.5, 2, 4, 6, or 24 hours post injection for the purpose of harvesting the organs of interest. Each organ was weighed and counted in a Wallac 1480 automated gamma counter (Perkin Elmer, Wellesley, MA). Mice used for imaging received a tail vein injection of 5×10^{12} virions of biotinylated phage followed by an injection of 7.40 MBq of ^{111}In -DTPA-SA. The mice were euthanized four hours post injection of the radiolabel with carbon dioxide. Imaging was performed by the Biomolecular Imaging Center in the Harry S. Truman VA Medical Center using a CTI Concord Microsystems microSPECT scanner (CTI Concorde Microsystems, Knoxville, TN). Data was gathered for 15 hours and the resulting images were reconstructed using a filtered backprojection reconstruction algorithm. The SPECT images were fused with conventional microCT (IMTEK, Inc., Freiburg Germany) images to validate regions of increased radiolabeled ligand uptake.

Three-step tumor targeting studies utilized groups of 3 mice. Each mouse received a tail vein injection of 1×10^{11} virions of biotinylated phage. Four hours post-injection the mice received a second injection containing 50 µg avidin (molar ratio of 10 cold avidin molecules to each

phage virion). The avidin was allowed to circulate within the mice for a period of twenty four hours. At the end of this incubation period the mice received the final injection of 0.37 MBq ^{111}In -DOTA-biotin. Thirty minutes post injection of the radiolabeled biotin the mice were sacrificed and processed the same as described for the two-step pretargeting studies. Mice used for the purpose of imaging received 7.40 MBq of ^{111}In -DOTA-biotin. All means with a t test value of $p < 0.05$ were considered to be significantly different.

3. Results

3.1. Immunohistochemical investigation of extravasation of phage from the vasculature

In this study, the previously *in vivo* selected G1 phage were utilized for the development of a novel phage-based pretargeting strategy for the SPECT/CT imaging of prostate cancer. The G1 phage clone was previously selected under conditions that were predicted to insure recovery of phage that extravasated the vasculature and bound directly to human PC-3 prostate carcinoma tumor cells. The procedure utilized for the *in vivo* selection of G1 phage included homogenization, washing, and gentle lysing of excised PC-3-derived human prostate carcinoma tumor tissue with a CHAPS detergent for the identification of phage that were bound directly to PC-3 tumor cells [27]. Here, the ability of phage to localize to the tumor and consequently bind PC-3 derived tumor cells *in vivo* was examined through immunohistological analysis of resected PC-3 tumor tissue from SCID mice injected with G1 phage. PC-3 human prostate carcinoma tumor bearing mice injected with biotinylated G1 phage were sacrificed four hours post injection followed by tissue harvesting and fixation. G1 phage particles were found in the liver and tumor tissues but not the muscle (Figure 1). G1 phage particles were located within much of the vasculature of both the liver and tumor tissues as well as bound to the surrounding tissue. Notably, extravasated G1 phage did not relocalize from the vasculature and spread into the surrounding tumor tissue in a homogenous pattern. In order to better determine the areas of phage binding we performed colocalization studies. Closer inspection of the localization of G1 phage within PC-3 human prostate carcinoma tumor tissue included colocalization studies of G1 phage with ErbB-2, a tumor cell marker, (Figure 2a) and PECAM-1, an endothelial cell marker (Figure 2b). ErbB-2 is an epidermal growth factor receptor which has been shown to be overexpressed in the PC-3 cell line as well as prostate carcinoma primary and metastatic tumors [39,40]. PECAM-1, also referred to as CD-31, is a cell adhesion molecule which is commonly utilized as an endothelial cell marker [41,42]. As shown in Figures 2a and 2b, G1 phage (shown in blue) could be visualized within the vasculature of the PC-3 carcinoma tissue and infiltrating the tumor tissue in an asymmetric fashion. Colocalization (shown as pink) of the G1 phage (blue) with ErbB-2 (red) as seen in Figure 2a demonstrated the binding of G1 phage directly to the PC-3 human prostate carcinoma tumor tissue. The uneven staining of PECAM-1 in Figure 1b confirmed the presence of discontinuous endothelium, also known as a “mosaic vessel”, within the tumor [43,44]. Conspicuously, the phage and PECAM-1 staining did not overlap or colocalize. These data demonstrated that the phage indeed extravasate the vasculature and bind directly to the PC-3 human prostate carcinoma tumor tissue *in vivo*. Thus, it was assumed that biotinylated-G1 phage would be able to localize and bind to prostate tumors *in vivo*, an important pre-requisite for pretargeting approaches. Having shown that the G1 phage displaying the foreign peptide, IAGLATPGWSHWLAL, can function as a pretargeting biological nanoparticle by binding prostate tumor cells *in vivo*, the distribution and tumor-targeting properties of the pretargeted biotinylated-G1 phage were investigated in both a two-step and three-step format.

The two-step pretargeting method utilized biotinylated-G1 phage and ^{111}In -DTPA-SA as previously described by Newton et al. [23]. The DTPA-SA conjugates generated contained, on average, 2 DTPA chelates per SA and the G1 phage were biotinylated with, on average, five biotins per phage particle. The added mass of 5 biotins did not significantly alter the final

mass of the phage particle (23.6 MDa). The two-step pretargeting method required, in brief, tumor bearing SCID mice to receive a tail vein injection of 1×10^{11} phage. The biotinylated-G1 phage were allowed to circulate within the mice for four hours, at which time the mice received a second tail vein injection containing ^{111}In -DTPA-SA (Figure 3). Each mouse was then sacrificed twenty four hours post injection of the ^{111}In -DTPA-SA for investigation of either the biodistribution of the radiolabel or for SPECT/CT imaging. In comparison, the three-step pretargeting protocol delivered the radiolabel via the small molecule ^{111}In -DOTA-biotin (~840 Daltons), instead of the much larger molecular weight ^{111}In -DTPA-SA (~60,000 Daltons) (Figure 3). The tumor-bearing SCID mice being treated with the three-step pretargeting method first received an intravenous injection of biotinylated-G1 phage. Four hours post injection of the biotinylated phage, the tumor bearing mice received a second injection containing avidin. Twenty four hours later the same mice then received the final injection of ^{111}In -DOTA-biotin. Half an hour post injection of the radiolabeled biotin, the mice were then subjected to biodistribution studies or SPECT/CT imaging.

3.2. Two-step pretargeting of PC-3 human prostate carcinoma

The first pretargeting method investigated was the two-step protocol and the pharmacokinetics of the ^{111}In -DTPA-SA within SCID mice bearing human PC-3 prostate carcinoma tumors were analyzed. The biotinylated-G1 phage were injected into SCID mice bearing human PC-3 xenografted tumors and allowed to circulate for four hours. Tumor bearing mice were sacrificed at 0.5, 2, 4, 6, and 24 hours post injection of ^{111}In -DTPA-SA. The distribution of the radiolabeled SA within the mice was determined by harvesting the tissues and organs of interest and using a gamma counter to determine the amount of radioactivity contained within each tissue sample. The clearance of ^{111}In radioactivity was found to be primarily through the urinary and hepatobiliary systems (Figure 4a). Tumor accumulation of the radiolabel began at 1.66 ± 0.39 %ID/g at 30 minutes post injection and decreased to 0.43 ± 0.11 %ID/g at six hours post injection. The final tumoral accumulation of ^{111}In -DTPA-SA at twenty four hours post injection was 0.67 ± 0.06 %ID/g (Figure 4a). It was assumed that the initial tumor uptake was, in part, due to the large amount of activity within the blood. However, at twenty four hours post injection, the retention of the radiolabel within the tumor was primarily attributed to the presence of biotinylated tumor-homing G1 phage. To validate this assumption, the biodistribution of ^{111}In -DTPA-SA only was investigated. At twenty four hours post injection, the tumor uptake and retention of ^{111}In -DTPA-SA without the presence of the biotinylated tumor-homing phage was 0.46 ± 0.02 %ID/g (Table 1) ($p = 0.0014$). Importantly, the tumor to blood ratio for the non-targeted ^{111}In -DTPA-SA at twenty four hours post injection was 1.19 compared to the tumor to blood ratio for the pretargeted ^{111}In -DTPA-SA of 1.40 (Table 1). These data are similar to those found previously in C57/BL6 mice bearing mouse melanoma tumors [23] and suggest that the tumor uptake and retention of the radiolabeled SA was due to the presence of biotinylated G1 phage within the PC-3 tumor.

3.3. Three-step pretargeting of PC-3 human prostate carcinoma

In an effort to improve the tumor uptake and clearance of the radiolabeled pretargeted agent a three-step strategy for imaging was developed. The three-step pretargeting approach was similar to the previously described two-step pretargeting scheme in that it utilized biotinylated phage, avidin, and ^{111}In -DOTA-biotin (Figure 3). For this procedure, a PC-3 human prostate carcinoma-bearing mouse first received a tail vein injection of biotinylated phage which was allowed to circulate for four hours, followed by a second tail vein injection of avidin. Twenty four hours after the injection of avidin, the same mouse received an injection containing ^{111}In -DOTA-biotin, which was then allowed to circulate and clear the mouse for varying amounts of time. Presumably, the small molecular weight of the radiolabeled biotin resulted in quick and efficient clearance of radiolabel from the mouse through the urinary system which resulted in much lower activity in non-target tissues (Figure 4b). Tumor uptake

was found to be 4.34 ± 0.26 %ID/g at 0.5 hours post injection followed by 1.43 ± 0.04 %ID/g and 0.94 ± 0.11 %ID/g at 0.75 hours and 1 hour post injection, respectively. Notably, the blood to tumor ratio at 0.5 hours post injection of ^{111}In -DOTA-biotin was 1.76 (Table 1). Again, the *in vivo* distribution of the pretargeted radiolabel alone was investigated to determine what amount of tumor uptake and retention of the radiolabel was due to the presence of tumor bound biotinylated G1 phage and avidin. Tumor uptake of non-pretargeted ^{111}In -DOTA-biotin only, was found to be 3.26 ± 0.46 %ID/g at 0.5 hours post-injection with a blood to tumor ratio of 1.62 ($p = 0.012$) (Table 1). Radiolabel uptake within the liver and lung tissues was also increased via the three-step pretargeting method. Liver uptake of ^{111}In -DOTA-biotin was 1.20 ± 0.11 %ID/g within the mice treated with the three-step pretargeting method versus that of 0.63 ± 0.19 %ID/g in the non-pretargeted mice. ^{111}In -DOTA-biotin within the lung tissue was found to be 2.09 ± 0.43 and 1.67 ± 0.53 %ID/g in three-step pretargeted and non-pretargeted mice, respectively. These data suggest that the tumor uptake of the radiolabel is due to the presence of biotinylated G1 phage/avidin complex bound to the PC-3 tumor tissue.

3.4. Direct comparison of two-step and three-step pretargeting methods via SPECT/CT imaging

The *in vivo* SPECT/CT imaging of solid PC-3 human prostate carcinoma tumors provided a more stringent comparison of the two pretargeting schemes. SCID mice bearing PC-3 prostate carcinoma tumors were utilized for two-step pretargeted imaging, and these mice were imaged twenty four hours post injection of the ^{111}In -DTPA-SA. The majority of the radioactivity was found within the liver. A representative two-step pretargeted SPECT/CT image is shown in Figure 5a. Three-step pretargeted imaging of solid PC-3 human prostate carcinoma tumors was also performed using tumor bearing SCID mice. These mice were imaged 0.5 hours post injection of 7.40 MBq of ^{111}In -DOTA-biotin. Tumor uptake of ^{111}In -DOTA-biotin was clearly visualized in the SPECT/CT images (Figure 5b). The specificity of the tumor uptake within mice treated with the three-step pretargeting strategy was further tested via a blocking study. For these studies, DOTA-biotin was labeled with stable, non-radioactive indium. The tumor uptake was successfully blocked using a 3 to 1 molar ratio of cold In-DOTA-biotin to ^{111}In -DOTA-biotin (Figure 5c). These data strongly suggest that the tumor uptake and retention of the radiolabeled biotin is due to the tumor bound biotinylated G1 phage/avidin complex.

Conclusion

The ability of G1 phage to bind directly to PC-3 tumor cells *in vivo* was examined via immunohistochemistry. Resected tumor tissue from PC-3 tumor bearing SCID mice injected with G1 phage was probed for the presence and localization of the phage. Biodistribution of G1 phage particles was similar to the reported biodistribution data of other types of M13 phage investigated, in that phage particles were found in the liver and tumor but not muscle at four hours post injection [24,45–48]. Further examination revealed co-localization of phage with the tumor cell marker, ErbB-2, but not the endothelial cell marker, PECAM-1. These results support the hypothesis that phage have the ability to extravasate the vasculature and enter into the surrounding tissue and confirm the notion that G1 phage bind specifically to prostate carcinoma cells both *in vitro* and *in vivo* [27]. The lack of PECAM-1 staining within certain areas of the mosaic vessel is not surprising. Similar phenomena have been described by Chang et al. and diTamaso et al. within SCID mice bearing LS174T human colon adenocarcinoma tumors [43,44]. Probing for phage within the PC-3 human prostate tumor revealed vasculature lined with phage but no co-localization with PECAM-1; this might be explained by the phage being trapped in the basement membrane. Baluk et al. described several layers of basement membrane, loosely associated with endothelial cells, and containing many projections and loops [49]. The blue stained phage retained in the loops and projections of the basement membrane can be seen in Figure 2B. Thus, the mode of phage extravasation may be passive

diffusion through the leaky, mosaic vessels present within the PC-3 tumor with excess phage being retained in the basement membrane. Future studies could be aimed at defining the precise mode of phage extravasation, but demonstration that G1 phage specifically bound to PC-3 tumor cells *in vivo* is a key step in their development as cancer imaging agents.

A novel phage-based pretargeting method was developed for the purpose of *in vivo* SPECT/CT imaging of solid human PC-3 prostate carcinoma tumors. The three-step pretargeting method involved the use of biotinylated G1 phage, followed by avidin, and ^{111}In -DOTA-biotin (Figure 3). The decision to utilize a four hour incubation period for the biotinylated phage was based upon previous work and unpublished observations made by our laboratory that the levels of phage within a tumor increase steadily for the first 30 minutes to 1 hour (depending upon the displayed peptide) and stabilize approximately by the fourth to sixth hour postinjection with the highest associated ratios of tumor to non-tumor signal [23,27]. The choice of ^{111}In -DTPA-SA instead of ^{111}In -DOTA-biotin for the two-step pretargeting strategy was based upon our experience that covalent attachment of streptavidin and/or avidin to phage was extremely inefficient presumably due to steric hinderance. Purification of the phage-avidin complex from non-modified phage was also difficult. Another variable included the use of avidin in the three-step method instead of SA (as in the two-step method), due to differences in blood half lives and total body clearance profiles for the two forms of biotin binding proteins. Avidin is a glycoprotein that is cleared from the blood as well as the whole body at a much faster rate than that of SA [32,50]. Thus, radiolabeled SA was employed for the two-step pretargeting method specifically because it has a longer blood half-life. It was hypothesized that more ^{111}In -DTPA-SA would bind to the tumor-bound biotinylated-G1 phage if the ^{111}In -DTPA-SA was circulating within the mouse for a longer period of time. In comparison, the use of the fast clearing avidin was utilized in the three-step pretargeting method because it was thought that it would reduce ^{111}In -DOTA-biotin accumulation in non-targeted tissues, such as liver. Also, each avidin molecule contains four biotin binding sites, thus a single biotinylated-G1 phage with a single bound avidin protein would still have the capability to capture three ^{111}In -DOTA-biotin molecules, and would thus function as a multimodal biological nanoparticle. Consequently, fewer avidin proteins would need to bind biotinylated-G1 phage than ^{111}In -DTPA-SA to reach a similar radiolabel tumor uptake. However, the potential number of ^{111}In -DOTA-biotin molecules able to bind to any one tumor bound phage/avidin complex may be affected by both endogenous biotin and biotinidase found within the serum of the animal. These two potential problems were addressed by purchasing biotinidase resistant DOTA-biotin [51] and by placing all mice used for these *in vivo* experiments on biotin free chow five days in advance of any experiment in order to reduce the levels of endogenous biotin within the serum [52]. Reduction of serum biotin levels aids in the prevention of the blocking of SA biotin binding sites which in turn increases the available binding sites for the radiolabeled biotin. Hamblett et al. report a 6.6 fold reduction of serum levels of biotin by feeding mice a biotin-deficient diet for just 1 day [52]. In comparison, we feed the mice biotin free chow for 5 days prior to any experiments.

Comparative biodistributions revealed that the tumor uptake of the three-step pretargeted ^{111}In -DOTA-biotin was greatly improved at 4.34 ± 0.26 %ID/g compared to that of 0.67 ± 0.06 %ID/g for the two-step pretargeted ^{111}In -DTPA-SA. Liver uptake at 0.5 hours post injection of ^{111}In -DOTA-biotin was 1.20 ± 0.11 %ID/g, which is a distinct improvement over the two-step pretargeting method which had a liver uptake of greater than 20 %ID/g even after twenty four hours post-injection. This high level of ^{111}In -DTPA-SA found in the liver was not due to the pharmacokinetics of SA. The liver retention of non-pretargated ^{111}In -DTPA-SA was found to be only 6.09 ± 0.95 %ID/g, thus the distribution of ^{111}In -DTPA-SA was shifted to the liver by the pharmacokinetics of the biotinylated phage. The liver and lung uptake of 0.63 ± 0.19 %ID/g and 1.67 ± 0.53 %ID/g, respectively, of non-pretargated ^{111}In -DOTA-biotin was lower than the levels resulting from the three-step pretargeted ^{111}In -DOTA-biotin. The

liver level of pretargeted ^{111}In -DOTA-biotin was 1.20 ± 0.11 %ID/g, a significantly higher value than the non-pretargeted ^{111}In -DOTA-biotin ($p = 0.0051$). Lung levels of pretargeted ^{111}In -DOTA-biotin was 2.09 ± 0.43 %ID/g ($p = 0.17$). This increase of uptake in liver and lungs suggests that the *in vivo* distribution of pretargeted ^{111}In -DOTA-biotin was altered by the presence of the biotinylated-G1 phage/avidin complex. This result is not surprising as phage are known to clear the body via the reticuloendothelial system, which includes lungs and liver [24]. Kidney uptake and retention of both the ^{111}In -DTPA-SA and ^{111}In -DOTA-biotin was found to be similar with values ranging from ~5 to ~10 %ID/g for all time points and compounds investigated. These values are similar to previously published data utilizing pretargeted DOTA-biotin conjugates [14,53].

SPECT/CT imaging of the two-step pretargeted human PC-3 prostate carcinoma tumor bearing SCID mice at twenty four hours post-injection of the ^{111}In -DTPA-SA was not successful in that no tumoral accumulation was evident. However, there was high signal from organs involved in the clearance of phage, such as the liver. In contrast, SPECT/CT images using the three-step pretargeting method with G1 phage revealed good PC-3 prostate tumor ^{111}In -DOTA-biotin uptake in xenografted SCID mice and reduced non-target organ uptake restricted to the kidneys and lungs. Importantly, the *in vivo* targeting specificity of the ^{111}In -DOTA-biotin was confirmed using a competition assay. SPECT/CT imaging of a SCID mouse bearing a PC-3 human prostate carcinoma tumor verified the ability of cold In-DOTA-biotin to block tumor accumulation of the radiolabeled ^{111}In -DOTA-biotin, strongly suggesting that the tumor accumulation of the ^{111}In -DOTA-biotin was due to the presence of tumor bound biotinylated G1 phage/avidin complex.

The two-step pretargeted phage imaging scheme has been performed previously by our laboratory using B16-F1 mouse melanoma tumors and biotinylated phage displaying modified melanocortin-1 receptor-homing peptides (MSH2.0) [23]. A SPECT/CT image was obtained of a B16-F1 tumor utilizing a two-step pretargeting method with biotinylated MSH2.0 phage. Specific tumor uptake was observed as early as four hours post injection; however, the tumor to muscle and tumor to blood ratios did not improve over time. The biodistribution data for the two-step MSH2.0 phage exhibited clearance through the gastrointestinal tract. Prolonged uptake of the radioactivity in the large and small intestine caused increased non-specific body cavity background, similar to what was observed in the two-step G1 phage image. It is likely the biodistribution and imaging with MSH2.0 phage would benefit from the three-step targeting, which would allow sufficient time for both the phage and the avidin/SA to clear prior to injecting the radiolabeled biotin compound. Recently, phage display has been used for the discovery of many cancer targeting peptides [54,55], however, only a handful have been developed into useful cancer imaging agents [40,56–58]. Thus, it is envisioned that this newly described technique would enable efficient analysis of the cancer targeting properties of newly discovered phage display-selected peptide sequences. The use of G1 human prostate tumor targeting phage in imaging substantiated our previously developed *in vivo* phage selection technique (as described in the introduction). Overall, when utilizing a phage-based tumor-targeting scheme for the *in vivo* imaging of cancer, the use of the three-step pretargeting method is likely the most effective form described to date.

Pretargeting has been utilized for more than twenty years, but the use of pretargeting with large macromolecules, such as 23.6 MDa phage, began only recently [16–19,23]. With the use of large nanoparticles in a pretargeting strategy, the simplest and/or most direct approach may not be the best. Although a three-step pretargeting approach is more laborious and time consuming it generates data with higher signal to noise ratios and the resulting images are far superior to those produced by the two-step pretargeting method. Thus, this method may have use as a diagnostic test or possibly the monitoring of response to a cancer treatment regimen.

The exploitation of phage as biological nanoparticles has many benefits. Not only are phage organic and nonpathogenic [45,48] they are self replicating, biological particles that are capable of displaying multiple tumor targeting peptides. In contrast, use of the more commonly employed nanoparticles with metallic cores result in documented side effects [59,60]. As such, phage might be thought of as multifunctional biologic nanoparticles capable of being covalently attached to numerous and various tags or labels. In general, the use of phage as self-replicating biological nanoparticles is a very safe, simple, and attractive solution for the development and implementation of *in vivo* imaging modalities.

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Muscle **Liver** **Tumor** **Figure 1.**

Immunolocalization of G1 phage within different tissues. A SCID mouse bearing a human PC-3 prostate carcinoma tumor received a tail vein injection containing 10^{11} virions of G1 phage. Four hours post injection, the mouse was sacrificed and the muscle, liver, and tumor tissues harvested. Presence of G1 phage within the different tissues was probed using an anti-phage antibody raised in rabbit, followed by an anti-rabbit antibody conjugated to AlexaFluor 488.

Figure 2.

Co-localization studies of G1 phage within PC-3 human prostate carcinoma tumors. Tumor tissue from a SCID mouse bearing a PC-3 tumor injected, four hours prior, with 10^{11} virions of G1 phage were probed for (A) phage (blue) and ErbB-2 (red). Areas of co-localization (pink) on the PC-3 tumor cells are denoted by a white arrow. (B & C) Presence of phage (blue) and PECAM-1 (green) were also probed but were found not to co-localize on vascular endothelial cells. Nuclei are stained red with propidium iodide.

Figure 3.
Schematic of two-step and three-step pretargeting methods.

Figure 4. Biodistribution studies of ^{111}In labeled compounds pretargeted to human PC-3 prostate tumors with biotinylated G1 phage. SCID mice bearing PC-3 tumors received injections of 10^{11} virions of biotinylated G1 phage. The G1 phage were allowed to circulate and clear the blood for four hours. The biodistribution of 1.85 MBq of ^{111}In -DTPA-SA and 0.37 MBq ^{111}In -DOTA-biotin pretargeted to PC-3 tumors in a two-step (A) or three-step (B) pretargeting strategy were then investigated at various time points post injection of the radiolabeled compound.

Figure 5.

SPECT/CT imaging studies of pretargeted ^{111}In labeled streptavidin and biotin in SCID mice bearing human prostate PC-3 carcinoma tumors. SCID mice bearing human PC-3 prostate carcinoma tumors received tail vein injections of 10^{11} virions of biotinylated G1 phage. (A) Four hours post injection of the biotinylated G1 phage, mouse A received an injection 7.40 MBq of ^{111}In -DTPA-SA for the purpose of two-step pretargeting by biotinylated G1 phage. The image was taken twenty four hours post injection of the radiolabel. (B & C) Mouse B and C received three-step pretargeting treatments. Four hours post injection of the biotinylated G1 phage both mice received an injection of avidin which was allowed to circulate and clear the body for twenty four hours. Mouse B then received a third injection 7.40 MBq of ^{111}In -DOTA-biotin, while the third injection given to mouse C contained both cold In-DOTA-biotin and ^{111}In -DOTA-biotin. All mice were euthanized before fifteen hours of scan data were obtained.

Table 1

Comparative biodistribution studies of Indium labeled compounds pretargeted and non pretargeted to human PC-3 prostate tumors with biotinylated G1 phage

Organ	Two-step pretargeted ¹¹¹ In-DTPA-SA		Non-pretargeted ¹¹¹ In-DTPA-SA	
	Average	Ratio	Average	Ratio
Blood	0.48 ±0.14	1.00	0.39 ±0.05	1.00
Tumor	0.67 ±0.06	1.40	0.46 ±0.02	1.19
Heart	0.67 ±0.15	1.40	0.31 ±0.06	0.79
Lung	1.30 ±0.17	2.71	0.48 ±0.08	1.24
Liver	21.55 ±2.35	44.90	6.09 ±0.95	15.61
Spleen	6.90 ±1.31	14.38	2.33 ±0.57	5.97
Stomach	0.46 ±0.03	0.96	0.50 ±0.20	1.27
Intestines	2.45 ±1.38	5.10	0.87 ±0.26	2.23
Kidneys	7.61 ±0.86	15.85	5.24 ±1.55	13.44
Muscle	0.24 ±0.01	0.50	0.11 ±0.01	0.29
Pancreas	0.58 ±0.11	1.21	0.32 ±0.06	0.83
Carcass	0.63 ±0.19	1.31	1.70 ±0.18	4.36
Bone	1.26 ±0.21	2.63	0.70 ±0.13	1.79
Skin	0.87 ±0.22	1.81	0.31 ±0.05	0.80
Brain	0.05 ±0.00	0.10	0.03 ±0.01	0.08

Organ	Three-step Pretargeted ¹¹¹ In-DOTA-biotin		Non-pretargeted ¹¹¹ In-DOTA biotin	
	Average	Ratio	Average	Ratio
Blood	2.47 ±0.80	1.00	2.01 ±0.75	1.00
Tumor	4.34 ±0.26	1.76	3.26 ±0.46*	1.62*
Heart	0.91 ±0.30	0.37	0.82 ±0.27	0.40
Lung	2.09 ±0.43	0.84	1.67 ±0.53	0.83
Liver	1.20 ±0.11	0.49	0.63 ±0.19	0.31
Spleen	0.76 ±0.07	0.31	0.53 ±0.27	0.26
Stomach	0.82 ±0.40	0.33	0.76 ±0.08	0.38
Intestines	0.78 ±0.15	0.31	0.98 ±0.41	0.49
Kidneys	8.66 ±1.81	3.51	7.85 ±1.84	3.90
Muscle	0.40 ±0.12	0.16	0.35 ±0.11	0.17
Pancreas	0.76 ±0.22	0.31	0.66 ±0.23	0.33
Carcass	1.41 ±0.58	0.57	3.52 ±2.14	1.75
Bone	0.27 ±0.08	0.11	0.21±0.07	0.10
Skin	0.96 ±0.16	0.39	1.22 ±0.24	0.61
Brain	0.10 ±0.03	0.04	0.07 ±0.02	0.04