Preloading with Unlabeled CA19.9 Targeted Human Monoclonal Antibody Leads to Improved PET Imaging with $^{89}$Zr-5B1

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Supporting Information

ABSTRACT: CA19.9 is one of the most commonly occurring and highest density antigens in >90% of pancreatic cancers, making it an excellent target for monoclonal antibody (mAb)-based imaging and therapy applications. Preloading of unlabeled antibodies to enhance targeting of a radiolabeled mAb has been previously described both for imaging and radioimmunotherapy studies for other targets. We investigated the effect of preloading with the unmodified anti-CA19.9 antibody SB1 on the uptake and contrast of the PET tracer $^{89}$Zr-5B1 in subcutaneous and orthotopic murine models of pancreatic cancer utilizing Capan-2 xenografts, known to both express CA19.9 and shed antigen into circulation. Biodistribution and PET imaging studies with $^{89}$Zr-5B1 alone showed high levels in the liver, spleen, and lymph nodes of mice with subcutaneous Capan-2 tumor xenografts when administered without preinjection of SB1. When unlabeled SB1 was administered prior to $^{89}$Zr-5B1, the tracer significantly enhanced image contrast and tumor to tissue ratios in the same model, and the improvement was related to the time interval between the injections. Moreover, tumors were clearly delineated in an orthotopic pancreatic cancer model using our optimized approach. Taken together, these data suggest that preloading with SB1 can improve $^{89}$Zr-5B1 imaging of disease in a Capan-2 mouse model and that exploration of preloading may have clinical utility for ongoing clinical investigations.

KEYWORDS: pancreatic cancer, PET imaging, CA19.9, SB1

INTRODUCTION

Pancreatic cancer remains one of the most deadly forms of cancer. In the United States, it currently accounts for 3% of new cancer cases and 7% of cancer deaths, and is projected to be the second leading cause of cancer deaths by 2030.1,2 Frontline therapies remain inadequate, and recent advances have only marginally improved outcomes for patients. There is a pressing need for new therapeutic strategies in the fight against pancreatic cancer, and a wide range of clinical trials are currently aiming to make inroads against the disease.3

Therapies based on monoclonal antibodies (mAb) are one promising option for molecularly targeted therapy of pancreatic cancer. The appeal is primarily due to the high affinity and specificity they may show for their target antigens. In addition to their potential as stand-alone immunotherapeutics, mAbs may also serve as a platform for delivering cytotoxic chemotherapies (antibody–drug conjugate; ADC) or therapeutic radionuclides (radioimmunotherapy; RIT).

CA19.9 is one of the most widely studied and oft employed serum biomarkers for pancreatic cancer, providing information regarding therapeutic response, overall survival, and the likelihood of recurrence in patients with established disease.4 However, the utility of serum CA19.9 levels as a management or diagnostic tool in pancreatic cancer is moderated by the fact that increased expression is also observed in a number of benign conditions and unrelated malignancies. Nonetheless, CA19.9 remains an attractive target for molecularly targeted therapeutics and diagnostics because it is the most common biomarker in pancreatic cancer and is preferentially expressed on the surface of cancer cells at extremely high concentrations.

For these reasons, a number of antibody-based agents targeting CA19.9 have been developed in the past decade.5 One of the most promising agents is SB1, which is a fully human, anti-CA19.9 mAb that is currently being investigated as both a stand-alone immunotherapeutic and a PET imaging agent for CA19.9-positive malignancies in preclinical models of cancer, including pancreatic cancer.6−11 However, imaging with an anti-CA19.9 directed mAb may be complicated by the fact that CA19.9 is also shed from the tumor into circulation. Circulating CA19.9 may create a pool of antigen that binds to the tracer mAb limiting its ability to bind to the target tissue. Antigen expression in nontumor tissue, including the blood, has been problematic for other $^{89}$Zr-labeled mAbs.12,13 In radio-
logical applications, the binding of radiolabeled mAb to circulating CA19.9 may also increase residence time in the blood, reducing tumor to tissue contrast and increasing the dosimetric burden in imaging or RIT applications.13

Circulating tumor antigens have been an obstacle in numerous clinical trials with 89Zr-labeled antibodies, including both 89Zr-rituxumab and 89Zr-trastuzumab.12–14 However, effective strategies for overcoming antigen sinks to provide images with enhanced contrast at target tissues have been developed. In the case of 89Zr-rituxumab, preloading with unlabeled rituximab prior to injection reduced uptake of the radiotracer in the spleen and circulating B cells, leading to improved image quality in patients with circulating CD20+ lymphocytes.12 Similarly, it was shown that injection of a large dose of unmodified trastuzumab immediately before 89Zr-trastuzumab, which in essence served to lower the specific activity at the time of injection, also led to improved image quality dependent upon total tumor burden of the patient.13 These “preloading” strategies offer a means to improve delivery of a targeted antibody agent to the tissue of interest (Figure 1).

![Figure 1. Illustration of the benefits of a preloading strategy in which unlabeled antibody is injected to bind circulating antigen prior to injection of the radiolabeled antibody allowing for decreased sequestration in the blood and increased uptake of the radiotracer.](image-url)

We modeled the presence of a serum CA19.9 antigen sink in subcutaneous and orthotopic murine models of pancreatic cancer using the Capan-2 cell line, which is known to both express and shed CA19.9 antigen in a mouse host.14 Using 89Zr-SB1 with and without preloading of unlabeled SB1, we investigated the effect of preloading on 89Zr-SB1 imaging in the context of circulating CA19.9. Our data suggest that the preloading of cold mAb improves the usefulness of 89Zr-SB1 as an imaging agent to visualize disease in this mouse model, which may be applicable to current ongoing clinical trials with MVT-2163, the clinical grade preparation of 89Zr-SB1.

### EXPERIMENTAL SECTION

#### CA19.9 ELISA

The CA19.9 levels in serum samples from mice with subcutaneous Capan-2 xenografts were analyzed using a commercially available solid-phase sequential ELISA kit (Sigma-Aldrich) per the manufacturer’s instructions. Blood (~200 μL) was collected from treatment naïve mice via a retro-orbital bleed, stored at 4 °C for 24 h in Eppendorf tubes, and separated by centrifugation at 2000g for 12 min. Analysis was carried out on undiluted as well as samples that were diluted 3-fold and 10-fold in phosphate-buffered saline (PBS) in order to ensure the CA19.9 concentrations in the sample fell within the calibration curve. All serum samples were stored at −20 °C and thawed slowly on ice prior to analysis.

#### Preparation of 89Zr-SB1

Human recombinant SB1 antibodies were purified from supernatant produced by a stable CHO-S production cell line grown in serum-free culture medium in a Wave bioreactor. Antibodies were purified by Protein A affinity chromatography (MabSelect, GE Healthcare) and buffer exchanged by dialysis into 10 mM phosphate, 150 mM NaCl, pH 6.0 buffer for storage at 4 °C. The purity was >95% (combined heavy and light chain) as estimated by SDS-PAGE under reducing conditions. SB1 antibodies (3.0 mg, 4.6 mg/mL; lot 140827) in PBS buffer (pH 6.0) was adjusted to pH 8.5 using sodium bicarbonate solution (200 mM) and functionalized with p-isothiocyanatobenzyl-desferrioxamine (DFOBz-NCS; Macrocyclics, Inc.) with a 1:4 mAb/DFO-Bz-NCS ratio. In brief, a volume of 8.0 μL of DFO-Bz-NCS (13.3 mM in dimethyl sulfoxide) was added to 650 μL of SB1, and the reaction was incubated at 37 °C for 1.5 h. The DFO-modified antibodies were purified via a PD10 desalting column (GE Healthcare) into PBS pH 7.4. This intermediate was radiolabeled as previously described,10 purified with a PD-10 gel-filtration column (GE Healthcare) into 0.9% saline solution, and used without further formulation. Radiochemical purity was assessed by instant thin layer chromatography (iTLC) on silica-impregnated paper strips that were read with an AR-2000 radioTLC plate reader (Bioscan Inc.) and analyzed with Winscan Radio-TLC software (Bioscan Inc.). 89Zr was produced at Memorial Sloan Kettering Cancer Center (MSK) using an EBCO TR19/9 variable-beam energy cyclotron (Ebc Industries Inc.) by the 89Y(p,n)89Zr reaction.16

#### Cell Culture and Animal Models

All of the animal experiments were done in accordance with a protocol approved by the MSK Institutional Animal Care and Use Committee, and all xenograft studies were performed in 6–8 week old female, athymic nude mice, strain Crl:Nu(NCr)-Foxn1Nid. Capan-2 cells were grown in Dulbecco’s modified essential medium (DMEM) modified to contain 4.5 g/L glucose and supplemented with 20% (v/v) heat-inactivated fetal calf serum, 100 IU penicillin, and 100 μg/mL streptomycin. Subcutaneous Capan-2 xenografts were inoculated into the right flank using 5 × 106 cells suspended in 150 μL of a solution containing a 1:1 mixture of Matrigel (Corning) and cell culture medium. Capan-2 tumors were grown for 21–28 days postimplantation to an average tumor size of 200 mm3 prior to imaging or biodistribution.

For orthotopic pancreas xenografts, athymic homozygous nude mice, strain Crl:Nu(NCr)-Foxn1Nid (Charles River Industries Inc.) by the 89Y(p,n)89Zr reaction.16

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Laboratories) age 6−8 weeks were used. Mice were anesthetized with 1−2% isoflurane gas in medical air at a rate of 2 L/min, and surgery was performed on a heated platform to help maintain body temperature. Bupivacaine, a local anesthetic agent, was injected intradermally in the area surrounding the incision line. Skin was prepped for surgery using alternating scrubs of povidone-iodine and 70% ethanol. A longitudinal incision (0.5−1 cm in length) was made in the skin and the peritoneum, allowing for the spleen and pancreas to be exteriorized. Capan-2 cells (2 × 10⁶ cells in 30 μL containing 1:1 cell media and Matrigel) were slowly injected into the parenchyma of the pancreas. The spleen and pancreas were returned to the peritoneal cavity, the peritoneal wall was closed using 4−0 Vicryl sutures, and the skin was closed using sterile wound clips. Meloxicam was administered prior to recovery and dosage repeated postsurgery as needed every 4−24 h. Tumors reached optimal size within 14−21 days postimplantation.

**Biodistribution.** Biodistribution studies were designed to first determine the effect of the amount of time between injection of the unlabeled SB1 and ⁸⁹Zr-SB1. Then, further studies were performed to determine the effect of injecting different amounts of cold SB1 at the optimal time point determined from the previous study. Finally, the uptake of the tracer from 24 to 120 h was followed, using the optimized approach.

Biodistribution studies were performed as previously reported.⁹,¹⁰ In brief, mice (n = 4−5 per time point) were injected via the lateral tail vein with ⁸⁹Zr-SB1 (∼30 μg, 135 ± 3 μCi) between 5 min and 24 h after the injection of unlabeled SB1 (40−250 μg) also via tail vein or without administration of SB1. Tissues of interest, including tumors in the subcutaneous xenograft model, were harvested between 24 and 120 h postinjection. The weight of each tissue sample was determined, and the localization of the tracer was analyzed by gamma counting and calibrating to standards of known activity. All activity concentrations from the biodistribution studies are expressed as the percentage of the injected dose per gram of tissue.

**PET and PET/CT Imaging.** A subset of mice (n = 3) used in the biodistribution studies were selected for PET imaging experiments, which were performed as previously described.⁹,¹⁰ Imaging studies in mice with subcutaneous xenografts were performed with a microPET Focus scanner (Concorde Microsystems), whereas PET/CT imaging of mice with orthotopic xenografts was performed on an Inveon PET/CT scanner (Siemens Healthcare Global) between 4 and 120 h postinjection of the radiotracer. All of the resulting images were analyzed using ASIPro VM software. Further details are provided in the Supporting Information.

**Statistical Analysis.** All biodistribution and tumor to tissue ratio are reported as the average value ± SD. Statistical analysis was performed using a two-tailed Student’s t test with p < 0.05 being considered significant.

## RESULTS

Serum samples isolated from mice bearing subcutaneous Capan-2 xenografts of mass ranging from 75 to 1400 mg were analyzed to quantify the circulating CA19.9 levels. We observed a correlation between the tumor size and CA19.9 serum levels, although serum CA19.9 levels were more variable in tumors with masses of >500 mg (Figure 2A). When divided into four subgroups based on tumors size, <250 mg, 250−500 mg, 500−750 mg, and >750 mg, the CA19.9 serum levels were significantly different between every set of groups with the exception of the 500−750 mg and >750 mg groups (Figure 2B). We chose to use animals bearing tumors between the size of 150 to 400 mg for imaging and biodistribution in order to provide a consistent and relevant model for our investigations. DFO-SB1 was radiolabeled with good specific activity (4.6 mCi/mg) and radiochemical purity (>98%), providing material for the imaging and biodistribution studies. To ensure comparability of results, approximately the same amount, both in terms of mass and activity, were injected for all experiments. Serial imaging experiments were performed in mice with subcutaneous Capan-2 xenografts without injection of SB1 prior to ⁸⁹Zr-SB1 to provide a point of comparison for subsequent experiments employing the preloading strategy. The level of radiotracer in tumor tissue was apparent in tomographic slices of each of the mice that were imaged, and biodistribution was performed at 120 h in the same cohort of mice confirmed good tracer uptake (25.8 ± 4.4%ID/g). The maximum intensity projections (MIPs) (Figures 3A and S1) showed that the highest activity concentration in all the mice that were imaged was in the ipsilateral lymph nodes as early as 4 h postinjection, and the biodistribution showed high levels in
liver and spleen, leading to low tumor to liver and tumor to spleen ratios.

To evaluate the effect of varying the time between injection of the SB1 and administration of $^{89}$Zr-SB1, a high dose (100 μg) of cold SB1 was injected 5 min, 1, 4, or 24 h prior to injection of $^{89}$Zr-SB1. Imaging (Figures 3 and S2) and biodistribution (Figure 4 and Table S1) at 120 h postinjection allowed for comparison of the various preloading schedules relative to no preloading of cold antibody. In mice injected with SB1 5 min prior to $^{89}$Zr-SB1, there was little to no perceptible difference relative to the cohort not administered SB1. However, biodistribution studies showed a marginal improvement in tumor uptake and in the tumor-to-liver and tumor-to-spleen ratios.

A more pronounced improvement in image quality, relative to $^{89}$Zr-SB1 alone, was observed in mice that were preloaded 1 h before $^{89}$Zr-SB1, although the results were variable between mice. The MIPs for mouse 2 and 3 from the 1 h cohort showed uptake of radiotracer primarily in the tumor tissue, whereas uptake in mouse 1 was highest in the ipsilateral lymph nodes (Figure 3C). Biodistribution data shows that the uptake and tumor to tissue ratios were improved relative to no preloading of cold SB1, but they were essentially identical to the 5 min cohort. The most pronounced improvement in terms of image quality was observed in mice preloaded 1 h prior to injection of $^{89}$Zr-SB1.

Figure 3. Maximum intensity projections of mice that were injected with $^{89}$Zr-SB1 without preloading (A) or with preloading of 100 μg of unlabeled SB1 at 5 min (B), 1 h (C), 4 h (D), or 24 h (E) prior to injection of $^{89}$Zr-SB1.

Figure 4. Bar graphs depicting activity concentration (A) and tumor to tissue ratios (B) in selected tissues as determined by biodistribution studies at 120 h postinjection of $^{89}$Zr-SB1.
quality, tumor-to-liver ratio, and tumor-to-spleen ratio was achieved by preloading 4 h before the radiotracer (Figure 3D).

In this case, both the tumor-to-liver and tumor-to-spleen ratios were significantly different than those obtained with no preloading (p < 0.01 and p < 0.05, respectively) (Figure 4 and Table S1). Additionally, each of the images acquired showed the highest activity concentration in the tumor tissue, rather than the lymph nodes.

Image quality after waiting 24 h between the injection of SB1 and $^{89}$Zr-SB1 was not as robust as the 4 h preloading cohort. Marginal improvement in image quality (Figure 3E) and contrast was achieved, but the improvement was belied by the essentially identical tumor uptake ($22.2 \pm 9.6\% \text{ID/g}$) relative to the no preloading cohort ($25.8 \pm 4.4\% \text{ID/g}$) and significantly lower uptake relative to the 4 h cohort ($42.2 \pm 14.9\% \text{ID/g}$). Waiting for 24 h to administer $^{89}$Zr-SB1 also results in lower tumor-to-liver and tumor-to-spleen ratios (Figure 4 and Table S1).

A biodistribution study was performed in mice with subcutaneous Capan-2 xenografts to assess the level of $^{89}$Zr-SB1 over time, between 24 and 120 h, in mice that received 100 $\mu$g of SB1 4 h prior to the radiotracer (Table 1). Using this optimized time point, we observed rapid uptake of radiotracer in the tumor tissue and a concomitant decrease of the activity concentration in the blood over time. High tumor-to-tissue contrast was achieved.

To study the effect of changing the dose level of cold antibody injected, 40, 100, or 250 $\mu$g of SB1 was injected 4 h prior to $^{89}$Zr-SB1, and the uptake at 120 h postinjection was assessed by biodistribution (Tables 1 and S2). Injection of 40 $\mu$g had similar effect on the distribution of $^{89}$Zr-SB1 compared to 100 $\mu$g, showing essentially identical average tumor uptake and marginally higher uptake in the liver and spleen. Similarly, increasing the amount of SB1 to 250 $\mu$g appeared to have similar overall distribution of $^{89}$Zr-SB1. The uptake values for all three cohorts showed no statistically significant difference in the uptake or tumor to tissue ratio in any organ at 120 h postinjection of $^{89}$Zr-SB1.

In order to assess this system in a model that simulates human disease dissemination, PET and PET/CT studies using the optimized parameters (preloading with 100 $\mu$g of unlabeled SB1 4 h prior to tracer administration) were performed in mice bearing tumors orthotopically implanted into the body of the pancreas. PET images showed rapid uptake in the tumor at 48 h and improved contrast at 120 h post injection (Figure 3A). PET/CT images acquired 120 h postinjection of $^{89}$Zr-SB1 provided anatomical reference (Figure 3B). Images of both mice show differing levels in the inguinal, axillary, and mesenteric lymph nodes, the latter having the highest activity concentration of the three.

## DISCUSSION

$^{89}$Zr-SB1 recently entered clinical trials as the investigational agent MVT-2163 for use as a staging and diagnostic tool for CA19.9 positive metastatic pancreatic cancer. Since CA19.9 can be found both as a soluble form circulating in the blood and as a highly expressed tumor surface antigen, there is the potential for improved images with preloading of unlabeled SB1, known as MVT-SB73 clinically, to bind circulating CA19.9. We sought to determine the best approach for image enhancement using preclinical models of pancreatic cancer that are known to shed CA19.9 into circulation.

For evaluation of the preloading method, we selected the Capan-2 xenograft model, a human pancreatic cancer cell line that has been reported to shed CA19.9 into the circulation,9,15. We evaluated the serum concentrations of CA19.9 from treatment naïve tumor bearing mice and found mouse serum levels to be proportional to tumor size. CA19.9 levels ranged from almost undetectable in animals with small tumors to approximately 800 U/mL in mice with large tumors. These levels correlate roughly to human levels that are low to moderate, with 0–37 U/mL being considered normal in healthy individuals. We chose to use mice with tumor volumes averaging 200 mm$^3$ because they generally shed a consistent and moderate level of CA19.9.

We established a baseline uptake of $^{89}$Zr-SB1 in our model via imaging experiments and terminal biodistribution in which no preloading of cold tracer was administered. This approach yielded images of reasonable quality, but the tumor-to-tissue uptake ratios in the liver and spleen were poor (1.5 ± 0.6 and 1.8 ± 0.8, respectively). Furthermore, mice with no preloading had the highest activity concentrations in the ipsilateral inguinal and axillary lymph nodes, which are prominent in the MIPs (Figure 3A). The uptake in the nodes was rapid, with high activity concentrations seen at 4 h postinjection (Figure S1), the earliest imaging time point. This is not unexpected for such systems as those organs are the primary route of clearance for antibodies and shed antigens. In humans, the mesenteric lymph

### Table 1. Biodistribution of $^{89}$Zr-SB1 in Mice Harboring Subcutaneous Capan-2 Xenografts That Were Injected with Cold SB1 4 h Prior to Injection of the Indicated Amount of Radiotracer; All Values Are Reported as %ID/g

| organ       | 100 $\mu$g preloading | 40 $\mu$g preloading | 250 $\mu$g preloading |
|-------------|------------------------|-----------------------|------------------------|
|             | 24 h | 48 h | 72 h | 120 h | 120 h | 120 h | 120 h |
| blood       | 12.5 ± 1.5               | 11.0 ± 2.2           | 7.3 ± 1.8            | 4.5 ± 2.2       | 4.7 ± 1.5       | 5.6 ± 1.3       |
| tumor       | 23.4 ± 6.6               | 36.6 ± 6.6           | 28.7 ± 9.9           | 44.0 ± 12.3     | 44.1 ± 11.5     | 54.7 ± 25.3     |
| heart       | 3.5 ± 0.8                | 3.2 ± 0.5            | 2.1 ± 0.1            | 1.5 ± 0.6       | 1.7 ± 0.5       | 1.8 ± 0.3       |
| lungs       | 7.2 ± 2.4                | 5.3 ± 1.3            | 3.4 ± 0.8            | 2.6 ± 1.0       | 3.0 ± 0.8       | 4.2 ± 1.2       |
| liver       | 6.8 ± 1.2                | 8.6 ± 2.2            | 9.5 ± 4.1            | 14.3 ± 5.8      | 16.1 ± 6.7      | 13.2 ± 3.1      |
| spleen      | 5.0 ± 1.6                | 5.4 ± 1.5            | 3.8 ± 0.7            | 7.9 ± 2.9       | 11.6 ± 7.7      | 7.6 ± 0.5       |
| pancreas    | 1.0 ± 0.1                | 1.0 ± 0.1            | 0.8 ± 0.1            | 0.6 ± 0.2       | 0.7 ± 0.1       | 0.8 ± 0.1       |
| stomach     | 0.6 ± 0.1                | 0.8 ± 0.2            | 0.7 ± 0.2            | 0.4 ± 0.2       | 0.7 ± 0.2       | 0.7 ± 0.1       |
| small intestine | 1.1 ± 0.1            | 1.0 ± 0.3            | 0.9 ± 0.3            | 0.6 ± 0.3       | 0.7 ± 0.1       | 0.8 ± 0.2       |
| large intestine | 0.7 ± 0.1            | 0.7 ± 0.1            | 0.8 ± 0.2            | 0.5 ± 0.1       | 0.6 ± 0.1       | 0.7 ± 0.2       |
| kidneys     | 6.8 ± 3.6                | 5.1 ± 0.4            | 4.8 ± 1.0            | 4.1 ± 1.0       | 4.2 ± 0.3       | 4.7 ± 0.7       |
| muscle      | 0.9 ± 0.1                | 0.8 ± 0.2            | 0.7 ± 0.1            | 0.4 ± 0.2       | 0.6 ± 0.2       | 0.6 ± 0.1       |
| bone        | 3.8 ± 0.7                | 5.1 ± 0.9            | 4.7 ± 1.9            | 6.6 ± 0.6       | 7.1 ± 0.3       | 8.2 ± 0.9       |
nodes are located proximal to the pancreas and the mesenteric artery, and interaction or encapsulation of the mesenteric artery determines a patient’s candidacy for surgical resection in nonmetastatic pancreatic cancer, therefore, a high contrast image in this region would be helpful for accurate disease staging via PET in humans.

Admittedly, the interpretation of these results is complicated. It is known that CA19.9 is shed from Capan-2 cells, and we have previously shown that SB1 bound to CA19.9 on the surface of Capan-2 cells is rapidly internalized, leading to retention of the labeled SB1 inside the cell and resultant tumor accumulation. As such, it is not easy to determine the source of the tracer that is localized to the lymph nodes, and indeed, there are several possible explanations. One possibility is that the tracer binds to circulating antigen and is sequestered in the lymph nodes. Another possibility is that the tracer binds at the tumor site where it is subsequently internalized and released into the lymphatic system. A third possibility is that Capan-2 cells, which are derived from a primary tumor having a low metastatic score, have metastasized to the lymphatic system, and the minimal loading dose required to see uptake of the radiotracer, suggesting that unlabeled 5B1 had insu

Previous studies with $^{89}$Zr-rituxumab indicated that the preloading could potentially cause blocking of the antigen in the tumor, reducing uptake of the radiotracer, suggesting that the amount of mass of unlabeled antibody relative to the size of the antigen sink would be a consideration for this approach. Also, the results of the trials with $^{89}$Zr-rituxumab and $^{89}$Zr-trastuzumab suggest that the timing of the preloading might influence image quality. Thus, we evaluated the impact of these two primary factors on the improvement of PET tracer uptake and PET image contrast for $^{89}$Zr-SB1 by studying the optimal timing between an injection of unlabeled SB1 and $^{89}$Zr-SB1, as well as the amount of unlabeled SB1 that was injected prior to the imaging agent.

The 5 min preloading time point was chosen to mimic coadministration of cold and labeled antibody similar to $^{89}$Zr-trastuzumab administration in humans. While this approach gave slightly improved tumor to liver and spleen ratios, the tumor uptake and contrast in the images was not significantly changed (Figure 3B). A potential explanation for this observation is that the unlabeled SB1 had insufficient time to distribute and bind the CA19.9 in circulation.

The time course described here suggests that waiting 4 h between injections will give the best results in terms of image quality and tumor to tissue activity concentration ratios. MIPs showed that the activity concentration was highest in the tumor in the 4 h cohort. In addition, the tumor to liver and spleen ratios were both higher than any other time point. This result has precedence as a 1–3 h lag between unlabeled rituximab and $^{89}$Zr-rituxumab showed improved tumor contrast. Given the improvement seen by preloading at 4 h, we sought to determine if allowing more time between injections would provide further improvement.

However, allowing 24 h between the SB1 and $^{89}$Zr-SB1 injections resulted in only marginally improved image quality and resulted in similar or even lower uptake relative to no preloading dose. Also, the tumor to liver and spleen ratios are much lower than with a 4 h preloading, suggesting that unlabeled compound may have bound in the tumor, blocking the subsequent binding of $^{89}$Zr-SB1.

With an optimal preloading time point established, we also investigated the effect of changing the amount of unlabeled SB1 that was preloaded 4 h prior to injection of $^{89}$Zr-SB1. Lowering the mass of unlabeled SB1 to 40 μg had essentially no effect on the activity concentrations in the tumor and other tissues (Table 1). The same was true of increasing the mass to 250 μg of SB1. This suggests that all doses saturate the CA19.9 in circulation, and the minimal loading dose required to see improved image quality in this model was not determined.

Finally, we evaluated a 100 μg preloading dose 4 h prior to injection of $^{89}$Zr-SB1 in a Capan-2 orthotopic xenograft model in which the tumors were implanted directly into the body of the pancreas (Figure 5). PET and PET/CT images showed that we are able to delineate the tumor tissue in the pancreas and xenografts, and the lack of any reports of subcutaneous Capan-2 xenografts metastasizing, the first two options seem much more plausible than the third, especially considering that accumulation in tumor tissue is increased by preloading, whereas localization to lymph nodes is drastically reduced. It is entirely possible that the tracer localized in the lymph nodes results from a combination of both processes. However, our knowledge of pancreatic lymph node drainage and associated pancreatic tumor metastases is limited, and this phenomena in the model at hand remains open to interpretation.

Figure 5. PET imaging studies in two mice with orthotopically implanted capan-2 xenografts that were administered 100 μg of unlabeled SB1 4 h prior to $^{89}$Zr-SB1. Tomographic slices and MIPs from PET scans at 48 and 120 h postinjection (A) as well as PET/CT images (B,C) acquired at 120 h postinjection of $^{89}$Zr-SB1 are shown.

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that there is little nonspecific uptake in healthy tissues. While these results are also promising, this is still not an ideal preclinical model for evaluating CA19.9 radiotracers because mice do not endogenously express CA19.9. Regardless, we believe these results may justify the use of a preloading strategy if and when circulating antigen reduces image quality in patients.

In summary, these studies represent a systematic evaluation of a preloading strategy for antibody based PET imaging in a system with shed, circulating antigen. We demonstrate that adopting a preloading strategy can improve tumor uptake, increase tumor-to-tissue ratios, and reduce background uptake in nontarget organs in both subcutaneous and orthotopic mouse models of pancreatic cancer. These data may inform a strategy that could prove beneficial in clinical trials of 89Zr-SB1, as well as future SB1-based RIT, which is currently under investigation.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.6b00130.

All images acquired for each mouse in this study as well as all activity concentration and uptake ratios are reported (PDF)

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Notes

The authors declare the following competing financial interest(s): Wolfgang Scholz is an employee of Mabvax Therapeutics.

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**ABBREVIATIONS**

PDAC, pancreatic ductal adenocarcinoma; CA19.9, carbohydrate antigen 19.9; PET, positron emission tomography

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