Predicting IGF-1R Therapy Response in Bone Sarcomas: Immuno-SPECT Imaging with Radiolabeled R1507

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Abstract

Purpose: To investigate whether indium-111–labeled R1507 (111In-R1507) immuno-SPECT (single-photon emission computed tomography), a novel noninvasive, in vivo screening method to visualize membranous insulin-like growth factor 1 receptor (IGF-1R) expression and accessibility, can be used to predict IGF-1R treatment (R1507) response in bone sarcomas.

Experimental Design: BALB/c nude mice were subcutaneously implanted with IGF-1R–expressing human bone sarcoma xenografts (OS-1, EW-5, and EW-8) which showed high, modest, or no response, respectively, to R1507, a monoclonal antibody targeting the extracellular domain of IGF-1R. An IGF-1R–negative tumor (OS-33), unresponsive to IGF-1R inhibitors, was examined as well. Mice were injected with 111In-R1507. Biodistribution and immuno-SPECT/computed tomography imaging studies were carried out 1, 3, and 7 days p.i. in mice with OS-1 and EW-5 xenografts and 3 days p.i. in mice with EW-8 and OS-33 xenografts.

Results: Biodistribution studies showed specific accumulation of 111In-R1507 in OS-1 and EW-5 xenografts (27.5 ± 6.5%ID/g and 14.0 ± 2.8%ID/g, 3 days p.i., respectively). Most importantly, 111In-R1507 uptake in IGF-1R positive, but unresponsive, EW-8 xenografts (6.5 ± 1.5%ID/g, 3 days p.i.) was similar to that of the IGF-1R–negative OS-33 tumor (5.5 ± 0.6%ID/g, 3 days p.i.). Uptake in normal tissues was low and nonspecific. Corresponding immuno-SPECT images clearly discriminated between high, modest, and nonresponding tumors by showing a homogeneous (OS-1), heterogeneous (EW-5), or nonspecific (EW-8 and OS-33) tumor uptake of 111In-R1507.

Conclusions: 111In-R1507 immuno-SPECT is an excellent method to visualize membranous IGF-1R expression and target accessibility in vivo in human bone sarcoma xenografts and may serve as an independent marker to predict IGF-1R therapy (R1507) response in bone sarcoma patients. Clin Cancer Res; 17(24); 7693–703. ©2011 AACR.

Introduction

Bone sarcomas comprise a heterogeneous group of tumors originating in or near the bone, with varying phenotypes. The 2 most commonly diagnosed primary malignant tumors of the bone, which mainly affect children and young adults, are osteosarcoma and Ewing sarcoma (1, 2). Current treatment regimens for these tumors consist of local therapy comprising surgery or radiotherapy (Ewing sarcoma) and polychemotherapy. Unfortunately, despite multimodal treatment, the final outcome has not improved significantly during the last decades and side effects of treatment are observed frequently. This disappointing situation shows a compelling need for a more effective and hopefully less toxic treatment modality to target bone sarcomas.

A new promising treatment option is blocking of insulin-like growth factor-1 receptor (IGF-1R) signaling. This transmembrane receptor tyrosine kinase plays an important regulatory role in several cancers, including osteosarcoma and Ewing sarcoma, in which increased levels of IGF-1R expression have been observed (3, 4). The activity of this receptor is mainly regulated by the presence and binding of the insulin-like growth factor-1 (IGF-1) ligand. In addition, the stimulatory effects of IGF-1 are influenced by circulating...
IGF-binding proteins (IGF-BP; ref. 5). Upon binding, IGF-1R undergoes rapid tyrosine phosphorylation, resulting in activation of the phosphoinositide 3-kinase/Akt and extracellular signal-regulated kinase pathways. These pathways are well known for their involvement in cellular proliferation, survival, invasion, and metastasis (6). In vitro and in vivo studies showed that both osteosarcoma and Ewing sarcoma cells are highly dependent on IGF-1R signaling, and activation of IGF-1R by IGF-1 stimulated osteosarcoma cell growth and metastatic behavior in vivo (7–9). Because the peak incidence of osteosarcoma and Ewing sarcoma coincides with the burst of growth hormone and IGF-1 release during puberty, a causal relationship has been suggested (4).

Consequently, several strategies have been developed to manipulate the IGF-1R pathway. One group of promising agents are the human monoclonal IGF-1R antibodies, which have shown to efficiently and effectively impair IGF-1R signaling in both preclinical and clinical studies. IGF-1R blocking resulted in marked antitumor activity in several bone sarcoma xenografts, and significant antitumor activity was observed in a variety of sarcoma patients, including osteosarcoma and Ewing sarcoma patients, with little to no side effects (10–16).

There are, however, still some limitations to the use of these anti-IGF-1R antibodies. One problem is that not every patient benefits from this novel treatment and that responses are often short lived.

Therefore, there is an urgent need for selection of patients that most probably will benefit from this novel treatment. In vitro data have indicated that IGF-1R expression is a prerequisite to respond to anti–IGF-1R therapy (17, 18). Therefore, one could base patient selection for IGF-1R–targeted therapy on the presence (and accessibility) of the IGF-1 receptor. Screening for the presence of a therapeutic target is generally carried out on a single historical tumor sample. However, this may be insufficient because IGF-1R expression can be heterogeneous throughout the tumor, and expression levels can change in time during tumor growth. IGF-1R–targeted therapy, or conventional cytotoxic treatment. In addition, patients can present with multiple tumor manifestations that show variable IGF-1R expression levels. In clinical care, however, it is unfeasible to carry out multiple biopsies. Furthermore, intuitively it seems appropriate to solely screen for membranous IGF-1R expression instead of total IGF-1R levels because IGF-1R antibody therapy solely targets receptors present on the cell membrane. Unfortunately, with current screening methods such as Western blot (WB) and even with immunohistochemistry (IHC), it remains difficult to specifically determine membranous IGF-1R expression in bone sarcoma samples. Another problem with current screening methods is that they do not account for in vivo target accessibility. Because physiologic factors may act as a barrier for adequate antibody targeting to the tumor, these factors must also be taken into account when predicting therapy response (19–22).

Recently, Heskamp and colleagues described a novel method to noninvasively visualize membranous tumor IGF-1R expression and accessibility in vivo in a breast cancer model with an indium-111 (111In)–labeled anti-IGF-1R antibody (R1507; 111In-R1507) and immuno-SPECT (single—photon emission computed tomography; ref. 23). However, although membranous IGF-1R expression was successfully shown in this model, it is still unclear whether membranous IGF-1R expression and accessibility is indeed an independent predictive response marker to IGF-1R–mediated therapy as other components of the IGF-1R pathway, including the presence of IGF-1 and IGF-BPs, have been implicated in predicting IGF-1R therapy response as well (24, 25). The aim of this study was to investigate whether membranous tumor IGF-1R expression and accessibility can be used to independently predict IGF-1R therapy response. To accomplish this, we examined the in vivo distribution of 111In-R1507 with immuno-SPECT in several bone sarcoma xenografts, including 2 osteosarcoma (OS-1 and OS-33) and 2 Ewing sarcoma xenografts (EW-5 and EW-8). The IGF-1R–positive OS-1 and IGF-1R–negative OS-33 xenografts previously showed high and no response to R1507 therapy, respectively (11). Interestingly, the IGF-1R–positive EW-5 and EW-8 xenografts showed modest and no response to R1507 therapy as shown in this manuscript (Supplementary Fig. S1). Because this selection includes a wide range of responses to R1507 therapy, independent of IGF-1R expression as assessed by conventional IHC or WB, these are excellent models to examine whether 111In-R1507
immuno-SPECT is suitable for predicting R1507 therapy response.

Materials and Methods

Mouse models

For all experiments, female BALB/c nude mice (6–8 weeks old) were used. Mice were housed under clean, nonsterile standard conditions in filter-topped cages (5–6 mice per cage), with free access to standard animal chow and water. Osteosarcoma and Ewing sarcoma xenografts (OS-1, OS-33, EW-5, and EW-8) were generously provided by the Pediatric Preclinical Testing Program (PPTP). During all experiments, xenografts were maintained by serial in vivo passages in 5 mice per xenograft (donor mice). For biodistribution and imaging studies, xenografts were harvested from donor mice and 4 × 4 × 4 mm tumor sections were subcutaneously (s.c.) implanted in the right or left flank of mice from the experimental groups. Experiments were carried out when tumors weighed 0.1 to 0.3 g. All experiments were approved by and carried out in accordance with the guidelines of the Institutional Animal Welfare Committee of the Radboud University Nijmegen.

IHC

All tumor xenografts were stained immunohistochemically to determine CD34, Ki-67, HIF-1α, and IGF-1R expression. Xenografts were fixed in 4% formalin and subsequently embedded in paraffin. Tumor sections (4 μm) were deparaffinized in xylol and rehydrated through a graded series of 95% and 70% alcohol and water series. Antigen retrieval was carried out by microwave heating of slides in a 10 mmol/L sodium citrate buffer, pH 6 for 10 minutes (Ki-67, HIF-1α by microwave heating of slides in a 10 mmol/L sodium citrate buffer, pH 6 for 10 minutes). After microwave heating, sections were incubated with monoclonal rabbit anti–Ki-67 (1:200; Neo Markers), polyclonal rabbit anti–HIF-1α, and IGF-1R (1:20; Monosan) overnight at 4°C. Endogenous peroxidase activity was blocked with 3% H2O2 for 10 minutes at room temperature (RT), and nonspecific binding was prevented by blocking with 20% normal goat serum or normal rabbit serum in PBS at RT for 30 minutes. Slides were incubated with primary antibody by PBS served as a negative control. Sections were then incubated with a goat–anti-rabbit or mouse–anti-rabbit biotinylated secondary antibody (1:200; Vector Laboratories; Pierce) in 50 mmol/L phosphate buffer, pH 7.2, at RT for 15 minutes. The reaction mixture was visualized with 3,3′-diaminobenzidine to visualize protein expression. Slides were counterstained with hematoxylin, dehydrated, and coverslipped.

 Autoradiography

Several EW-5 tumors were subjected to autoradiography to visualize intratumoral distribution of 111In-R1507. After dissection, EW-5 xenografts were fixed in 4% formalin, embedded in paraffin, and 10-μm tumor sections were cut. Sections were exposed to a phosphor imaging plate, incubated for approximately 1 minute, and subsequently the plate was scanned in the PhosphorImager (Fuji; BAS-1800 II).

Radiolabeling

R1507, a fully human monoclonal antibody directed against an epitope on the extracellular domain of the human IGF-1R, was obtained from Roche Diagnostics and radioiodinated with 111In (23). Prior to the 111In-labeling, R1507 was conjugated with isoiothiocyanatobenzyl–diethylenetriaminepentaacetic acid (ITC–DTPA; Macrocyclis) in 0.1 mol/L NaHCO3, pH 9.5, with a 14-fold molar excess of ITC–DTPA for 1 hour at RT. Unconjugated ITC–DTPA was removed by dialysis against 0.25 mol/L ammonium acetate buffer, pH 5.4. The R1507–DTPA conjugate was labeled with 111In (Covidien BV;) in 0.1 mol/L 2-(N-morpholino)ethanesulfonic acid buffer, pH 5.4, at RT for 30 minutes. For biodistribution studies, the specific activity was 0.2 MBq/μg and 11.1 MBq/μg for immuno-SPECT studies. Labeling efficiency was determined by instant thin-layer chromatography on TEC Control chromatography strips (Biodex), with 0.1 mol/L citrate buffer, pH 6.0, as the mobile phase. If labeling efficiency was below 95%, the reaction mixture was purified on a PD-10 column (GE Healthcare;) eluted with PBS, 0.5% bovine serum albumin (BSA). In all experiments, radiochemical purity of 111In-R1507 exceeded 98%.

 Radiiodination of R1507.

R1507 was radiolabeled with iodine-125 (125I), as described previously (23). The radioiodination with 125I (PerkinElmer;) to a specific activity of 0.4 MBq/μg in a IODOGEN–coated vial (Thermo Scientific; Pierce) in 50 mmol/L phosphate buffer, pH 7.2, at RT for 15 minutes. The reaction mixture was purified on a PD-10 column, eluted with PBS, 0.5% BSA. The radiochemical purity of 125I-R1507 exceeded 99%.

 In vitro characteristics of 111In-R1507 and 125I-R1507.

The immunoreactive fractions of 111In-R1507 and 125I-R1507 as determined by the Lindmo assay were 99% and 88%, respectively, as previously described by Heskamp and colleagues (23; results not shown).

Biodistribution studies

Tumor-bearing (OS-1 and EW-5) mice were intravenously injected with a mixture of 125I- and 111In-labeled R1507 (0.2 MBq each). The total injected protein dose was adjusted to either 3 μg (optimal dose; dose finding experiments in Supplementary Fig. S2) or 300 μg (control to block the IGF-1R in vivo) per mouse by the addition of unlabeled R1507 (23). At days 1, 3, and 7 (n = 6 per group) postinjection, mice were euthanized using O2/CO2 asphyxiation and relevant tissues such as tumor, blood, muscle, femur, lung, liver, kidney, spleen, duodenum, and pancreas were dissected, blotted dry, and weighed. Activity was measured in a shielded 3-inch well-type gamma-counter (Wizard; Pharmacia-LKB). To correct for physical decay and calculate uptake of the radiolabeled antibody in each sample as a fraction of the injected dose, aliquots of the injected dose were measured in a shielded 3-inch well-type gamma-counter (Wizard; Pharmacia-LKB).
were counted simultaneously. The results are expressed as percentage of the injected dose per gram (% ID/g).

**Immunoo-SPECT**

For immuno-SPECT, OS-1 and EW-5 tumor-bearing mice received an intravenous injection of 3 µg $^{111}$In-labeled R1507 (20 MBq; $n = 6$). At 1 and 3 days postinjection, mice were anesthetized with isoflurane/O$_2$ inhalation and scanned for 0.5 to 1 hour using the U-SPECT-II gamma camera (MILabs) and the 1.0-mm diameter pinhole rat collimator tube. At day 7, mice were euthanized, scanned for 2 hours, and $^{111}$In-R1507-uptake was determined ex vivo, as described above. Mice bearing OS-33, EW-8, and OS-1/OS-33 tumors were injected with 3 µg $^{111}$In-labeled R1507 as well (20 MBq; $n = 4$ per group), and scanned on day 3 postinjection followed by dissection of relevant tissues. An additional group of OS-33 tumor-bearing mice ($n = 6$) was coinjected with 300 µg unlabeled R1507, scanned on day 3, and dissected.

Scans were reconstructed with MILabs reconstruction software, using an ordered-subset expectation maximization algorithm, with a voxel size of 0.375 mm. Representative three dimensional (3D) images are displayed. Tumor-to-background ratios are represented by tumor-to-liver ratios as determined from the ex vivo biodistribution studies.

**Computed tomography scan**

Computed tomography (CT) scans were carried out directly after immuno-SPECT imaging. Mice were scanned for 8 minutes using the U-CT scanner (MILabs), and images were reconstructed using MILabs reconstruction software. To determine the exact location of $^{111}$In activity, CT- and immuno-SPECT scans were coregistered. 3D images were created using Siemens Inveon Research Workplace software (Siemens).

**Results**

**Characterization of bone sarcoma xenografts**

Because target expression and target accessibility are both important factors for predicting therapy response, all tumors were stained immunohistochemically for IGF-1R and CD34 expression. Figure 1A shows that the 2 osteosarcoma xenografts consist of multiple tumor cells surrounded by an extracellular matrix and several blood vessels, without any necrotic areas. The OS-33 tumor showed an even more apparent tumor vasculature and extracellular matrix component as than the OS-1 tumor. In contrast, the 2 Ewing sarcoma models showed virtually no extracellular matrix and were characterized by multiple necrotic lesions surrounded by hypoxic borders, as shown by Ki-67 and HIF-1α staining patterns. Blood vessels were located in the center of viable cell areas in Ewing tumors. IGF-1R expression was abundantly present in the cytoplasm of OS-1, EW-5, and EW-8 tumors and some membranous staining was detected as well. In EW-5 and EW-8 xenografts, IGF-1R expression tended to be higher at hypoxic areas on the border of necrotic lesions than IGF-1R expression in more vital areas close to blood vessels. The OS-33 tumor was IGF-1R negative (Fig. 1B).

**Biodistribution and pharmacodynamics of radiolabeled R1507 in mice with OS-1 and EW-5 xenografts**

The biodistribution of $^{111}$In-R1507 and $^{125}$I-R1507 in nude mice bearing s.c. OS-1 and EW-5 tumor xenografts was determined 1, 3, and 7 days postinjection, and the results are summarized in Fig. 2.

From day 1 onward, $^{111}$In-R1507 efficiently and specifically accumulated in the OS-1 tumor (15.7 ± 4.0%ID/g), and uptake levels improved even further on days 3 and 7 (27.5 ± 6.5%ID/g and 25.8 ± 5.8%ID/g, respectively). In EW-5 tumors, $^{111}$In-R1507 showed the same pattern about tumor accumulation over time, although tumor uptake levels were less than observed in OS-1 tumors. $^{111}$In-R1507 uptake levels in EW-5 tumors on days 1, 3, and 7 were 8.4 ± 1.4%ID/g, 14.0 ± 2.8%ID/g, and 15.2 ± 3.7% ID/g, respectively. Coadministration of an excess unlabeled R1507 (300 µg) resulted in both tumors in a significant decrease in tumor uptake at all time points, indicating IGF-1R mediated, specific accumulation of the antibody in the tumor. In normal tissues, uptake of $^{111}$In-R1507 was generally low and nonspecific because uptake levels were similar in the presence and absence of an excess unlabeled R1507. Only blood and well-perfused organs showed some uptake in the OS-1 and EW-5 models at day 1, but all these levels decreased gradually over time as a result of blood clearance. Consequently, highest tumor-to-blood and tumor-to-liver ratios were observed at day 7 in both OS-1 (4.5 ± 1.4 and 8.1 ± 2.5, respectively) and EW-5 models (2.0 ± 0.3 and 4.8 ± 0.9, respectively).

In contrast, tumor accumulation of $^{125}$I-R1507 remained relatively low in both xenografts. In OS-1 tumors, highest uptake was seen at day 1 (6.9 ± 1.5%ID/g), followed by a decrease in accumulation on days 3 and 7 (6.6 ± 2.5%ID/g and 3.1 ± 0.8%ID/g, respectively). The same pattern was observed in EW-5 tumors, with relatively high uptake on day 1 (3.9 ± 0.5%ID/g) and a subsequent decrease in accumulation on days 3 and 7 (3.8 ± 0.7%ID/g and 2.2 ± 0.5%ID/g, respectively). In both bone sarcomas, tumor uptake of $^{125}$I-R1507 was significantly lower than $^{111}$In-R1507 at all time points ($P < 0.05$). Because the $^{125}$I-label washes out of the cell after receptor internalization, whereas $^{111}$In does not, this indicates rapid internalization and degradation of the receptor–antibody complex (23, 26). Tumor-to-blood and tumor-to-liver ratios of $^{125}$I-R1507 in OS-1 tumors were highest at day 7 (1.2 ± 0.6) and day 1 (3.0 ± 0.7, respectively). In EW-5 tumors, tumor-to-blood and tumor-to-liver ratios of $^{125}$I-R1507 were both highest at day 7, which were 0.4 ± 0.1 and 1.7 ± 0.4, respectively.

Because $^{111}$In-R1507 clearly showed superior tumor-targeting properties compared with $^{125}$I-R1507 in both osteosarcoma and Ewing sarcoma models, we used $^{111}$In-R1507 in the immuno-SPECT studies.
Immuno-SPECT/CT of $^{111}$In-R1507 in mice with OS-1 and EW-5 xenografts

Membranous IGF-1R expression was visualized in OS-1 and EW-5 tumors with $^{111}$In-R1507 immuno-SPECT. CT scans were acquired to visualize animal anatomy. Representative immuno-SPECT/CT images acquired at days 1, 3, and 7 postinjection of $^{111}$In-R1507 in an OS-1 tumor are shown in Fig. 3A. From day 1 onward, the subcutaneous OS-1 tumor was clearly visualized and $^{111}$In-R1507 was distributed homogeneously throughout the whole tumor. Tumor-to-background contrast improved in time due to clearance of $^{111}$In-R1507 from the blood. Virtually no uptake was seen in normal tissues on days 3 and 7. After the final scan on day 7, ex vivo biodistribution revealed a tumor uptake of 18.6%ID/g and a tumor-to-blood ratio of 4.7. Uptake in other organs remained low, with a tumor-to-liver ratio of 7.9.

Interestingly, the EW-5 tumors showed quite a different uptake pattern as shown in Fig. 3B. On day 1, only modest $^{111}$In-R1507 uptake was shown which was confined to a few small areas of the tumor. Despite an increase in tumor uptake on days 3 and 7, $^{111}$In-R1507 distribution remained very heterogeneous throughout all EW-5 tumors at all time points. Ex vivo biodistribution of the displayed tumor on day 7 showed a tumor uptake of 9.5%ID/g and a tumor-to-blood ratio of 2.1. Uptake in other organs remained low, with a tumor-to-liver ratio of 3.0.

To investigate whether the heterogeneous distribution pattern of $^{111}$In-R1507 was the result of loss of IGF-1R expression in certain areas of the tumor, tumors were
subjected to IHC after dissection. To directly compare $^{111}$In-R1507 tumor uptake with IHC, autoradiography was carried out as well on slides directly adjacent to those used for IHC to visualize the regions of $^{111}$In-R1507 uptake in those specific tumor slides. In the EW-5 tumor, autoradiography showed a heterogeneous uptake pattern of $^{111}$In-R1507. However, IHC revealed a homogeneous distribution of IGF-1R expression throughout the EW-5 tumor samples, indicating that loss of IGF-1R expression was not the cause of the heterogeneous uptake pattern (Fig. 3D and E).

Immuno-SPECT/CT of $^{111}$In-R1507 in mice with OS-33 and EW-8 xenografts

To show the specificity of the $^{111}$In-R1507 antibody in vivo, mice with IGF-1R-negative OS-33 osteosarcoma...
xenografts were subjected to immuno-SPECT/CT as well. Mice bearing OS-33 tumors were imaged at day 3 postinjection, showing little to no uptake of $^{111}$In-R1507 in the tumor, as shown in Fig. 3C. Uptake in the OS-33 tumor was $5.5 \pm 0.6\%$ID/g and tumor-to-blood and tumor-to-liver ratios were $1.2 \pm 0.4$ and $1.4 \pm 0.5$, respectively (Fig. 4). These low levels of tumor accumulation were the result of nonspecific localization because administration of an excess unlabeled R1507 (300 $\mu$g) resulted in similar uptake levels ($5.9 \pm 0.4\%$ID/g).
For additional specificity testing, EW-8 xenografts were subjected to immuno-SPECT/CT and imaged at day 3 post-injection as well. These tumors were selected because EW-8 xenografts showed apparent IGF-1R expression on IHC and/or WB, but lack response to IGF-1R antibody-mediated therapy. Figure 3C clearly shows that there is virtually no tumor uptake of 111In-R1507 in EW-8 tumors on immuno-SPECT scans, similar to the OS-33 tumors.

Ex vivo biodistribution supported these findings by showing a tumor uptake of 6.5 ± 1.5%ID/g and a tumor-to-blood ratio of 1.4 ± 0.2. Uptake in other organs was low, with a tumor-to-liver ratio of 1.5 ± 0.3. The EW-8 tumors used in the imaging experiments were still IGF-1R positive on IHC. Table 1 summarizes tumor uptake and distribution of 111In-R1507 in all bone sarcoma xenografts used in this study.

**Immuono-SPECT/CT of 111In-R1507 in mice with OS-1/OS-33 double xenografts**

Because patients can present with multiple tumor manifestations showing variable IGF-1R expression levels, we checked whether 111In-R1507 was able to visualize IGF-1R expression accurately in mice with multiple lesions. The IGF-1R-positive OS-1 xenograft was implanted in the right flank, whereas the IGF-1R-negative OS-33 xenograft was implanted in the left flank. Mice were imaged 3 days postinjection of the radiolabeled antibody. Figure 3C clearly shows that 111In-R1507 is able to distinguish between IGF-1R-positive (OS-1) and IGF-1R-negative (OS-33) xenografts. Uptake in the OS-1 tumor was 17.7%ID/g, whereas the uptake in the OS-33 tumor was 5.7%ID/g. 111In-R1507 uptake in normal tissues remained low, with an uptake of 6.2%ID/g and 4.3%ID/g in blood and liver, respectively.

**Discussion**

In this study, we showed for the first time in various bone sarcoma models that in vivo membranous IGF-1R expression levels and target accessibility, as assessed by 111In-R1507 immuno-SPECT, positively correlate to the degree of response to the IGF-1R inhibitor R1507. The IGF-1R positive and highly IGF-1R therapy responsive OS-1 xenograft showed specific accumulation of 111In-R1507 throughout the whole tumor. Interestingly, the EW-5 xenograft which is also IGF-1R positive, but only modestly responsive to anti–IGF-1R therapy, showed a very heterogeneous tumor distribution pattern of 111In-R1507. Consequently, 111In-R1507 tumor uptake in EW-5 tumors was significantly lower than observed in OS-1 tumors. More importantly, the IGF-1R-positive EW-8 tumor that is unresponsive to anti–IGF-1R therapy showed significantly lower tumor uptake of 111In-R1507 than OS-1 and EW-5 tumors. Moreover, 111In-R1507 uptake levels in the EW-8 tumors were comparable with those of the IGF-1R–negative and nonresponsive OS-33 xenografts. Altogether, these findings strongly indicate that 111In-R1507 immuno-SPECT may be used as an independent method to predict IGF-1R therapy response.

Although we were able to correlate 111In-R1507 immuno-SPECT directly to the degree of R1507 therapy response, it remains a challenge to extrapolate these results to response to other IGF-1R inhibitors. At present, various IGF-1R

<table>
<thead>
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<th>Xenograft</th>
<th>IGF-1R status</th>
<th>Response to R1507*</th>
<th>Tumor 111In-R1507 uptake on day 3 p.i.</th>
<th>111In-R1507 distribution on immuno-SPECT</th>
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<tr>
<td>OS-1</td>
<td>Positive</td>
<td>High</td>
<td>27.5 ± 6.5%ID/g</td>
<td>Homogeneous</td>
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<tr>
<td>EW-5</td>
<td>Positive</td>
<td>Intermediate</td>
<td>14.0 ± 2.8%ID/g</td>
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<tr>
<td>EW-8</td>
<td>Positive</td>
<td>Low</td>
<td>6.5 ± 1.5%ID/g</td>
<td>Nonspecific</td>
</tr>
<tr>
<td>OS-33</td>
<td>Negative</td>
<td>Low</td>
<td>5.5 ± 0.6%ID/g</td>
<td>Nonspecific</td>
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*Data from Kolb et al. (11) and Supplementary Figure S1.

**Table 1.** 111In-R1507 tumor uptake and distribution in bone sarcoma xenografts

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Figure 4. Biodistribution of 111In-R1507 in mice with OS-33 xenografts. Mice were injected with 3 μg 111In-R1507 (n = 4; 20 MBq) or coinjected with 300 μg unlabeled R1507 (n = 6; 20 MBq) and were dissected at day 3 postinjection. Values are presented as mean%ID/g ± SD.
inhibitors have been tested upon the bone sarcoma xenografts in this study. Although in general similar response patterns are seen as with R1507, some discrepancies exist. EW-8 tumors for instance are not as consistently resistant to IGF-1R-mediated therapy as OS-33 tumors because low antitumor responses were observed during BMS-754807 therapy (27). This may be explained by the fact that IGF-1R is present in EW-8 tumors, in contrast to the completely IGF-1R-negative OS-33 xenograft. Although not always easy to target, compounds that differ structurally from R1507 may show superior tumor penetrating capacities resulting in improved receptor targeting. Another possible explanation for improved targeting with BMS-754807 is that this compound inhibits insulin receptor (IR) signaling as well (27). Because evidence is growing toward an oncogenic role for IR signaling in (bone) cancer, and IR is abundantly expressed in Ewing sarcomas, additional IR inhibition may result in superior antitumor effects compared with IGF-1R targeting alone (28, 29). In addition, OS-1 has shown an intermediate response to the IGF-1R inhibitors IMC-A12 and BMS-754807, whereas EW-5 xenografts showed a high response to SCH-717454 (27, 30, 31). Nevertheless, although these discrepancies exist, $^{111}$In-R1507 immuno-SPECT is still able to separate the major responders (high and moderate response) from the virtually nonresponders (low to no response) about IGF-1R-mediated therapy. Only for R1507 therapy, $^{111}$In-R1507 immuno-SPECT is able to give more detailed information about the degree of tumor response.

In this study, we focused on membranous IGF-1R expression as a marker for predicting IGF-1R therapy response. Although previous studies reported that there is no straight forward correlation between IGF-1R expression levels and response to IGF-1R therapy, these findings were based on total IGF-1R expression levels as determined by WB (11, 12). Because IGF-1R therapy solely targets IGF-1 receptors present on the cell membrane, it would be more appropriate to specifically screen for membranous IGF-1R expression as shown in this study.

With the imaging technique described in this article, we tackle several limitations that currently exist in determining (membranous) IGF-1R expression levels in a tumor. First of all, with commercially available antibodies directed against IGF-1R, it remains difficult to determine the level of membrane-bound receptors with IHC on sarcoma samples. High levels of cytoplasmic expression are frequently observed in human bone sarcoma biopsies and could cause difficulties to detect specific membranous expression. Even if membranous IGF-1R expression could clearly be shown by IHC, some shortcomings exist that make this technique unsuitable for the prediction of response to IGF-1R treatment. One problem is that bone sarcomas are characterized by various cell populations. Osteosarcomas for instance consist of a mixture of some of the following cell types: osteoblasts, osteoclasts, fibroblast-like cells, chondroblast-like cells, extracellular matrix cells, endothelial cells, and infiltrated immune cells such as macrophages. IGF-1R staining of a section of one tumor sample does therefore not necessarily represent IGF-1R expression levels in the whole tumor. Furthermore, metastatic bone sarcoma patients often present with multiple lesions showing variable target expression levels. It is however unfeasible to carry out multiple biopsies.

Another major problem with current screening methods is that they may show IGF-1R expression levels, but fail to show in vivo target accessibility. As is known the effect of antibody therapy not only depends on the presence of the receptor on tumor cells, but also on tumor physiology. Tumor interstitial pressure, vascular permeability, microvessel density, necrosis, and tumor perfusion are important factors that contribute to whether or not an antibody reaches its target (19–21, 32). Although immunostaining can give some information about these factors, such as CD34 staining for tumor vasculature, this only represents a small region of the tumor. Furthermore, information about tumor interstitial pressure and tumor perfusion cannot be obtained by IHC. In addition, osteosarcomas the presence and amount of extracellular matrix is also an important factor to take into account (22). If tumor cells are surrounded by a calcified, solid bone matrix, these tumor cells may be less accessible for the antibody. The large necrotic areas frequently observed in Ewing sarcomas may severely impede antibody diffusion as well. In this study, we specifically chose to implant human osteosarcoma and Ewing sarcoma xenografts consisting of several cell types including extracellular matrix components instead of injecting cultured bone sarcoma cells to mimic a human bone tumor as closely as possible. In the in vivo osteosarcoma and Ewing sarcoma xenograft models, the physiologic factors are taken into account and immuno-SPECT shows whether the antibody is able to target the IGF-1 receptor.

The importance of screening for target accessibility instead of merely screening for target expression in bone sarcomas was shown in our experiments. Although the OS-1 tumors predominantly showed a homogeneous tumor distribution of $^{111}$In-R1507, EW-5 tumors showed a very heterogeneous uptake pattern of $^{111}$In-R1507 on immuno-SPECT scan. IHC revealed that this was not due to loss of IGF-1R expression. Instead, $^{111}$In-R1507 was not able to reach or bind to receptors in certain areas of these tumors, possibly due to high interstitial fluid pressure and/or low vascular permeability. This may very well explain why in previous studies the response of EW-5 tumors to IGF-1R antibody therapy was only modest, despite marked IGF-1R expression on IHC and WB. More evidence about the importance of target accessibility was found in the distribution of $^{111}$In-R1507 throughout EW-8 tumors. Despite being IGF-1R positive on IHC and WB, EW-8 tumors showed virtually no uptake of $^{111}$In-R1507, which correlates directly to the lack of response to R1507. These findings emphasize that not only target expression, but also target accessibility is an important factor to predict therapy response. When a bone sarcoma patient shows $^{111}$In-R1507 only in a small region of the tumor, IGF-1R monotherapy may not be sufficient. Interestingly, IGF-1R therapy
combined with rapamycin already proved to be very effective in both osteosarcoma and Ewing sarcoma xenografts. In EW-5 xenografts, the combination of figitumumab and rapamycin showed even supraadditive effects (12). In addition, inhibition of IGF-1R may significantly potentiate the antitumor activity of conventional chemotherapeutic drugs (33).

One major problem about IGF-1R-targeted therapy is that tumors have the potential to develop resistance to IGF-1R antibodies. The mechanisms underlying this phenomenon are currently under investigation, and in vitro studies have previously shown that low expression levels of IGF-1R were associated with (de novo) resistance (24). A recent study further investigated mechanisms of acquired resistance using a small-molecule tyrosine kinase inhibitor of the IGF-1R/IR family kinases, and showed chronic loss of IGF-1R expression during treatment. This decrease in IGF-1R expression can result in a decreased dependency on the IGF-1R pathway for growth. As a result, other receptors, such as platelet-derived growth factor-receptor α or AXL, are upregulated and cells subsequently rely on downstream signaling of these receptors (34). These findings indicate that it is not only important to screen for IGF-1R expression levels prior to IGF-1R therapy, but also to monitor these levels during IGF-1R therapy. This illustrates that imaging IGF-1R expression during IGF-1R antibody-targeted therapy is warranted. It must, however, be noted that the IGF-1R antibody used for imaging needs to be directed against another, noncompetitive epitope of the IGF-1R than the IGF-1R antibody used for treatment to avoid saturation of the receptor with therapeutic IGF-1R antibody, precluding IGF-1R imaging. In this way, receptor downregulation can be monitored and may predict resistance due to chronic loss of IGF-1R. Because the imaging method described in this article is noninvasive, it can be safely used to monitor treatment response at multiple time points.

In this study, we used the fully human monoclonal antibody R1507 directed against the human IGF-1 receptor. Because R1507 does not cross-react with murine IGF-1R, the high tumor-to-background ratios seen in our models may be lower in patients because radiolabeled R1507 will also recognize IGF-1R expressed in normal human tissues, such as muscle, cartilage, and bone (35). However, a recent pediatric phase I study using R1507 showed that even in young children (aged ≥2 and <18 years) drug-related toxicity was minimal (10). Therefore, we believe that IGF-1R expression levels expressed in normal tissues would not dramatically interfere with immuno-SPECT imaging of tumor IGF-1R expression. This, however, remains to be investigated.

In summary, we have shown that 111In-R1507 immuno-SPECT imaging is an excellent method to visualize membranous IGF-1R expression and target accessibility in vitro in human bone sarcoma xenografts. More importantly, we showed a positive correlation between 111In-R1507 immuno-SPECT and the degree of R1507 therapy response, whereas IGF-1R expression of the same tumors on IHC or WB did not show such a correlation. Although antitumor responses of the used bone sarcoma xenografts are not always as consistent when using other IGF-1R inhibitors, 111In-R1507 immuno-SPECT is able to distinguish major responders (high and moderate response) from the virtually nonresponders (low to no response). These findings confirm that this novel technique is superior to currently used conventional screening methods in determining IGF-1R expression levels and predicting response to IGF-1R therapy. We therefore would plea for introducing 111In-R1507 immuno-SPECT for the indication and monitoring of IGF-1R-targeted therapies in bone sarcoma patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank the PPTP for generously providing the bone sarcoma xenografts and Roche Diagnostics for providing R1507. Debby Smits, Bianca Lemmens-van de Weem, and Renty Lemmens-Hermans provided technical assistance.

Grant Support

This work was supported by Stichting Vanderes. Part of the work was supported through NIH funds, an USPIS award from the National Cancer Institute (CA23099), a SARC grant, and a personal research grant of the Dutch Research Council (016.096.010). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 10, 2011; revised September 21, 2011; accepted October 11, 2011; published OnlineFirst October 28, 2011.
Predicting IGF-1R Therapy Response in Bone Sarcomas


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