Determining Maximal Tolerable Dose of the Monoclonal Antibody BR96 Labeled with $^{90}$Y or $^{177}$Lu in Rats: Establishment of a Syngeneic Tumor Model to Evaluate Means to Improve Radioimmunotherapy

Linda Mårtensson, Zhongmin Wang, Rune Nilsson, Tomas Ohlsson, Peter Senter, Hans-Olov Sjögren, Sven-Erik Strand, and Jan Tennvall

**Abstract**

**Purpose:** To evaluate therapeutic strategies, it is essential to use biological models reflecting important aspects of the clinical situation. The aim of the present study was to compare the maximal tolerable dose of the monoclonal antibody BR96 labeled with $^{90}$Y or $^{177}$Lu in immunocompetent rats. Maximal tolerable dose was defined as the highest activity that allows 100% of the animals to survive without clinical signs, such as infections, bleeding, or diarrhea, and with <20% loss in body weight.

**Experimental Design:** Increasing activity levels of BR96 labeled with $^{90}$Y or $^{177}$Lu were administered to groups of rats. Blood parameters, body weight, and general performance were monitored for 8 weeks.

**Results:** Two days postinjection, all groups had decreased leukocyte counts down to 5% to 15% of initial values. Initiation of recovery (at 14–21 days) showed a dose-response relationship. All groups, except the group given the highest activity of $^{90}$Y, had complete resolution in their leukopenia. The decrease in platelets was delayed to days 7 to 14 postinjection with a dose-dependent response regarding both severity of the nadir (10–40% of initial value) and the start of recovery. Animals in the groups given the highest activities of both $^{90}$Y and $^{177}$Lu exhibited skin infections on day 21.

**Conclusions:** The results showed good reproducibility and dose-dependent toxicity for both radionuclides, indicating that the maximal tolerable dose for $^{177}$Lu–BR96 (1,000 MBq/kg) is 1.7 times that for $^{90}$Y–BR96 (600 MBq/kg) in rats. This model makes it feasible to evaluate strategies to escalate therapeutic doses to tumors without increasing normal tissue toxicity.

**A major limiting factor in the application of radioimmunotherapy to solid tumors is low tumor-to-normal tissue activity ratio. The slow antibody accretion in solid tumors limits delivery of effective tumor-absorbed doses at acceptable normal tissue toxicity, especially to the bone marrow. Various strategies have been developed to overcome the low tumor-to-normal tissue ratios and reduce the toxicity in normal organs (1–3). To evaluate new therapeutic strategies, it is essential to use biological models reproducing important aspects of clinical treatment. The use of human tumors in immunodeficient mice has several shortcomings in radioimmunotherapy, e.g., the relatively large size of the tumors and the immunocompromised status of the animals. Due to the small body size, the cross dose from surrounding tissue will significantly increase the absorbed dose to an organ when administering long-range $\beta$-emitters, such as yttrium $^{90}$Y (ref. 4). We instead used an immunocompetent rat model that better reflects the clinical situation.

Lutetium $^{177}$Lu is a relatively recently used radionuclide in radioimmunotherapy thought to be a promising alternative or complement to the more established use of $^{90}$Y. The decay properties of $^{177}$Lu versus $^{90}$Y (6.7 and 2.7 days, respectively) is better suited to the pharmacokinetics of monoclonal antibodies (mAb) as the toxic effects on the bone marrow will be delayed. The aim of the present study was to determine and compare the maximal tolerable doses of the tumor binding mAb BR96 labeled with $^{90}$Y or $^{177}$Lu. The study was done by administration of escalating activities of $^{90}$Y or $^{177}$Lu in rats and monitoring the myelotoxicity, body weight, and performance for a period of 2 months. Maximal tolerable dose was defined as the highest activity (MBq/kg) that allows 100% of the animals to survive without clinical signs of toxicity, such as infections, bleeding.
or diarrhea, and with <20% loss in body weight. Comparative studies of the maximal tolerable dose of mAbs labeled with $^{90}$Y or $^{177}$Lu have been done in mice (5, 6); however, to the best of our knowledge, such a study has not been carried out in rats.

### Materials and Methods

**Monoclonal antibody BR96 and conjugation with 1,4,7,10-tetraazacyclododecane-N,N',N''-tetraacetic acid and avidin**

The monoclonal antibody BR96. BR96 (Seattle Genetics, Inc. Seattle, WA) is a chimeric (mouse/human) mAb binding the tumor-associated glycoprotein Lewis Y (Le$^a$). Le$^a$ is expressed on the majority of human epithelial tumors including breast, gastrointestinal tract, non–small-cell lung, cervix, ovary, and some melanomas (7). As the majority of tumor-associated mAbs, BR96 also reacts with some normal tissue, primarily human cells of the gastrointestinal tract (7).

Conjugation with 1,4,7,10-tetraazacyclododecane-N,N',N''-tetraacetic acid and avidin. BR96 was conjugated with the trifunctional chelator 1033 (MitraTag, Mitra Medical AB, Lund, Sweden), carrying a acetic acid and biotin moiety (8). Before conjugation, BR96 was transferred by dialysis into a 50 mmol/L HEPES, 1 mmol/L diethylenetriaminepentaacetic acid buffer (pH 8.5). Conjugation was done by adding 80 µg of 1033 per milligram of BR96 and incubating for 2 hours at room temperature and overnight at 4°C. After conjugation, the conjugate was transferred to 0.25 mol/L ammonium acetate storage buffer (pH 5.3). The number of 1033 molecules per BR96 molecule was determined by the 4'-hydroxyazobenzene-2-benzoic acid photometric method (9). This assay is based on the binding of the dye 4'-hydroxyazobenzene-2-benzoic acid to avidin and the ability of biotin to displace the dye in stoichiometric proportions.

### Radiolabeling and quality control of radioimmunoconjugate

**Radiolabeling and quality control.** The same procedure was used for labeling with $^{90}$YCl$_3$ (MDS Nordion, Vancouver, Canada) and $^{177}$LuCl$_3$ (MDS Nordion, Vancouver, Canada). Both the 1033-BR96 stored in 0.25 mol/L ammonium acetate buffer and the radionuclide solutions were preheated at 45°C for 10 minutes. The 1033-BR96 solution was then added to the radionuclide-containing vials and incubated at 45°C for 15 minutes. The reaction was quenched with an excess of diethylenetriaminepentaacetic acid for 5 minutes.

The radiochemical purity of the labeled immunoconjugates was determined by instant TLC (1 × 9 cm silica gel impregnated glass fiber sheet, eluted in 0.1 mol/L EDTA). High-performance liquid chromatography [7.8 × 300 mm molecular sieving column, Phenomenex SEC S3000 (Phenomenex, Torrance, CA), eluted in 0.05 mol/L sodium phosphate at 1.0 mL/min] was used to control the radiochemical purity and signs of aggregation or fragmentation.

**Avidin-binding fraction test.** To ensure that the labeling had not affected the biotin moiety of the 1033 molecule, the avidin-binding ability of the radioimmunoconjugates was assessed. An adsorption column packed with ~0.3 ml Mitra Avidin-Agarose (Mitra Medical) was used. A 50 µL sample of radioimmunoconjugate was added to the column and incubated for 10 minutes at room temperature. The column was washed eight times with 0.5 ml PBS containing 0.05% Tween 20, and each washing was collected separately in tubes. The activity in the column and in each tube was measured in a NaI(TI) scintillator. The avidin-binding fraction was expressed as the percentage of radioactivity in the column in relation to the sum of the radioactivity in the tubes and the column.

**Animals**

In this study, immunocompetent rats of the Brown Norwegian strain (Harlan, Horst, the Netherlands) were used. As shown by immunohistochemistry, Brown Norwegian rats express the BR96 epitope in some normal tissues, such as the gastrointestinal epithelium, hence mimicking the human situation. The animals were kept under standard conditions and fed with standard pellets and fresh water.

### Experimental design

The studies were conducted in compliance with Swedish legislation on animal rights and protection. Twenty-seven male Brown Norwegian rats weighing 230 to 250 g were used in this study. Twenty-four of these rats were injected i.v. with escalating activities of $^{90}$Y–1033-BR96 or $^{177}$Lu–1033-BR96, calculated for each group as MBq/kg. Three rats were used as controls (Table 1).

Blood samples were collected twice a week for 8 weeks postinjection and WBC counts, RBC counts, and platelet counts were analyzed in a Medonic Cell Analyzer-Vet CAS30 Vet (Boule Medical, Stockholm, Sweden). At the time of blood sampling, the weight and physical condition of the animals were monitored. Toxicity was evaluated by monitoring animals for loss of body weight, decline in general condition, and hematologic toxicity.

### Results

#### Preparation of radioimmunoconjugates

After conjugation, the number of 1033 molecules conjugated per BR96 molecule was determined to be, on average, 2.9. The specific activity was 970 MBq/mg for $^{90}$Y–1033-BR96 and 1,830 MBq/mg for $^{177}$Lu–1033-BR96. Instant TLC showed the radiochemical purity to be 96% for $^{90}$Y–1033-BR96 and 98% for $^{177}$Lu–1033-BR96. No signs of aggregation or fragmentation were observed with high-performance liquid chromatography. The avidin-binding fraction exceeded 90% for both radioimmunoconjugates at the time of injection.

### Maximal tolerable dose

**Body weight loss.** During the first 5 days postinjection, animals lost weight (Table 2), reaching a nadir on day 5. For the groups of animals receiving $^{90}$Y–1033-BR96, the loss of weight was more profound than in the groups receiving $^{177}$Lu–1033-BR96 and dose related in contrast to the $^{177}$Lu–1033-BR96 groups where no dose-dependence was seen. The control animals had a 3% weight gain during the corresponding time interval. After day 5, the injected animals started to gain weight as the control animals.

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Table 1. Experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Radionuclide</th>
<th>Administered quantity of BR96 (µg)</th>
<th>Mean and range of administered activity (MBq/kg body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$^{90}$Y</td>
<td>87</td>
<td>340 (338-342)</td>
</tr>
<tr>
<td>2</td>
<td>$^{90}$Y</td>
<td>113</td>
<td>468 (466-473)</td>
</tr>
<tr>
<td>3</td>
<td>$^{90}$Y</td>
<td>138</td>
<td>623 (617-628)</td>
</tr>
<tr>
<td>4</td>
<td>$^{177}$Lu</td>
<td>100</td>
<td>515 (500-525)</td>
</tr>
<tr>
<td>5</td>
<td>$^{177}$Lu</td>
<td>150</td>
<td>667 (666-669)</td>
</tr>
<tr>
<td>6</td>
<td>$^{177}$Lu</td>
<td>100</td>
<td>827 (818-836)</td>
</tr>
<tr>
<td>7</td>
<td>$^{177}$Lu</td>
<td>150</td>
<td>1,000 (992-1,007)</td>
</tr>
<tr>
<td>8</td>
<td>$^{177}$Lu</td>
<td>150</td>
<td>1,185 (1,173-1,205)</td>
</tr>
<tr>
<td>9 (Controls)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

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7 H.O. Sjögren and I. Hellström, unpublished data.
Blood parameters. Myelotoxicity was monitored by quantification of WBC, RBC, and platelet counts. For both $^{90}$Y–1033-BR96 and $^{177}$Lu–1033-BR96, all groups showed a similar decrease in WBC 2 days postinjection (5-15% of initial values; Figs. 1 and 2). A clear dose-response relationship was seen regarding the recovery in WBC (Figs. 1 and 2). Animals receiving the lowest activity (340 MBq/kg) of $^{90}$Y–1033-BR96 started to recover at day 14, and the group receiving the highest activity (625 MBq/kg) started to recover at day 24 (Fig. 1). Animals belonging to the latter group had not fully recovered to their original WBC (75%) after 2 months. This group developed wound-like skin infections in the facial area and were, therefore, treated with antibiotics on days 35 to 45. A similar pattern was seen for animals receiving $^{177}$Lu–1033-BR96 (Fig. 2). The rats receiving the highest activity (1,185 MBq/kg) of $^{177}$Lu–1033-BR96 developed the same type of skin infections. One of these rats had to be sacrificed for ethical reasons on day 28. Individual variation between the rats within each group was low (Figs. 1 and 2).

A dose-response relationship was seen for the decrease in RBC in rats injected with $^{90}$Y–1033-BR96 (50-95% of initial values), reaching nadir on day 25 postinjection. For rats injected with $^{177}$Lu–1033-BR96, a substantial decrease (46% of initial values) was only seen in the group receiving the highest activity (1,185 MBq/kg), with nadir on day 25 postinjection. Platelet counts showed a clear dose-response relation for both $^{90}$Y–1033-BR96 and $^{177}$Lu–1033-BR96 (Figs. 3 and 4). Platelets started to decline on day 7 postinjection and started to recover on days 14 to 28 postinjection. All animals had recovered their initial platelet counts after 2 months.

Discussion

The present study shows that it is possible to administer 600 MBq/kg $^{90}$Y–1033-BR96 and 1,000 MBq/kg $^{177}$Lu–1033-BR96 without exceeding the maximal tolerable dose in immunocompetent rats, with good reproducibility. An activity-dependent myelotoxicity (MBq/kg) was observed for both radionuclides. We defined the maximal tolerable dose as the highest activity (MBq/kg) that allows 100% of the animals to survive without clinical signs of toxicity, such as infections, bleeding, or diarrhea, and with >20% loss in body weight. This definition differs from that in most studies in mice, where the maximal tolerable dose is generally defined only as 100% survival. Because these rats are immunocompetent in contrast to nude mice, infections can be used to identify the maximal tolerable dose. The dose-limiting factor in the present study was prolonged “suppression” of WBC, resulting in skin infections. For all treated groups, WBC toxicity reached its nadir ($<0.5 \times 10^9$ leukocytes/L, ~90% reduction) 2 days postinjection, independently of the administered activity (MBq/kg) or radionuclide. When the nadir was sustained for >28 days, infections occurred, and, in these cases, the toxicity was only reversible to 75%. Platelet count depression was reversible in all groups and no bleeding was detected during the nadir.

In mice, the maximal tolerable dose for mAbs labeled with $^{177}$Lu has been determined to be 10.2 and 18.5 MBq per animal for 6- to 7-week-old mice in two different studies (5, 6). The more recent results obtained by Brouwers et al. (6) correlate well with our results in rats if the mean weight of a mouse is approximated to 20 g (925 MBq/kg). In these studies, the maximal tolerable dose for the same mAbs labeled with $^{90}$Y was determined to be 3.9 and 5.6 MBq per animal, which corresponds to 195 and 280 MBq/kg (5, 6). Our results indicate that the maximal tolerable dose (MBq/kg) of mAb labeled with $^{90}$Y in rats is more than twice that in mice. This can probably be explained by the difference in the particle range of...
the two radionuclides. When $^{90}$Y, with a long particle range (maximum penetration depth 12 mm), is injected into the small body of a mouse, the cross dose from surrounding tissue will increase the absorbed dose in the bone marrow compared with a rat with a larger body volume. For $^{177}$Lu, the contribution of the cross dose to the total absorbed dose in the bone marrow will be lower because of the short particle range (maximum penetration depth, 1.5 mm). The weight loss during the first 5 days was also clearly related to the administered activity, but was more pronounced for the animals receiving $^{90}$Y. The reason for this is probably greater exposure of the intestine to radiation due to the longer particle range, as discussed above.

It has been shown that when mAbs are labeled with $^{90}$Y or $^{177}$Lu, the accretion of the two radionuclides in tumor and normal organs is nearly identical (10). Dosimetry calculations based on the longer half-life of $^{177}$Lu relative to $^{90}$Y (6.7 versus 2.7 days) predict that $^{177}$Lu-labeled mAbs should be able to deliver higher absorbed doses to the tumor at the maximal tolerable dose (11). However, the particle range has important implications for the curability of tumors and it should be considered that each radionuclide has an optimal tumor size for cure (12).

Because myelotoxicity generally is dose-limiting in radioimmunotherapy, it is more relevant to investigate the bone marrow toxicity of radiolabeled mAbs in immunocompetent animal models than in immunodeficient animal models, such as the nude mouse. In this study, non–tumor-bearing Brown Norwegian rats were used as rapid tumor growth would decrease the observation period and might influence the parameters monitored. A colon carcinoma cell line expressing the BR96 epitope has been established in the Brown Norwegian strain (13) and will be used in future studies to monitor therapeutic effects.

Therapeutic studies in this syngeneic tumor model have several advantages compared with studies in immunodeficient xenogeneic models. The induction of vascularization and stroma tissue support is more adequate by syngeneic tumor cells than by xenogeneic cells, and the immune response to the tumor is similar to that of the animal in which the original tumor developed. As a consequence, the infiltration of the tumor into surrounding normal tissue and metastasizing at other locations are more similar to the clinical situation than in immunodeficient xenograft tumor models. Also, the fact that the epitope for BR96 is expressed in some normal tissues is more relevant to the clinical situation regarding toxicity.
evaluation in normal organs. In addition, the tumor accretion of radiolabeled mAb (%ID/g) in many xenografted mouse models reaches ~50% ID/g (5, 6, 10), which does not correspond to clinical situations where the tumor accretion is 0.001% to 0.01% ID/g (1). In this aspect our model, which has a tumor accumulation of radiolabeled tumor-specific mAb of ~2% ID/g (14–16), is more relevant to the clinical situation.

To evaluate whether the low tumor-to-normal tissue ratio can be improved and the maximal tolerable dose increased, studies evaluating the therapeutic potential of radioimmunotherapy with BR96 in combination with extracorporeal affinity adsorption treatment are ongoing in this syngeneic tumor model. Extracorporeal affinity adsorption treatment eliminates circulating radiolabeled antibodies from the blood at a predetermined time after injection. Extracorporeal affinity adsorption treatment is based on the biotin-avidin system utilizing the high-affinity interaction between avidin and biotin. Antibodies are conjugated with 1033, as described above. By passing whole blood through an on-line column coated with avidin, ~95% of the radioimmunoconjugate is trapped in the column and eliminated from the circulation. In this rat tumor model, we have previously shown significantly increased tumor-to-normal tissue ratios with $^{111}$In-labeled mAb BR96 after extracorporeal affinity adsorption treatment (17). Based on these results from radioimmunotargeting, it is anticipated that extracorporeal affinity adsorption treatment will augment the maximal tolerable dose of radioimmunoconjugates when combined with extracorporeal affinity adsorption treatment, which could result in improved therapeutic results in solid tumors with radioimmunotherapy.

The longer physical half-life of $^{177}$Lu versus $^{90}$Y suggests that it will be possible to delay the onset of extracorporeal affinity adsorption treatment, allowing greater accumulation of the radioimmunoconjugate in the tumor without unacceptable exposure to normal tissue.

**Acknowledgments**

We thank Professor Scott Wilbur (University of Washington, Seattle, WA) for developing the MitraTag and Lars Lindgren for excellent technical assistance.

**References**

Means to Improve Radioimmunotherapy

Establishment of a Syngeneic Tumor Model to Evaluate

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