Maximum Tolerated Dose and Large Tumor Radioimmunotherapy Studies of $^{64}$Cu-labeled Monoclonal Antibody 1A3 in a Colon Cancer Model

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Abstract

The purpose of this study was 2-fold: to determine the maximum tolerated dose (MTD) of $^{64}$Cu-bromoacetamidobenzyl-1,4,8,11-tetraazacyclotetradecane-$N',N''$-$N''$-$N''$-$N''$-tetraacetic acid (BAT)-2-iminothiolane (2IT)-monoclonal antibody (MAb) 1A3 in hamsters, and second, to determine the therapeutic efficacy of $^{64}$Cu-BAT-2IT-MAb 1A3 at various dose levels in hamsters with large (600 mg), 7-day-old GW39 human colorectal carcinoma tumors. In the MTD studies, non-tumor-bearing hamsters were injected with varying amounts of $^{64}$Cu-BAT-2IT MAb 1A3 (>10 mCi) normalized to mCi injected/kg of hamster body weight. Results indicated that the MTD was 150 mCi of Cu-64/kg of body weight. Hamsters receiving higher doses (170-190 mCi/kg) lost greater than 20% of their body weight, and all died between 8 and 13 days ($n = 3$). All hamsters receiving doses $\leq 150$ mCi/kg (120-150 mCi; $n = 3$) survived to the experimental end point (6 weeks) with an overall gain in weight. WBC and platelet counts were depressed in all animals 7 days after treatment but returned to normal values in the survivors by 2 weeks. For larger tumor therapy studies, two 3.5 mCi doses separated by 24 or 48 h with 44% (4 of 9) and 25% (2 of 8) survival, respectively. In every large tumor experimental group, 100% of animals experienced tumor growth inhibition compared to saline control animals. To-gether, the MTD and the large tumor therapy studies confirm that $^{64}$Cu-labeled agents are excellent candidates for radioimmunotherapy trials.

Introduction

RIT$^3$ combines the tumor targeting properties of anticancer MAbs with the cytotoxic properties of therapeutic radionuclides to cause cancer remission or regression in patients. RIT is a rapidly growing area of basic research and clinical practice, and to date, most success with this form of cancer therapy has been with hematological malignancies (i.e., leukemias and lymphomas: Refs. 1–6), which are more radiosensitive and more accessible to systemically administered radiopharmaceuticals than most solid tumors. Success with solid tumors has been limited because low tumor uptake of radiopharmaceuticals has necessitated using high doses, which in turn cause bone marrow toxicity (7–10). There is mounting evidence, however, that RIT may be useful as an adjuvant therapy to control micrometastatic disease (11–13).

An important aspect in selecting a radionuclide for RIT is the energy of the $\beta^-$ decay, which determines the range of the particle in tissue. The most abundant $\beta^-$ decay of $^{131}$I (0.606 MeV; 82%) has a maximum range in tissue of about 2–3 mm, whereas $^{90}$Y (2.27 MeV; 100%) has a much larger $\beta^-$ decay energy and consequently a maximum range in tissue of ~11 mm. Thus, for tumors with diameters less than 1 cm, much of the radiation of a $^{90}$Y decay may not be deposited in the tumor, but in surrounding tissue. There are a number of other $\beta^-$ emitting radionuclides that are currently being evaluated in humans, including $^{67}$Cu, $^{188}$Re, $^{186}$Re, and $^{177}$Lu (6, 13–15).

Our research in the field of RIT has focused on the use of $^{64}$Cu as a radionuclide for RIT and targeted radiotherapy. $^{64}$Cu decays by $\beta^-$ (0.655 MeV; 19%), $\beta^-$ (0.573 MeV; 40%), and electron capture (1.68 MeV; 43%), which enables diagnostic imaging by positron emission tomography and radiotherapy with the same isotope. In RIT studies, we have shown that both $^{64}$Cu- and $^{67}$Cu-labeled MAB 1A3 ($^{64}$Cu; $t_{1/2} = 62$ h; 100% $\beta^-$) exhibited complete tumor growth inhibition in a well-established animal model (16). An interesting aspect of our studies is that $^{64}$Cu and $^{67}$Cu demonstrated very similar lethal efficiency despite different decay schemes (16, 17). We also showed that considerably lower absorbed doses were required for $^{64}$Cu- and $^{67}$Cu-labeled MAbs to effect complete tumor remission compared to doses reported for both $^{131}$I- and $^{90}$Y-labeled MAbs in similar animal models (16).

In this study, we determined the MTD of $^{64}$Cu-BAT-2IT-1A3 in hamsters. Additionally, the efficacy of $^{64}$Cu-BAT-2IT-

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2 To whom requests for reprints should be addressed, at Department of Surgery, Box 8109, Washington University School of Medicine, St. Louis, MO 63110. Phone: (314) 454-8039; Fax: (314) 454-5049. E-mail: ConnettJ@msnotes.wustl.edu.
3 The abbreviations used are: RIT, radioimmunotherapy; BAT, bromoacetamidobenzyl-1,4,8,11-tetraazacyclotetradecane-$N',N''$,$N''$-$N''$-tetraacetic acid; MAb, monoclonal antibody; MTD, maximum tolerated dose; 2IT, 2-iminothiolane.
Materials and Methods

MAb 1A3. MAb 1A3 is a mouse MAb of the IgG1, κ isotype that binds to antigen(s) extracted in the methanol phase of a Folch extract, suggesting a lipid component (18). This MAb reacts strongly with colon and rectal carcinomas and weakly, if at all, with normal colon tissue or with other normal tissue (19). No binding to cancers other than colorectal cancer has been observed in extensive histology studies with this MAb (20, 21). In vitro studies have shown that MAb 1A3 is internalized by target cells, and approximately $10^6$ antigen receptors are expressed on LS174T colon cancer cells.

Preparation of $^{64}$Cu-BAT-2IT-1A3. MAb 1A3 was purified from serum-free medium by Invitron (St. Louis, MO) with proprietary methods. Copper-64 ($t_{1/2} = 12.8$ h), was produced on a biomedical cyclotron at Washington University (St. Louis, MO) according to published methods (specific activity, 6,000–50,000 Ci/mmol; Ref. 22). Dose fractionation studies are possible because copper-64 can be made on a daily basis in our facility (23). All solutions were made using distilled deionized water ([Milli-Q7 (Millipore; Bedford, MA); >18 M resistivity]. Ammonium citrate ($NH_4Cit$) was purchased from Fluka Chemical Co. (Ronkonkoma, NY). All other chemicals were obtained from Aldrich Chemical Co. (Milwaukee, WI). The synthesis of BAT was accomplished as described in the literature with minor alterations (24). BAT was conjugated to 1A3 with the linking agent 2IT and labeled with $^{64}$Cu as previously described (25). The purity of $^{64}$Cu-BAT-2IT-1A3 was analyzed by fast protein liquid chromatography as previously described (25). The immunoreactivity of $^{64}$Cu-BAT-2IT-1A3 was determined by use of 1A3 antigen expressing GW39 human colon cancer cell suspensions as targets under conditions of antigen excess (26). Radioactivity was measured with a Beckman gamma counter (Beckman Instruments, Fullerton, CA).

Animal Model. The GW39 human colon cancer xenografts were propagated in 7-9-week-old male Golden Syrian hamsters (27). Tumor cell suspensions with >90% viability were injected in the right thigh of hamsters. The tumor grows exponentially in this model for 10–14 days, after which time, some necrosis becomes apparent. All MAbs or control buffers were given by intracardiac injection. All animal experiments were performed in compliance with guidelines specified by Washington University Animal Studies Committee.

MTD Studies. MTD is defined as the highest possible dose resulting in no animal deaths and less than 20% weight loss. Non-tumor-bearing hamsters were injected with varying amounts of $^{64}$Cu-BAT-2IT-1A3 (>10 mCi/nm) normalized to mCi injected/kg of hamster body weight. The animals ($n = 16$) were weighed and observed daily for any change in gross physical appearance. Weight loss greater than 20%, lethargy, scruffy coat, and diarrhea were interpreted as indications of toxicity. Samples of blood from study animals ($n = 8$) were taken by retroorbital bleeding 1 or 2 days before treatment and approximately every week thereafter for 6 weeks for analysis of hematology and liver and kidney enzyme levels. At sacrifice, hamsters were examined macroscopically for any organ toxicity.

RIT Studies. RIT studies were performed on hamsters carrying large, 7-day-old tumors and injected with 2–15 mCi (0.5–1.4 mg) of $^{64}$Cu-BAT-2IT-1A3. Control animals received saline only. Hamsters were sacrificed when tumors were approximately 10 g or after surviving tumor free for 4 months. The 10 g end point was selected for humane reasons. During the first month of therapy hamsters were weighed twice a week; 5–10 hamsters were used for each condition; data from repeat experiments were combined. At time of sacrifice, tumors were weighed and normal hamster tissues were examined.

Statistical Methods. To compare survival among different treatment groups, the SAS Lifetest procedure was used to generate Kaplan-Meier probability density plots; these data...
Table 1  Dose of Cu-64-1A3 (mCi/kg of hamster body weight)

Blood samples were taken by retroorbital bleeding 1 or 2 days before treatment and then every week thereafter for 6 weeks. DT, days after therapy; WBC, (10^3/mm^3); RBC, (10^6/mm^3); plat, platelets (10^3/mm^3); tp, total protein (g/dl); alp, alkaline phosphatase (units/liter); hgb, hemoglobin (g/dl); NA, not applicable. Values are mean ± S.D.

<table>
<thead>
<tr>
<th></th>
<th>≤150 (n = 5) (DT)</th>
<th>&gt;150 (n = 3) (DT)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>-2</td>
<td>7</td>
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<tr>
<td>WBCs</td>
<td>7.72 ± 1.4</td>
<td>5.03 ± 0.7</td>
</tr>
<tr>
<td>RBCs</td>
<td>9.59 ± 0.4</td>
<td>9.9 ± 0.03</td>
</tr>
<tr>
<td>plat</td>
<td>491 ± 86</td>
<td>372 ± 157</td>
</tr>
<tr>
<td>tp</td>
<td>6.22 ± 0.2</td>
<td>6.43 ± 0.2</td>
</tr>
<tr>
<td>alp</td>
<td>141 ± 8</td>
<td>170 ± 12</td>
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<tr>
<td>hgb</td>
<td>17.7 ± 0.5</td>
<td>17.9 ± 0.2</td>
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were then analyzed using log-rank, Wilcoxon, and −2log(LR) statistics, and the P values were reported.

Results

Chemistry. The specific activity of 64Cu-BAT-2IT-1A3 was 5.5 ± 2.2 mCi/mg with a radiochemical purity of 90.7 ± 8.8%. Fast protein liquid chromatography showed that ~9% of the labeled material was high molecular weight aggregates. No free copper-64 was detected. The immunoreactivity of 64Cu-BAT-2IT-1A3 was 79.3 ± 8.0%.

MTD Studies. The MTD was 150 mCi of 64Cu/kg of hamster body weight (Fig. 1). Hamsters receiving higher doses (170–190 mCi/kg) lost greater than 20% of their body weight, and all died between 8 and 13 days (n = 3; Fig. 2). Hamsters receiving doses ≤150 mCi/kg (120–150 mCi/kg; n = 13) survived to the experimental end point (6 weeks) with an overall gain in weight. As Table 1 shows, in the group of hamsters receiving 120–150 mCi/kg, WBC counts dropped to 30% of pretreatment values at 1 week posttreatment but were back up to normal by 2 weeks posttreatment. Platelets dropped to 20% of controls by 1 week posttreatment, but these also returned to normal by 2 weeks posttreatment. A slight drop in liver enzymes was seen at 1 week (to 80% of control values), but these were normal at 2 weeks posttreatment. No significant changes in total protein or RBCs were detected in survivors.

Animals receiving doses above the MTD (170–190 mCi/kg) demonstrated severe depression of white blood counts (6% of control values) and platelets (28% of control values) and died before these rebounded.

RIT Studies. Therapeutic amounts of 64Cu-BAT-2IT-1A3 were administered to hamsters carrying large (600 mg), 7-day-old GW39 human colorectal tumors. All treatment groups were significantly different from controls (P < 0.0001). Initial results treating 7-day-old tumors with 2 or 3 mCi of 64Cu-BAT-2IT-1A3 (Fig. 3) showed significant tumor growth inhibition (P < 0.0001) compared to the saline control group, but the tumors regrew, and all treated hamsters were sacrificed by 13 weeks (16). The results of the MTD studies suggested that we could significantly increase the doses in these large tumor studies without causing toxicity. Hamsters receiving a single dose of 7 or 15 mCi of 64Cu-BAT-2IT-1A3 showed significantly (P < 0.03) improved survival rates compared to the groups receiving 2 and 3 mCi and the saline-treated control hamsters. At the end point of the experiment (4 months following treatment), 40% of hamsters (8 of 20)
treated with 7 mCi of $^{64}$Cu-BAT-2IT-1A3 and 62.5% of hamsters (5 of 8) treated with 15 mCi of $^{64}$Cu-BAT-2IT-1A3 were alive and tumor free. In contrast, 100% of hamsters (44 of 44) treated with saline had to be sacrificed by 5 weeks because their tumors had grown to $\geq$10 g.

In another set of experiments, the 7-mCi dose was fractionated into two 3.5-mCi doses (Fig. 4). Forty-four % (4 of 9) of hamsters receiving two 3.5-mCi doses of $^{64}$Cu-BAT-2IT-1A3 given 24 h apart survived to the experimental end point (4 months), compared to 25% (2 of 8) survival when the interval between doses was increased to 48 h. These experiments demonstrated that at doses well below the MTD, fractionating the dose gave no advantage with respect to survival over giving a single 7-mCi dose. Furthermore, the differences in survival curves were not significant. However, advantages of dose fractionation may be better demonstrated at total doses near or greater than the MTD.

In all RIT treatment groups, hamsters appeared healthy. They gained weight at the same rate as untreated controls and had no gross visceral abnormalities or histological abnormalities of the liver or kidneys.

The effectiveness of each treatment protocol was also evaluated by determining the mean life span of each hamster group (Table 2). There clearly was a dose response with respect to survival giving a single 7-mCi dose. Furthermore, the differences in survival curves were not significant. However, advantages of dose fractionation may be better demonstrated at total doses near or greater than the MTD.

Table 2: Mean life span (weeks)

<table>
<thead>
<tr>
<th>Control (saline)</th>
<th>Single-dose therapies (mCi)</th>
<th>Split doses (3.5 + 3.5 mCi)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>3.8 $\pm$ 0.8</td>
<td>6.8 $\pm$ 2.2</td>
<td>7.1 $\pm$ 3.2</td>
</tr>
</tbody>
</table>

Dosimetry. The tumor doses in hamsters bearing large, 7-day-old tumors injected with 7 or 3 mCi of $^{64}$Cu-BAT-2IT-1A3 were calculated to be 220 and 330 rads (110 rads/mCi), respectively (16). Thus, hamsters treated with 7 or 15 mCi of $^{64}$Cu-BAT-2IT-1A3 received tumor doses of approximately 770 and 1650 rads, respectively. Normal organ absorbed doses for $^{64}$Cu-labeled 1A3 show the critical organ for $^{64}$Cu-BAT-2IT-1A3 was the upper large intestine (0.51 rads/mCi; Ref. 16). Although the critical organ is the gut, the organ most critically affected by the therapy is likely to be bone marrow. The large drop in WBC counts in our experiments suggest that animal deaths at doses exceeding the MTD are due to radiation-induced bone marrow suppression (Table 1).

Discussion

Successful RIT of solid tumors depends on a number of factors, including choice of radionuclide, choice of MAbs, the use of intact MAbs or fragments, and tumor size (7, 8). As tumors grow larger, the impediments to penetration by MAbs also increase (28, 29). Previously, we demonstrated that 2 mCi of $^{64}$Cu-BAT-2IT-1A3 caused tumor regression with no regrowth out to the 7 month experimental end point in greater than 80% of hamsters with small, 2-day-old (~300-400 mg) tumors (16). Our present results extend these studies to hamsters with large, 7-day-old tumors (~600 mg). To optimize RIT in this model system, the MTD of $^{64}$Cu-labeled MAb 1A3 was determined.

Our MTD studies demonstrate that we can safely administer 150 mCi of $^{64}$Cu/kg of body weight. Because the hamsters in our studies averaged 100 g, the 2-mCi dose, which caused tumor regression of small tumors (16), occurred at only 13% of the MTD, emphasizing the effectiveness of $^{64}$Cu-labeled therapeutic agents. Although other RIT studies have shown solid tumor regression in animal models, most of these successes occurred at or near the MTD (8, 30–38). In the context of these results, the therapeutic efficacy of $^{64}$Cu-BAT-2IT-1A3 in small GW39 tumor-bearing hamsters is remarkable. The ability of $^{64}$Cu-BAT-2IT-1A3 to cause tumor regression well below the MTD suggests that this is a good candidate for clinical RIT trials. Additionally, at the MTD of 150 mCi/kg, the hematopoietic toxicity appears mild: although there was a depression of WBCs and platelets 1 week after treatment, recovery was rapid and complete by 2–3 weeks in the absence of bone marrow transplants or cytokine enhancement. This is in contrast to most other radioisotopes (e.g., $^{131}$I, $^{90}$Y, and $^{188}$Re) used in RIT in which suppression of WBCs and platelets occurred for longer times (30, 32, 34, 35, 39) and with greater severity (34, 40). Our results support the suggestion that copper has no apparent affinity for bone or bone marrow (6, 41), thus limiting myelotoxicity, which is usually the dose-limiting toxicity for RIT, and explaining at least in part why $^{64}$Cu-BAT-2IT-1A3 is an effective therapeutic agent at doses well below the MTD.

One hypothesis for the therapeutic efficacy of $^{64}$Cu-BAT-2IT-1A3 is a possible synergy between nonradioactive and radioactive copper in the nucleus. It has been demonstrated that copper ions are important structural elements of the nuclear matrix of cells (42, 43). In other studies, isolated nuclei treated with copper (II) ions were more sensitive to radiation-induced DNA double strand breaks when compared with untreated nuclei (44, 45). In our studies, it is feasible that copper binding to chromatin opened up the DNA and made it more susceptible to damage by radionuclides. Alternatively, or in concert with the above hypothesis, it is possible that $^{64}$Cu can replace cold copper in the nuclear matrix and upon transmutation to its decay products (giving off recoil energy) cause destabilization in the anchor region and thus inhibit repair mechanisms. Studies have been carried out to determine the extent of delivery of $^{64}$Cu-labeled agents to the nuclei of tumor cells. It has been shown that when $^{64}$Cu-TETA-octreotide was delivered to somatostatin receptor positive AR42J cells grown in culture, the amount of $^{64}$Cu found to localize in the nucleus reached a maximum of...
nearly 40% at 4 h postdelivery (46). This is in sharp contrast to 111In-DTPA-octreotide, in which only 4.5% of the 111In was delivered to the nucleus at the same time point. These preliminary studies demonstrate that there may be mechanisms that preferentially traffic copper ions to the cell nucleus. It has not yet been determined whether 64Cu dissociates from the TETA chelate inside the tumor cells and then localizes in the nucleus, but this is a likely possibility.

Our RIT results in hamsters with large (600 mg) tumors at the time of treatment are encouraging. Even suboptimal doses (3 mCi) of 64Cu-BAT-2IT-1A3 were able to extend the mean life span of tumor-bearing hamsters when compared to control hamsters (16). Previous dosimetry estimates suggest that with a 3-mCi dose in this large tumor model, tumors received 330 rads compared to an absorbed dose of 586 rads for tumors that regressed in our previous small tumor study (16). Larger doses (7 and 15 mCi) were more effective at inhibiting growth of these larger tumors (40 and 62.5% survival, respectively) and delivered estimated tumor doses of 770 and 1650 rads. The fact that even at the MTD we did not effect 100% cure reflects some of the inherent difficulties in targeting large tumors, such as decreased uptake and tumor penetration of MAb into larger tumors (11, 47–49).

Our dose fractionation experiments demonstrated that the split dose (3.5 + 3.5 mCi) with a 24-h interval was at least as effective as the single 7-mCi dose with respect to both mean life expectancy and overall survival. The split dose with the 48-h interval was less effective, suggesting that because of the 12.8-h half-life of 64Cu, shorter intervals between treatments will be more effective. Also in our RIT doses, with each administration being at the MTD (39). However, the timing between doses in this study was critical to prevent lethality. Future multiple-dose studies might incorporate the use of cytokines to decrease myelosuppression and to safely decrease the interval between multiple doses at or near the MTD (51).

In summary, we have been successful at producing large amounts of 64Cu on a biomedical cyclotron at Washington University on a daily basis (22, 23) so that high dose therapy studies with or without dose fractionation are feasible. We have shown that 64Cu-BAT-2IT-1A3 is capable of causing tumor regression in small tumors (300–400 mg) at doses that are only 13% of the MTD. Our ability to cause complete tumor regression in over 60% of hamsters with large, 600-mg solid GW39 colorectal tumors and to effect tumor growth inhibition in 100% of these animals further documents the therapeutic potential of 64Cu-BAT-2IT-1A3. We are hopeful that more rigorous dose fractionation experiments with and without immunotherapeutic enhancers will lead to further RIT successes with 64Cu-labeled agents.

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References


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