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Research Paper

Pre-clinical evaluation and efficacy studies of a melanin-binding IgM antibody labeled with ^{188}Re against experimental human metastatic melanoma in nude mice

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Abbreviations: RIT, radioimmunotherapy; TCEP, tris(2-carboxyethyl) phosphine hydrochloride; DTT, dithiothreitol

Key words: clinical trial, melanoma, melanin-binding antibody, radioimmunotherapy, 188-Rhenium

Purpose: **Currently there is no satisfactory treatment for metastatic melanoma. Radioimmunotherapy (RIT) uses the antigen-antibody interaction to deliver lethal radiation to target cells. Recently we established the feasibility of targeting melanin in tumors with 188-Rhenium (^{188}Re)-labeled 6D2 mAb to melanin. Here we carried out pre-clinical development of ^{188}Re-6D2 to accrue information necessary for a Phase I trial in patients with metastatic melanoma.**

Results: **TCEP proved to be effective in generating a sufficient number of -SH groups on 6D2 to ensure high radiolabeling yields with ^{188}Re and preserved its structural integrity. ^{188}Re-6D2 was quickly cleared from the blood with the half-life of approximately 5 hrs and from the body—with the half-life of 10 hrs. The doses of 0.5, 1.0 and 1.5 mCi significantly ($p < 0.05$) slowed down A2058 tumor growth in nude mice, also causing release of melanin into the extracellular space which could provide additional target for repeated treatments. Transient effects of RIT on WBC and platelet counts resolved by day 14 post-treatment.**

Experimental design: **Tris(2-Carboxyethyl) Phosphine Hydrochloride (TCEP) was evaluated as potential agent for generation of -SH groups on 6D2 mAb. TCEP-treated 6D2 mAb was radiolabeled with ^{188}Re and its radiochemical purity and stability was measured by ITLC and HPLC and its immunoreactivity—by melanin-binding ELISA. The pharmacokinetics, therapeutic efficacy and acute hematologic toxicity studies were performed in nude mice bearing lightly pigmented A2058 human metastatic melanoma tumors.**

Conclusions: **We have developed radiolabeling and quality control procedures for melanin-binding ^{188}Re-6D2 mAb which made possible currently an on-going Phase I clinical trial in patients with metastatic melanoma.**

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Introduction

Melanoma poses an increasing health problem that affects about 40,000 patients each year in the United States and an estimated 100,000 world-wide. While primary melanomas that are localized to the skin can be successfully treated by surgical removal, there is no satisfactory treatment for metastatic melanoma, a condition that currently has an estimated five year survival of 6%. Targeted radionuclide therapy has evolved into an efficient modality for cancer patients in whom standard anti-neoplastic therapies have failed.[1] One type of targeted radionuclide therapy-radioimmunotherapy (RIT) takes advantage of the specificity of the antigen-antibody interaction to deliver localized lethal doses of radiation to target cells using radiolabeled antibodies.[2,3] The clinical success of FDA-approved drugs such as Zevalin® and Bexxar® (anti-CD20 monoclonal antibodies labeled with 90-Yttrium (^{90}Y) and 131-Iodine (^{131}I), respectively) for the treatment of relapsed or refractory B-cell non-Hodgkin lymphoma (NHL) demonstrates the potential of RIT as an anti-neoplastic strategy. Encouraging reports on the use of RIT as an initial treatment for follicular lymphoma[4] support the use of RIT as first-line therapy for this malignancy. Hence, the increasing acceptance of RIT for certain lymphomas combined with the development of a technical infrastructure to support this type of therapy have created a favorable environment for the development of radionuclide therapy for metastatic melanoma provided that suitable targets can be identified.

Melanoma owes its name to the presence of the pigment melanin. Given that even amelanotic melanomas contain some melanin, this pigment presents a potential target for development of radionuclide therapy of metastatic melanoma. Historically, melanin was not considered a target for RIT because it is an intracellular pigment outside the reach of a specific antibody. Because melanomas are rapidly growing, cell turnover releases melanin pigment into the extracellular space that can be targeted for delivery of cytotoxic

radiation by radiolabeled melanin-binding antibodies. Experimental results have established the feasibility of targeting melanin released from dead melanoma cells in tumors with radiolabeled antibodies[5] and peptides.[6] Furthermore, this strategy is attractive because melanin in normal tissues is not accessible to the antibody by virtue of its intracellular location. To test this hypothesis we employed a murine IgM mAb known as 6D2 generated from mice immunized with melanin produced by the fungus, *Cryptococcus neoformans*.[7] This antibody also binds human melanin since both fungal and human melanins have structural similarities[8] and are negatively charged. Nude mice bearing MNT1 pigmented human melanoma tumors were treated with mAb 6D2 labeled with 1.5 mCi of the β-emitter 188-Rhenium (^{188}Re). Mice treated with radiolabeled mAb 6D2 manifested inhibition of tumor growth and prolonged survival. MAb 6D2 bound tumor melanin but did not bind to normal melanized tissues in C57BL6 black mice. The mechanism of melanoma targeting with mAb 6D2 involved antibody binding to extracellular melanin released during tumor cell turnover or to dying tumor cells with damaged or permeable membranes. These results provided the basis for the pre-clinical development of radiolabeling of ^{188}Re-6D2 mAb. Consequently we carried out additional pre-clinical development of ^{188}Re-6D2, including pharmacokinetics, efficacy and acute hematologic toxicity studies in a metastatic human melanoma model in mice to accrue additional information necessary to support a Phase I trial in patients with metastatic melanoma.

Results

Influence of TCEP concentration and incubation time on mAb 6D2 SH group generation. The efficiency of TCEP in generating -SH groups was estimated from the radiolabeling yields with ^{188}Re, while the structural integrity of TCEP-treated mAb 6D2 was assessed with non-reducing SDS-PAGE and size exclusion HPLC. Lower concentrations of TCEP (2:1 and 10:1 TCEP to 6D2 molar ratios) were inefficient at generating -SH groups resulting in low radiolabeling yields with ^{188}Re of 25% (Table 1). In contrast, TCEP to 6D2 molar ratios of 50:1 and 100:1 led to 70–72% radiolabeling yields (Table 1). However, at the 100:1 molar ratio there was some fragmentation of 6D2 mAb relative to that observed for lower concentrations (Fig. 1). Consequently, a 50 molar excess of TCEP over 6D2 mAb was selected for subsequent experiments.

In experiments assessing incubation time, we noted that 5- and 15-min incubations resulted in 41–42% radiolabeling yields with ^{188}Re, 30- and 60-min incubations increased the yield to 71–72%, while longer incubation times up to 240 min produced no further yield (Table 2). SDS-PAGE analysis of mAb 6D2 samples treated with TCEP for different times and then labeled with "cold" Re demonstrated that after short incubation times with TCEP (up to 30 min) most of 6D2 remained as an intact IgM molecule while 120 min incubation caused significantly more fragmentation of the IgM (Fig. 2A). We therefore selected an incubation time of 30 min and a 50:1 molar ratio of TCEP to mAb 6D2 as the optimal conditions for generating -SH groups on 6D2 mAb. Interestingly, however, although non-reducing SDS-PAGE showed fragments in antibody preparations labeled with "cold" Re (Fig. 2A), the antibody eluted from the size exclusion HPLC column as a single peak at 6.7 min similar to that of the intact native 6D2 (Fig. 2B) for all preparations (Fig. 2C–F). Blaunstein et al.[10] observed the same inconsistency between SDS-PAGE and size exclusion HPLC of TCEP-treated murine IgG

Table 1 Influence of TCEP molar excess over 6D2 mAb on radiolabeling yields with ^{188}Re

TCEP molar excess over 6D2	Radiolabeling yield, %
0	10
2	25
10	25
50	72
100	70

Incubation was carried out at room temperature for one hour.

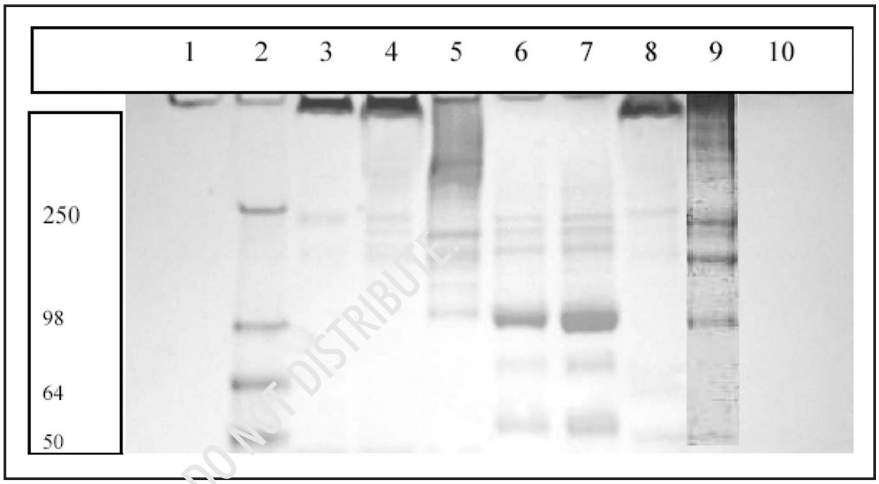

Figure 1. Structural integrity of 6D2 mAb after treatment with TCEP. For comparative purposes samples of 6D2 treated with DTT as in[5] are also shown. Non-reducing SDS-PAGE (4–20% Tris-Glycine gel) was used. Lane # 1: empty; Lane # 2: Pre-stained MW markers: 250 kDa myosin, 98 kDa BSA, 64 kDa glutamic dehydrogenase, 50 kDa alcohol dehydrogenase; Lane # 3: affinity purified 6D2 standard; Lane # 4: 6D2:TCEP, 1:10 molar ratio; Lane # 5: 6D2:TCEP, 1:100 molar ratio; Lane # 6: 6D2 treated with DTT; Lane # 7: the same; Lane # 8: mouse myeloma IgM standard; Lane # 9: 6D2:TCEP, 1:50 molar ratio; Lane # 10: empty.

Table 2 Influence of incubation time of 6D2 mAb with 50 molar excess of TCEP on radiolabeling yields with ^{188}Re

Time of reduction with TCEP, min	Radiolabeling yield, %
5	42
15	41
30	72
60	71
120	66
240	33

Incubation at room temperature.

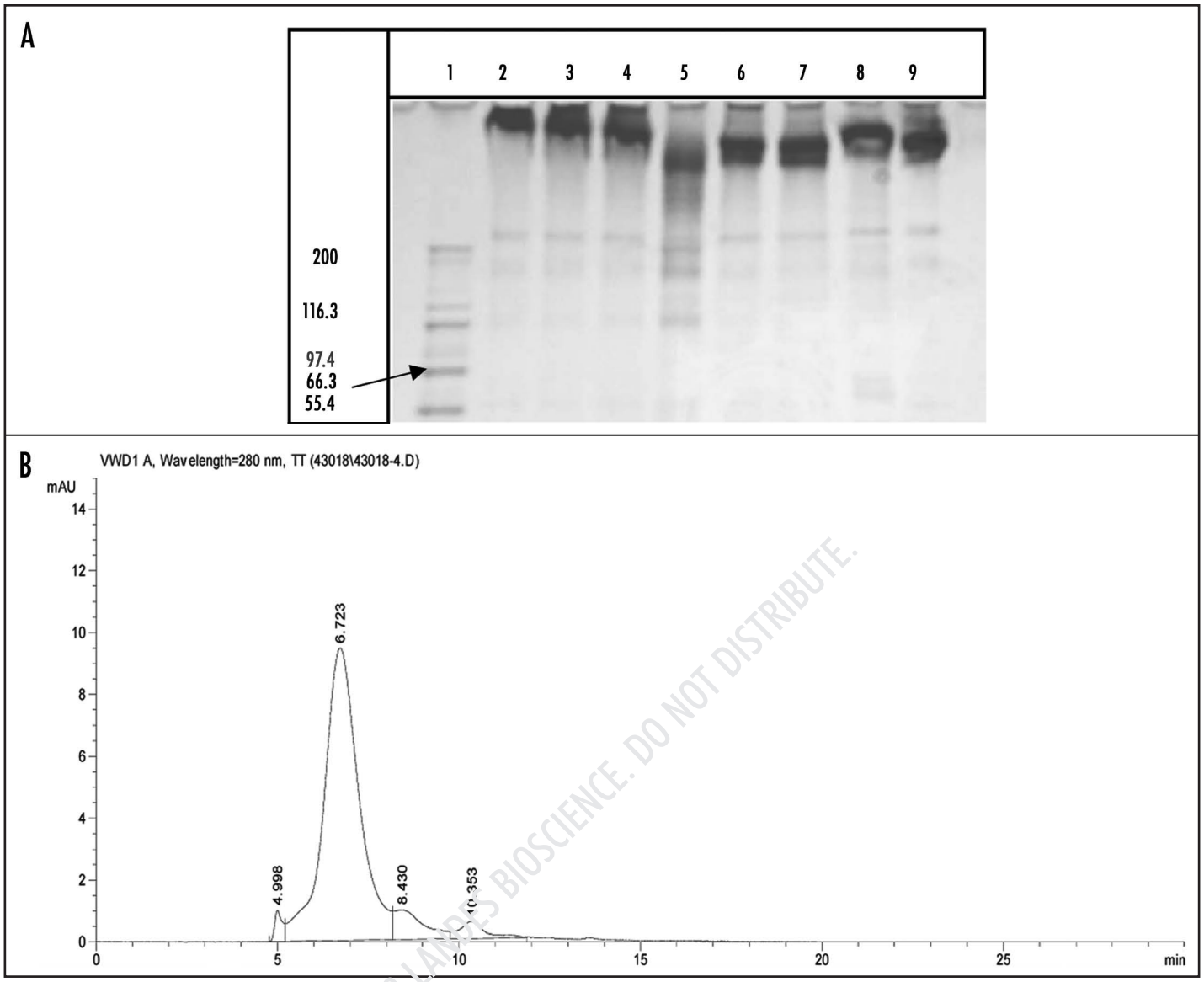

Figure 2. Structural integrity of 6D2 mAb after treatment with TCEP at a TCEP:mAb ratio of 50:1 for various periods of time. The samples were subsequently labeled with "cold" rhenium. Non-reducing SDS-PAGE (4–20% Tris-Glycine gel) was used. (A) SDS-PAGE: Lane # 1: Pre-stained MW markers Myosin 200 kDa, B glactosides 116.3 kDa, Phosphorylase b 97.4, Bovine Albumin 66.3 kDa, Glutamic Dehydrogenase, 55.4 kDa; Lane # 2: 6D2, 5 min treatment; Lane # 3: 6D2, 15 min treatment; Lane # 4: 6D2, 30 min treatment; Lane # 5: 6D2, 120 min treatment; Lane # 6: 6D2, 30 min treatment; Lane # 7: 6D2, 60 min treatment; Lane # 8: Sigma Std. IgM; Lane # 9: 6D2 reference standard. (B–E) size exclusion HPLC of 6D2: (B) 6D2 reference standard; (C) 6D2, 5 min treatment; (D) 6D2, 30 min treatment; (E) 6D2, 120 min treatment.

radiolabeled with ^{99m}Tc, and Michaelsen et al.[11] reported similar findings for a human IgG1. In both reports, the discordance between SDS-PAGE and HPLC did not affect the immunoreactivity of mAbs towards their respective antigens. The fragmentation apparent by SDS-PAGE might reflect electrophoretic forces pulling apart an antibody held together by fewer disulfide bridges as a result of TCEP treatment. The size exclusion HPLC, on the other hand, allows detection of the antibody in its form in solution.

Radiochemical purity, stabilization of ^{188}Re-6D2 with L-ascorbic acid and "cold" 6D2 and melanin-binding ELISA. Because radiolabeling yields with ^{188}Re of TCEP-treated 6D2 were 71–72%, post-radiolabeling purification was necessary. After passing ^{188}Re-6D2 through a size exclusion HiPrep column, the radiochemical purity of ^{188}Re-6D2 was 92–93% with <1% radiocolloids, as determined by SG-ITLC. Approximately 70% of the total amount of the antibody was recovered from the HiPrep column. The purified antibody was immediately stabilized with 0.2 mg/mL L-ascorbic acid, which is widely used as a radioprotector for radiopharmaceuticals. In addition to its radioprotective properties, L-ascorbic acid also decreased the pH of ^{188}Re-6D2 in saline from 5.3 to 4.8; the lower pH may have further stabilized the ^{188}Re radiolabel on the antibody.[12] The radiochromatographic profile of the purified ^{188}Re-6D2 stabilized with L-ascorbic acid is shown in Figure 3A. We also evaluated the stability of ^{188}Re radiolabel on the antibody after freezing the radiolabeled mAb at -80°C immediately after preparation and thawing 24 hr later. Both SG-ITLC (91% radiochemical purity) and radiochromatography (Fig. 3B) showed the ^{188}Re radiolabel remained attached to the antibody. The addition of "cold"

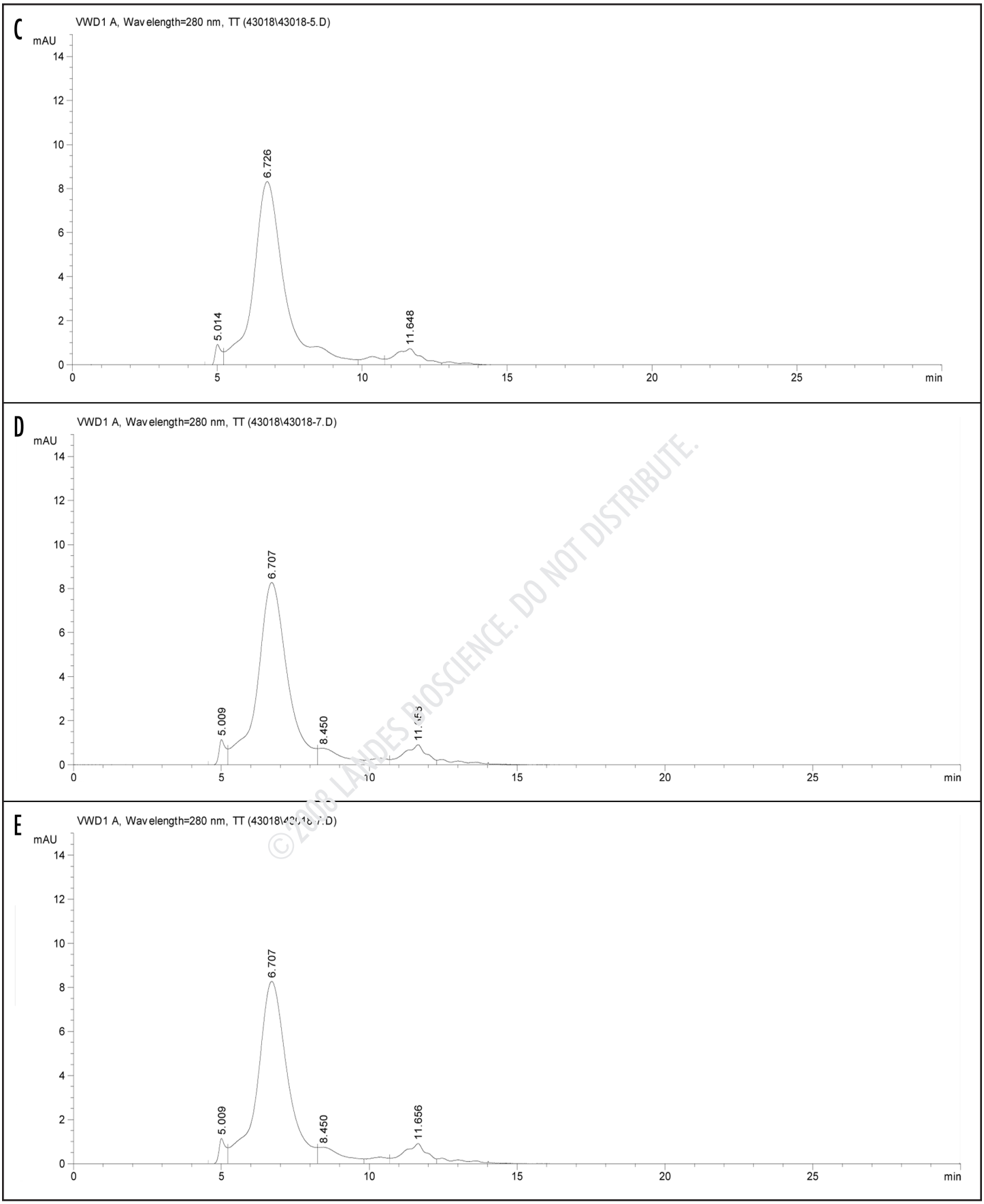

Figure 2 C–E. See legend, page 1118.

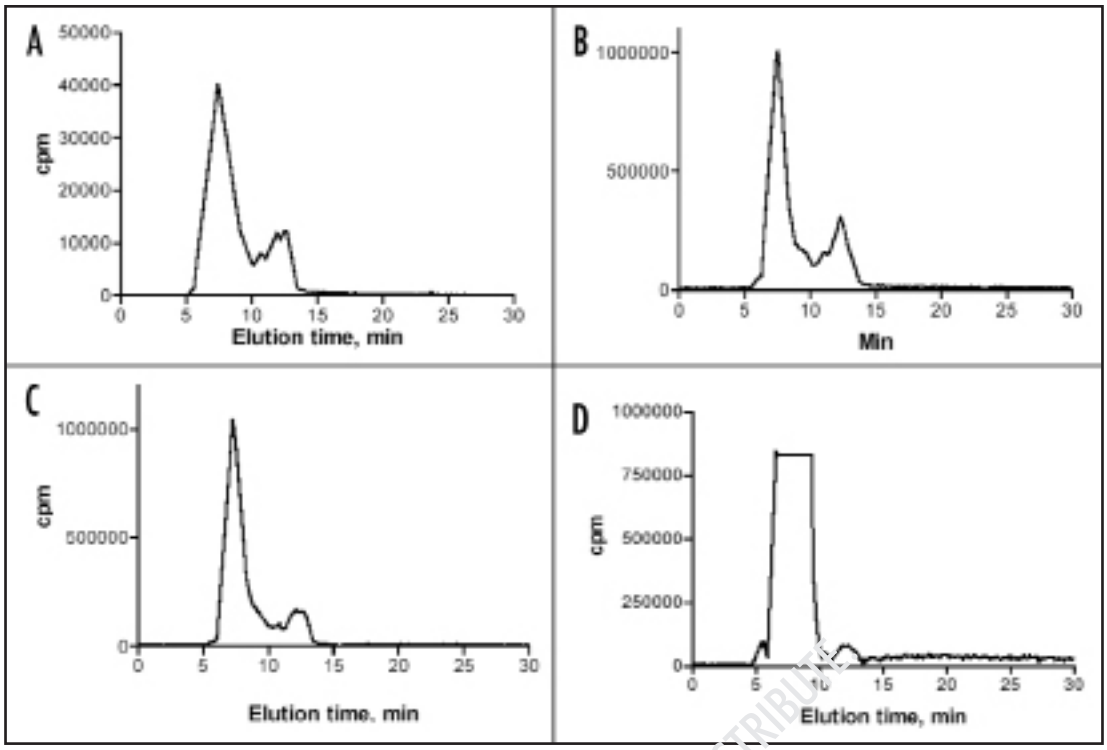

Figure 3. Radiochromatographic profiles of ^{188}Re-6D2 preparations: (A) eluted from HiPrep column and stabilized with 0.2 mg/mL L-ascorbic acid; (B) the same preparation as in (A) but frozen for 24 hr in a resin vial; (C) stabilized with 0.2 mg/mL L-ascorbic acid and "cold" 6D2, stored at 4°C for six hours and passed through infusion set; (D) eluted from HiPrep column with 0.2 mg/mL L-ascorbic acid in saline.

6D2 to ^{188}Re-6D2 immediately after purification on the HiPrep column increased the overall amount of mAb in the preparation to 10 mg and improved the radiochromatographic profile (Fig. 3C), possibly due to more complete recovery of the antibody fraction from the HPLC column because of the higher concentration (0.2 mg/mL versus 0.25 mg/mL in preparations without addition of "cold" 6D2). In an attempt to further increase the radiochemical purity of the final product, 0.2 mg/mL L-ascorbic acid was added to the saline used for elution of HiPrep column. This modification of the purification procedure further increased the radiochemical purity of ^{188}Re-6D2 to 97% by ITLC and radiochromatography (Fig. 3D).

Immunoreactivity of radiolabeled mAb is an important quality control parameter to ensure the fidelity of radiolabeled mAb in binding its antigen. The immunoreactivity of ^{188}Re-6D2 was evaluated by melanin-binding ELISA using 96-well plates coated with synthetic melanin. ^{188}Re-6D2 bound to melanin to the same degree as native 6D2 (Fig. 4A). Storage of ^{188}Re-6D2 for six hours at 4°C (proposed shelf-life of ^{188}Re-6D2 in the clinical trial) followed by passing it through the infusion set did not cause any significant decrease in its immunoreactivity ($p = 0.06$) (Fig. 4B). Likewise, overnight freezing of ^{188}Re-6D2 at -80°C in a resin vial did not affect its ability to bind to melanin ($p = 0.07$) (Fig. 4C).

Biodistribution and WBAR of ^{188}Re-6D2. The biodistribution of ^{188}Re-6D2 was evaluated in nude mice bearing tumors derived from the A2058 human metastatic melanoma cell line. ^{188}Re-6D2 was quickly cleared with a half-life of approximately five hours from the blood and 10 hours from the body (Fig. 5A). The kidney uptake was significant at two hours post-IV injection (18% ID/g); however, it rapidly decreased to 5.6% at 24 hrs and to 3% ID/g at 48 hrs (Fig. 5B). Overall, the clearance of ^{188}Re-6D2 from all major organs was rapid and mirrored the clearance from the blood (Figs. 5B and 6). Due to 8% of free ^{188}Re-perrhenate in the preparation, the WBAR at four hours showed some uptake in the thyroid (Fig. 6A) that disappeared at 24 hours (Fig. 6B) due to the inability of ^{188}Re-perrhenate to accumulate in thyroid tissue.[13] Tumor uptake was modest with a maximum uptake of 1.94% ID/g reached at four hours and decreasing to 0.51 and 0.21%, at 24 and 48 hrs, respectively (Figs. 5B and 6). However, this was clearly melanin-specific uptake since the tumor to muscle ratio for these tumors located in a muscle bed grew progressively from 2 at 5 min post-injection to 5 at 4 hr, and stayed around 5 at 24 and 48 hrs.

Therapy for A2058 human metastatic melanoma tumors in nude mice, tumor histology and evaluation of acute hematologic toxicity of ^{188}Re-6D2. We evaluated the ability of ^{188}Re-6D2 to affect the growth of human metastatic melanoma tumors in nude mice by administering increasing amounts of ^{188}Re-6D2. While the lowest dose of 0.15 mCi had no effect on tumor progression relative to untreated mice, the doses of 0.5, 1.0 and 1.5 mCi significantly ($p < 0.05$) slowed tumor growth (Fig. 7A). The radiation treatment caused widespread necrosis of the tumors in the groups treated with higher doses. On day 35 post-treatment, the tumors of the untreated mice and mice treated with 1.0 and 1.5 mCi were removed and analyzed histologically. In untreated mice the tumors were large with central necrosis. The tumor cells had a high mitotic index (5 per 400X field) and there were low numbers of infiltrating lymphocytes and macrophages around the tumor (Fig. 7B). In contrast, the tumors from mice treated with ^{188}Re-6D2 had more extensive central necrosis than the tumors from control mice and very few viable tumor cells (Fig. 7C). The neoplastic cells present within the tumors from treated animals had far fewer mitotic figures

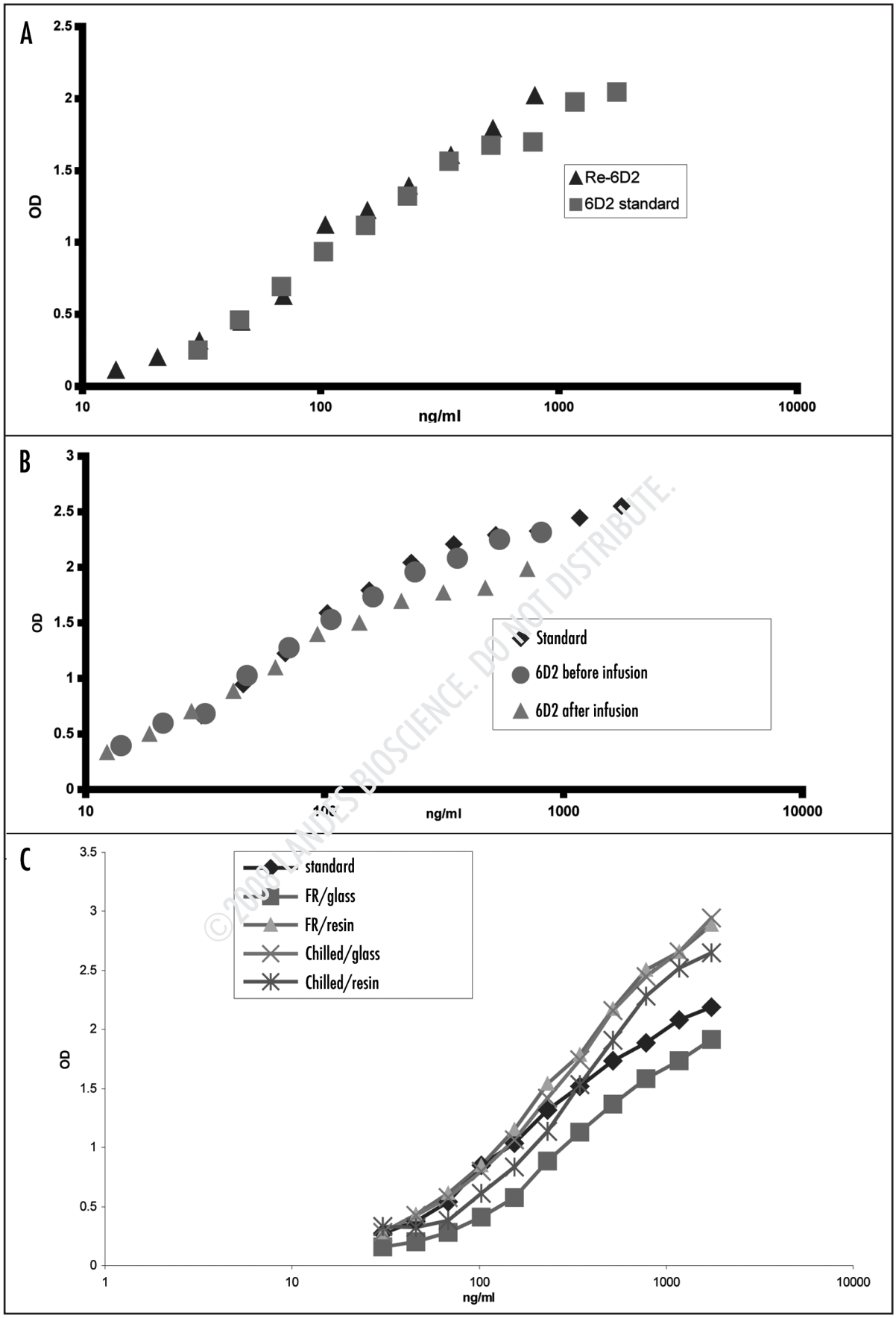

Figure 4. Binding of ^{188}Re-6D2 to melanin by ELISA: (A) immediately after preparation; (B) after passing through an infusion set 6 hrs after preparation; (C) after overnight storage at 4°C and -80°C in glass and resin vials.

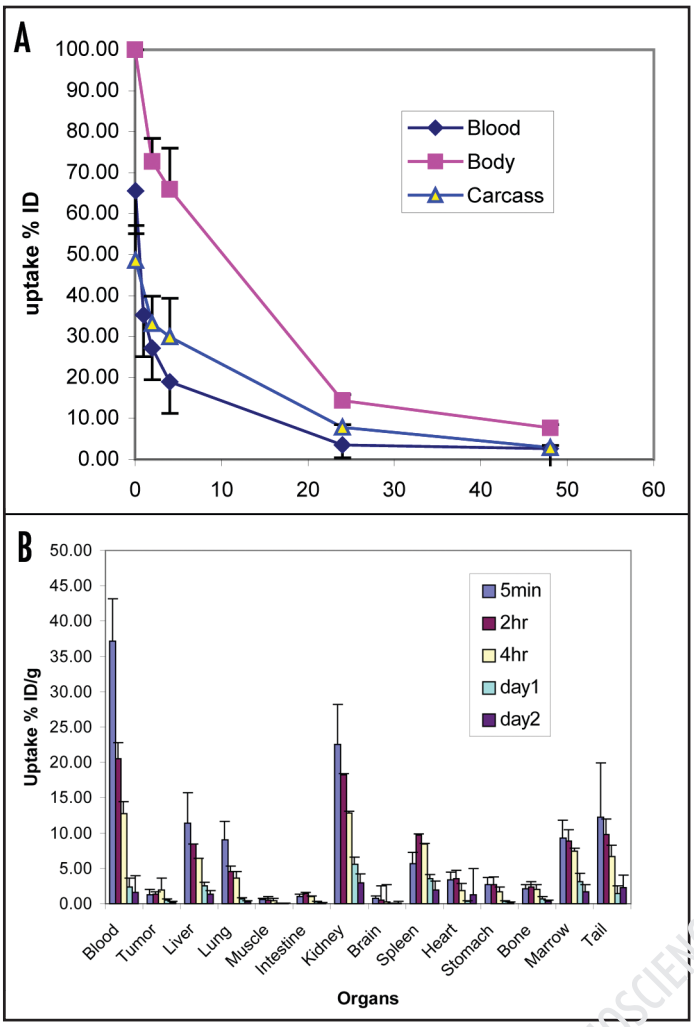

Figure 5. Biodistribution of ^{188}Re-6D2 in nude mice bearing A2058-derived melanoma tumors after IV administration: (A) blood, whole body and carcass clearance; (B) distribution in major organs and tumors.

(0.4/400X field) and more individual cell apoptosis and necrosis than did the tumors from control animals. In addition, one tumor from a treated mouse had extensive fibrovascular granulation tissue with infiltrates of lymphocytes, macrophage, plasma cells, and a few neutrophils inside the tumor itself (not seen in control tumors). Interestingly, while only a few granules of melanin were visible in control tumors (Fig. 7B) abundant melanin deposit were found in treated tumors (Fig. 7C). The better visibility of melanin in treated tumors was due to increased extracellular melanin, presumably, as a result of the treatment with radiolabeled mAb.

The hematologic toxicity of the radiolabeled mAb treatment was evaluated by measuring platelet and WBC counts in blood. A drop in platelet and WBC count in groups treated with the high doses was detected on days 3 and 7 post-treatment (Fig. 8); however, these counts normalized by Day 14. The body weight of mice in all groups was stable (results not shown).

Discussion

The lack of effective clinical treatment for metastatic melanoma and positive results from treatment of human melanoma in a mouse model with ^{188}Re-labeled melanin-binding mAb 6D2,[5] encouraged the development of this mAb for clinical evaluation in patients with metastatic melanoma. RIT has experienced a renaissance and in the last 5 years there have been reports of renewed effort in developing RIT for the treatment of melanoma (reviewed in ref. 14). We have chosen ^{188}Re radionuclide to deliver cytotoxic radiation to the melanoma cells, as it has long range in tissue needed for "cross-fire" approach utilized in targeting melanin. It is a safer radionuclide than ^{131}I or ^{90}Y because of its much shorter physical half-life (16.9 hr) and by virtue of not being a thyroid-accumulating (as opposed to ^{131}I) or bone marrow accumulating (as opposed to ^{90}Y) radionuclide. In addition, there is clinical experience with ^{188}Re-labeled anti-NCA and anti-CD66 mAbs, which proved to be safe and effective in leukemia patients.[12,15]

The development of ^{188}Re-6D2 for clinical use required the development of a robust and reproducible radiolabeling procedure and quality control techniques. Since the mAb is radiolabeled with ^{188}Re by attachment to -SH groups—it was important to identify a suitable reducing agent and to develop conditions that maximized labeling and minimized damage to the immunoglobulin molecule. In earlier studies, dithiothreitol (DTT) was utilized for generation of -SH groups on 6D2 mAb.[5] However, DTT competes with mAb for binding ^{188}Re, and separation of reduced mAb from excess of DTT is required before the radiolabeling step is initiated. Since radiolabeled mAbs usually require a final purification step to reach >90% radiochemical purity, the incorporation of an additional purification step into the manufacturing protocol was potentially cumbersome. To avoid this problem, we sought a reducing agent that would not compete for ^{188}Re with the antibody. Consequently, we evaluated a phosphine-based agent Tris(2-Carboxyethyl) Phosphine Hydrochloride (TCEPHCl), which is a soluble salt that is stable during prolonged storage in solution and does not react with ^{188}Re. These studies demonstrated TCEP ability to generate a sufficient number of -SH groups on 6D2 mAb while preserving its structural integrity.

The biodistribution and WBAR of ^{188}Re-6D2 radiolabeled by this new procedure showed that it behaved essentially the same as ^{188}Re-6D2 mAb radiolabeled after reduction with DTT.[5] For example, the 5-hr half-life of blood clearance of ^{188}Re-6D2 in the present study is comparable to the 6.5-hr half-life of the DTT -reduced mAb.[5] The patterns of clearance from the kidneys and all other major organs were also very similar for TCEP- and DTT-reduced mAb 6D2 as well as to those of control ^{188}Re-IgM performed in our previous study.[5] The kidney clearance of ^{188}Re-6D2 mAb is unusual for a molecule of this size (~900,000 Da) which is not a subject to glomerular filtration. The melanin-binding domain of 6D2 includes avery high number of positively charged aminoacids (Dadachova E, et al. unpublished observations) which makes it possible for some of the ^{188}Re-6D2 mAb in circulation to bind to the negatively charged membranes of the cells in glomeruli without being filtered.

The major difference between the two studies was in the tumor type induced in nude mice. In the prior study,[5] we used dark eumelanotic primary human melanoma cell line MNT1, whereas in the current study we used the lightly colored pheomelanotic metastatic human melanoma cell line A2058. In our previous study we proved the specific binding of 6D2 mAb to both eumelanotic

and pheomelanotic cell lines by immunofluorescence, immunohistochemistry, and by in vitro binding of ^{188}Re-6D2 and control ^{188}Re-IgM mAbs to the cells with no observable binding for control ^{188}Re-IgM. The tumor uptake in the earlier study,[5] estimated by scintigraphic imaging, was higher (approximately 15 and 5% ID/g at 3 and 24 hr, respectively) than in the current study (approximately 2 and 0.5 % ID/g at 4 and 24 hr, respectively), presumably due to the type of melanin contained in the tumor. MNT1 tumors contain black eumelanin,[8] which is very close in structure to the fungal melanin used as an antigen to develop 6D2 mAb.[7] In contrast, A2058 tumor cells are pigmented with pheomelanin, a yellow or reddish-brown melanin-type pigment.[8] Both pheomelanin and eumelanin are negatively charged and share some structural similarity,[8] with both types found in melanomas where eumelanin is the predominant pigment in primary tumors, while pheomelanin is associated with metastatic melanomas and disease progression.[16] Although the predominance of pheomelanin in A2058 cells resulted in less binding of ^{188}Re-6D2 in the current study, patient tumors will most likely be represented by both eumelanin and pheomelanin since melanoma is a very heterogeneous disease.[17,18] Also, it is important to note that tumor uptake of mAbs to intracellular targets is a more complex issue than the tumor uptake of mAbs to conventional surface antigens. In this regard, Epstein and colleagues, one of the first groups to consider extravasated intracellular antigens as targets for RIT,[19] did not observe specific uptake of radiolabeled TNT-1 mAb to intranuclear histones in human cervical carcinomas in mice compared to a control mAb, yet they observed therapeutic results and preferential targeting within the tumor.[19]

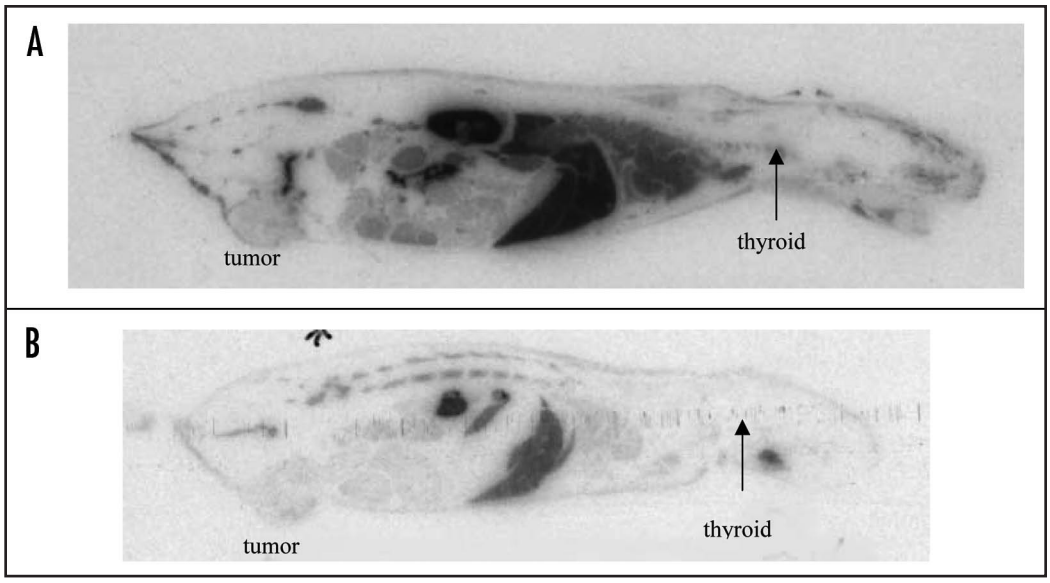

Figure 6. Whole body autoradiography of ^{188}Re-6D2 in A2058 human melanoma-bearing nude mice after IV administration: (A) 4 hr; (B) 24 hr.

The aim of the therapy studies was to prove that ^{188}Re-6D2 mAb radiolabeled via TCEP reduction is capable of delivering therapeutic radiation to melanoma tumors in the same manner as DTT-treated mAb.[5] In our previous study we have already proven that the therapeutic effect of ^{188}Re-6D2 mAb was due to its melanin-specific binding to the tumor as control ^{188}Re-6D2 mAb did not have any therapeutic effect in tumor-bearing nude mice. In the current study, despite a modest uptake of ^{188}Re-6D2 in tumor tissue, there was significant retardation in A2058 tumor growth not only for the highest dose of 1.5 mCi ^{188}Re-6D2 (the dose used in previous study),[5] but also for the lower two doses. The ability of low amounts of melanin to provide a sufficient target for melanin RIT was recently predicted by computer modeling which showed that the doses of radiation delivered to the melanoma tumor in a patient will be remarkably similar within a 1,000-fold range of tumor melanin concentration of 76–0.076 μM, with 76 μM melanin concentration being that of the highly melanized MNT1 tumors.[20] Melanin is a unique intracellular antigen because it is a chemically resistant pigment that can accumulate in tumor tissues to provide more target material for repeated treatments. In fact, histological evaluation of A2058 tumors in control and RIT-treated mice showed that most of the melanin in the treated tumors became extracellular (Fig. 7C), providing potential abundant target for the second round of RIT. Treatment modalities that increase the amount of accessible melanin in the tumors by killing cells and releasing their melanin contents might be fractionated RIT,[21] chemotherapy with dacarbazine or similar agents,[22] or alternating magnetic field cancer therapy.[23]

In summary, we have developed radiolabeling and quality control procedures for ^{188}Re-labeled melanin-binding 6D2 mAb for use in a clinical trial in patients with metastatic melanoma. The radiolabeled antibody had the immunoreactivity of the native mAb, was stable over time, had fast clearance from the blood and major organs and manifested preferential tumor uptake. Furthermore, we have established the efficacy of RIT with mAb 6D2 against an aggressive, lightly pigmented melanoma model derived from a metastatic tumor and confirmed that the doses employed were associated with limited and transient hematological toxicity. The efficacy in pheomelanin-pigmented cells derived from an aggressive tumor is important given the variability of melanoma tumors and the likelihood that metastatic lesions will be less pigmented than primary lesions. These results provide critically useful information for the manufacture of a clinical lot of the mAb and further support for this approach to treating human melanoma.

Materials and Methods

Antibody, melanoma cell line and radioisotope. Fungal melanin binding MAb 6D2 previously described in[7] was produced by Goodwin Biotech-nology Inc., (Plantation, FL). The mAb 6D2 was purified using an anti-murine IgM antibody (for studies involving

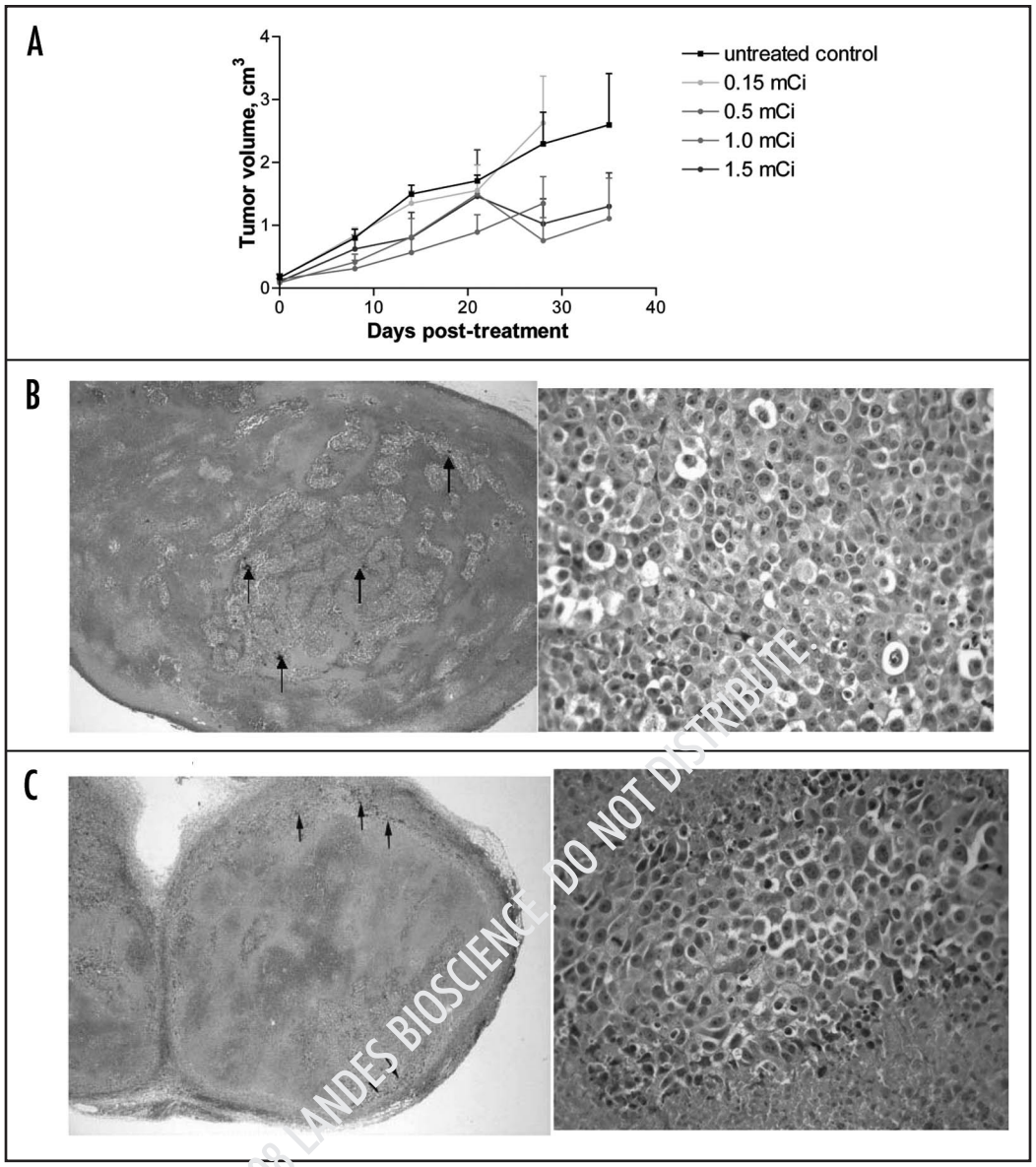

Figure 7. Therapy of A2058 human melanoma-bearing nude mice with various doses of ^{188}Re-6D2: (A) tumor volumes; (B and C) histology of the tumors: (B) tumor from untreated mouse; (C) tumor from a mouse treated with 1.5 mCi. Tissues were stained with hematoxylin and eosin. Melanin granules are marked with black arrows. Left in (B and C) 25X magnification; right: 400X magnification.

the generation of sulfhydryl groups with TCEP) or a multicolumn purification system (for all other studies). Purity of the 6D2 from these two processes was >95% via HPLC-SEC. The melanoma cell line was the A2058 certified cell line derived from a lymph node metastasis from a patient with malignant melanoma (American Type Culture Collection, Manassas, VA). The cells were maintained as monolayers in Dulbecco's Modified Eagle's Medium with four mM L-Glutamine, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, supplemented with 10% fetal bovine serum and 5% penicillin-streptomycin solution at 37°C and 5% carbon dioxide, and harvested by using 0.25% (w/v) Trypsin-EDTA solution. The cells were washed in serum-free Dulbecco's Modified Eagle's Medium before inoculation into nude mice.

^{188}Re as sodium perrhenate $Na^{188}ReO_4$ was eluted from ^{188}W/^{188}Re generator (Oak Ridge National Laboratory, Oak Ridge, TN) by passing 15 mL 150 mM NaCl solution through a generator at a rate of 2–5 mL/min. The eluate was collected into the sterile vial and ^{188}Re activity was measured in the dose calibrator.

Generation of sulfhydryl (-SH) groups on 6D2 via reduction of disulfide bonds with TCEP. Tris(2-Carboxyethyl) Phosphine Hydrochloride (TCEPHCl, Pierce) was evaluated as a reducing agent for generating -SH groups on the proteins via reduction of disulfide bonds. We first evaluated the influence of TCEP molar excess over 6D2 mAb on the mAb structural integrity and on radiolabeling yields. Four hundred μg (140 μL) samples of 6D2 mAb (2.857 mg/mL initial concentration, in 20 mM sodium phosphate buffer + 150 mM NaCl, pH = 7.5) were incubated for 1 hr at room temperature with 0, 2, 10, 50 and 100 molar excess of TCEP (1.5 mM solution in PBS) over 6D2 mAb. After incubation, each sample was split into two 200 μg aliquots. One aliquot was treated with 15 mM N-Ethylmaleimide (Pierce) in PBS to protect the generated -SH groups from recombining and analyzed by non-reducing SDS-PAGE

(4 and 4–20% tris-glycine non-reducing gels). The other aliquot was radiolabeled with ^{188}Re to evaluate the influence of TCEP molar excess over 6D2 on radiolabeling yields.

The kinetics of generating -SH groups on 6D2 via TCEP reduction at constant TCEP to 6D2 molar ratio was studied by incubating 6D2 at the above concentration with 50 molar excess of TCEP (1.5 mM solution in PBS) over 6D2 for 5 min–4 hr at room temperature. Each sample was then split into two aliquots. The first aliquot was labeled with "cold" Sodium Perrhenate following the radiolabeling procedure described below and analyzed by non-reducing SDS-PAGE and size exclusion HPLC on a TSK4000 column (TosoHaas, Japan) eluted with PBS at 1 mL/min. The eluted protein was detected by UV absorption at 280 nm wavelength. The second aliquot was radiolabeled with ^{188}Re, and the influence of reduction time with TCEP on radiolabeling yields was determined.

Radiolabeling with ^{188}Re, quality control and purification and stabilization of the final product. Reduction of perrhenate Na^{188}ReO$_4$. Sodium gluconate (1.5 mL of a 0.5 g/mL solution) and stannous chloride (0.1 mL of a 150 mg/mL solution in 1 M HCl) were added sequentially to 15 mL saline containing Na^{188}ReO$_4$ eluted from the ^{188}W/^{188}Re generator. The reaction mixture was incubated at 37°C for 1 hr and the percentage of ^{188}Re reduction was determined with 10 cm SG-ITLC strips (silica gel instant thin layer chromatography, Gelman Sciences) developed with acetone. In this system, reduced ^{188}Re stays at the point of application while unreduced perrhenate moves with the solvent front. The strips were cut in half and counted in a gamma counter.

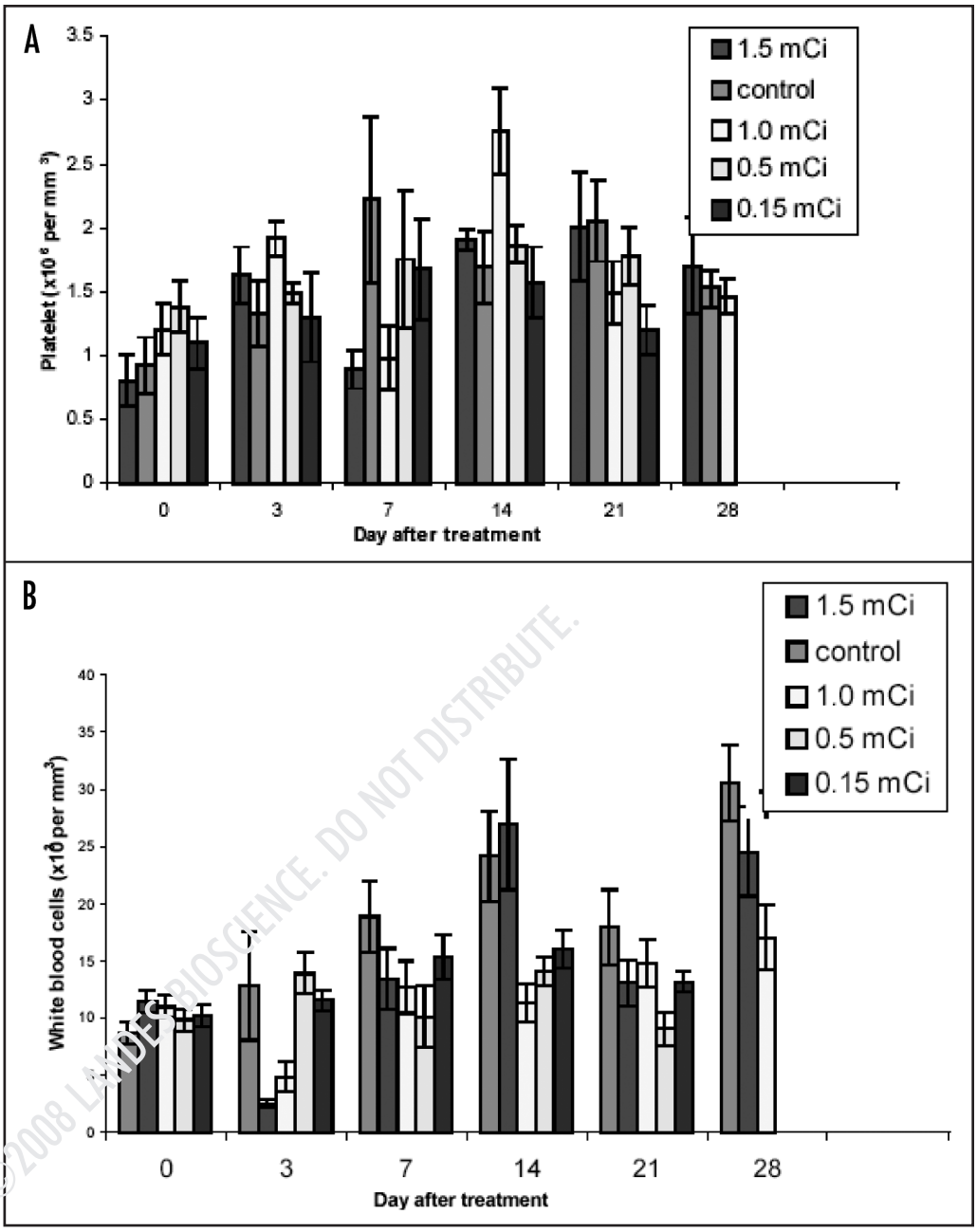

Figure 8. Platelet and white blood counts in A2058 human melanoma-bearing nude mice treated with various doses of ^{188}Re-6D2: (A) platelet; (B) white blood count.

Radiolabeling of TCEP-treated 6D2 mAb with ^{188}Re. Thirty min after the start of ^{188}Re reduction, 0.1 mL of 5 mM TCEP (50:1 TCEP to 6D2 molar ratio) was added to the vial containing 10 mg (5 mg/mL) 6D2 and incubated at room temperature for 30 min. After this hr of reduction, the ^{188}Re was added to the 6D2/TCEP mixture and incubated at 37°C for 1 hr. The percentage of incorporation of ^{188}Re into 6D2 was determined using SG-ITLC developed with saline (where radiolabeled 6D2 stays at the point of application while small molecular ^{188}Re-containing species travel with the solvent front). The amount of radiocolloids in the preparation was quantified with SG-ITLC strips pre-saturated with BSA. When these strips are developed in ethanol:NH_4OH:H_2O (2:1:5), the radiolabeled proteins move with the solvent front while radiocolloids stay at the point of application. The strips were cut in half and counted in a gamma counter.

Purification of ^{188}Re-6D2 on a size exclusion column, melanin-binding ELISA and stabilization with L-ascorbic and "cold" 6D2. Radiolabeled ^{188}Re-6D2 was loaded onto a pre-equilibrated HiPrep size exclusion column (GE Healthcare, Sweden) with a peristaltic pump and eluted with approximately 25 mL saline. The purified ^{188}Re-6D2 was immediately analyzed for: (1) percentage of ^{188}Re incorporation into 6D2 by SG-ITLC developed with saline and by size exclusion HPLC on a TSK4000 column with radioactivity

detected by a Bioscan Flow Count detector; (2) ability to bind to melanin by a melanin-binding ELISA (Huntington, NJ). The purified ^{188}Re-6D2 was stabilized with 20 mg/mL L-ascorbic acid for a final concentration of 0.2 mg/mL. In some experiments, "cold" 6D2 was added as well to bring the total amount of 6D2 up to 10 mg. The influence of overnight freezing at -80°C on radiochemical purity and immunoreactivity of radiolabeled mAb was evaluated by ITLC, HPLC and melanin-binding ELISA.

Animal model of human metastatic melanoma. All animal studies were carried out in accordance with the guidelines of the Institutes for Animal Studies at the Albert Einstein College of Medicine and of the University of California, Davis. For pharmacokinetics and whole body autoradiography (WBAR) studies, forty 11-week old female nude mice were implanted subcutaneously with 5 x 10^6 A2058 cells in each of two abdominal sites and used for experiments 14 days later. For therapy/acute hematologic toxicity evaluation studies, 5–6 week-old female nude mice were implanted subcutaneously with 8 x 10^6 A2058 cells into the left flank and used for therapeutic experiments 12 days after tumors reached the size of approximately 0.15 cm^3 (0.02–0.4 cm^3).

Pharmacokinetics and whole body autoradiography (WBAR). For pharmacokinetic experiments, 36 nude mice bearing A2058 cell-derived melanoma tumors were given either 30 μCi ^{188}Re-6D2 (total amount of 6D2 150 μg/100 μL) and sacrificed at 5 min, 2 hr, 4 and 24 hrs; or 120 μCi ^{188}Re-6D2 (total amount of 6D2 150 μg/100μL) and sacrificed at 48 hr. Blood clearance samples (2 μL) were collected at 5 min, 1 hr, 2, 4, 24 and 48 hr from the dorsal tail vein and counted in a sodium iodide gamma well counter (Packard, Downers Grove, IL). Decay-corrected radioactivity in the blood was expressed as % ID, using a weight-based theoretical blood volume. Whole body activity was measured at the time of injection, and at 2, 4, 24 and 48 hrs using the iso-responsive sodium iodide detector system (Picker Nuclear, North Haven, CT); the counts were decay corrected and expressed as % ID. Pharmacokinetic data for other tissues were obtained by removing and weighing the tissues and counting them in the same gamma well counter. The concentration of radioactivity in each organ was expressed as % ID/g.

For WBAR, four mice were given 120 μCi ^{188}Re-6D2 (total amount of 6D2 150 μg/300μL); two mice were sacrificed at 4 hr and two at 24 hr. Mice were anesthetized by intravenous injection of 60 mg/100 μL aqueous solution of sodium pentobarbital and flash frozen in a hexane-dry ice bath. The frozen mice were embedded in frozen 4% carboxymethylcellulose, and sagittal sections were taken at -20°C with a Leica Polycut. Sections of 50 μm thickness were taken to show tumors, spleen, kidney, liver and the midline of the vertebral column. The sections were desiccated, and autoradiograms were prepared by exposing the sections to x-ray film (Kodak BioMax MS, Rochester, NY).

Treatment of A2058 tumor-bearing mice with increasing doses of ^{188}Re-6D2 and evaluation of acute hematologic toxicity. The nude mice bearing A2058 cell-derived melanoma tumors were randomized into five groups of six mice. Groups 1–4 received IV injections of 0.15, 0.5, 1.0 and 1.5 mCi ^{188}Re-6D2. The control group was untreated. Mice were weighed and tumor volumes were measured immediately before administration of radiolabeled mAb and weekly thereafter. Tumors were measured in three dimensions with calipers, and tumor volume was calculated by multiplying the product of the three perpendicular diameters by 0.5, assuming an elliptical geometry. To evaluate the acute hematologic toxicity of RIT, platelet and WBC counts were measured on the day of therapy and on days 3, 7, 14, 28;[9] after administration of ^{188}Re-6D2. Blood samples from each animal were collected from the tail vein and individually diluted 1:200 into 25% (v/v) ammonium oxalate for platelet counts, and 1:20 into 2% (v/v) acetic acid—for WBC counts. The diluted blood cells were counted using a hemocytometer and light microscopy at magnification X 100 (WBCs) or X 450 (platelets).

Tumor histology. To assess the effects of radiolabeled mAb on the tumors, the tumors from the treated and control mice were removed at the end of the therapy study and fixed in 10% neutral buffered formalin. Tissues were routinely processed, paraffin embedded, cut to 5 μm, and stained with hematoxylin and eosin (H&E) for histological evaluation.

Statistical analysis. The Wilcoxon rank sum test was used to compare tumor sizes and platelet and WBC counts between different treatment groups in therapy studies. Differences were considered statistically significant when p values were <0.05.

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