Radiolabeled Melanin-Binding Peptides are Safe and Effective in Treatment of Human Pigmented Melanoma in a Mouse Model of Disease

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ABSTRACT

The incidence of melanoma is rising, and therapeutic options for metastatic melanoma are limited. We report the results of experimental melanoma therapy with 188-Rhenium-labeled melanin-binding decapeptide ([188]RE-HYNIC-4B4) and a comprehensive safety evaluation of this treatment. [188]RE-HYNIC-4B4 bound only to nonviable eumelanotic MNT1 and pheomelanotic SK-28-MEL human melanoma cells in vitro, *as determined by immunofluorescence, which is consistent with the inaccessibility of intracellular melanin in live cells and suggests specificity for tumors with a significant amount of extracellular melanin. Administration of 1 mCi [188]RE-HYNIC-4B4 to MNT1 tumor-bearing mice significantly slowed tumor growth, with the therapeutic effect being a result of specific binding to tumor melanin, as irrelevant [188]RE-labeled decapeptide did not produce therapeutic gain. Repeated doses of [188]RE-HYNIC-4B4 had a more profound effect on tumor growth than a single dose. Treatment of tumors with 0.3–0.4 cm diameter was more effective than of larger ones (0.5–0.7 cm). There was no difference in uptake of [188]RE-HYNIC-4B4 in melanized tissues of black C57BL6 mice and no histologically apparent damage to these tissues in comparison with white BALB/C mice. Treatment of C57BL6 mice with [188]RE-HYNIC-4B4 did not change their behavior, as established by SHIRPA protocol, and did not cause damage to neurons and glial cells. These results indicate that radiolabeled melanin-binding peptides are efficient and safe in treatment of melanoma and could be potentially useful against this tumor.*

Key words: melanoma, melanin, melanin-binding peptide, nude mice, radiolabeled peptides, 188-Rhenium, therapy

INTRODUCTION

The incidence of melanoma is rising,[1] and it is an important cause of cancer among young patients (30–50 years of age). This disease currently affects ~40,000 new patients each year in the United States, and there are an estimated 100,000 cases worldwide.[2,3] Metastatic melanoma is al-

most always fatal,[4] with a median survival time of 8.5 months and an estimated 5-year survival of 6%.[4] Therapeutic options for metastatic melanoma are limited, and there has been little change in the prognosis of this disease in the past 25 years.[5] We have recently demonstrated the feasibility of targeting melanin, an intracellular melanocyte pigment, to deliver cytotoxic radiation to human melanoma cells *in vivo* using a fungal melanin-binding monoclonal antibody (mAb 6D2) with promising therapeutic results.[6] During the last decade, radiolabeled peptides that bind to different receptors on the tumors have been investigated as potential therapeutic agents, both in preclinical and clinical settings.[7] Advantages of radiolabeled peptides over mAbs include relatively straightforward chemical synthesis, versatility, easier radiolabeling, rapid clearance from the circulation, faster penetration and more uniform distribution into tissues, and less immunogenicity.[8] On the other hand, peptides have a very short serum half-life and lack the effector functions conferred by an antibody constant region.

While studying melanogenesis of human pathogenic fungus *Cryptococcus neoformans in vivo*, we identified several fungal melanin-binding peptides using phage display libraries.[9] Because both fungal and tumor melanins are negatively charged,[10–12] and fungal melanin-binding peptides were characterized by a high proportion of positively charged and aromatic residues, we hypothesized that these peptides would also bind to tumor melanin and could be used to deliver therapeutic radionuclides to melanoma tumors. In this paper, we report the results of experimental melanoma therapy with 188-Rhenium (^{188}Re)-labeled fungal melanin-binding decapeptide 4B4 and a comprehensive safety evaluation of this treatment. The results indicate that radiolabeled melanin-binding peptides have activity against melanoma and that these reagents could be potentially useful against this tumor.

MATERIALS AND METHODS

Peptide Synthesis and Radiolabeling

The melanin-binding decapeptide 4B4 (YERK-FWHGRH)[9] was synthesized from D-amino acids with N terminal biotin by conventional solid-state peptide synthesis (SPPS) techniques on an automatic peptide synthesizer, using standard Fmoc/HBTU chemistry on ring amide MBHA resin in the Laboratory for Macromolecular Analysis and Proteomics (Albert Einstein College of Medicine; Bronx, NY). For labeling with ^{188}Re, the 4B4 peptide and irrelevant control decapeptide PA1[9] were synthesized from D-amino acids with HYNIC (hydrazinonicotinamide) ligand at the N terminus using Fmoc reagent 6-Fmoc-hydrazino-nicotinic acid (Trilink Biotechnology, Inc.). The peptides were characterized by ES-MS, and molecular mass of ^{188}Re-HYNIC-4B4 was found to be 1550.

^{188}Re in the form of sodium perrhenate Na^{188}ReO$_4$ was eluted from a ^{188}W/^{188}Re generator (Oak Ridge National Laboratory; Oak Ridge, TN), and HYNIC-4B4 and HYNIC-PA1 peptides were radiolabeled with ^{188}Re-gluconate by incubation for 1 hour at room temperature while protected from light, according to Abrams et al.[13] Incorporation of radioactivity into the peptides was determined by instant thin-layer chromatography with silica gel–impregnated glass fibers (ITLC-SG) developed with saline. In this system, ^{188}Re-labeled peptides had an $R_f = 0$, whereas ^{188}Re-gluconate and ^{188}Re-perrhenate moved with the solvent front. If needed, the radiolabeled peptides were purified on a SEP-PAK18 chromatographic column, according to Westlin et al.[14]

Serum Stability of ^{188}Re-HYNIC-4B4 and ^{188}Re-HYNIC-PA1.

^{188}Re-HYNIC-4B4 was incubated in mouse serum at 37°C, and aliquots were withdrawn at 0, 0.5, 1, 2, 3, 4, and 5 hours and analyzed on a size-exclusion high-performance liquid chromatography (HPLC) column eluted with phosphate buffered saline (PBS), pH 7.2, at 1 mL/min. Peptide and proteins were monitored by an ultraviolet (UV) detector at 280 nm; 1-mL fractions were collected and counted in a dose calibrator.

Melanoma Cells

Human lightly pigmented melanoma cells SK-MEL-28 (ATCC) were grown in complete growth medium (ATCC) supplemented with 10% fetal bovine serum (FBS) and 110 μM L-tyrosine to promote melanin formation. Highly pigmented human melanoma cells MNT1[15] were grown in MEM/20% FBS medium. The percentage of viable cells in the samples was determined to be $96 \pm 1\%$ by Trypan blue exclusion assay.

Binding of [188]Re-HYNIC-4B4 to SK-MEL-28 and MNT1 cells

The binding of [188]Re-HYNIC-4B4 to SK-MEL-28 and MNT1 cells was evaluated by incubating radiolabeled peptide (20 ng/mL) with $0.2–2.0 \times 10^6$ cells. Peptide binding to both whole and osmotically lysed cells was evaluated. After incubation for 1 hour at 37°C, the cells were collected by centrifugation, the supernatant was removed, the cell pellet washed with PBS, and the pellet and the supernatant were counted in a gamma counter to calculate the percentage of peptide binding to the cells. To prove that the binding of the peptide was specific, cells were also preincubated with an excess (2 μg/mL) of unlabeled HYNIC-4B4.

Immunofluorescence of MNT1 Cells

The binding of the 4B4 peptide to melanoma cells *in vitro* was analyzed by immunofluorescence, as in Nosanchuk et al.[9] Approximately 10^6 melanoma cells were blocked for nonspecific binding by incubation in SuperBlock (Pierce; Rockford, IL) for 1 hour at 37°C. Biotinylated 4B4 was then incubated with the cells for 1 hour, followed by the addition of streptavidin conjugated with fluorescein isothiocyanate (FITC). The slides were viewed with an Olympus AX70 microscope (Melville, NY) equipped with a FITC filter. Irrelevant biotinylated decapeptide PA1[9] was used as a negative control.

HPLC Analysis of Melanin from SK-28-MEL and MNT1 Melanoma Cells

Melanin from MNT1 and SK-MEL-28 melanoma cells was purified using a modified methodology for isolating melanin from fungal cells.[10] Briefly, the cells were subjected to the sequence of enzymatic digestion—boiling in 6 M HCl, extensive dialysis against deionized water, and drying at 50°C. Purified melanin was subjected to acidic permanganate oxidation, as described by Ito and Wakamatsu,[16,17] and the oxidation products were analyzed by reverse-phase HPLC using a Shimadzu LC-600 chromatography system, Hamilton PRP-1 C_{18} column (250×4.1 mm dimensions, 7-μm particle size), and Shimadzu SPD-6AV UV detector. The mobile phase was 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). At 1.0 mL/min, the elution gradient was (min, %B): 0, 0; 1, 0; 12, 25; 14, 25; 16, 0. The UV detector was set at a 255-nm absorbance. Pyrrole-2,3,5-tricarboxylic acid (PTCA), 1,3-thiazole-2,4,5-tricarboxylic acid (TTCA), and 1,3-thiazole-4,5-dicarboxylic acid (TDCA) were used as standard compounds. Chromatograms of TDCA, TTCA, and PTCA standards yielded peaks at 6.1, 7.1, and 11.0 minutes, respectively.

Animal Models

All animal studies were carried out in accordance with the guidelines of the Institute for Animal Studies at the Albert Einstein College of Medicine (Bronx, NY). For biodistribution and therapy studies in tumor-bearing mice, the tumors were induced by injecting approximately 10^6 MNT1 human melanoma cells into the right flank of female nude mice. Four (4) weeks after implantation, the tumors reached 0.3–0.7 cm in diameter. Comparative biodistribution of [188]Re-HYNIC-4B4 in the eyes and skin on the tails was performed in white BALB/c and black C57BL6 female mice that have black eyes and melanized skin on their tails. Toxicity of therapeutic doses of [188]Re-HYNIC-4B4 to melanized normal tissues (eyes, skin, and melanized neurons in substantia nigra), as well as to the brain, was evaluated in C57BL6 female mice.

Biodistribution of [188]Re-HYNIC-4B4 in MNT1 Tumor-Bearing Nude Mice and in C57BL6 Mice

To assess the uptake of [188]Re-HYNIC-4B4 peptide in the tumor and normal organs, MNT1 tumor-bearing nude mice were injected intravenously i.v. with 2 μg (50 μCi) [188]Re-HYNIC-4B4. Four (4) mice per time interval were sacrificed at 30 minutes, 1, 2, 3, and 24 hours postinjection, their major organs removed, blotted to remove blood, weighted, and counted in a gamma counter. Comparative biodistribution in the eyes and tail skin was similarly done in normal white BALB/c and black C57BL6 mice sacrificed 1 and 24 hours after i.v. administration of 2 μg (50 μCi) [188]Re-HYNIC-4B4.

Therapy of MNT1 Tumor-Bearing Mice with [188]Re-HYNIC-4B4

Two therapy experiments were conducted in MNT1 tumor-bearing mice. Ten (10) mice per group were used in both studies. During the initial study, mice with tumors of 0.5–0.7 cm in diameter were used. Mice in the first group were

treated intraperitoneally (i.p.) with 1 mCi [188]Re-HYNIC-4B4 (2 μg), the second group received 2 × 1 mCi [188]Re-HYNIC-4B4 20 days apart to investigate the effect of multiple treatments on tumor progression, and a third group was left untreated. In a follow-up study, we investigated the influence of tumor size on the therapy results and introduced another control in form of [188]Re-labeled irrelevant decapeptide HYNIC-PA1. Mice with tumors 0.3–0.4 cm in diameter were used. Mice in the first group were treated i.p. with 2 × 1 mCi [188]Re-HYNIC-4B4 10 days apart, the second group received 2 × 1 mCi [188]Re-HYNIC-PA1 10 days apart, and the third group was left untreated. The size of the tumor (which was assumed to be a half-sphere) was measured with calipers in three dimensions every 4 days, and the tumor volume was calculated as the product of these measurements multiplied by 0.5. Mice were observed for tumor regrowth for 3 months (first study) or 2 months (second study). To assess the effects of radiolabeled peptide on the tumor cells, MNT1 tumors from [188]Re-HYNIC-4B4 treated and control mice were removed at the end of the second study. Tumor tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, cut and stained with hematoxylin and eosin (H&E). Prussian Blue stain was used to identify iron pigment. Masson-Fontana stain and melanin bleaching were used to corroborate the H&E identification of melanin pigment.

Tumor Dosimetry

In our dosimetry calculations, we utilized the dosimetric model for a laboratory mouse, which takes into consideration self-doses for the organs and the cross-organ doses resulting from beta-radiation "cross-fire".[18] The biodistribution data were used to obtain cumulative activities by generating time-activity curves, followed by integration of the area under the curve (Prism software, GraphPad; San Diego, CA).

Toxicity to the Kidneys and Normal Melanized Tissues Post-Treatment with [188]Re-Labeled Melanin-Binding Peptide

As nephrotoxicity after radiolabeled peptide therapy[19] was of concern, we assessed toxicity to the kidneys of the mice treated with 2 × 1 mCi [188]Re-HYNIC-4B4 and sacrificed at the conclusion of the 3-month study. To investigate whether radiation damage was induced by [188]Re-HYNIC-4B4 in melanin-containing normal tissues, 5 C57BL/6 mice received 2 × 1 mCi [188]Re-HYNIC-4B4 10 days apart. At 2 months after administration of [188]Re-HYNIC-4B4, mice were sacrificed and their eyes and melanized skin from the tails were removed. Kidney and other tissues were prepared for histology, as described above. Healthy nude and C57BL/6 mice were similarly studied for comparison.

Behavioral and Histological Assessment of Toxicity to the Brain

As peptides are small molecules that can penetrate the blood-brain barrier, and as the brain contains melanized tissues, we considered the possibility of subtle damage that would manifest itself in behavioral changes. Consequently, we performed behavioral assessments of 5 C57BL/6 mice that received 2 × 1 mCi [188]Re-HYNIC-4B4 10 days apart and compared them with 5 untreated controls. Afterward, we carried out histological evaluation of the subjects' substantia nigra for the possibility of tissue damage. The behavioral assessment was done using the Primary screen SHIRPA Protocol,[20] which is widely used for screening drug candidates by pharmaceutical laboratories. This method provides a behavioral and functional profile by observational assessment of mice and includes evaluation of gait, posture, motor control and coordination, changes in excitability and aggression, salivation, lacrimation, piloerection, muscle tone, and temperature. It also tests for a gross measure of analgesia. All parameters are scored to provide a quantitative assessment that enables comparison of results over time. The behavior of treated and control animals was assessed at 1 week and 2 months post-treatment with [188]Re-HYNIC-4B4. After the second assessment with the SHIRPA Protocol, the mice were sacrificed by CO_2 asphyxiation and perfused through the heart with PBS, followed by 4% paraformaldehyde (PFA). The brain was then extracted, fixed with 4% PFA overnight, paraffin embedded, sectioned, and the slides were stained with toluidine blue.

Statistics

The Wilcoxon Rank Sum test was used to compare organ uptake in biodistribution studies. A two-tailed Student's *t* test for unpaired data was employed to analyze differences in cell binding during *in vitro* studies and in tumor sizes during therapy studies. Differences were considered statistically significant when P-values were <0.05.

RESULTS

Melanin-Binding Peptide Synthesis, Radiolabeling, and Serum Stability

Fmoc-HYNIC reagent provided a convenient way of synthesizing melanin-binding and irrelevant control peptides modified with HYNIC ligand at the N-terminus. Radiolabeling of these peptides with [188]Re resulted in 55%–65% labeling yields, with the remainder of the radioactivity being perrhenate. Purification using SEP-PAK18 chromatographic cartridges led to 95%–99% radiochemical purity. Utilization of D-amino acids, a well-known technique to extend the plasma half-life of peptides, as well as the introduction of HYNIC moiety into the molecules, proved to be useful for serum stability of both decapeptides, as at 5 hours incubation in mouse serum, 70%–75% of [188]Re activity was still associated with the peptides and not with plasma proteins (results not shown).

[188]Re-HYNIC-4B4 Peptide Bound only to Nonviable Melanoma Cells *In Vitro*

Peptide binding to melanin in MNT1 cells was studied by immunofluorescence (Fig. 1A). The biotinylated 4B4 peptide bound only to nonviable melanoma cells (Fig. 1A, arrowhead), which comprised 3%–5% of the total cells in culture, as measured by the dye-exclusion assay. Nonviable cells apparently released their melanin or had permeable cell membranes that allowed access by
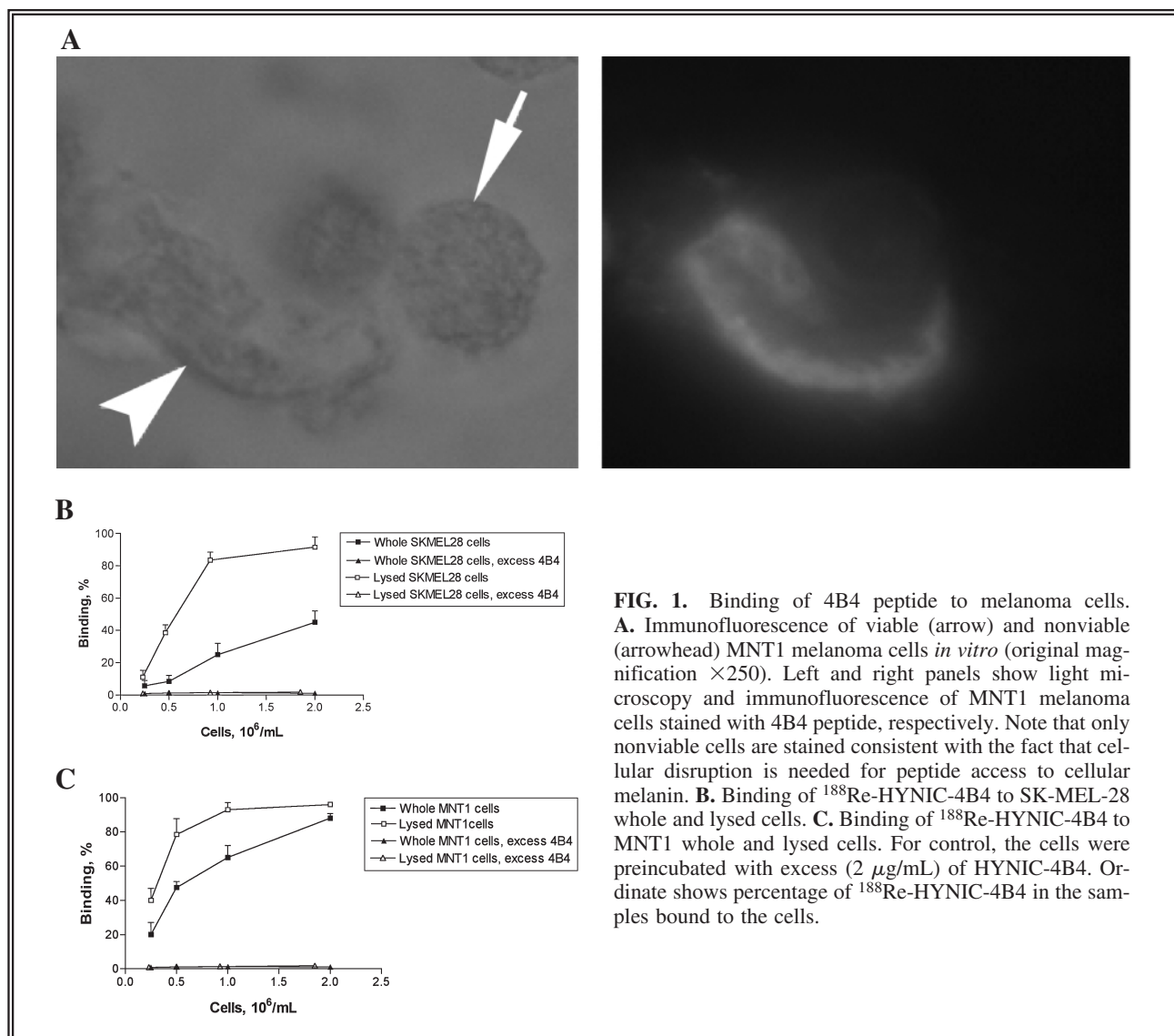

FIG. 1. Binding of 4B4 peptide to melanoma cells. **A.** Immunofluorescence of viable (arrow) and nonviable (arrowhead) MNT1 melanoma cells *in vitro* (original magnification ×250). Left and right panels show light microscopy and immunofluorescence of MNT1 melanoma cells stained with 4B4 peptide, respectively. Note that only nonviable cells are stained consistent with the fact that cellular disruption is needed for peptide access to cellular melanin. **B.** Binding of [188]Re-HYNIC-4B4 to SK-MEL-28 whole and lysed cells. **C.** Binding of [188]Re-HYNIC-4B4 to MNT1 whole and lysed cells. For control, the cells were preincubated with excess (2 μg/mL) of HYNIC-4B4. Ordinate shows percentage of [188]Re-HYNIC-4B4 in the samples bound to the cells.

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the peptide to melanin (Fig. 1A, left panel). No binding was observed to viable cells with intact cell membranes (Fig. 1A, arrow). Control biotinylated decapeptide PA1 did not bind to either viable or dead MNT1 cells (results not shown).

[188]Re-HYNIC-4B4 readily bound to both types of melanoma cells employed in this study— slightly pigmented SK-28-MEL cells and highly pigmented MNT1 cells (Fig. 1B,C). The binding was specific for melanin, as preincubating the cells with an excess of unlabeled HYNIC-4B4 effectively blocked the subsequent binding of [188]Re-HYNIC-4B4. To investigate whether there was a correlation between the amount of extracellular melanin and the peptide binding, we compared binding to whole cells with that to lysed cells. For both cell lines, significantly higher binding to lysed cells was observed.

HPLC Analysis of Melanin from SK-28-MEL and MNT1 Melanoma Cells

Reverse-phase HPLC analysis of the yellowish-brown melanin from SK-MEL-28 cells and the black melanin from MNT1 cells was done to investigate the structural motifs of melanins from different cell lines that may be responsible for the binding of 4B4 peptide. The background solution (consisting of 1.85% Na_2SO_3 and 0.012% $KMnO_4$ in 0.18 M H_2SO_4) used for oxidation of melanin had peaks that eluted at 2.7 and 15.7 minutes (Fig. 2A), and the PTCA and TDCA standards eluted at 11 and 6.2 minutes, respectively (results not shown). Oxidized melanin from MNT1 cells yielded one peak at 11 minutes that was assigned to PTCA (Fig. 2B). The chromatogram of the oxidized melanin from SK-28-MEL cells had both PTCA and TDCA peaks that were smaller than the PTCA peak in the MNT1 chromatogram, consistent with the lower quantity of melanin in the SK-28-MEL cells (Fig. 2C). The absence of a TDCA peak in the MNT1 chromatogram may be explained by the fact that black coloration of the tumors is caused by the presence of eumelanin, whereas TDCA is primarily a product of pheomelanin oxidation.[17]

Biodistribution of [188]Re-HYNIC-4B4 in MNT1 Tumor-Bearing Mice

[188]Re-HYNIC-4B4 was cleared rapidly from the blood with only 0.5% injected dose per (ID/g) remaining in circulation at 24 hour postinjection (Fig. 3A). Interestingly, a transient increase in uptake of practically all major organs was observed
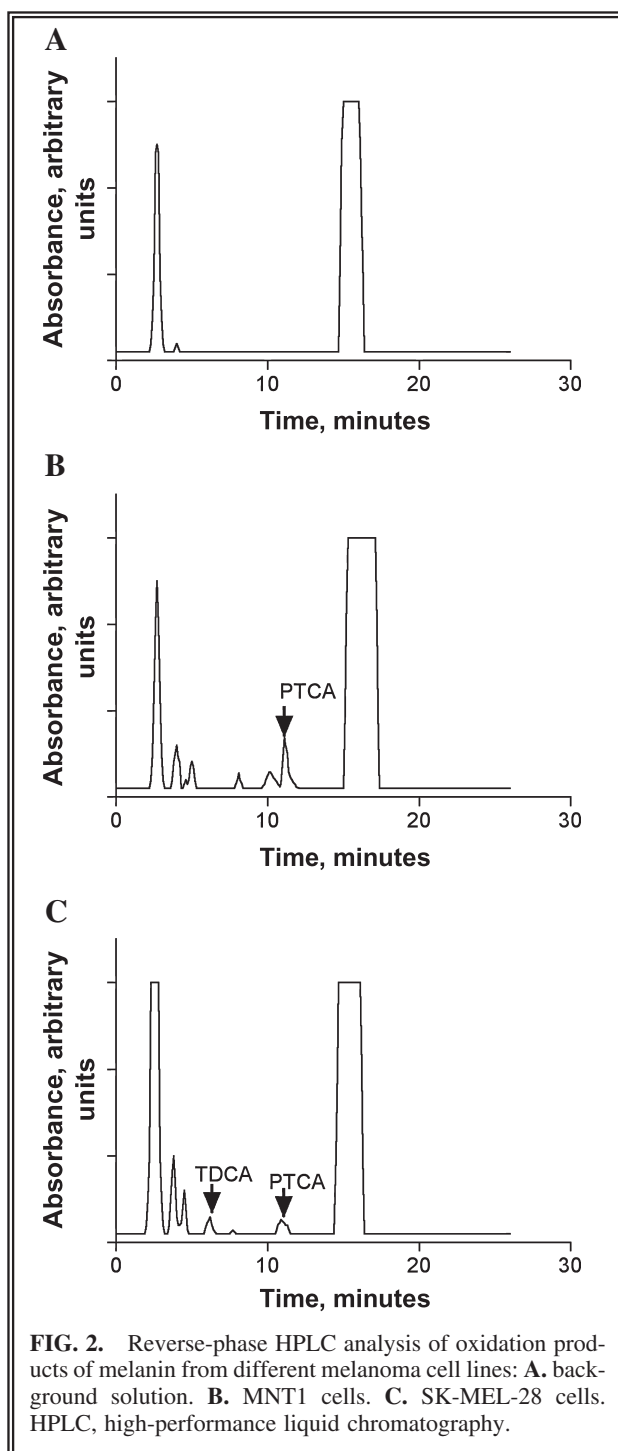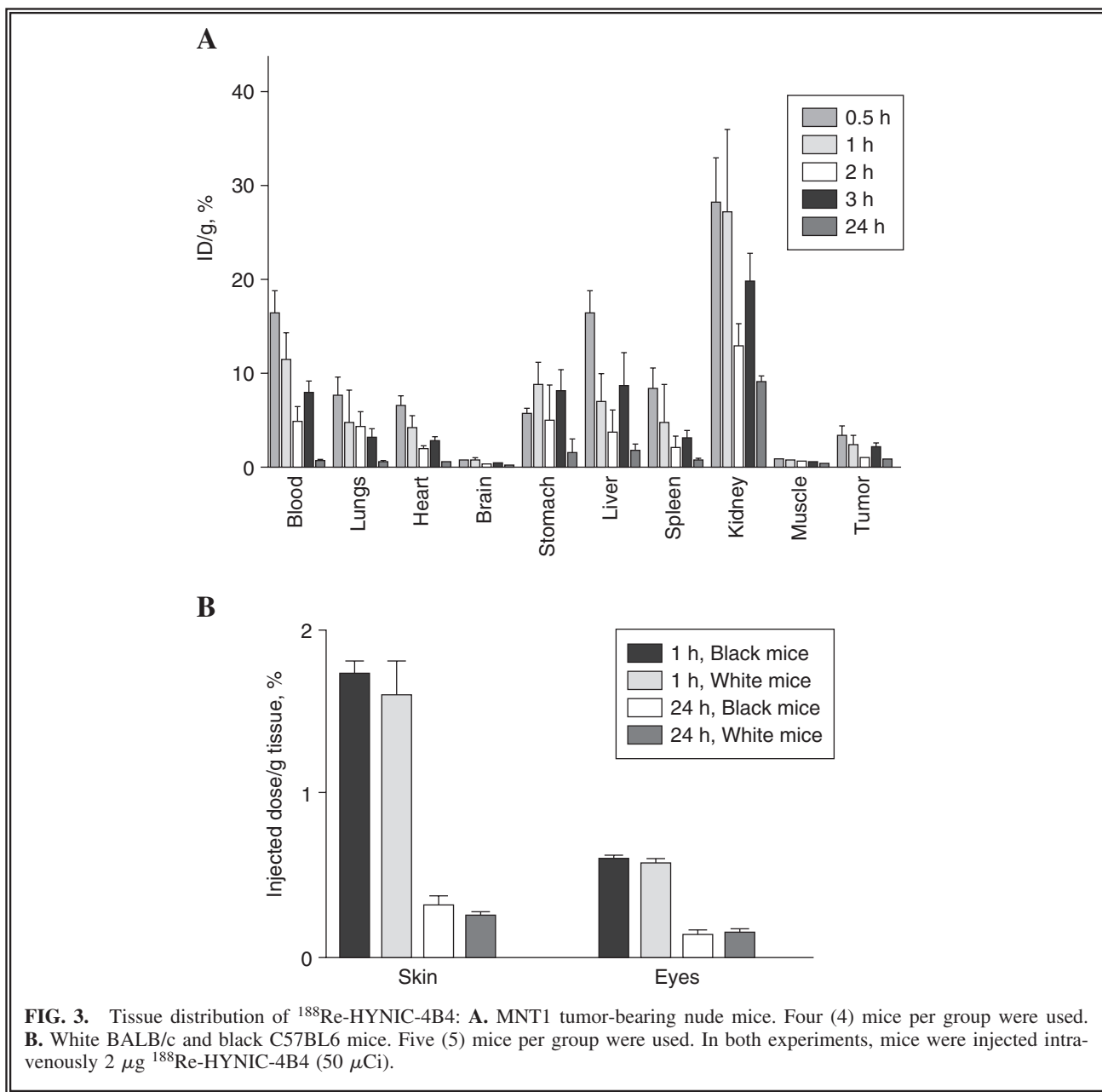

FIG. 2. Reverse-phase HPLC analysis of oxidation products of melanin from different melanoma cell lines: **A.** background solution. **B.** MNT1 cells. **C.** SK-MEL-28 cells. HPLC, high-performance liquid chromatography.

at 3 hour postinjection, which might be explained by the redistribution of activity, possibly from the intestinal compartment. The kidney uptake was high, with ~30% ID/g at 0.5–1 hour postinjection, which decreased to 10% ID/g at 24 hour, closely resembling the biodistribution pattern of

FIG. 3. Tissue distribution of [188]Re-HYNIC-4B4: **A.** MNT1 tumor-bearing nude mice. Four (4) mice per group were used. **B.** White BALB/c and black C57BL6 mice. Five (5) mice per group were used. In both experiments, mice were injected intravenously 2 μg [188]Re-HYNIC-4B4 (50 μCi).

melanin-binding mAb [188]Re-6D2.[6] The tumor uptake of [188]Re-HYNIC-4B4 was highest at the earlier time intervals (~4.5% ID/g) and decreased approximately ninefold to 0.5% ID/g at 24 hour. The clearance of the peptide from the liver and spleen paralleled its clearance from the tumor. At all times, uptake of [188]Re-HYNIC-4B4 in the tumor was 10 times higher than in the surrounding muscle tissue.

No statistically significant difference was observed between uptake of [188]Re-HYNIC-4B4 in the eyes and the skin of black C57BL6 mice in comparison with that in white BALB/c mice (Fig. 3B), which is consistent with the inaccessibility of melanin pigment in healthy melanized tissues to [188]Re-HYNIC-4B4 peptide.

Therapy of MNT1 Melanoma in Nude Mice with [188]Re-HYNIC-4B4 and Tumor and Kidney Dosimetry

In the initial study to examine the effect of radiolabeled melanin-binding peptide on MNT1 tumors, three groups of 10 MNT1 tumor-bearing

nude mice with 0.5–0.7 cm in diameter were treated i.p. with: (1) 1.0 mCi [188]Re-HYNIC-4B4; (2) 2 × 1.0 mCi [188]Re-HYNIC-4B4 20 days apart; or (3) left untreated. The tumors grew in the untreated group, and the last surviving mouse had to be sacrificed on day 52 because of the size of its tumor. A somewhat slower tumor growth was observed in the group that received one treatment with [188]Re-HYNIC-4B4, and significantly slower growth occurred ($P = 0.01$) in the group treated twice (Fig. 4A).

In the second therapy study, we investigated the influence of tumor size on the therapy results, as well as any possible effect on tumor growth from radiation delivered by nonspecifically bound decapeptide. Injection of mice bearing 0.3–0.4 cm in diameter tumors with 2 × 1.0 mCi [188]Re-HYNIC-PA1 10 days apart did not have any therapeutic effect on the tumor, whereas 2 × 1.0 mCi [188]Re-HYNIC-4B4 administered according to the same regimen completely arrested the growth of the tumors until day 20 post-treatment, with tumors subsequently resuming growth

at a significantly slower rate than the larger tumors in the first study. These results demonstrate that the tumoricidal effect of [188]Re-HYNIC-4B4 was a result of its specific binding to melanin in the tumor and that tumors with smaller diameters were more susceptible to treatment with radiolabeled peptide than larger ones. The dose delivered to the MNT1 tumor by 1 mCi [188]Re-HYNIC-4B4 was estimated to be 300 cGy.
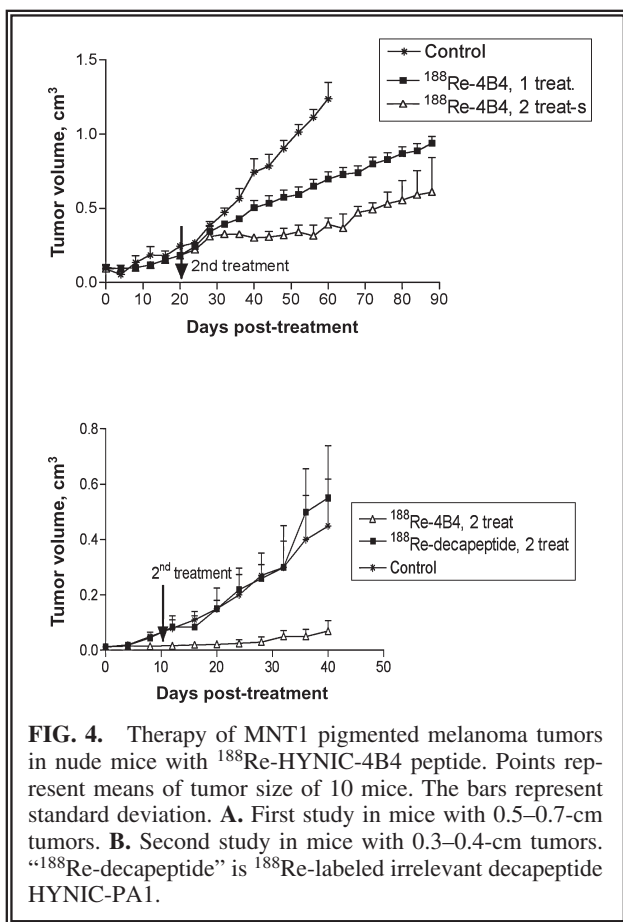
Control mice injected with MNT1 cells all developed tumors that were composed of malignant melanoma cells (Fig. 5A). In those mice in which tumors stopped growing post-treatment with 2 × 1 mCi [188]Re-HYNIC-4B4 in the second study, no residual malignant melanoma cells were identifiable (Fig. 5B). Only areas of fibrosis with phagocytic histiocytes associated with intra- and extracellular melanin pigment and a rare iron granule were found in the areas where the MNT1 cells had been injected (Fig. 5C,D).

Histological Evaluation of [188]Re-HYNIC-4B4 Toxicity to Kidneys and Normal Melanized Tissues

The kidneys of [188]Re-HYNIC-4B4-treated mice revealed normal glomeruli and tubules without signs of fibrosis, vasculitis, or neoplasm (Fig. 5E). No histological damage was apparent in the eyes (Fig. 5F) and melanocytes in the skin (data not shown) of C57BL6 mice sacrificed 2 months post-treatment.

Behavioral and Histological Assessment of Brain Toxicity of [188]Re-HYNIC-4B4

C57BL/6 mice treated with 2 × 1 mCi [188]Re-HYNIC-4B4 and control mice were subjected to comprehensive behavioral assessment using the SHIRPA Protocol at 1 week and 2 months post-treatment. Each parameter of animal behavior was scored on a scale assigned in SHIRPA to this particular parameter. Results of the assessment at 1 week post-treatment are presented in Table 1. There were no significant differences in the behavior of control and treated mice, with the possible exception of the touch-escape response, which was more vigorous in [188]Re-HYNIC-4B4-treated mice. Interestingly, treated mice were, on average, 1.2 g heavier than control mice at the 1-week evaluation. The second behavioral assessment performed 2 months post-treatment showed that the body weight equalized between the control and treated group, and no significant differences in behavioral parameters were observed. At

FIG. 4. Therapy of MNT1 pigmented melanoma tumors in nude mice with [188]Re-HYNIC-4B4 peptide. Points represent means of tumor size of 10 mice. The bars represent standard deviation. **A.** First study in mice with 0.5–0.7-cm tumors. **B.** Second study in mice with 0.3–0.4-cm tumors. "[188]Re-decapeptide" is [188]Re-labeled irrelevant decapeptide HYNIC-PA1.
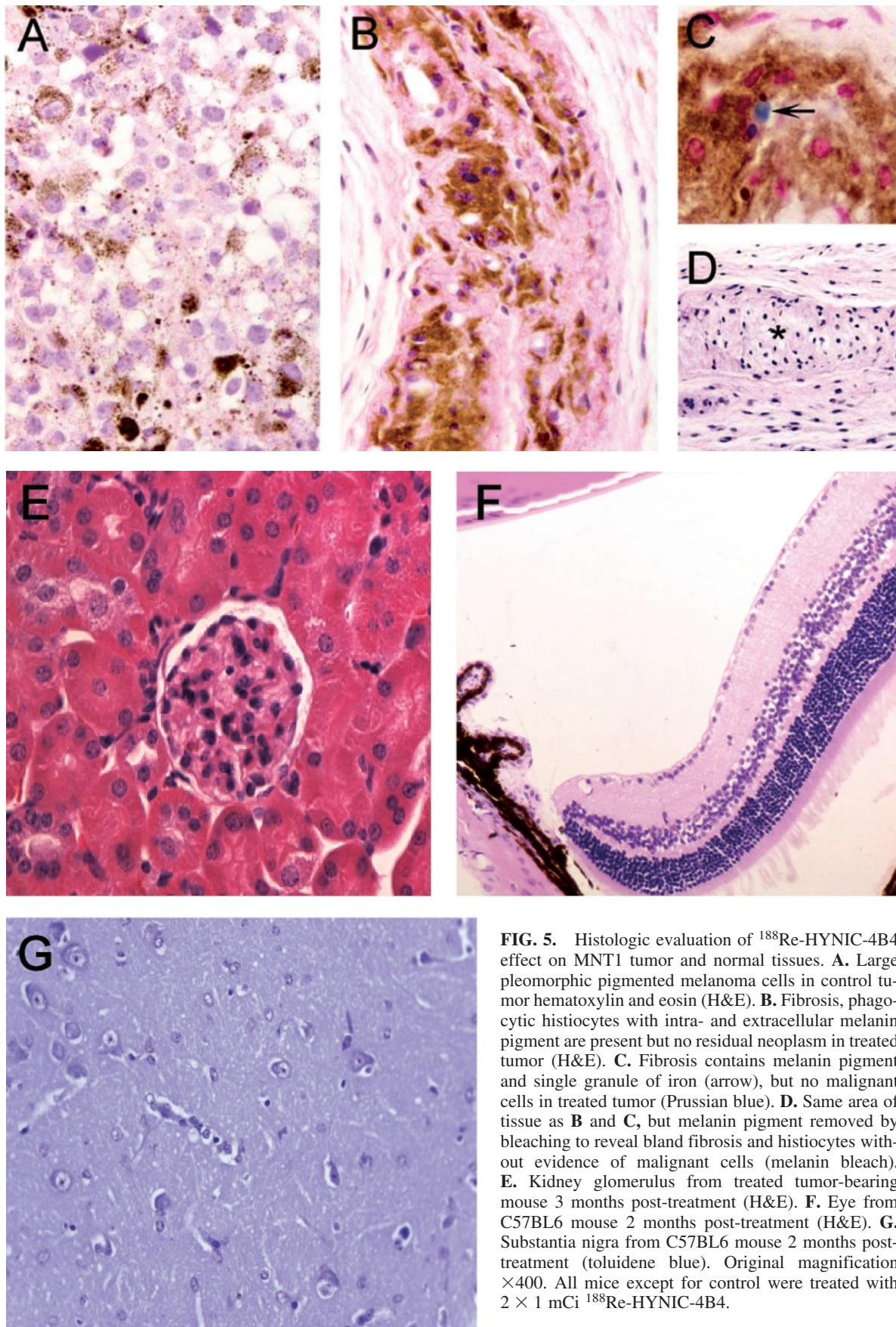
FIG. 5. Histologic evaluation of [188]Re-HYNIC-4B4 effect on MNT1 tumor and normal tissues. **A.** Large pleomorphic pigmented melanoma cells in control tumor hematoxylin and eosin (H&E). **B.** Fibrosis, phagocytic histiocytes with intra- and extracellular melanin pigment are present but no residual neoplasm in treated tumor (H&E). **C.** Fibrosis contains melanin pigment and single granule of iron (arrow), but no malignant cells in treated tumor (Prussian blue). **D.** Same area of tissue as **B** and **C,** but melanin pigment removed by bleaching to reveal bland fibrosis and histiocytes without evidence of malignant cells (melanin bleach). **E.** Kidney glomerulus from treated tumor-bearing mouse 3 months post-treatment (H&E). **F.** Eye from C57BL6 mouse 2 months post-treatment (H&E). **G.** Substantia nigra from C57BL6 mouse 2 months post-treatment (toluidene blue). Original magnification ×400. All mice except for control were treated with 2 × 1 mCi [188]Re-HYNIC-4B4.

Table 1. Behavioral Observation Profile Performed According to SHIRPA Protocol for Control and Treated with 2×1 mCi Doses of [188]Re-HYNIC-4B4 C57BL6 Mice (5 Mice per Group) 1 Week Post-Treatment

	Control mice	Treated mice
Parameter		
Weight, g	18 (0.3)	19.2 (0.7)
Body position	4.4 (0.5)	4.4 (0.5)
Spontaneous activity	2.4 (0.5)	2.2 (0.4)
Respiration rate	2 (0)	2 (0)
Tremor	0.8 (0.4)	1 (0)
Parameter		
Transfer arousal	4.8 (0.4)	4.8 (0.4)
Locomotor activity	4.6 (1.1)	5 (0.7)
Palpebral closure	0 (0)	0 (0)
Pilorection	0 (0)	0 (0)
Startle response	0.6 (0.9)	0.6 (0.9)
Gait	0 (0)	0 (0)
Pelvic elevation	1.8 (0.4)	2 (0))
Tail elevation	1 (0)	1 (0)
Touch escape	1.4 (0.5)	2.4 (0.9)
Positional passivity	0 (0)	0 (0)
Parameter		
Trunk curl	0.6 (0.5)	1 (0)
Limb grasping	1 (0)	1 (0)
Visual placing	3.4 (0.5)	3.2 (0.4)
Grip strength	3.2 (0.8)	2.8 (0.8)
Body tone	1 (0)	1 (0)
Pinna reflex	1 (0)	1 (0)
Corneal reflex	1 (0)	1 (0)
Toe pinch	2.8 (0.4)	2.8 (0.4)
Wire maneuver	0.6 (0.5)	0.4 (0.5)
Parameter		
Lacrimation	0 (0)	0 (0)
Salivation	0 (0)	0 (0)
Provoked biting	0.4 (0.5)	0.6 (0.5)
Righting reflex	0 (0)	0 (0)
Negative geotaxis	0 (0)	0 (0)
Irritability	0 (0)	0.2 (0.4)
Aggression	0 (0)	0 (0)
Vocalization	0 (0)	0.2 (0.4)

Each parameter of mouse behavior was scored on a scale assigned to SHIRPA to this particular parameter; mean values with standard deviation in parentheses for each group are given.

the end of 2 months of observation, mice were killed and their brains examined for histological evidence of neuronal damage (Fig. 5G). No difference in neurons and glial-cell morphology was found between [188]Re-HYNIC-4B4-treated and control mice.

DISCUSSION

Radiolabeled peptides provide valuable treatment options for patients with metastatic cancers when standard therapies do not exist or are not effective. Results from preclinical and clinical multi-center studies have shown an effective therapeutic response when using radiolabeled somatostatin analogs to treat receptor-positive tumors.[7] There are also data on intratumoral administration of [188]Re-labeled somatostatin analog RC160 in melanoma patients with solitary skin metastases, which resulted in complete ablation of small (<1 cm in diameter) tumors.[21] Hence, there are clinical precedents for the potential usefulness of radiolabeled peptides in cancer therapy.

Most melanomas are pigmented by the presence of melanin, some of which is extracellular as a result of cellular turnover. Thus, extracellular melanin presents a promising target for drugs carrying a cytotoxic "payload" of radiation, if such therapies spare other melanotic tissues. A variety of substances, such as melanin binders, melanin precursors, and binders to melanogenesis-related proteins (MRPs), could potentially serve as "delivery vehicles" of radionuclides for the treatment of melanoma (reviewed in Dadachova and Casadevall[22]). One of the MRPs, a melanocyte-stimulating hormone (MSH) receptor, is currently under investigation with the effort directed primarily at the synthesis of metal-cyclized alpha-MSH peptide analogs, such as Re-(Arg(11))CCMSH.[23] Very recently the first therapy with [188]Re-(Arg(11))CCMSH in B16/F1 murine melanoma and TXM13 human melanoma-bearing mouse models was reported to result in the reduction of the tumor growth rate and prolonged survival in the treatment groups.[24] However, the safety of such treatment toward normal melanized tissues is unclear as relevant toxicity studies have not been reported.

We have recently established the feasibility of targeting melanin released from dead melanoma cells in tumors with radiolabeled melanin-binding antibodies to achieve a therapeutic effect.[6] In normal tissues, melanin is contained intracellularly in melanosomes. However, as melanoma tumors have rapid cell turnover, there appears to be significant tissue stores of extracellular melanin, which could be targeted by melanin-binding compounds. In contrast to conventional tumor antigens, melanin is insoluble, resistant to degradation, and can be expected to accumulate in targeted tissues. Hence, melanin may be a par-

ticularly attractive target because the efficacy of therapy could increase with each subsequent treatment cycle, as cells are killed and more melanin is released into the extracellular space. In contrast to melanin-binding mAbs, peptides have significantly lower molecular mass, which imply the possibility of delivering radionuclides deeper and more uniformly into the tumor during therapy.

Melanin-binding decapeptide 4B4 bound only to nonviable melanoma cells, as determined by immunofluorescence. This observation is consistent with the inaccessibility of intracellular melanin in live cells and suggests specificity for tumors with significant cell turnover where melanin would be released extracellularly.

In vitro cell binding showed that radiolabeled [188]Re-HYNIC-4B4 bound to both MNT1 human melanoma cells that were highly pigmented with eumelanin,[15] and to the lightly pigmented SK-MEL-28 human melanoma cells that were pigmented with pheomelanin.[25] HPLC studies of melanin from MNT1 and SK-MEL-28 melanoma cells revealed similar products of oxidative degradation, though their ratio was different. Thus, melanin-binding peptides can be used for targeting melanins of various types. This observation is important for metastatic melanoma—as both types of melanins are found in melanomas—but eumelanin is the predominant pigment in primary tumors, whereas pheomelanin is associated with progression of the disease.[26]

Administration of radiolabeled melanin-binding peptide to MNT1 tumor-bearing mice induced a therapeutic effect, despite a modest uptake of peptide by the tumor (4%–5% ID/g). Administration of [188]Re-HYNIC-4B4 significantly slowed tumor growth, whereas the estimated dose delivered to the tumor by the 1-mCi dose was only 300 cGy. An explanation may be that some biological effect of the peptide, as well as that deep penetration of the small peptide into the tumor and "cross-fire" irradiation of distant cells by the energetic beta-emissions of [188]Re, provided relatively homogenous irradiation of the tumor. In fact, we have recently demonstrated that a dose of 330 cGy can be effective in breast tumors treated with ^{18}F-FDG, which also distributes in the tumors relatively homogeneously.[27] The therapeutic effect of [188]Re-HYNIC-4B4 was a result of its specific binding to melanin in the tumor. Repeated doses of [188]Re-HYNIC-4B4 had a more profound effect on tumor growth than a single dose, suggesting the potential effectiveness of multiple administrations of radiolabeled peptide. Treatment of smaller tumors (0.3–0.4 cm in diameter) in comparison to larger ones (0.5–0.7 cm) was more effective. The dependence of the efficacy of treatment with radiolabeled peptides on the size of the tumor has been reported for (^{90}Y-DOTA)(0),Tyr(3)]octreotide treatment of somatostatin receptor-positive rat pancreatic CA20948 tumors in Lewis rats.[28]

There was no histological evidence for kidney toxicity, despite a high kidney uptake of [188]Re-HYNIC-4B4. Clinical trials of radiolabeled peptides suggest that the risk of nephrotoxicity is a function of such characteristics of the peptide molecule as its mass, charge, and clearance pathways, as well as of the chemical and physical characteristics of the radionuclide.[19] In this regard, [188]Re, with its relatively short half-life (16.9 hours versus 2.8 days for ^{90}Y), may have advantages over ^{90}Y used so far in most of the patient trials with radiolabeled peptides. Also, coadministration of positively charged amino acids might reduce the kidney uptake of [188]Re-HYNIC-4B4. Evaluation of potential toxicity to normal melanized tissues is important for any melanin-targeting pharmaceutical, especially those with small molecular mass, which can potentially allow the peptide to penetrate membranes of melanocytes. In this regard, melanin precursors, such as 4-S-cysteaminylphenol (4-S-CAP), that have demonstrated antimelanoma activity also caused depigmentation of follicular melanocytes in C57BL black mice,[29] and iodobenzamides have been found to colocalize with pigmented cells in the eyes and the skin.[30] The absence of toxicity of [188]Re-HYNIC-4B4 toward eyes and melanized skin in C57BL6 mice is consistent with the inability of [188]Re-HYNIC-4B4 to penetrate intact cell membrane, probably owing to its positive charge. We also did not observe any toxic effects of [188]Re-HYNIC-4B4 on the cells in substantia nigra in C57BL6 mice. Though it can be argued that neuromelanin is present in mice in limited amounts,[31] the absence of histologically apparent toxicity to neurons and glial cells, combined with the low uptake of [188]Re-HYNIC-4B4 in the brain and absence of changes in behavior of [188]Re-HYNIC-4B4-treated mice, suggest that this approach may prove to be safe.

CONCLUSIONS

In conclusion, we report promising therapeutic results in a mouse model of human melanoma

with a melanin-binding decapeptide radiolabeled with [188]Re. The treatment was not toxic to normal melanotic tissues and was tolerated by the kidney. Combination therapy using melanin-binding peptides and antibodies could provide significant advantages over either modality, given the differences in the serum half-life and tissue penetration of these molecules.

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REFERENCES

1. Rigel DS. Malignant melanoma: Incidence issues and their effect on diagnosis and therapy in the 1990s. *Mayo Clin Proc* 1997;72:367.
2. Grin-Jorgensen CM, Rigel DS, Friedman RJ. The world-wide incidence of malignant melanoma. In: Balch CM, Houghton AN, Milton GW, Sober AJ, Soong SJ, eds. *Cutaneous Melanoma, Ed. 2*, Philadelphia: J.B. Lippincott Co., 1992:27.
3. Liu T, Soong SJ. Epidemiology of malignant melanoma. *Surg Clin N Am* 1996;76:1205.
4. Sun W, Schuchter LM. Metastatic melanoma. *Curr Treat Options Oncol* 2001;2:193.
5. Mandara M, Nortilli R, Sava T, et al. Chemotherapy for metastatic melanoma. *Exp Rev Anticancer Ther* 2006;6: 121.
6. Dadachova E, Nosanchuk JD, Shi L, et al. Dead cells in melanoma tumors provide abundant antigen for targeted delivery of ionizing radiation by a monoclonal antibody to melanin. *Proc Natl Acad Sci USA* 2004;101: 14865.
7. Krenning EP, Kwekkeboom DJ, Valkema R, et al. Peptide receptor radionuclide therapy. *Ann NY Acad Sci* 2004;1014:234.
8. Blok D, Feitsma RI, Vermeij P, et al. Peptide radiopharmaceuticals in nuclear medicine. *Eur J Nucl Med* 1999;26:1511.
9. Nosanchuk JD, Valadon P, Feldmesser M, et al. Melanization of *Cryptococcus neoformans* in murine infection. *Mol Cell Biol* 1999;19:745.
10. Rosas AL, Nosanchuk JD, Gomez BL, et al. Isolation and serological analyses of fungal melanins. *J Immunol Meth* 2000;244:69.
11. Nosanchuk JD, Casadevall A. Cellular charge of *Cryptococcus neoformans*: Contributions from the capsular polysaccharide, melanin, and monoclonal antibody binding. *Infect Immun* 1997;65:1836.
12. Blower PJ, Clark K, Link EM. Radioiodinated methylene blue for melanoma targeting: Chemical characterisation and tumour selectivity of labelled components. *Nucl Med Biol* 1997;24:305.
13. Abrams MJ, Juweid M, ten Kate CI, et al. Technetium-99m-human polyclonal IgG radiolabeled via the hydrazinonicotinamide derivative for imaging focal sites of infection in rats. *J Nucl Med* 1990;31:2022.
14. Westlin J-E, Janson ET, Ahlström H, et al. Scintigraphy using a 111-In-labeled somatostatin analogue for localization of neuroendocrine tumors. *Antibody, Immunoconj Radiopharm* 1992;5:367.
15. Kushimoto TA, Basrur V, Valencia J, et al. A model for melanosome biogenesis based on the purification and analysis of early melanosomes. *Proc Natl Acad Sci USA* 2001;98:10698.
16. Ito S, Fujita K. Microanalysis of eumelanin and pheomelanin in hair and melanomas by chemical degradation and liquid chromatography. *Anal Biochem* 1985;144:527.
17. Wakamatsu K, Ito S. Advanced chemical methods in melanin determination. *Pigment Cell Res* 2002;15:174.
18. Hui TE, Fisher DR, Kuhn JA, et al. A mouse model for calculating cross-organ beta doses from yttrium-90-labeled immunoconjugates. *Cancer* 1994;73(Suppl):951.
19. Lambert B, Cybulla M, Weiner SM, et al. Renal toxicity after radionuclide therapy. *Radiat Res* 2004;161: 607.
20. Rogers DC, Fisher EM, Brown SD, et al. Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. *Mamm Genome* 1997;8:711.
21. Bender H, Guhlke S, Ezzidin S, et al. Local ablation of solitary metastases of melanoma by [188]Re labeled somatostatin analogue RC160. [abstract] *J Nucl Med* 2001;42(Suppl):312.
22. Dadachova E, Casadevall A. Melanin as a potential target for radionuclide therapy of metastatic melanoma. *Future Oncol* 2005;1:541.
23. Miao Y, Whitener D, Feng W, et al. Evaluation of the human melanoma targeting properties of radiolabeled alpha-melanocyte stimulating hormone peptide analogues. *Bioconjug Chem* 2003;14:1177.
24. Miao Y, Owen NK, Fisher DR, et al. Therapeutic efficacy of a [188]Re-labeled alpha-melanocyte-stimulating hormone peptide analog in murine and human melanoma-bearing mouse models. *J Nucl Med* 2005;46:121.
25. Rice L, Wainwright M, Phoenix DA. Phenothiazine photosensitizers: III. Activity of methylene blue derivatives against pigmented melanoma cell lines. *J Chemother* 2000;12:94.
26. Hearing V. The melanosome: The perfect model for cellular responses to the environment. *Pigment Cell Res* 2000;13(Suppl 8):23.

27. Moadel RM, Weldon RH, Katz EB, et al. Positherapy: Targeted nuclear therapy of breast cancer with ^{18}F-2-deoxy-2-fluoro-D-glucose (^{18}F-FDG). *Cancer Res* 2005;65:698.

28. de Jong M, Breeman WA, Bernard BF, et al. Tumor response after [(90)Y-DOTA(0),Tyr(3)]octreotide radionuclide therapy in a transplantable rat tumor model is dependent on tumor size. *J Nucl Med* 2001; 42:1841.

29. Yukitake J, Otake H, Inoue S, et al. Comparison of *in vivo* antimelanoma effect of enantiomeric alpha-methyl- and alpha-ethyl-4-S-cysteaminylphenol. *Melanoma Res* 2004;14:115.

30. Chehade F, De Labriolle-Vaylet C, Michelot J, et al. Distribution of I-BZA (N-2-diethylaminoethyl-4-iodobenzamide) in grafted melanoma and normal skin: A study by secondary ion mass spectroscopy (Noisy-le-grand). *Cell Mol Biol* 2001;47:529.

31. Lindquist NG, Larsson BS, Lyden-Sokolowski A. Autoradiography of [14C]paraquat or [14C]diquat in frogs and mice: Accumulation in neuromelanin. *Neurosci Lett* 1988;93:1.