Phage Display Library Derived Peptides that Bind to Human Tumor Melanin as Potential Vehicles for Targeted Radionuclide Therapy of Metastatic Melanoma

Robertha C. Howell,[†] Ekaterina Revskaya,[†] Valeria Pazo,[‡] Joshua D. Nosanchuk,[§,||] Arturo Casadevall,[§,||] and Ekaterina Dadachova*[,†,§]

Department of Nuclear Medicine, Department of Microbiology and Immunology, and Department of Medicine, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461, Jacobi Medical Center, Bronx, New York 10461. Received October 22, 2006; Revised Manuscript Received July 31, 2007

Metastatic melanoma remains an incurable disease, and there is a great need for novel therapeutic modalities. We have recently identified melanin as a target for radionuclide therapy of melanoma and demonstrated the feasibility of this approach using a 188-rhenium (^{188}Re)-radiolabeled melanin-binding decapeptide to fungal melanin known as 4B4. Although the results indicated that radiolabeled melanin-binding decapeptide had activity against melanoma, that peptide also manifested high kidney uptake and this might become a concern during clinical trials. We hypothesized that by identifying peptides with different amino acid composition against tumor melanin we might be able to decrease their kidney uptake. Using the Heptapeptide Ph.D.-7 Phage Display Library, we identified three heptapeptides that bind to human tumor melanin. These peptides were radiolabeled with ^{188}Re via HYNIC ligand, and their comprehensive biodistribution in A2058 human metastatic melanoma tumor-bearing nude mice was compared to that of ^{188}Re-4B4 decapeptide. While tumor uptake of heptapeptides was quite similar to that of ^{188}Re-4B4 decapeptide, there was dramatically less uptake in the kidneys at both 3 h (6% ID/g vs 38%) and 24 h (2% ID/g vs 15%) postinjection. Administration of one of the generated heptapeptides, ^{188}Re-HYNIC-AsnProAsnTrpGlyProArg, to A2058 human metastatic melanoma-bearing nude mice resulted in significant retardation of the tumor growth. Immunofluorescence showed that in spite of their relatively small size heptapeptides were not able to penetrate through the membranes of viable melanoma cells and bound only to extracellular melanin, which provides assurance that they will be safe to healthy melanin-containing tissues during radionuclide therapy. Thus, these heptapeptides appear to have potentially significant advantages for targeted therapy of melanoma relative to existing melanin-binding peptides.

INTRODUCTION

The incidence of melanoma is rising (*1*), and it is an important cause of cancer among young patients (30–50 years). This disease currently affects ~40 000 new patients each year in the US, and there are an estimated 100 000 cases worldwide (*2, 3*). Metastatic melanoma is almost always fatal (*4*) with the 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 for this disease in the past 25 years (*5*).

Most melanomas are pigmented by the presence of melanin. Some melanomas are called "amelanotic" because they are not black or darkly pigmented. However, even amelanotic melanomas contain some melanin (*6, 7*), which makes this pigment a convenient target for the development of radionuclide therapy of metastatic melanoma. The multiplicity of compounds which could potentially serve as "delivery vehicles" of radionuclides to melanoma tumors can be divided into three groups: molecules that bind to melanin, molecules that are melanin precursors, and molecules that bind to melanogenesis-related proteins (reviewed in ref (*8*)).

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 (*9*). Later, we expanded this approach to peptides by performing experimental melanoma therapy with 188-rhenium (^{188}Re)-labeled fungal melanin-binding decapeptide 4B4 (*10*) and carried out a comprehensive safety evaluation of this treatment (*11*). The results indicated that radiolabeled melanin-binding peptides have activity against melanoma and that these reagents could be potentially useful against this tumor. Peptides are attractive antitumor agents because they are easily synthesized, are nonimmunogenic, provide rapid clearance from the circulation, and penetrate tissues more thoroughly than larger molecules such as antibodies (*12*). On the other hand, peptides are rapidly degraded by the enzymes in the bloodstream and lack the effector functions conferred by an antibody constant region. The problem of rapid clearance can be ameliorated somewhat by synthesis using D-amino acids, but there is always a concern that peptides can bind to kidney cells and produce nephrotoxicity. This is a particular concern for melanin-binding peptides, because these are usually cationic (*10*), and thus can bind to the negatively charged kidney basement membrane. Clinical trials of radiolabeled peptides suggest that the risk of nephrotoxicity is a function of such characteristics of a peptide molecule as its mass, charge, and clearance pathways as well

* Ekaterina Dadachova PhD, Department of Nuclear Medicine, 1695A Eastchester Road, Bronx, NY 10461, Ph: 718-405-8485; FAX: 718-405-8457; E-mail: edadacho@aecom.yu.edu.

[†] Department of Nuclear Medicine,Albert Einstein College of Medicine of Yeshiva University.

[‡] Jacobi Medical Center.

[§] Department of Microbiology and Immunology, Albert Einstein College of Medicine of Yeshiva University.

[||] Department of Medicine, Albert Einstein College of Medicine of Yeshiva University.

10.1021/bc060330u CCC: $37.00
Published on Web 10/02/2007

as the chemical and physical characteristics of the radionuclide (*13*). When radiolabeled fungal melanin-binding peptides were used for experimental melanoma therapy, they showed high kidney uptake (*11*). Although there was no long-term nephrotoxicity, this issue could become a concern during clinical trials. Thus, there is a clear need to improve melanin-binding peptides by decreasing their kidney uptake. Here, we describe the isolation and characterization of melanin-binding peptides from peptide libraries with dramatically reduced kidney-binding capacity.

EXPERIMENTAL PROCEDURES

Reagents and Cells. The Ph.D.-7 Phage Display library Kit including the Heptapeptide Phage Display Library, −28 gIII sequencing primer, −96 gIII sequencing primer, and *E. coli* ER2738 host strain were purchased from New England Biolabs. The library has complexity in excess of two billion independent clones, which is sufficient to contain most if not all of the $20^7 = 1.28 \times 10^9$ possible heptapeptide sequences. The Detection Module Recombinant Phage Antibody System used to quantify bound phage was purchased from Amersham Biosciences. QIAPrep Spin M13 Prep columns were purchased from Qiagen. HRP-labeled anti-M13 monoclonal conjugate was purchased from GE Healthcare. HRP-labeled Streptavidin for quantification of the biotinylated peptides and subsequent binding strength determination was purchased from Fisher Scientific. Highly pigmented human melanoma cells MNT1 (*14*) were a gift from Dr. V. Hearing (NIH, Bethesda, MD). Human metastatic pigmented melanoma cells A2058 cells (ATCC, Mannassas, VA) were grown in Dulbecco's Modified Eagle's Medium with 4 mM L-glutamine, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate. This medium was supplemented with a 10% fetal bovine serum and 5% penicillin–streptomycin solution. Cells were grown at 37 °C in 75 cm^2 cell culture flasks. Subculturing was done at 80–95% confluence at subcultivation ratios of 1:6 and 1:12. Cell layers were dispersed with 0.25% (w/v) Trypsin–EDTA (1×). The cell line Hep 3B2.1–7 (*Homo sapiens* hepatatocellular carcinoma) was purchased from ATCC (cat no HB-8064). Cells were grown routinely in Eagle's Minimum Essential Medium (EMEM) (ATCC) containing 10% FBS (Sigma) at 37 °C in 5% CO_2 incubator.

Preparation of Plates for Melanin-Specific ELISA. Melanin from MNT-1 human melanoma cells was purified as in ref (*11*), and suspended at 2 mg/mL concentration in deionized H_2O. Approximately 150 μL was added to each well of 96 well plates and the solution gently agitated overnight. The melanin-coated wells were then baked at 80 °C for ½ h and blocked with 2% dry milk for 1 h followed by 5 mg/mL BSA in deionized H_2O.

Panning Procedure. Ten microliters of the original phage library (New England Biolabs) was diluted to 100 μL with TBST (TBS + 0.1% [v/v] Tween-80) and the resulting 110 μL pipeted onto one melanin-coated well. The plate was incubated at room temperature for 1 h with gentle agitation. The phages were then discarded and the well washed, to remove nonbinders, 10 times with TBST and excess liquid blotted after each wash. The bound phage was eluted with 0.2 M glycine–HCl (pH 2.2) in 1 mg/mL BSA by rocking the plate gently at room temperature for 10 min. The eluate was pipeted into a microcentrifuge tube, neutralized with 15 μL of 1 M Tris–HCl (pH 9.1) and titered onto LB/IPTG/Xgal plates to determine the concentration of the phage eluate. It was amplified by incubating at 37 °C with vigorous shaking with an early log phase ER2738 culture for 4.5 h. Purification was performed by first centrifuging the culture at 9000 rpm at 4 °C to pellet the ER2738 cells. The supernantant was removed and treated with 1/6 equivalent volume of PEG/NaCl. After cooling overnight at 4 °C, the resulting suspension was again centrifuged at 9000 rpm. The resulting pellet was collected and resuspended in 1 mL TBS. Further centrifugation pelleted residual cells, and 80% of the suspension was collected. One-sixth of equivalent volume of PEG/NaCl was added and the solution incubated on ice for 1 h. The suspension was microcentrifuged at 4 °C for 20 min and the supernatant removed and discarded. The pellet was resuspended in 200 μL TBS. The concentration of the phage was determined by titering onto LB/IPTG/Xgal plates. The process was repeated twice using the obtained eluate (volume-corrected to yield 2×10^{11} phages) for panning with reduced panning time and increasing Tween concentration).

Isolating Clones. The final eluate was plated onto a LB/IPTG/Xgal plate to yield approximately 50 individual clones. Twenty-four clones from the plate were chosen at random and amplified by incubation with 1 mL of early log phase ER2738 culture. The resulting amplified eluates were purified as described above and each plated titered and plated again to yield a plate with approximately 20 individual plaques. Four plaques from each plate were amplified in 1 mL of early log phase ER2378 and the 4 resulting cultures pooled. The cultures were centrifuged at 9000 rpm for 10 min and 80% of the supernatant was removed. The removed supernatant was centrifuged again and 80% of the supernatant removed for extraction of phage DNA. The phage DNA was extracted by elution on QIAPrep Spin M13 Prep. The resulting DNA was amplified by standard molecular biology techniques. Twenty-four phages were selected for DNA sequencing, which was performed at the AECOM DNA Sequencing Core Facility.

Selection of Phages with the Best Melanin-Binding Properties. The phages with the best melanin-binding properties were selected by analyzing the concentration of the phages in the wells coated with melanin as compared to the concentrations in the noncoated wells. To obtain the concentrations of phage, the wells were first treated with an HRP/anti-M13 monoclonal antibody conjugate over a period of 1 h until binding had occurred. Subsequently, the excess conjugate was removed and the wells were washed. Quantitative amounts of HRP substrate (ABTS) and peroxide were added, and after a period of 10 to 30 min, changes in the color of the solution were analyzed for absorbance at 492 nm. The changes in absorbance at 492 nm are directly related to the amount of HRP enzyme present and as such to the amount of bound phage.

Synthesis of Biotinylated and HYNIC-Conjugated Heptapeptides. The best phage binders were selected as above, and the displayed heptapeptides were synthesized from L-amino acids with biotin at the N terminus by Peptides International (KY) for immunofluorescence experiments. To minimize enzymatic degradation *in vivo*, for radiolabeling purposes the same heptapeptides were synthesized by Anaspec from D-amino acids with a hydrazinonicotinamide (HYNIC) chelate appended to the N terminus. An additional heptapeptide, AspGlyAlaTrpMetGlyAla, whose sequence was derived from the sequence of previously described irrelevant decapeptide P601G AspGlyAlaSerTyrSer-TrpMetTyrGlyAla (*10*), was also synthesized in a biotinylated form and with HYNIC ligand as a random control. Biotinylated and HYNIC-conjugated heptapeptides were analyzed by HPLC on a VYDAC 250 mm analytical column equipped with a supporting guard column using the following gradient (A, 0.05% TFA in H_2O; B, 0.05% TFA in acetonitrile; 10–60% A over 50 min at 1 mL/min). The resulting HPLC confirmed the HPLC data obtained from the manufacturers.

Immunofluorescence of A2058 Melanoma Cells. The binding of the biotinylated heptapeptides to A2058 melanoma cells *in vitro* was analyzed by immunofluorescence as in ref (*10*). Approximately 10^6 melanoma cells were blocked for nonspecific binding by incubation in SuperBlock (Pierce, Rockford, IL) for 1 h at 37 °C. Biotinylated heptapeptides were then incubated with the cells for 1 h followed by addition of streptavidin

conjugated to fluorescein isothiocyanate (FITC). The slides were viewed with an Olympus AX70 microscope (Melville, NY) equipped with a FITC filter. Irrelevant biotinylated heptapeptide AspGlyAlaTrpMetGlyAla was used as a negative control.

Radiolabeling of Heptapeptides. The ^{188}Re-perrhenate eluted in saline from ^{188}W/^{188}Re generator was reduced by adding $SnCl_2$ (20 μL of 20 mg/mL 0.1 M HCl) to a suspension of 20 mg sodium glucoheptonate and 400 μL ^{188}Re-perrhenate. The resulting suspension was thoroughly mixed and incubated for 1 h at 37 °C. The reduction was assessed by SG-ITLC in dry acetone. One hundred microliters of a freshly prepared solution of 2 mg HYNIC–heptapeptide dissolved in 1 mL NH_4OAc (0.15 M, pH 4) was placed in a 1.5 mL microcentrifuge tube. To this solution was added approximately 600 μCi of reduced ^{188}Re (up to 400 μL). The resulting solution was incubated in the dark at room temperature for 1 h. The extent of radiolabeling was checked by SG-ITLC developed in 0.15 M NH_4OAc, pH 4. If necessary, the ^{188}Re-HYNIC–heptapeptides were purified from unreacted ^{188}Re on the reverse-phase C18 column. The column was primed with methanol followed by equilibration with NH_4OAc. The sample was then loaded onto the column and the column washed with 5 × 250 μL 0.125 M NH_4OAc followed by 2 × 250 μL 0.5 M NH_4OAc. The radiolabeled HYNIC–heptapeptides were eluted with 2 × 3 mL MeOH. The methanol was removed by evaporation under a nitrogen stream. SG- ITLC (0.15 M NH_4OAc, pH 4) was used to confirm the radiochemical purity of ^{188}Re-HYNIC–heptapeptides.

Binding of ^{188}Re-HYNIC–Heptapeptides to MNT1 and A2058 Melanoma Cells. To investigate the binding of radiolabeled heptapeptides to MNT1 human pigmented melanoma cells, the whole cells in increasing amounts from 1.3 to 15 million per sample were placed into the microcentrifuge tubes preblocked with 1% BSA to prevent nonspecific protein binding in 0.5 mL PBS, and radiolabeled heptapeptides in 0.19 nM concentration were added. In blocking experiments, the cells were preblocked with 10 μg per sample of the respective "cold" heptapeptide. The samples were incubated at 37 °C for 1 h. The suspensions were centrifuged at 1500 rpm for 20 min to pellet the cells, the supernatant was transferred into a different tube, and the radioactivity of the cells and of the supernatant was then counted in a gamma counter. For measuring the binding of heptapeptides to the lysed cells, the experiments were conducted as above, but MNT1 or A2058 cells were osmotically lysed immediately before the binding experiment. For estimation of association equilibrium constants K_a, the radiolabeled heptapeptides were added in increasing amounts (from 0.053 nM to 0.53 nM) to the lysed MNT1 or A2058 lysed cells (5 × 10^6 cells per centrifuge tube). After 1 h incubation at 37 °C, the activity in the tubes was counted in a gamma counter, the cells were collected by centrifugation, and the pellets were counted. Scatchard analysis was used to compute the heptapeptide association equilibrium constants K_a as in ref (*15*).

Biodistribution of ^{188}Re-HYNIC–Heptapeptides. All animal studies were carried out in accordance with the guidelines of the Institute for Animal Studies at the Albert Einstein College of Medicine. For biodistribution in tumor-bearing mice, melanoma tumors were induced by injecting approximately 8 × 10^6 A2058 human melanoma cells in 100 μL of the cell-growing medium into the right flank of female nude mice. Ten days after implantation, the tumors reached 0.3–0.7 cm in diameter. To study the biodistribution of four different ^{188}Re-HYNIC–heptapeptides and ^{188}Re-HYNIC–4B4 decapeptide (*11*) as a control, 30 tumor-bearing mice were divided into 5 groups of 6 mice each. Each group was further subdivided into 2 groups of 3 mice each. Each radiolabeled peptide was administered to 2 groups of mice at a dosage of 40 μCi (50 μg) in 100 μL via the tail vein. After 3 h and 24 h, respectively, the first and second groups of each set of mice were sacrificed and internal organs and the tumor taken, blotted from blood, weighed, counted in a gamma counter, and percent Injected dose per gram calculated.

To prove the specificity of peptide uptake in melanoma tumors, the biodistribution of ^{188}Re-HYNIC-AsnProAsnTrpGlyProArg was studied in nude mice bearing two different xenografts—human hepatocellular carcinoma and A2058 metastatic melanoma. For this purpose, 8 × 10^6 A2058 human melanoma cells and 10^7 Hep 3B human hepatocellular carcinoma cells in 100 μL of the cell-growth medium were injected into the right and left flanks, respectively, of female nude mice. The biodistribution experiment was performed essentially as above except that only tumors, blood, and muscle were taken.

To investigate the influence of D-lysine coinjection on the uptake of ^{188}Re-HYNIC–heptapeptides in kidneys and other major organs, one group of six female CD-1 mice were injected with 40 μCi (50 μg) of ^{188}Re-HYNIC-ThrThrHisGlnPheProPhe in 100 μL via the tail vein, while another group received the same heptapeptide coinjected with D-lysine (400 mg/kg body weight). Three mice from each group were sacrificed at 3 and 24 h, and the biodistribution was performed as above.

Therapy of A2058 Tumor-Bearing Mice with ^{188}Re-HYNIC-AsnProAsnTrpGlyProArg Heptapeptide. The A2058 tumors were initiated in nude mice as described above. When the tumors reached 0.7–0.8 cm in diameter, the mice were randomized into four groups of six mice. Groups 1–3 received IP injections of 1.0 mCi ^{188}Re-HYNIC-AsnProAsnTrpGlyProArg heptapeptide, a matching amount of "cold" HYNIC-AsnProAsnTrpGlyProArg heptapeptide (0.2 μg), or 1.0 mCi ^{188}Re-HYNIC-4B4 decapeptide, respectively. Control group # 4 was left untreated. On day 7, the treatment of animals in groups 1–3 was repeated with the same doses. The size of the tumors was measured in three dimensions with calipers immediately before administration of peptides and on days 3, 6, 14, 17, 21, 25, 32, and 35. The tumor volume was calculated by multiplying the product of the three perpendicular diameters by 0.5.

Correlative Histology/Immunofluorescence of A2058 Melanoma Tumors. To establish the colocalization of heptapeptides with melanin in melanoma tumors, consecutive slides of A2058 melanoma tumors were stained with hematoxylin and eosin (H&E) or were incubated with the biotinylated AsnProAsnTrpGlyProArg heptapeptide for 1 h followed by addition of streptavidin conjugated to fluorescein isothiocyanate (FITC). The slides were viewed with an Olympus AX70 microscope (Melville, NY) equipped with a FITC filter. Irrelevant biotinylated heptapeptide AspGlyAlaTrpMetGlyAla was used as a negative control.

Scintigraphic Imaging. Two mice out of each group treated with radiolabeled peptides in therapy experiments were anesthetized with Isoflurane and scintigraphically imaged for 2 min on a Siemens gamma camera equipped with *ICON* image processing software.

Statistical Analysis. The nonparametric Wilcoxon rank sum test was used to compare organs uptake in biodistribution studies and tumor sizes in therapy studies. The Student's *t* test for unpaired data was employed to analyze differences in binding between heptapeptides. The differences were considered statistically significant when P values were <0.05.

RESULTS

Selection of Tumor Melanin-Binding Phages and Synthesis of Heptapeptides. Twenty-four unique peptides from 24 individually isolated clones were identified (Table 1). Although no consensus sequence was identified throughout these peptides, the sequence "aromatic"−*−* "aromatic" occurred in approximately 25% of the peptides (where * represents an

Table 1. Amino Acid Sequences of Peptides from Isolated M13 Clones[a]

Clone ID		AA Sequence	Single Letter Sequence	Features of Constituent Amino Acids in Peptides of Isolated Clones (aromaticity and charge)	
1	A8	ThrHisPheAlaThrMetPhe	THFATMF	*+*****	*aa***a
2	A4	LeuGlnProThrMetHisLys	LQPTMHK	*****++	*****a*
4	A9	PheLysProProAlaSerLeu	FKPPASL	*+*****	a******
5	A2	AlaGluPheAlaPheAsnPhe	AEFAFNF	*-*****	**a*a*a
6	B6	AsnAlaProSerArgAsnGln	NAPSRNQ	****+**	*******
7	B3	AsnProArgProLeuSerIle	NPRPLSI	**+****	*******
8	A6	SerHisTyrValAsnSerGln	SHYVNSQ	*+*****	*aa****
9	2C	GluGlyLeuHisGlnArgLys	EGLHQRK	***+*++	***a***
10	1A	HisProGlnHisAlaGlyGly	HPQHAGG	+**+***	a**a***
11	4B	HisThrSerHisLeuSerCys	HTSHLSC	+**+***	a**a***
12	*3A*	*TrpProSerPheProIlePro*	*WPSFPIP*	*******	*a**a***
13	3C	ThrProAlaLeuGlnPhePro	TPALQFP	*******	*****+*
14	2D	SerPheThrTrpAlaProAsp	SFTWAPD	******-	*a*a***
15	*2A*	*AlaProProTyrThrAlaTyr*	*APPYTAY*	*******	***a**a
16	4C	SerTrpProSerHisValPro	SWPSHVP	****+**	*a**a**
17	1D	HisProAsnLeuLeuSerPro	HPNLLSP	+******	a******
18	4A	AsnGluSerProProAsnPro	NEYPPNP	*-*****	*******
19	3D	AsnProAsnTrpGlyProArg	NPNWGPR	******+	***a***
20	1B	ThrArgArgGluThrProAla	TRRETPA	*++_***	*******
21	4D	HisThrThrHisHisArgAsn	HTTHHRN	+**+++*	a**aa**
22	2B	ValGluThrTrpArgProAsn	VETWRPN	*-**+**	***a***
23	3B	GluArgPheIleAspThrGln	ERFIDTQ	-+**-**	**a****
24	*1C*	*ThrThrHisGlnPheProPhe*	*TTHQFPF*	**+***	**a*a*a

[a] The peptides with the best binding are highlighted in green, "a" represents an aromatic amino acid, +/− the charge of the amino acid, and * an irrelevant amino acid.

irrelevant amino acid and "aromatic" an aromatically based amino acid). More specifically, the aromatic amino acids were represented by histidine, a positively charged amino acid, in 50% of these cases. Histidine was also found extensively throughout the majority of the other peptide sequences. The best tumor melanin-binding phages were identified by comparing the binding of the phages to the melanin-coated well versus the non-melanin-coated wells (first round of selection, Figure 1a). From this group of clones, 8 of the best binders were chosen for further study (selected phages are highlighted in green in Table 1). These phages were then subjected to the second round of selection by performing ELISA for fourfold serial dilutions of the phage starting from 1.2×10^{12} pfu/mL. Of 8 phages, the 3 best binding phages were identified on the basis of comparison of the binding curves obtained from the melanin-coated wells and the uncoated wells (Figure 1b). As a result, 3 heptapeptides—HisThrThrHisHisArgAsn, AsnProAsnTrp GlyProArg, and ThrThrHisGlnPheProPhe—identified as 4D, 3D, and 1C in Table 1, respectively, were synthesized in biotinylated form and HYNIC-conjugated form for further in vitro and in vivo evaluation.

Heptapeptides Binding To Melanoma Cells *in Vitro*. The binding of heptapeptides to A2058 melanoma cells in culture was studied by immunofluorescence. Figure 2a shows that the biotinylated HisThrThrHisHisArgAsn heptapeptide bound only to nonviable melanoma cells. Nonviable cells apparently released their melanin or had permeable cell membranes that allowed access of the peptide to melanin (Figure 2a, right panel). No binding was observed to viable cells with intact cell membranes (Figure 2a, left panel). Control biotinylated hep-

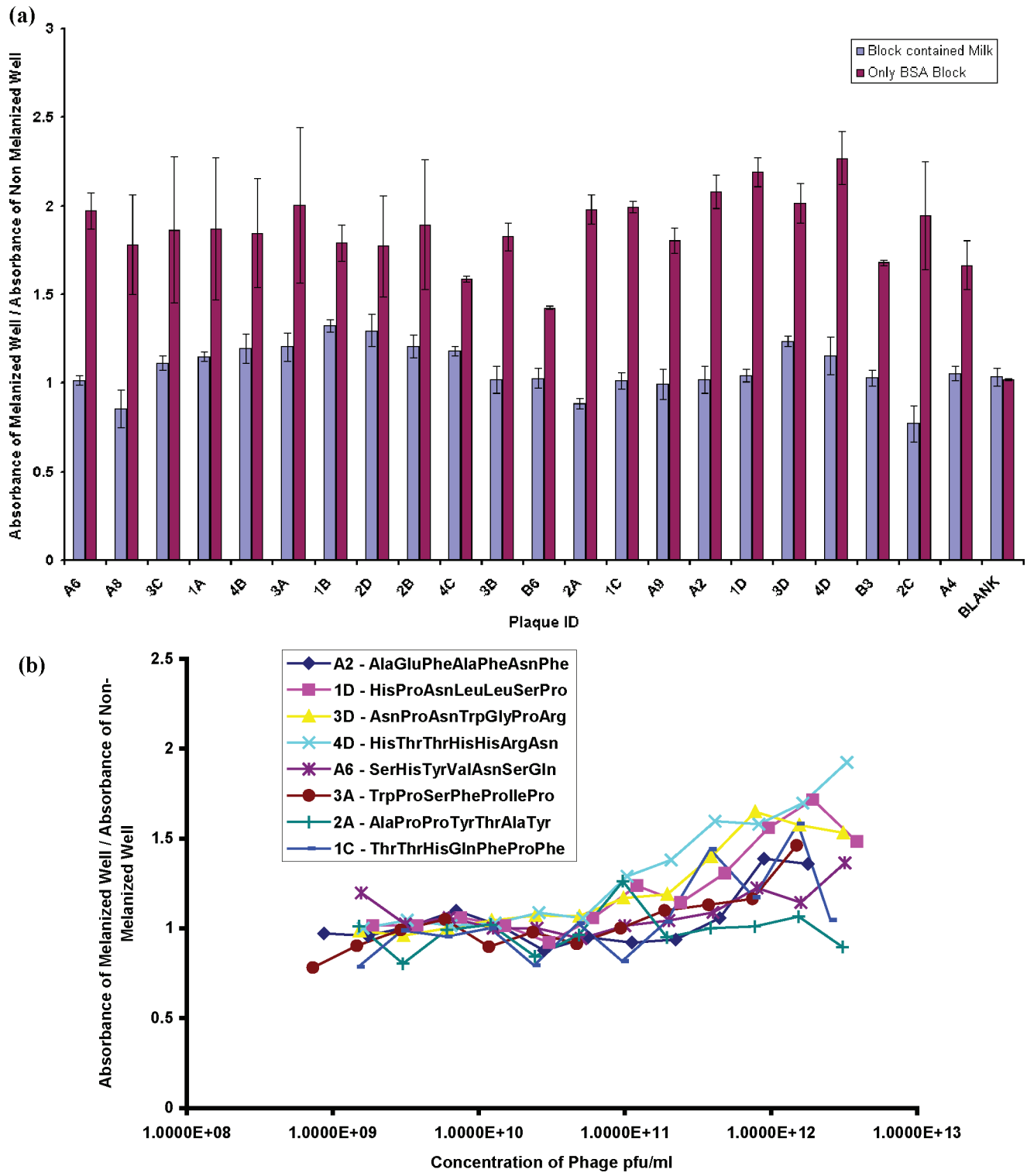

Figure 1. Selection of human tumor melanin binding phages from PhD-7 library. (a) First round of selection: plot of the absorbance ratios for the phages in melanized to nonmelanized wells. The wells were coated with melanin derived from MNT1 melanoma cells. Different blocking conditions were employed to ensure maximal blocking of nonspecific binding. (b) Second round of selection: binding curves resulting from ELISA of the best binding phages showing the ratio of the absorbance of the melanized to nonmelanized wells. Dilutions were fourfold starting at 1×10^{12} pfu/mL.

tapeptide AspGlyAlaTrpMetGlyAla did not bind to either viable or dead A2058 cells (results not shown).

Radiolabeling of HYNIC-Conjugated Heptapeptides. Labeling of HYNIC-conjugated heptapeptides with ^{188}Re resulted in an average of 92% yields for HisThrThrHisHisArgAsn, ThrThrHisGlnPheProPhe, AsnProAsnTrpGlyProArg, and AspGlyAlaTrpMetGlyAla heptapeptides allowing for their use in cell binding and biodistribution experiments without further purification.

Binding of Radiolabeled Heptapeptides To Mnt1 and A2058 Melanoma Cells. First, we investigated whether ^{188}Re-labeled AsnProAsnTrpGlyProArg, ThrThrHisGlnPheProPhe,

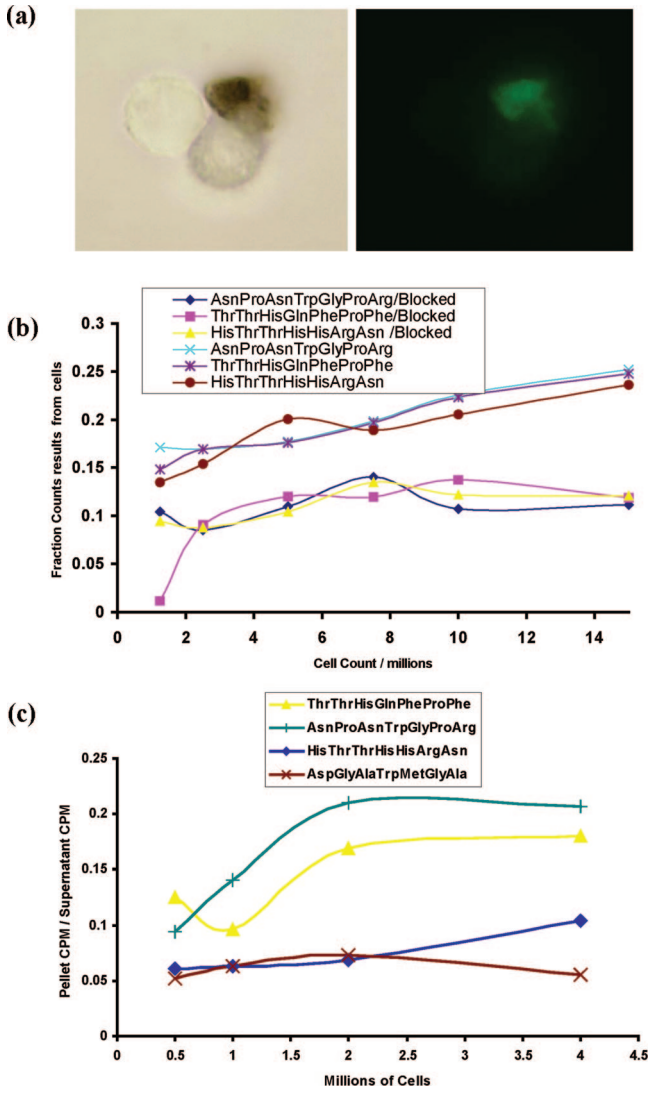

Figure 2. Binding of the heptapeptides to MNT1 and A2058 human melanoma cells. (a) Immunofluorescence image of biotinylated HisThrThrHisHisArgAsn heptapeptide binding to A2058 melanoma cells in culture. (b) Binding of heptapeptides to the whole MNT1 highly melanized melanoma cells. In blocking experiments, the MNT1 cells were preblocked with 10 μg per sample of the respective "cold" heptapeptide. (c) Binding of ^{188}Re-labeled HYNIC–heptapeptides to lysed A2058 cells.

and HisThrThrHisHisArgAsn heptapeptides would bind to whole highly melanized with eumelanin MNT1 melanoma cells (*11*), as melanin from those cells was used for selection of phages. The preparations of whole MNT1 cells contain approximately 10% nonviable cells (as per Trypan blue dye assay) in which melanin is accessible to the peptides. The binding to the MNT1 cells was proportional to the overall number of cells per sample and was decreased 2-fold by preblocking the cells with an excess of "cold" heptapeptides, proving that the binding of ^{188}Re-labeled peptides was specific (Figure 2b). Interestingly, the binding of all three of the above heptapeptides to MNT1 cells was very similar. The osmotic lysis of MNT1 released melanin from all cells in preparations, which increased the percentage binding to almost 100% (results not shown). Scatchard analysis of the binding to the lysed cells allowed estimation of the K_a for AsnProAsnTrpGlyProArg, ThrThrHisGlnPheProPhe, and HisThrThrHisHisArgAsn as $(7.1 \pm 0.5) \times 10^7$ M^{-1}. As A2058 metastatic melanoma cells are only slightly pigmented with pheomelanin, the binding of peptides to the lysed A2058 cells was significantly lower than binding to the

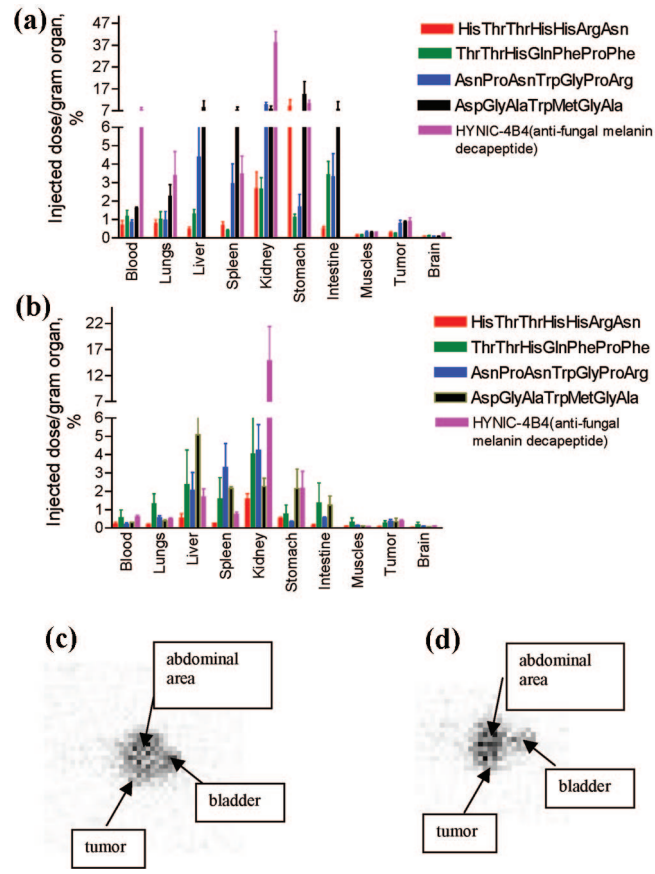

Figure 3. Biodistribution and scintigraphic imaging of ^{188}Re-labeled HYNIC–heptapeptides and ^{188}Re-labeled HYNIC–4B4 decapeptide in nude mice bearing A2058 human melanoma tumors (a) 3 h biodistribution post IV injection; (b) 24 h biodistribution post IV injection; (c) 1 h scintigraphic image of a mouse injected IP with ^{188}Re-HYNIC-AsnProAsnTrpGlyProArg; (d) 3 h scintigraphic image of a mouse injected IP with ^{188}Re-HYNIC-AsnProAsnTrpGlyProArg. The mice were imaged laying on their backs.

lysed MNT1 cells with the highest value of 20% observed for AsnProAsnTrpGlyProArg (Figure 2c). The unrelated control peptide AspGlyAlaTrpMetGlyAla practically did not bind to A2058 cells, while HisThrThrHisHisArgAsn binding reached only 10% at the high cell number (Figure 2c). The two best binders to the A2058 cell line, AsnProAsnTrpGlyProArg and ThrThrHisGlnPheProPhe, had K_a values of 1.5 and 1.1×10^7 M^{-1}, respectively.

Biodistribution and Scintigraphic Imaging of ^{188}Re-HYNIC–Heptapeptides in A2058 Melanoma Tumor-Bearing Mice. The biodistribution of AsnProAsnTrpGlyProArg, ThrThrHisGlnPheProPhe, and HisThrThrHisHisArgAsn heptapeptides and irrelevant AspGlyAlaTrpMetGlyAla was compared to that of fungal melanin-binding decapeptide 4B4 (Figure 3a,b). A dramatic difference in kidney uptake was observed between heptapeptides and 4B4 decapeptide at both 3 h (2.5–12% ID/g vs 38%) and 24 h (1.8–2.4% ID/g vs 15%) postinjection ($P = 0.001$). Melanin-specific heptapeptides showed greater stability *in vivo* than irrelevant heptapeptide AspGlyAlaTrpMetGlyAla or 4B4 decapeptide as judged by the lower stomach uptake of tumor melanin-specific heptapeptides ($P = 0.02$). Though the tumor to blood ratios were less than 1 for the heptapeptides used in biodistribution, for AsnProAsnTrpGlyProArg peptide tumor to blood ratio was almost 1 (0.92), which was a significant improvement in comparison to 4B4 decapeptide (tumor to blood ratio 0.12). Tumor uptake of both heptapeptides and decapeptide was modest at 0.5–1% ID/g at 3 h and decreased approximately 5-fold to 0.1–0.2% ID/g at

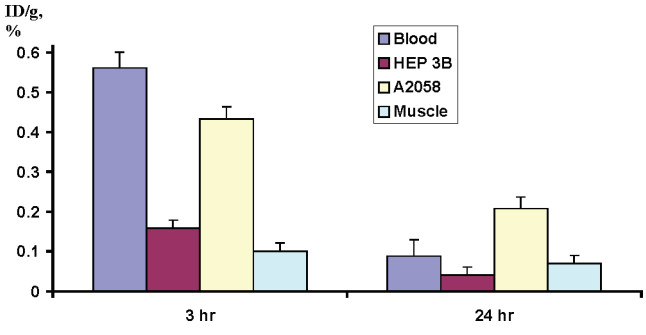

Figure 4. Comparative biodistribution at 3 and 24 h of ^{188}Re-HYNIC-AsnProAsnTrpGlyProArg heptapeptide in nude mice bearing two different xenografts: hepatocellular carcinoma and A2058 melanoma.

24 h. Importantly for therapy, however, the tumor to muscle uptake for these subcutaneous tumors was around 3. Overall, the best biodistribution pattern in terms of low kidney uptake, high stability, and relatively stable tumor uptake was observed for AsnProAsnTrpGlyProArg and ThrThrHisGlnPheProPhe heptapeptides, which was consistent with their higher binding to A2058 melanoma cells *in vitro*. In order to visualize tumor uptake, we imaged mice injected with the ^{188}Re-HYNIC-AsnProAsnTrpGlyProArg peptide on a gamma camera (Figure 3c,d). At both 1 and 3 h postinjection, most of the activity was localized to the abdominal cavity with practically no activity in the blood pool, thus confirming very fast clearance of the peptide from the blood. The tumor was visible at both 1 h (Figure 3c) and 3 h images (Figure 3d).

Biodistribution of ^{188}Re-HYNIC-AsnProAsnTrpGlyProArg in Nude Mice with Hepatocellular Carcinoma and A2058 Melanoma Xenografts. To confirm the specificity of heptapeptide uptake in melanoma tumors, the biodistribution of ^{188}Re-HYNIC-AsnProAsnTrpGlyProArg heptapeptide was also performed in mice bearing two different tumor xenografts on their flanks. The uptake of heptapeptide in A2058 melanoma tumors was 3 times higher than in hepatocellular carcinoma at 3 h postinjection and 5 times higher at 24 h (Figure 4).

Effect of D-Lysine Coinjection on Biodistribution of Heptapeptides. Co-injection of ThrThrHisGlnPheProPhe with D-lysine (400 mg/kg) did not reduce kidney uptake of the peptide. In fact, lysine coinjection was found to increase kidney uptake: At 3 h postinjection, there was 4 times more peptide in the kidneys of mice coinjected with D-lysine compared to control mice ($P = 0.003$) (Figure 5). Even after 24 h, the profound effect was still evident in the mice to whom D-lysine was administered compared to controls (3-fold difference).

Correlative Histology/Immunofluorescence of A2058 Melanoma Tumors. In order to establish the colocalization of heptapeptides with melanin in melanoma tumors, consecutive slides of A2058 melanoma tumors were stained with H&E or were incubated with the biotinylated AsnProAsnTrpGlyProArg heptapeptide. Biotin-AsnProAsnTrpGlyProArg localized in the melanin-rich areas of A2058 tumors (Figure 6a), while irrelevant control heptapeptide did not (results not shown).

Therapy of A2058 Tumor-Bearing Mice with ^{188}Re-HYNIC-AsnProAsnTrpGlyProArg Heptapeptide. To examine the effects of radiolabeled melanin-binding heptapeptides on A2058 tumors, we selected ^{188}Re-HYNIC-AsnProAsnTrpGlyProArg, as this peptide showed the best biodistribution pattern in terms of relatively high tumor uptake, low kidney uptake, and good stability *in vivo*. Three groups of six A2058 tumor-bearing nude mice with tumors of 0.7–0.8 cm in diameter were treated IP with (1) 2 × 1.0 mCi ^{188}Re-HYNIC-AsnProAsnTrpGlyProArg; (2) 2 × "cold" HYNIC-AsnProAsnTrpGlyProArg (0.2 μg each injection); or (3) 2 × 1.0 mCi ^{188}Re-HYNIC–4B4 decapeptide 7 days apart. Mice in the control group were left untreated. Two treatments with peptides were given because this regimen proved effective in the earlier study with ^{188}Re-HYNIC–4B4 decapeptide in MNT1 melanoma tumor-bearing mice (*11*). ^{188}Re-HYNIC–4B4 decapeptide was used as a control because of its success in treatment of MNT1 tumors (*11*) and comparability to ^{188}Re-HYNIC-AsnProAsnTrpGlyProArg uptake in A2058 tumors in this study. The A2058 tumors grew aggressively in the untreated group, and the last surviving mouse had to be sacrificed on day 35 because of the size of its tumor. Significantly slower ($P << 0.01$) tumor growth was observed in all treatment groups with ^{188}Re-HYNIC-AsnProAsnTrpGlyProArg slowing the tumor growth more efficiently ($P = 0.02$) than "cold" HYNIC-AsnProAsnTrp GlyProArg or ^{188}Re-HYNIC–4B4 decapeptide from day 25 posttreatment. Interestingly, "cold" HYNIC-AsnProAsnTrp GlyProArg was almost as efficient in slowing down tumor growth as ^{188}Re-HYNIC–4B4 decapeptide.

DISCUSSION

During the past decade, radiolabeled peptides that bind to different receptors on a variety of tumors have been investigated as potential therapeutic agents in both the preclinical and clinical settings (*16*). Preclinical studies of the radiolabeled peptides in melanoma treatment have concentrated on melanocyte-stimulating hormone (MSH) receptor with efforts directed primarily at the synthesis of metal-cyclized α-MSH peptide analogues such as Re-(Arg(11))CCMSH (*17*). 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 (*18*). We have recently demonstrated that ^{188}Re-labeled fungal melanin-binding decapeptide 4B4 was effective against experimental MNT1 human melanoma and was safe for normal melanized tissues (*11*). As no human melanin-binding peptides have been reported, for the proof-of-principle experiments targeting melanin in the tumors with radiolabeled melanin-binding peptides (*11*), we utilized the peptides that were originally generated to fungal melanin for purposes unrelated to cancer research (*10*). Although no nephrotoxicity was noted in those studies, the high kidney uptake of the 4B4 fungal melanin-binding peptide prompted the search for the peptides with lower kidney uptake. In this study, we generated the peptides to human melanin in an attempt to provide the structural solution to the problem of high kidney uptake.

During the past decade, phage display has been extensively used in cancer research (reviewed in ref (*19*)). Phage display can be utilized for the fast identification of biologically active ligands for molecules already determined to be important in cancer development, for creating a profile of peptide ligands for a particular cancer cell type, and for the identification of organ and tumor homing peptides. The limitations of the phage display approach are conformational differences between the peptides fused to phage coat proteins and chemically synthesized peptides, which can result in inferior binding of chemically synthesized peptides to their target when compared to the original phages (*20*).

The availability of the commercially available PhD-7 phage display library allowed us to generate heptapeptides that were subsequently selected for their tumor melanin-binding properties by ELISA. These heptapeptides were less aromatic and less positively charged than melanin-binding decapeptides recovered by screening on fungal melanin (*10*). Surprisingly, in spite of their small size, the heptapeptides were not able to penetrate through the membranes of viable A2058 human melanoma cells and bound only to the melanin in nonviable cells or released from the dead cells as demonstrated by immunofluorescence.

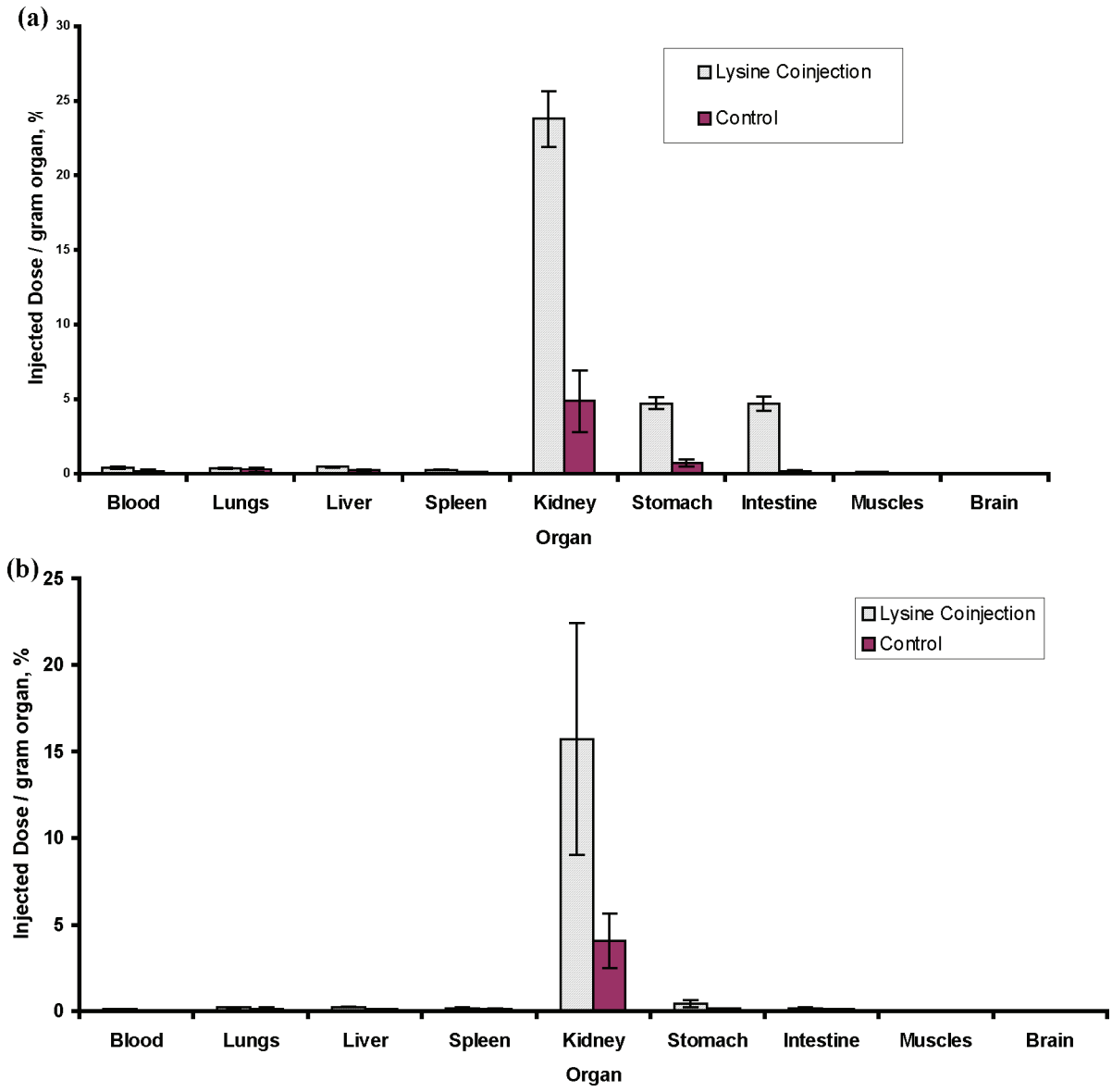

Figure 5. Comparative biodistribution of ^{188}Re-labeled HYNIC-ThrThrHisGlnPheProPhe heptapeptide in non-tumor-bearing CD-1 mice injected IV with peptide alone or coinjected with D-lysine (400 mg/kg body weight): (a) 3 h postinjection; (b) 24 h postinjection.

This result is encouraging, as the inability of heptapeptides to penetrate through cell membranes will minimize toxicity to healthy melanized cells during therapy.

When administered to tumor-bearing mice, both ^{188}Re-labeled heptapeptides and ^{188}Re-labeled decapeptide 4B4 exhibited tumor uptake in A2058 human metastatic melanoma tumors. This is encouraging, as the panel of heptapeptides was developed against black eumelanin from MNT1 melanoma tumors (*8*) and decapeptide 4B4 against black fungal melanin (*10*). In contrast, A2058 tumor cells are pigmented with pheomelanin, a yellow or reddish-brown melanin-type pigment. Both pheomelanin and eumelanin are negatively charged and share some structural similarity, with both types found in melanomas where eumelanin is the predominant pigment in primary tumors, while pheomelanin is associated with metastatic melanomas and progression of the disease (*21*). The predominance of pheomelanin in A2058 cells resulted in relatively low binding of heptapeptides to the tumors. Since melanoma is a very heterogeneous disease, melanin in the patients' tumors will be most likely represented by both eumelanin and pheomelanin, and even so called "amelanotic" melanomas do contain some melanin to ensure targeting with heptapeptides (*22, 23*). In addition, it is worthwhile to note that in a very recent study Newton et al. used phage display methodology to generate α-MSH peptide analogues for the purpose of imaging melanoma tumors (*24*) and observed relatively low uptake of peptides and phages in the tumors. The unrelated control peptide AspGlyAlaTrpMetGlyAla also showed some tumoral accumulation in our biodistribution study. However, it was unstable *in vivo*, which was manifested by high stomach, liver, and intestine uptake. Its high stomach uptake was most likely due to ^{188}Re-perrhenate presence resulting from *in vivo* decomposition of peptide. As pigmented cells can express sodium iodide symporter (NIS) (*25*), the uptake in the tumor seen for AspGlyAlaTrpMetGlyAla may actually be the binding of ^{188}Re-perrhenate to NIS. The specificity of heptapeptides for tumor melanin was proven by preferential uptake of ^{188}Re-HYNIC-AsnProAsnTrpGlyProArg in A2058 melanoma xenografts in mice bearing hepatocellular carcinoma and melanoma xenografts simultaneously and by colocalization of biotin-AsnProAsnTrpGlyProArg with melanin on tumor slides.

In our earlier studies, we estimated the number of nonviable cells in experimental melanoma tumors by subjecting the cells from melanoma tumors grown in nude mice to Trypan blue

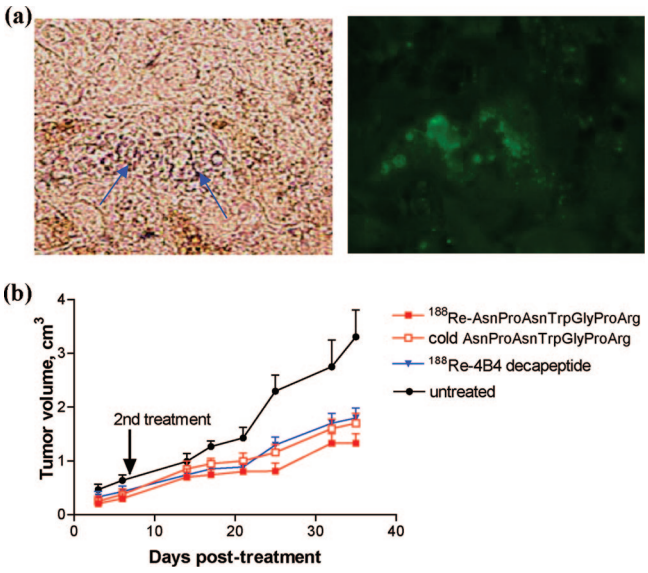

Figure 6. Colocalization of ^{188}Re-HYNIC-AsnProAsnTrpGlyProArg heptapeptide with melanin in melanoma tumors and therapy of A2058 tumor-bearing mice with the same heptapeptide. (a) Left: H&E stained A2058 melanoma tumor slide. Melanin deposits are shown with blue arrows. Right: immunofluorescence with biotinylated AsnProAsnTrp GlyProArg. (b) A2058 tumor-bearing nude mice with tumors of 0.7–0.8 cm in diameter were treated IP with (1) 2 × 1.0 mCi ^{188}Re-HYNIC-AsnProAsnTrpGlyProArg; (2) 2 × "cold" HYNIC-AsnProAsnTrpGlyProArg (0.2 μg each injection); or (3) 2 × 1.0 mCi ^{188}Re-HYNIC–4B4 decapeptide 7 days apart. Mice in the control group were left untreated. Points represent means of tumor size of 6 mice. The bars represent standard deviation.

assay. Approximately 7% of all cells (melanoma and connective tissue) turned to be nonviable in non-necrotic tumors, and up to 40% of cells were nonviable in necrotic tumors (Dadachova et al., unpublished observations). Apparently, this number of cells was sufficient to provide the target for the radiolabeled peptides. In spite of modest tumor uptake, significantly slower ($P << 0.01$) tumor growth was observed in all treatment groups with ^{188}Re-HYNIC-AsnProAsnTrpGlyProArg slowing the tumor growth more efficiently ($P = 0.02$) than "cold" HYNIC-AsnProAsnTrpGlyProArg or ^{188}Re-HYNIC–4B4 decapeptide from day 25 posttreatment. "Cold" HYNIC-AsnProAsnTrp GlyProArg was almost as efficient in slowing down tumor growth as ^{188}Re-HYNIC–4B4 decapeptide, suggesting that the peptide has a therapeutic effect independent of the attached radionuclide. The mechanism for this phenomenon is unknown, but in prior studies, we also observed that "cold" melanin-binding mAb 6D2 had some therapeutic effect on melanoma tumors (*9*). We speculate that melanin-binding peptides and mAbs might be absorbed into the surface of melanin where they become immunogenic and elicit an immune response that triggers localized inflammation that produces an antitumor effect. The explanation for higher efficacy of the radiolabeled heptapeptide may be the combination of the biological effect of the peptide with the deep penetration of the small peptide into the tumor and "cross-fire" irradiation of distant cells by the energetic β-emissions of ^{188}Re providing relatively homogenous irradiation of the tumor. Also, in the future, it might be possible to increase tumor uptake of these heptapeptides using site-directed mutagenesis or peptide arrays.

Since the purpose of the study was to generate melanin-binding peptides with low kidney uptake, we paid particular attention to uptake of radiolabeled heptapeptides in this organ. The kidney uptake of heptapeptides described in this study was on average 7 times less than that for ^{188}Re-labeled decapeptide 4B4. This is a dramatic improvement in the biodistribution pattern of melanin-binding peptides, given that kidneys are a major concern for toxicity during therapy with radiolabeled peptides (*13*). Interestingly, an attempt to further decrease kidney uptake of the ^{188}Re-labeled heptapeptide pharmacologically by coadministration of positively charged amino acid D-lysine, which was supposed to block the negative charge on cell membranes in the proximal tubules and thus prevent heptapeptide retention after glomerular filtration, paradoxically increased its uptake by several fold. Interestingly, the same phenomenon was observed when ^{188}Re-labeled decapeptide 4B4 was coadministered to mice with D-lysine (E. Dadachova, unpublished observations). The explanation of this phenomenon is yet to be found, as molecular mechanisms of renal reabsorption of peptides used in radionuclide therapy have not been clarified. For example, radiolabeled somatostatin peptide analogues have been used in clinical practice since the late 1980s, but only very recently has evidence been presented to suggest that the molecular mechanism of [^{111}In-DTPA]octreotide uptake in renal proximal tubules involves multiligand scavenger receptor megalin (*26*).

In conclusion, by utilizing phage display technique, we generated a panel of tumor melanin-binding heptapeptides which when radiolabeled with ^{188}Re demonstrated 7-fold lower kidney uptake in mice than previously reported ^{188}Re-labeled fungal melanin-binding decapeptide. Administration of one of the generated heptapeptides, ^{188}Re-HYNIC-AsnProAsnTrpGlyProArg, to A2058 human metastatic melanoma-bearing nude mice resulted in significant retardation of the tumor growth. In spite of their relatively small size, heptapeptides were not able to penetrate through the membranes of viable melanoma cells and bound only to extracellular melanin, which provides assurance that they will be safe to healthy melanin-containing tissues during radionuclide therapy of melanoma. Thus, these heptapeptides can be potentially developed into efficient and safe delivery vehicles for targeted radionuclide therapy of melanoma.

ACKNOWLEDGMENT

The authors would like to thank NIH-designated Albert Einstein College of Medicine Cancer Center for providing funding for this work; Dr. M. Torres for help with the phage display library; Dr. L. Mintz for advice on the peptides synthesis; and Dr. X-G. Wang for supplying hepatocellular carcinoma cells.

LITERATURE CITED

(1) Rigel, D. S. (1997) Malignant melanoma: Incidence, issues and their effect on diagnosis and therapy in the 1990s. *Mayo Clin. Proc. 72* (4), 367–371.
(2) Grin-Jorgensen, C. M., Rigel, D. S., and Friedman, R. J. (1992) The worldwide incidence of malignant melanoma. In *Cutaneous Melanoma*, 2nd ed. (Balch C. M., Houghton A. N., Milton G. W., Sober A. J., and Soong S. J., Eds.) pp 27–39, Lippincott Co., Philadelphia.
(3) Liu, T., and Soong, S. J. (1996) Epidemiology of malignant melanoma. *Surg Clin. N. Am. 76* (6), 1205–1222.
(4) Sun, W., and Schuchter, L. M. (2001) Metastatic melanoma. *Curr. Treat. Options Oncol. 2* (3), 193–202.
(5) Schuchter, L. (2004) Are we at a turning point for melanoma therapy? 2nd International Melanoma Research Congress, Phoenix, Arizona, November 13–16, 2004; p 2.
(6) Cohen-Solal, K. A., Crespo-Carbone, S. M., Namkoong, J., Mackason, K. R., Roberts, K. G., Reuhl, K. R., and Chen, S. (2002) Progressive appearance of pigmentation in amelanotic melanoma lesions. *Pigment Cell Res. 15* (4), 282–289.
(7) Busam, K. J., Hester, K., Charles, C., Sachs, D. L., Antonescu, C. R., Gonzalez, S., and Halpern, A. C. (2001) Detection of clinically amelanotic malignant melanoma and assessment of its

margins by in vivo confocal scanning laser microscopy. *Arch. Dermatol. 137* (7), 923–929.
(8) Dadachova, E., and Casadevall, A. (2005) Melanin as a potential target for radionuclide therapy of metastatic melanoma. *Future Oncol. 1* (4), 541–549.
(9) Dadachova, E., Nosanchuk, J. D., and Shi, L., et al. (2004) Dead cells in melanoma tumors provide abundant antigen for targeted delivery of ionizing radiation by a monoclonal antibody to melanin. *Proc. Natl. Acad. Sci. U.S.A. 101* (41), 14865–14870.
(10) Nosanchuk, J. D., Valadon, P., Feldmesser, M., and Casadevall, A. (1999) Melanization of Cryptococcus neoformans in murine infection. *Mol. Cell Biol. 19* (1), 745–750.
(11) Dadachova, E., Moadel, T., Schweitzer, A. D., Bryan, R. A., Zhang, T., Mints, L., Revskaya, E., Huang, X., Ortiz, G., Nosanchuck, J. S., Nosanchuck, J. D., and Casadevall, A. (2006) Radiolabeled melanin-binding peptides are safe and effective in treatment of human pigmented melanoma in a mouse model of disease. *Cancer Biother. Radiopharm. 21* (2), 117–129.
(12) Blok, D., Feitsma, R. I., Vermeij, P., and Pauwels, E. J. (1999) Peptide radiopharmaceuticals in nuclear medicine. *Eur. J. Nucl. Med. 26* (11), 1511–1519.
(13) Lambert, B., Cybulla, M., Weiner, S. M., Van De Wiele, C., Ham, H., Dierckx, R. A., and Otte, A. (2004) Renal toxicity after radionuclide therapy. *Radiat. Res. 161* (5), 607–611.
(14) Kushimoto, T. A., Basrur, V., Valencia, J., Matsunaga, J., Viera, W. D., Ferrans, V. J., Muller, J., Appella, E., and Hearing, V. J. (2001) A model for melanosome biogenesis based on the purification and analysis of early melanosomes. *Proc. Natl. Acad. Sci. U.S.A. 98*, 10698–10703.
(15) Lindmo, T., Boven, E., and Cuttitta, F. (1984) Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. *J. Immunol. Methods 72*, 77–89.
(16) Krenning, E. P., Kwekkeboom, D. J., Valkema, R., Pauwels, S., Kvols, L. K., and de Jong, M. (2004) Peptide receptor radionuclide therapy. *Ann. N.Y. Acad. Sci. 1014*, 234–245.
(17) Miao, Y., Whitener, D., Feng, W., Owen, N. K., Chen, J., and Quinn, T. P. (2003) Evaluation of the human melanoma targeting properties of radiolabeled alpha-melanocyte stimulating hormone peptide analogues. *Bioconjugate Chem. 14* (6), 1177–1184.
(18) Miao, Y., Owen, N. K., Fisher, D. R., Hoffman, T. J., and Quinn, T. P. (2005) Therapeutic efficacy of a 188Re-labeled alpha-melanocyte-stimulating hormone peptide analog in murine and human melanoma-bearing mouse models. *J. Nucl. Med. 46* (1), 121.
(19) Romanov, V. I. (2003) Phage display selection and evaluation of cancer drug targets. *Curr. Cancer Drug Targets 3* (2), 119–129.
(20) Landon, L. A., Zou, J., and Deutscher, S. L. (2004) Is phage display technology on target for developing peptide-based cancer drugs? *Curr. Drug Discovery Technol. 1* (2), 113–32.
(21) Hearing, V. (2000) The melanosome: the perfect model for cellular responses to the environment. *Pigment Cell Res. 13* (Suppl 8), 23–34.
(22) Cohen-Solal, K. A., Crespo-Carbone, S. M., and Namkoong, J., et al. (2002) Progressive appearance of pigmentation in amelanotic melanoma lesions. *Pigment Cell Res. 15* (4), 282–289.
(23) Busam, K. J., Hester, K., and Charles, C., et al. (2001) Detection of clinically amelanotic malignant melanoma and assessment of its margins by in vivo confocal scanning laser microscopy. *Arch. Dermatol. 137* (7), 923–929.
(24) Newton, J. R., Miao, Y., Deutscher, S. L., and Quinn, T. P. (2007) Melanoma imaging with pretargeted bivalent bacteriophage. *J. Nucl. Med. 48* (3), 429–36.
(25) Slominski, A., Wortsman, J, Kohn, L., Ain, K. B., Venkataraman, G. M., Pisarchik, A., Chung, J. H., Giuliani, C., Thornton, M., Slugocki, G., and Tobin, D. J. (2002) Expression of hypothalamic-pituitary-thyroid axis related genes in the human skin. *J. Invest. Dermatol. 119* (6), 1449–55.
(26) de Jong, M., Barone, R., Krenning, E., Bernard, B., Melis, M., Visser, T., Gekle, M., Willnow, T. E., Walrand, S., Jamar, F., and Pauwels, S. (2005) Megalin is essential for renal proximal tubule reabsorption of (111)In-DTPA-octreotide. *J. Nucl. Med. 46* (10), 1696–1700.

BC060330U