Characterization and Development of a Peptide (p160) with Affinity for Neuroblastoma Cells

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Drug-targeting strategies can increase the efficacy and reduce the side effects and toxicity of conventional chemotherapy or may lead to new radiolabeled molecules useful for diagnosis and therapy. To identify and characterize new carrier molecules, we evaluated a peptide that had been identified by phage display technology. **Methods:** The peptide p160 (VPWMEPAYQRFL) was prepared by solid-phase peptide synthesis and radiolabeled with ^{125}I or ^{131}I. The radiolabeled peptide and derivatives of it were used to study binding and internalization in vitro and to assess their distribution in tumor-bearing mice. **Results:** Cell-binding assays on the human neuroblastoma cell line WAC 2 indicated the affinity and specificity of ^{125}I-labeled p160 toward neuroblastoma cells. Binding of the ^{125}I-labeled p160 was inhibited up to 95% by the unlabeled peptide. Furthermore, 50% of the total bound activity was internalized into the neuroblastoma cells. Biodistribution studies on nude mice showed a higher tracer accumulation in tumors than in most organs. Perfusion of the animals reduced uptake in all tissues, whereas tumor uptake remained constant. Fluorescence-activated cell-sorting studies with fluorescein isothiocyanate–labeled p160 demonstrated an increased fluorescence signal. Investigation of the binding properties of the fragments p160-8-1, p160-8-2, and p160-8-3 indicated that the sequence EPAYQR might be of significance for the binding of p160. **Conclusion:** These data indicate that the p160 peptide is an attractive candidate for the development of a neuroblastoma-specific vector that can be used for drug targeting or radiopeptide-based diagnosis and therapy.

Key Words: peptide; tumor affinity; neuroblastoma

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S pecific targeting of chemotherapeutic drugs to tissues of interest, with little uptake by healthy tissues, is of great importance in the development of cancer therapy. One strategy is peptide receptor targeting. Because numerous human cancers overexpress peptide receptors, it is possible to identify peptide ligands that can target them and serve either as carriers for the delivery of cytotoxic drugs or as vehicles for the delivery of radiopharmaceuticals for peptide receptor radiation therapy.

The most prominent peptide for receptor targeting in oncologic applications is octreotide (Sandostatin; Novartis), a somatostatin receptor subtype 2–binding peptide (1). Somatostatin receptors are overexpressed in many human cancers such as neuroendocrine tumors and other solid tumors but also in peritumoral and tumoral blood vessels (2,3). The high affinity of octreotide to the somatostatin receptor subtype 2 and its potential to transport radionuclides and therapeutic agents into somatostatin receptor–expressing tissues (4,5) has allowed its clinical use for diagnosis and radiopeptide therapy (6). Moreover, modifications of the peptide have led to derivatives with even better binding and metabolic properties (7). For example, Tyr^3-octreotide has a higher affinity for somatostatin subtype 2 receptors than does native octreotide. Furthermore, oxidation of the C-terminal threoninol to threonine led to the development of octreotate, a peptide that shows a higher internalization rate into somatostatin receptor–positive tumor cells and a lower accumulation in tissues such as liver or kidney (8).

The successful clinical application of octreotide and its derivatives underlines the important role of peptide-targeted systems for cancer diagnosis and therapy. Further support is given by the favorable pharmacokinetic properties of peptides, such as the rapid clearance from blood because of their small size and the lack of immunogenicity, representing obvious advantages over antibodies, which are widely used as vehicles for tumor targeting. Therefore, the identification and development of new peptides with specific targeting abilities and reduced background binding are major challenges in cancer-related peptide research. A highly efficient technology for the identification of new tumor affine peptides is the screening of phage display libraries (9). This technology, based on the principle that

bacteriophages can present specific binding ligands on their surface, has been used extensively to select peptides for targeting organs, tumors, or cell types (10–13).

A peptide that has been identified through random peptide phage display is p160 (VPWMEPAYQRFL). P160 consists of 12 amino acids and was isolated via selection rounds of a phage library on the neuroblastoma cell line WAC 2 (14). The binding of the bacteriophage t160, displaying the peptide p160, was inhibited by the chemically synthesized peptide, revealing that the phage binding to WAC 2 cells is mediated through the displayed peptide. Furthermore, an internalization of t160 in WAC 2 neuroblastoma cells could be shown by confocal light microscopy.

Neuroblastoma is a malignant embryonic tumor in young children (15). Many neuroblastomas (approximately 40%) are diagnosed as metastatic disease and, even with intensive therapy, are usually associated with poor survival, which has not improved in the past decade. Delivery of cytotoxic drugs or radionuclides into the tumor and metastases could facilitate a targeted therapy with increased drug concentration in the tumor and fewer side effects on vital organs, as is so important for small children.

In this study, we investigated the in vitro and in vivo properties of the neuroblastoma affine peptide p160. Binding, internalization, and biodistribution studies were performed, using the neuroblastoma cell line WAC 2 as the target. The affinity, binding kinetics, and serum stability of p160 were determined, and different derivatives of the p160 peptide were synthesized and tested to study its cellular handling and optimize its properties for drug targeting.

MATERIALS AND METHODS

Reagents

All standard reagents and solvents for the peptide synthesis were purchased from Merck. The chemicals for peptide synthesis were supplied from Novabiochem. Triisopropylsilane was obtained from Fluka. Radioisotope Na^{125}I and Na^{131}I were obtained from Amersham Pharmacia Biotech.

Peptides

All peptides were analyzed and purified by high-performance liquid chromatography (HPLC) on a P-580 system (Gyncotech) equipped with a variable SPD 6-A ultraviolet detector and a C-R5A integrator (both Shimadzu). The columns were LiChrosorb RP-select B (5 μm, 250 × 4 mm; 10 μm, 250 × 10 mm; Merck). All analytic runs were performed with a linear gradient over 30 min of 5%–95% acetonitrile in water at a flow rate of 0.7 mL/min. All preparative runs were performed with the same gradient and a flow rate of 4 mL/min. The mass of the products was determined by mass spectrometry analysis on a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-3; Kratos Instruments). Lyophilization was performed on an α$_{1–2}$ lyophilizator (Heraeus-Christ).

The peptides p160 (VPWMEPAYQRFL), D-p160 (all amino acids in D-isoform), p160-8-1 (EPAYQRFL), p160-8-2 (WMEPAYQR), p160-8-3 (VPWMEPAY), 2Nle-p160-8-2 (W-Nle-EPAYQR), and β-Ala-p160-8-2 (WMEP-β-Ala-YQR) were obtained by solid-

phase peptide synthesis using Fmoc coupling protocols according to the Merrifield strategy (16). The peptides were synthesized manually using an in-house–manufactured solid-phase peptide synthesis reactor on 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin (Rink amide resin). All peptides were radiolabeled with ^{125}I and ^{131}I using the chloramine-T method (17). The products were purified by HPLC and analyzed by analytic HPLC. The iodinated product was free of unlabeled precursor and free iodine. The labeled peptides were either used immediately or stored at −80°C until use. For fluorescence-activated cell sorting, fluorescein isothiocyanate (FITC) was coupled via an additional lysine at the COOH terminus of p160.

Cell Lines

All cell lines were cultivated at 37°C in a 5% CO$_2$ incubator. The human neuroblastoma cell line WAC 2 was cultured in RPMI 1640 with GlutaMAX (Invitrogen) containing 10% fetal calf serum (Invitrogen) and a 25 mmol/L concentration of HEPES. Human umbilical vein endothelial cells (HUVEC) were isolated as described (18,19). HUVEC cells were incubated on gelatin (1%)-coated cell culture flasks using medium 199 (Invitrogen) containing 20% fetal calf serum, 2 mmol of glutamine per liter, 100 IU of penicillin per milliliter, 100 IU of streptomycin per milliliter, and 2 ng of bFGF per milliliter (Roche Diagnostics).

In Vitro Binding Experiments and Competition Experiments

For binding assays, 3×10^5 WAC 2 neuroblastoma cells were seeded into 6-well plates and cultivated in 3 mL of incubation medium at 37°C for 24 h. The medium was replaced by 1 mL of fresh medium (without fetal calf serum) containing $1−2 \times 10^6$ cpm of ^{125}I-labeled p160 peptide ($3.6–7.2 \times 10^{−10}$ mol/L), and incubation was performed for 1 h at 37°C. To determine specific versus nonspecific binding, we incubated the cells with the unlabeled p160 peptide at concentrations varying from $10^{−4}$ to $10^{−12}$ mol/L. After incubation, the medium was removed and the cells were washed 3 times with ice-cold phosphate-buffered saline (PBS) to remove the unbound radioactive labeled peptide. The cells were then lysed with 0.5 mL of a 0.3 mol/L concentration of NaOH, and the radioactivity was measured with a γ-counter and calculated as the percentage uptake per 10^6 cells. HUVEC were used as a negative control. In vitro binding studies were also performed using the peptide D-p160 and octreotide as negative control competitors for binding of the radioligand ^{125}I-p160. To investigate the kinetic properties of the peptide p160, we incubated ^{125}I-labeled p160 with WAC 2 cells for different periods. In vitro binding experiments on WAC 2 cells were also performed, using the ^{125}I-labeled peptides p160-8-1, p160-8-2, p160-8-3, 2Nle-p160-8-2, and β-Ala-p160-8-2. The binding properties of the peptide β-Ala-p160-8-2 were also investigated in HUVEC.

Internalization Experiments

For internalization experiments, subconfluent cell cultures of WAC 2 cells were incubated with ^{125}I-p160 for 60 min at 37°C and 4°C. Cellular uptake was stopped by removing medium from the cells and washing them with 3 mL of ice-cold PBS. To remove the radioactivity bound on the surface of the cells, we incubated them with 1 mL of glycine-HCl, 50 mmol/L, in PBS (pH 2.8) for 10 min at room temperature. The cells were then washed with 3 mL of ice-cold PBS and lysed with 0.5 mL of NaOH, 0.3 mol/L. The surface-bound and internalized radioactivity was measured in a γ-counter and calculated as the percentage uptake per 10^6 cells.

Metabolic Studies

The stability of p160 and β-Ala-p160-8-2 was tested in human serum. The peptide was incubated at 37°C in human serum at a concentration of 5×10^{-4} mol/L. At time points varying from 5 min to 6 h, samples were taken and an equal volume of acetonitrile was added to precipitate serum proteins, which were pelleted by centrifugation. The supernatant was analyzed by reversed-phase HPLC. Samples of metabolic products of p160 in human serum were isolated and analyzed by MALDI-TOF mass spectrometry [20].

Fluorescence-Activated Cell Sorting (FACS)

For fluorescence-activated cell sorting, 1×10^6 WAC 2 cells were seeded into 6-well plates and cultivated in 3 mL of incubation medium at 37°C for 24 h. The medium was replaced by 1 mL of fresh medium (without fetal calf serum) containing FITC-Lys-p160 at a concentration of 10^{-6} mol/L. After 20 min of incubation at 37°C, the medium was removed and the cells were washed with 3 mL of incubation medium. The cells were transferred in 1.5-mL Eppendorf tubes and centrifuged at 1,000 rpm for 10 min. The pellet was resuspended in cell wash medium, and fluorescence-activated cell sorting was performed. To determine specific versus nonspecific binding, we also incubated the cells in the presence or absence of the unlabeled p160 peptide at a concentration of 10^{-4} mol/L. To determine whether the binding of FITC-Lys-p160 was mediated through the peptide, we also performed FACS analysis with WAC 2 cells incubated with FITC at the same concentration (10^{-6} mol/L). To discriminate between peptide-bound labeled cells and autofluorescence of unlabeled cells, we measured the autofluorescence of WAC 2 cells. Fluorescence up to the measured intensity was considered autofluorescence and determined through a cutoff line. Cells in which the fluorescence was higher than the cutoff value were considered labeled by FITC-Lys-p160. FACS analysis was performed in a Galaxy Pro flow cytometer (Partec) equipped with a mercury vapor lamp (100 W) and filter combinations for 4′,6-diamidino-2-phenylindol and a 488-nm argon laser with filter combination for FITC. Histogram and dot blot analysis was done with the FlowMax analysis software (Partec).

In Vivo Experiments

In vivo experiments were performed on 9-wk-old female BALB/c *nu/nu* mice carrying subcutaneously transplanted WAC 2 tumors. For transplantation of the tumors, WAC 2 cells were grown to 90% confluence, harvested with PBS/ethylene diamine tetraacetic acid, resuspended in Falcon Matrigel-Matrix (BD), and kept on ice. Twp hundred microliters of the Matrigel-Matrix cell suspension were injected subcutaneously into the anterior region of the mouse trunk. The tumors were allowed to grow for about 2 wk, until they reached approximately 1.0 cm³ in size. ^{131}I-Labeled p160 was injected (approximately 1 MBq) via the tail vein into the mice, and 1 h after injection the animals were sacrificed. Tumor, blood, and selected tissues (heart, spleen, liver, kidney, muscle, and brain) were removed, drained of blood, and weighed, and the radioactivity was measured with a γ-counter (LB 951G; Berthold Technologies). The percentage injected dose per gram of tissue (%ID/g) was calculated. To determine uptake in blood-free organs, we performed perfusion experiments. For the perfusion studies, tumor-bearing mice were anesthetized with an intraperitoneal 5-mg injection of Ketanest (Parke-Davis) and 400 mL of 0.2% Rompun (BayerVital). A catheter was put in the ascending aorta through a small cut in the left ventricle of the

heart of the mouse, and perfusion was performed with 25 mL of 0.9% NaCl through a cut in the liver. After perfusion, samples of tumor and control organs were removed and weighed, the radioactivity was measured with a γ-counter, and the percentage dose per gram of tissue was calculated. Organ distribution experiments of ^{131}I-labeled β-Ala-p160-8-2 without perfusion were also performed on BALB/c *nu/nu* mice carrying subcutaneously transplanted WAC 2 tumors. The time kinetic biodistribution of β-Ala-p160-8-2 was demonstrated after 5, 15, and 60 min of circulation of the peptide in the bloodstream of the animals.

Data Analysis and Statistics

Statistical comparisons between groups were performed using the unpaired Student *t* test on the SIGMASTAT program (Jandel Scientific). A *P* value of 0.05 or less was considered statistically significant.
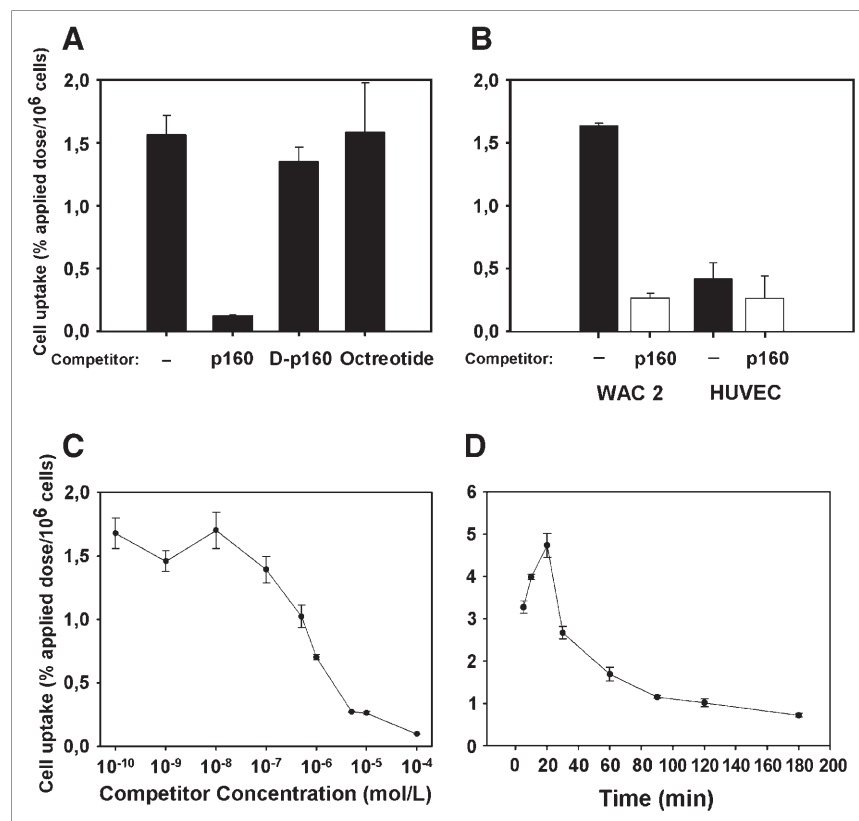
RESULTS

In Vitro Characteristics of p160

To characterize the binding of p160, we incubated ^{125}I-p160 with WAC 2 cells and HUVEC for 1 h at 37°C. To demonstrate specific binding, we performed the incubation in the presence of unlabeled p160 peptide (10^{-4} mol/L) as a competitor and in the presence of the peptides D-p160 (10^{-4} mol/L) and octreotide (10^{-4} mol/L) as negative control competitors. ^{125}I-Labeled p160 showed a binding capacity of about 1.6% of the applied dose per 10^6 cells. The unlabeled peptide p160 caused an up to 95% decrease in binding of ^{125}I-p160, whereas D-p160 or octreotide had no effect at the same concentration (Fig. 1A). The binding capacity of ^{125}I-p160 on HUVEC was measured as 0.5% of the applied dose per 10^6 cells, and the binding was not competitively abolished by the unlabeled p160 peptide (Fig. 1B). The affinity of p160 was further evaluated using different concentrations of the competitor. At a competitor concentration of 10^{-4} mol/L, up to 95% of the binding of ^{125}I-p160 was inhibited. At concentrations below 10^{-9}, the bound activity reached the level of uncompeted binding. The concentration at which 50% of the binding of ^{125}I-p160 was inhibited was calculated as 1.66 ± 0.988 μmol/L (Fig. 1C). To investigate the kinetic properties of p160 binding, we performed time-course binding experiments on WAC 2 cells for incubation times varying from 5 min to 3 h. p160 showed a time-dependent increase in binding for an incubation period up to 20 min. Thereafter, the amount of bound peptide decreased to a level of 15% of the maximal uptake after 3 h of incubation (Fig. 1D).

To evaluate the specific binding of p160 to WAC 2 cells, we performed a FACS analysis. For the FACS analysis, lysine was added to the C-terminus of p160 to allow labeling with FITC. The FACS analysis revealed that the FITC-labeled p160 peptide bound to the neuroblastoma WAC 2 cells, whereas the control dye FITC alone did not change the fluorescence signal of the WAC 2 cells at the same concentration. Incubation of WAC 2 cells with FITC-labeled p160 in the presence of unlabeled p160 at a

FIGURE 1. Binding and competition of ^{125}I-labeled p160. (A) Specific binding of ^{125}I-p160 to neuroblastoma WAC 2 cells. Nonspecific binding was determined in presence of unlabeled p160 (10^{-4} mol/L). The peptides octreotide and D-p160 were used at same concentration (10^{-4} mol/L) as negative control competitors. (B) Binding of ^{125}I-p160 with and without excess of unlabeled peptide (10^{-4} mol/L) in WAC 2 and HUVEC cells. Incubation was performed for 1 h at 37°C. (C) Competition for bound ^{125}I-p160 by unlabeled p160 peptide. WAC 2 cells were incubated with $1-2 \times 10^6$ cpm radioligand and increasing concentrations of unlabeled p160 peptide. (D) In vitro cell accumulation of ^{125}I-p160 in WAC 2 cells as function of time. Incubation was performed for periods from 5 to 180 min. All experiments were performed in triplicate; SD is shown.

concentration of 10^{-4} mol/L resulted in an up to 80% decrease in the fluorescence signal (Fig. 2).

In vitro internalization studies were performed to determine the rate of internalization of ^{125}I-p160 in WAC 2 cells. To distinguish between surface-bound peptide and internalized peptide, we removed all outside bound peptide by including an acidic wash step in the washing procedure. After a 1-h incubation of the cells with ^{125}I-p160 at 37°C, the internalized radioactivity was measured as almost 50% of the total bound activity, suggesting that half the bound peptides were internalized at that time point. Internalization experiments were also performed at 4°C and demonstrated a reduction in binding of the ^{125}I-p160 to the background level, indicating no internalization at that temperature (Fig. 3).

Stability and Fragment Analysis

The in vitro stability of p160 in human serum was investigated through incubation of p160 at a concentration of 10^{-4} mol/L in human serum and HPLC analysis of serum samples taken at different times. The experiments revealed a complete degradation of p160 by serum proteases after 4 h. Degradation products detected by HPLC were isolated, and their mass was determined by mass spectrometry. The main products of the serum degradation of p160 appeared after a 2-h incubation of the peptide in human serum and had masses of 1,276 g/mol, 1,120 g/mol, and 1,022 g/mol. The first fragment (fragment 1) corre-

sponded to the sequence VPWMEPAYQR (1,276 g/mol), which lacks the 2 C-terminal amino acids Phe and Leu. The second fragment (fragment 2) corresponded to the sequence VPWMEPAYQ (1,120 g/mol) and resulted from further degradation of the C-terminal amino acid Arg. Finally, the third fragment (fragment 3) corresponded to the sequence EPAYQRFL (1,022 g/mol). In contrast to the other fragments, this one contained all C-terminal amino acids, but the N-terminal amino acids Val-Pro-Trp-Met had been cleaved off.
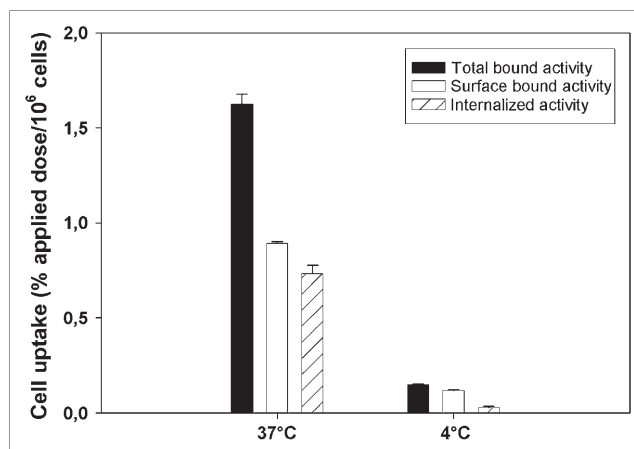
Binding Characteristics of Fragments

To further characterize the binding site in the sequence of p160, we synthesized different variations of the degradation fragments, labeled them with ^{125}I, and investigated them on WAC 2 cells. Binding of the ^{125}I-labeled fragments p160-8-1 (EPAYQRFL), p160-8-2 (WMEPAYQR), and p160-8-3 (VPWMEPAY) was compared with binding of ^{125}I-p160 (Table 1). This comparison revealed that the fragment p160-8-1, which is the degradation product fragment 3, bound the WAC 2 cells to the same extent as did native p160. The peptide p160-8-2, which contains the middle part of p160 and was mostly conserved during degradation, also bound WAC 2 cells with the same capacity as did full-length p160. The peptide p160-8-3, containing the first 8 N-terminal amino acids, showed only 15% of the binding of p160. Furthermore, modified sequences of the peptide p160-8-2 were investigated and
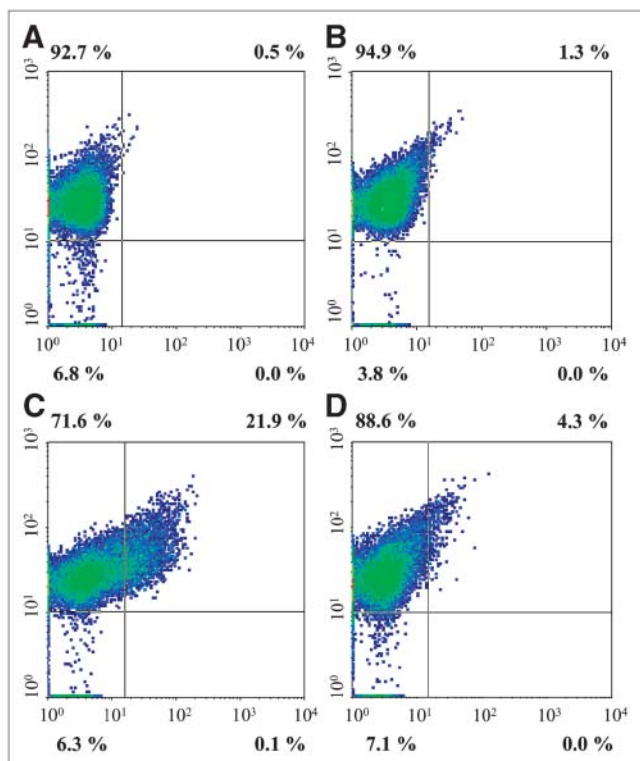
FIGURE 2. Fluorescence-activated cell-sorting analysis. (A) Autofluorescence of WAC 2 cells. (B) Fluorescence of WAC 2 cells after incubation with FITC (10^{-6} mol/L) for 20 min at 37°C. (C) Fluorescence of WAC 2 cells after incubation with FITC-Lys-p160 (10^{-6} mol/L) for 20 min at 37°C. (D) Fluorescence of WAC 2 cells after incubation with FITC-Lys-p160 (10^{-6} mol/L) for 20 min in presence of unlabeled p160 at concentration of 10^{-4} mol/L.

FIGURE 3. Binding and internalization of ^{125}I-p160 in WAC 2 cells. Cells were grown for 24 h and incubated with $1-2 \times 10^6$ cpm radioligand for 1 h at 37°C or at 4°C. After being washed with acid buffer (pH 2.8), cells were lysed and internalized radioactivity was measured.

compared with the binding of p160. Because the methionine in the sequence of p160-8-2 is oxidized to methionine sulfoxide during the iodine-labeling procedure, it was substituted by the amino acid norleucine (Nle). The resulting peptide, Nle-p160-8-2, showed improved binding to WAC 2, with 73% more binding capacity than that of p160. To improve the stability, we synthesized and investigated a peptide with an exchange of alanine to β-alanine in the sequence of p160-8-2. This peptide, called β-Ala-p160-8-2,

showed a more than 2-fold increased binding capacity to WAC 2 cells when compared with native p160 (Table 1).

To investigate if the increased binding affinity of β-Ala-p160-8-2 still ensured binding specificity, we performed binding experiments of ^{125}I-β-Ala-p160-8-2 on WAC 2 cells. ^{125}I-β-Ala-p160-8-2 showed a binding capacity of up to 6% to WAC 2 cells (Fig. 4A). This binding could be inhibited by the addition of a 5×10^{-5} mol/L concentration of the unlabeled compound and by the addition of unlabeled p160, although β-Ala-p160-8-2 could not inhibit binding of ^{125}I-p160 as effectively as could p160 itself. The binding kinetics of ^{125}I-β-Ala-p160-8-2 were, in comparison to those of p160, slower and showed maximum binding at 30 min of incubation (Fig. 4B). Stability data with ^{125}I-β-Ala-p160-8-2 in human serum showed 1 degradation product appearing very early after 5 min of incubation, but this metabolite remains stable for more than 6 h (Fig. 4C). Experiments using HUVEC showed that β-Ala-p160-8-2 still bound preferentially to WAC 2 cells and showed no binding to HUVEC (data not shown).

TABLE 1
Ratios of Binding of Fragments p160-8-1, p160-8-2, p160-8-3, Nle-p160-8-2, and βAla-p160-8-2 to Binding of p160 on Neuroblastoma WAC 2 Cells

| Fragment	Name	Sequence	Ratio (binding fragment/binding p160)
	p160	Val-Pro-Trp-Met-Glu-Pro- Ala -Tyr-Gln-Arg-Phe-Leu	
1	p160-8-1	Glu-Pro- Ala -Tyr-Gln-Arg-Phe-Leu	1.20
2	p160-8-2	Trp-Met-Glu-Pro- Ala -Tyr-Gln-Arg	1.04
3	p160-8-3	Val-Pro-Trp-Met-Glu-Pro- Ala -Tyr	0.12
4	Nle-p160-8-2	Trp-Nle-Glu-Pro- Ala -Tyr-Gln-Arg	1.73
5	βAla-p160-8-2	Trp-Met-Glu-Pro-βAla-Tyr-Gln-Arg	2.56

All peptides were labeled with ^{125}I, and incubation time was 1 h.

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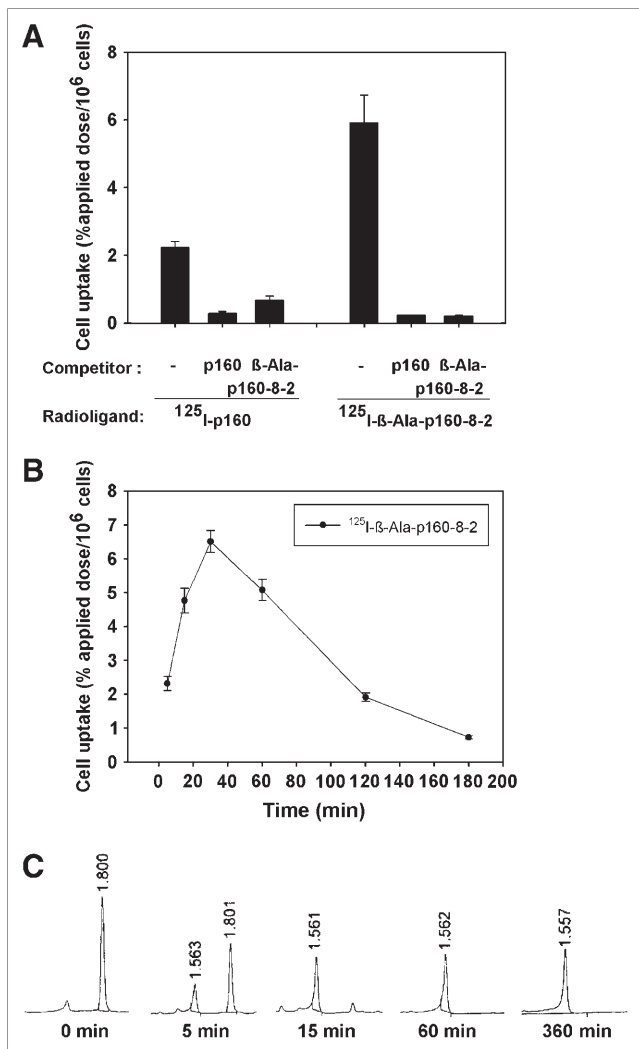
FIGURE 4. Binding and competition of ^{125}I-labeled p160 and ^{125}I-β-Ala-p160-8-2. (A) Specific binding of ^{125}I-p160 and ^{125}I-β-Ala-p160-8-2 to WAC 2 cells. As competitors, unlabeled p160 and β-Ala-p160-8-2 were used at concentration of 5×10^{-5} mol/L. Incubation was performed for 1 h at 37°C. (B) Time kinetics of ^{125}I-β-Ala-p160-8-2 binding to WAC 2. (C) Unlabeled β-Ala-p160-8-2 was incubated in human serum, and aliquots were removed for subsequent analysis at indicated time points. Experiments A and B were performed in triplicate; SD is shown.

In Vivo Experiments of p160 and β-Ala-p160-8-2

Biodistribution experiments of ^{131}I-labeled p160 were performed on female BALB/c *nu/nu* mice carrying WAC 2 tumors. The biodistribution in WAC 2 tumor-bearing mice at 1 h after intravenous injection of ^{131}I-p160 showed a tumor accumulation of 4% injected dose per gram of tissue. Uptake in tumor was higher than uptake in heart, spleen, liver, muscle, and brain and almost the same as uptake in kidney and lung (Fig. 5). Only the blood value (6.5%) was higher than the tumor value, and lung and kidney showed accumulations similar to that in tumor. To reduce blood background in tumor and other organs, we performed biodistribution studies, followed by perfusion of the mice
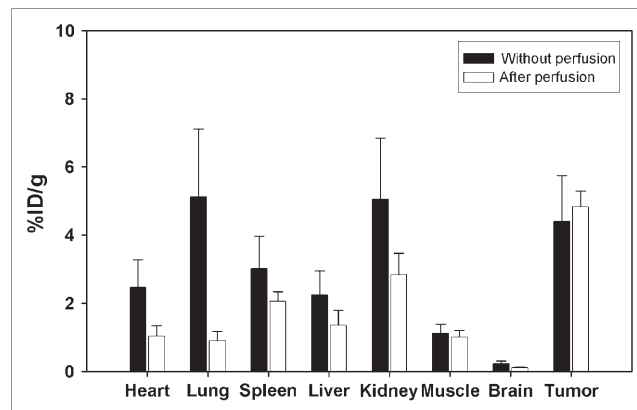
FIGURE 5. In female BALB/c *nu/nu* mice carrying WAC 2 tumors, organ distribution of p160 after 1-h circulation of ^{131}I-labeled p160 ($n = 4$ animals per experiment) and after perfusion ($n = 6$ animals per experiment).

with 0.9% NaCl. The perfusion experiments showed a reduction in uptake in most organs but not in tumor, resulting in a higher ratio of tumor uptake to organ uptake. Heart, lung, liver, and kidney showed a statistically significant decrease, $P < 0.05$, in unperfused organs, compared with perfused organs.

Biodistribution experiments of ^{131}I-labeled β-Ala-p160-8-2 without perfusion at 1 h after intravenous injection of the peptide in neuroblastoma tumor–bearing mice also showed a higher uptake in tumor than in most organs. The tumor accumulation reached a level of 3% of the injected dose per gram of tissue. Uptake was higher in tumor than in heart, spleen, muscle, and brain. However, kidney showed a higher accumulation, which reached a value of 7.1 %ID/g. The time kinetic biodistribution of ^{131}I-labeled β-Ala-p160-8-2 revealed a decrease in uptake over time in healthy tissues but not in tumor, where the uptake remained almost constant (Fig. 6).
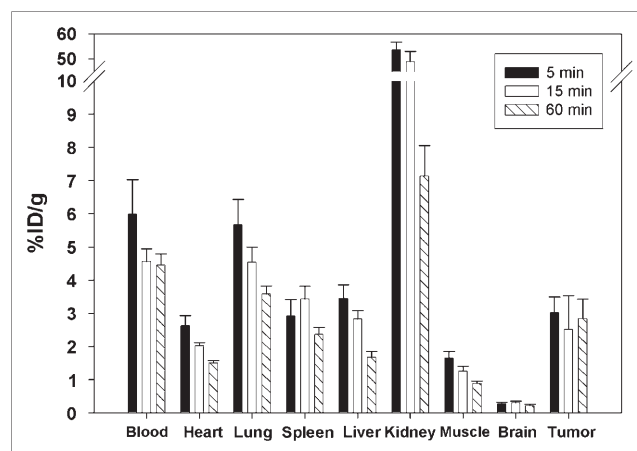
FIGURE 6. Time kinetic organ distribution of βAla-p160-8-2 in female BALB/c *nu/nu* mice carrying WAC 2 tumors. Incubation was performed for 5, 15, and 60 min ($n = 3$ animals per experiment) with ^{131}I-labeled βAla-p160-8-2.

DISCUSSION

P160 is a linear dodecapeptide identified by Zhang et al. (*14*) through random phage display. This peptide shows high binding efficiency and selectivity to human neuroblastoma WAC 2 cells. Cellular binding of p160 on WAC 2 cells could be mediated through a specific receptor. This hypothesis is strongly supported by the results of the competition experiments in vitro and by the results of the fluorescence-activated cell sorting. Particularly, competition experiments demonstrated that uptake of ^{125}I-p160 in WAC 2 cells lessened with increasing concentrations of the unlabeled p160 peptide as a competitor. Furthermore, this competition was performed to be specific, because unspecific competitors such as D-p160 and octreotide were not able to competitively abolish binding of the ^{125}I-labeled p160 peptide. In addition, fluorescence-activated cell-sorting studies demonstrated an increased accumulation of FITC-Lys-p160 in WAC 2 cells that was also inhibited in the presence of the unlabeled peptide. The hypothesis that a receptor-mediated process might be involved in the binding of p160 was sustained by internalization studies. These experiments revealed internalization of the peptide into the WAC 2 cells. Moreover, internalization of the peptide was suppressed at 4°C, a result that is expected for a receptor-mediated endocytotic process. A previous publication (*21*) showed that the p160 peptide also binds to MDA-MB 435 and MCF-7 breast carcinoma cells. Although MDA-MB 435 binds p160 with 1.6% cell uptake after 1 h—the same extent as the WAC 2 cells—MCF-7 cells bind p160 up to 7% cell uptake. In addition to the binding experiments, fluorescence microscopy also showed the internalization. Time kinetics of p160 binding with both the MDA-MB 435 cells and the MCF-7 cells were similar, with an initial high and fast uptake and a decrease in signal over time. This decrease in signal could reflect degradation of the peptide itself by cell-surface peptidases and degradation of the internalized peptide in the lysosomal compartment. Or the decrease could reflect a deiodination process resulting in a loss of labeled peptide.

The use of an agent as a targeting vehicle requires that the agent bind selectively to the tissue of interest. This requirement is fulfilled when the uptake by the healthy tissues is lower than that by the target. The biodistribution data of ^{131}I-labeled p160 showed that p160 has potential in this respect. After intravenous administration in WAC 2 tumor–bearing mice, ^{131}I-p160 showed a higher uptake in tumor than in most other organs. Only in blood and in kidneys was the uptake almost the same as in tumor. The high uptake by the kidneys may be due to renal excretion of the peptide. A possible explanation for the elevated blood values is an interaction of p160 with serum proteins such as albumin. Furthermore, a rapid degradation of the peptide by serum proteases might also be a reason for the high blood radioactivity values, because such degradation might lead to ^{131}I-labeled fragments that cannot bind to the tumor and thereafter circulate in the bloodstream before being ex-

creted. In addition, deiodination of the labeled peptide can result in high concentrations of free ^{131}I that might circulate in the bloodstream and be responsible for the high radioactivity values in blood (*22*). The contribution of the blood pool to the radioactivity values in normal organs was demonstrated by the perfusion experiments. Perfusion significantly decreased uptake in normal tissues but not in tumor, resulting in higher tumor-to-organ ratios. The selective decrease in radioactivity uptake after perfusion was noticed mainly in highly perfused organs such as lung and kidney. This result and the fact that the radioactivity level in the tumor remained almost constant after perfusion indicate a specific and selective in vivo accumulation of ^{131}I-p160 in WAC 2 tumors. Therefore, although perfusion experiments have no diagnostic or therapeutic relevance, they reveal important characteristics of the peptide and its binding properties in the different tissues.

Investigation of the metabolic properties of p160 revealed that the peptide is not stable in human serum. P160 can rapidly be degraded by serum proteases. Degradation of p160 might lead to fragments that do not share the high affinity of the native peptide to neuroblastoma cells. Therefore, stabilization of p160 is a major issue of further investigation because it might lead both to enhanced accumulation in the tumor and to a decrease in the blood pool. This stabilization can be realized by targeted modifications in the sequence of p160—for example, by exchange of amino acids with unnatural amino acids that cannot be recognized by serum proteases, such as D-amino acids or *N*-methylated amino acids. A prerequisite for these targeted modifications is identification of the degradation sites in the sequence of p160. Mass analysis of the p160 fragments after incubation in human serum revealed masses corresponding to the sequences VPWMEPAYQR, VPWMEPAYQ, and EPAYQRFL, indicating that the first peptidic bonds to be degraded are those between the amino acids ^{10}Arg-^{11}Phe, ^{9}Gln-^{10}Arg, and ^{4}Met-^{5}Glu.

Identification of the binding site in the sequence of a receptor-binding peptide is of great importance because of the possibility that peptides with enhanced affinity and selectivity to target cells and improved metabolic properties could be synthesized. A prominent example is the amino acid sequence Arg-Gly-Asp. This sequence was found to account for the ability of fibronectin to bind cells (*23*). Further investigation revealed that the Arg-Gly-Asp motif is present in many extracellular matrix components and is capable of targeting integrins both to angiogenic endothelial cells and to tumor cells (*24,25*), resulting in the development of analogs with improved properties for tumor imaging (*26,27*) and tumor targeting (*28,29*). In the case of p160, investigation of the binding properties of fragments p160-8-1, p160-8-2, and p160-8-3 indicated that the sequence EPAYQR might be of significance for the binding of p160 in WAC 2 cells. This hypothesis is strongly supported by the fact that the peptides p160-8-1 (EPAYQRFL) and p160-8-2 (WMEPAYQR) have almost the same binding capacity in

WAC 2 cells, when compared with the binding of p160, whereas the peptide p160-8-3 (VPWMEPAY) shows a significantly lower affinity to neuroblastoma cells. Furthermore, the role of individual amino acids was demonstrated through investigation of the properties of modified analogs of p160-8-2. The enhanced uptake in WAC 2 neuroblastoma cells after replacement of ^{2}Met by norleucine and of ^{5}Ala by β-alanine might be explained by various mechanisms. Those modifications might either increase the affinity and internalization of the peptide or improve the metabolic stability of p160.

CONCLUSION

Peptides with a high affinity for tumors can be used as lead structures for efficient targeting of chemotherapeutic drugs or imaging agents to the tissue of interest. The cytotoxic analogs of luteinizing hormone-releasing hormone are a prominent example of peptide targeting. These analogs contain doxorubicin linked to [D-Lys^6]-luteinizing hormone-releasing hormone, which has been used for the targeting of prostate cancers (*30*). Our data with the p160 peptide show that p160 might be a promising candidate for this use. Binding, internalization, and biodistribution experiments demonstrate that binding of p160 in neuroblastoma cells might be mediated through a specific receptor. Therefore, p160 is a promising carrier molecule for the delivery of therapeutic agents to neuroblastoma.

REFERENCES

1. Bauer W, Briner U, Doepfner W, et al. SMS 201-995: a very potent and selective octapeptide analogue of somatostatin with prolonged action. *Life Sci.* 1982;31:1133–1140.
2. Reubi JC. Peptide receptors as molecular targets for cancer diagnosis and therapy. *Endocr Rev.* 2003;24:389–427.
3. Watson JC, Balster DA, Gebhardt BM, et al. Growing vascular endothelial cells express somatostatin subtype 2 receptors. *Br J Cancer.* 2001;85:266–272.
4. Nagy A, Schally AV, Halmos G, et al. Synthesis and biological evaluation of cytotoxic analogs of somatostatin containing doxorubicin or its intensely potent derivative, 2-pyrrolinodoxorubicin. *Proc Natl Acad Sci U S A.* 1998;95:1794–1799.
5. Benali N, Cordelier P, Calise D, et al. Inhibition of growth and metastatic progression of pancreatic carcinoma in hamster after somatostatin receptor subtype 2 (sst2) gene expression and administration of cytotoxic somatostatin analog AN-238. *Proc Natl Acad Sci U S A.* 2000;97:9180–9185.
6. de Jong M, Breeman WA, Bakker WH, et al. Comparison of (111)In-labeled somatostatin analogues for tumor scintigraphy and radionuclide therapy. *Cancer Res.* 1998;58:437–441.
7. Henze M, Dimitrakopoulou-Strauss A, Milker-Zabel S, et al. Characterization of ^{68}Ga DOTA-D-Phe^1-Tyr^3-octreotide kinetics in patients with meningiomas. *J Nucl Med.* 2005;46:763–769.
8. Bodei L, Cremonesi M, Zoboli S, et al. Receptor-mediated radionuclide therapy with ^{90}Y-DOTATOC in association with amino acid infusion: a phase I study. *Eur J Nucl Med Mol Imaging.* 2003;30:207–216.
9. Landon LA, Deutscher SL. Combinatorial discovery of tumor targeting peptides using phage display. *J Cell Biochem.* 2003;90:509–517.
10. Pasqualini R, Ruoslahti E. Organ targeting in vivo using phage display peptide libraries. *Nature.* 1996;380:364–366.
11. Zitzmann S, Mier W, Schad A, et al. A new prostate carcinoma binding peptide (DUP-1) for tumor imaging and therapy. *Clin Cancer Res.* 2005;11:139–146.
12. Arap W, Pasqualini R, Ruoslahti E. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. *Science.* 1998;279:377–380.
13. Barry MA, Dower WJ, Johnston SA. Toward cell-targeting gene therapy vectors: selection of cell-binding peptides from random peptide-presenting phage libraries. *Nat Med.* 1996;2:299–305.
14. Zhang J, Spring H, Schwab M. Neuroblastoma tumor cell-binding peptides identified through random peptide phage display. *Cancer Lett.* 2001;171:153–164.
15. Westermann F, Schwab M. Genetic parameters of neuroblastomas. *Cancer Lett.* 2002;184:127–147.
16. Wellings DA, Atherton E. Standard Fmoc protocols. *Methods Enzymol.* 1997;289:44–67.
17. Crim JW, Garczynski SF, Brown MR. Approaches to radioiodination of insect neuropeptides. *Peptides.* 2002;23:2045–2051.
18. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins: identification by morphologic and immunologic criteria. *J Clin Invest.* 1973;52:2745–2756.
19. Schmidt K, Hoffend J, Altmann A, et al. Transfer of the sFLT-1 gene in Morris hepatoma results in decreased growth and perfusion and induction of genes associated with stress response. *Clin Cancer Res.* 2005;11:2132–2140.
20. Kuhnast B, Bodenstein C, Haubner R, et al. Targeting of gelatinase activity with a radiolabeled cyclic HWGF peptide. *Nucl Med Biol.* 2004;31:337–344.
21. Askoxylakis V, Zitzmann S, Mier W, et al. Preclinical evaluation of the breast cancer cell-binding peptide, p160. *Clin Cancer Res.* 2005;11:6705–6712.
22. Behe M, Du J, Becker W, et al. Biodistribution, blood half-life, and receptor binding of a somatostatin-dextran conjugate. *Med Oncol.* 2001;18:59–64.
23. Pierschbacher MD, Ruoslahti E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature.* 1984;309:30–33.
24. Ruoslahti E. RGD and other recognition sequences for integrins. *Annu Rev Cell Dev Biol.* 1996;12:697–715.
25. Zitzmann S, Ehemann V, Schwab M. Arginine-glycine-aspartic acid (RGD)-peptide binds to both tumor and tumor-endothelial cells in vivo. *Cancer Res.* 2002;62:5139–5143.
26. Chen X, Hou Y, Tohme M, et al. Pegylated Arg-Gly-Asp peptide: ^{64}Cu labeling and PET imaging of brain tumor alphavbeta3-integrin expression. *J Nucl Med.* 2004;45:1776–1783.
27. Haubner R, Wester HJ, Weber WA, et al. Noninvasive imaging of alpha(v)beta3 integrin expression using ^{18}F-labeled RGD-containing glycopeptide and positron emission tomography. *Cancer Res.* 2001;61:1781–1785.
28. Haubner R, Wester HJ, Burkhart F, et al. Glycosylated RGD-containing peptides: tracer for tumor targeting and angiogenesis imaging with improved biokinetics. *J Nucl Med.* 2001;42:326–336.
29. Janssen ML, Oyen WJ, Dijkgraaf I, et al. Tumor targeting with radiolabeled alpha(v)beta(3) integrin binding peptides in a nude mouse model. *Cancer Res.* 2002;62:6146–6151.
30. Letsch M, Schally AV, Szepeshazi K, Halmos G, Nagy A. Preclinical evaluation of targeted cytotoxic luteinizing hormone-releasing hormone analogue AN-152 in androgen-sensitive and insensitive prostate cancers. *Clin Cancer Res.* 2003;9:4505–4513.