A New Phage-Display Tumor-Homing Peptide Fused to Antiangiogenic Peptide Generates a Novel Bioactive Molecule with Antimelanoma Activity

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Abstract
Phage-display peptide libraries have been widely used to identify specific peptides targeting in vivo tumor cells and the tumor vasculature and playing an important role in the discovery of antitumor bioactive peptides. In the present work, we identified a new melanoma-homing peptide, (CVNHPAFAC), using a C7C phage-display library directed to the developing tumor in syngeneic mice. Phage were able to preferentially target melanoma in vivo, with an affinity about 50-fold greater than that with normal tissue, and the respective synthesized peptide displaced the corresponding phage from the tumor. A preferential binding to endothelial cells rather than to melanoma cells was seen in cell ELISA, suggesting that the peptide is directed to the melanoma vasculature. Furthermore, the peptide was able to bind to human sonic hedgehog, a protein involved in the development of many types of human cancers. Using a new peptide approach therapy, we coupled the cyclic peptide to another peptide, HTMYYHYQHHL-NH2, a known antagonist of VEGFR-2 receptor, using the GYG linker. The full peptide CVNHPAFACCYHTMYHYQHHL-NH2 was effective in delaying tumor growth (P < 0.05) and increasing animal survival when injected systemically, whereas a scramble-homing peptide containing the same antagonist did not have any effect. This is the first report on the synthesis of a tumor-homing peptide coupled to antiangiogenic peptide as a new anticancer therapeutics. Mol Cancer Res; 1–8. ©2011 AACR.

Introduction
Melanoma is a highly aggressive cancer with poor prognosis in the metastatic stage. The incidence is rapidly increasing in the world with approximately 68,000 new cases of invasive melanoma diagnosed in the United States in 2009 (1). Malignant melanoma is responsible for 80% of total skin cancer deaths despite the fact that it accounts for only 4% of dermatologic cancers (2). The main goal in cancer treatment is to target cancer cells or its vasculature and preserve normal tissues, thus implying that specific delivery ligands are required (3).

Phage-display peptide libraries have been widely used to identify specific peptides and proteins targeting cancer cells in vitro and in vivo (4–7) and tumor vasculature (8). The methodology consists in the selection of peptides or proteins displayed on phage libraries that recognize a ligand, followed by phage DNA sequencing, to determine the peptide sequence. The use of synthetic peptides, based on phage selective binding, is valuable in cancer therapy because of their low interaction with the immune system, potential large-scale production, reproducibility, and good tumor and tissue penetration (9).

Tumor blood vessels differ from vessels of normal tissues by expressing different antigens or overexpressing others, thus implying that specific delivery ligands are required for effective antitumor treatment. Angiogenesis is essential for tumor development; therefore, the discovery of specific inhibitors is an important strategy in cancer therapy. A small peptide called RGD-4C (CDCRGDCFC) and targeting tumor vasculature was complexed with doxorubicin and shown to be very effective in treatment of mice bearing carcinomas (10). In 2002, Hetian and colleagues selected a novel peptide by phage display designated K237 (HTMYHYQHHL) that bound to VEGF receptor-2 (VEGFR-2) and interfered with VEGF binding (11). This peptide also exhibited in vivo activity when injected at peritumor sites. Using an in vivo biopanning technique, Bussolati and colleagues (12) identified a specific peptide CVGNDNSSC that bound to tumor endothelial cells and when conjugated with saporin was able to induce apoptosis of tumor cells in vivo and disruption of the vessel network.

In the melanoma model, there are few therapeutic studies using the phage-display methodology, including treatment with whole phage (13), a human melanin-binding peptide
and a peptide targeting a desintegrin that is important for melanoma adhesion (15). In the present work, our objective was to identify specific tumor-homing peptides by in vivo phage display and to couple it with an anti-angiogenic peptide aiming at a new therapeutic approach. This is the first description of an in vivo phage-display experiment in melanoma model and the conjugation of a targeting peptide to a bioactive one for antitumor activity.

Materials and Methods

Mice and cultured cell lines

Eight-week-old male C57BL/6 mice were obtained from the Center for Development of Experimental Models (CEDEME), Federal University of São Paulo (UNIFESP). Animal experiments were carried out according to the UNIFESP Ethics Committee for Animal Experimentation.

The B16F10 murine melanoma cell line is syngeneic in C57BL/6 mice and was originally obtained from the Ludwig Institute for Cancer Research, São Paulo branch. The melanotic subline NEX2 (B16F10-Nex2) was isolated at the Experimental Oncology Unit (UNONEX). Tumor cell lines and human umbilical vein endothelial cells (HUVEC) were maintained in complete medium consisting of RPMI-1640, pH 7.2, supplemented with 10 mmol/L HEPES, 24 mmol/L sodium bicarbonate, 10% heat-inactivated fetal calf serum (FCS) from Gibco, and 40 μg/mL gentamicin sulfate (Hipolabor Farmacêutica) at 37°C in a humidified atmosphere with 5% CO₂.

Phage library

A peptide phage library, PhD C7C, was purchased from New England Biolabs. This library consists of 7 random amino acid peptides flanked by cysteine residues from the N-terminal sequences of the M13 PIII coat protein. All phage protocols, including phage amplification, titration, and DNA isolation, were carried out as recommended by the manufacturer. The control biopanning provided with the kit was used.

In vivo biopanning

In vivo biopanning was carried out according to Matsuo and colleagues (16). Briefly, 5 x 10⁴ melanoma cells were injected subcutaneously in the right flank of mice. When tumors reached approximately 1,000 mm³, mice were injected intravenously with 10¹¹ plaque forming units (pfu) of the random 7C7 library in the tail vein. After 60 minutes, mice were sacrificed by cervical displacement, and the kidney, spleen, and tumor mass were excised for phage titration. Cells were resuspended in PBS and passed through a 70-μm cell strainer (Becton Dickinson). To remove blood contaminants, cells were resuspended in ice-cold 0.2% NaCl and hemolyzed in 1 volume of ice-cold 1.6% NaCl. After several washes with PBS, tumor cells, kidney, and spleen tissues were weighted, and phages were eluted with 0.1 mol/L glycine, pH 2.2, for 10 minutes, neutralized with 1 mol/L Tris, pH 9.1, and titrated. The ratio of pfu per gram of tissue was used to analyze the fold increase of individual selected phages in comparison with the C7C peptide library. The same procedure was used in the peptide competition assay by coinjecting 500 μg of the selected peptide along with the corresponding phage.

Peptide synthesis

Peptides were synthesized by the solid-phase and classical solution methods of peptide synthesis. All the obtained peptides were purified by semipreparative liquid chromatography on an Econosil C18 column. The molecular masses of synthesized peptides were confirmed by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry with a ToFSpec-E from Micromass spectrometer.

Cell ELISA

To test the binding of phage C, biotin-peptide C, or biotin-scramble peptide with B16F10-Nex2 cells or HUVECs, 10⁴ cells were plated on milk-white 96-well plates by centrifugation (1,500 × g for 10 minutes) and fixed with 0.5% of glutaraldehyde overnight at 4°C. After neutralization with 0.5 mol/L glycine for 1 hour at room temperature, plates were blocked with PBS containing 1% bovine serum albumin (BSA) for 3 hours at 37°C, followed by incubation with 10⁷ pfu of phage C or the phage library and different concentrations of biotin-peptide C or biotin-scramble peptide in PBS containing 0.1% BSA overnight at 4°C. Cells were washed with PBS-Tween 0.1% and incubated with a rabbit serum polyclonal anti-M13 (1:1,000) or streptavidin-peroxidase in the case of the biotin-labeled peptides in PBS containing 0.1% BSA overnight at 4°C. After washing, wells were incubated with secondary anti-rabbit peroxidase antibody (GE Healthcare) for 1 hour at 37°C, washed again, and luminescence (Pierce) measured on a plate reader. Phage specificity was determined by the ratio of luminescence of phase C and that of the phage library.

ELISA

A milk-white 96-well plate was coated with 1 μg/mL of human recombinant sonic hedgehog protein (R&D Systems) in carbonate bicarbonate buffer (50 mmol/L at pH 8.6) overnight at 4°C. Plate was washed with PBS-Tween (0.1%) and blocked with PBS containing 1% of BSA for 2 hours at room temperature. The biotin-peptide C or biotin-scramble peptides were added to the wells at the final concentration of 5 μmol/L in PBS-BSA 0.1% and incubated for 2 hours at room temperature with gently agitation. After several washes, avidin-peroxidase in the same buffer were added and incubated for 1 hour at room temperature. The plate was washed again, and the relative luminescence units (RLU) were measured in a plate reader. The specific RLU for each biotin-peptide was determined by the subtraction of sonic hedgehog from the BSA-coated wells.

Cell viability assay

B16F10-Nex2 or HUVECs were seeded at 5 x 10³ per well into 96-well plates (Corning Costar Co) 12 hours later.
previously to the incubation with serially diluted peptides to a final volume of 100 µL in complete RPMI medium containing 10% FCS. After 24 hours, cells were detached with PBS-EDTA solution, and viable cells were determined in a Neubauer chamber with the exclusion dye Trypan Blue.

**In vitro angiogenesis assay**

The *in vitro* angiogenesis assay was conducted as described elsewhere (17). Briefly, HUVECs (5 × 10^4 cells per well) were suspended in 100 µL of RPMI medium supplemented with 0.2% of FCS containing 10 µmol/L of the peptides and plated on a BD Matrigel Matrix (BD Biosciences) and incubated at 37°C for 18 hours. Images were taken with a high-performance digital microscope camera (ProgRes C14 Plus; Jenoptik) coupled to a Nikon inverted microscope. The percentage of angiogenic structures were determined from 2 independent experiments carried out in triplicates.

**Treatment of subcutaneous murine melanoma**

The protocol used was the same described elsewhere (18). Basically, 5 × 10^4 B16F10-Nex2 cells were inoculated subcutaneously on the right flank of C57Bl/6 mice (n = 5). Mice treatment was carried out on day 1 after tumor cell challenge, during 3 weeks in alternate days intraperitoneally with doses of 100 µmol/L ranging from 10 to 100 µmol/L of peptides. Tumor volume determination and animal sacrifice were carried out as described earlier. The tumor volume (in mm^3) was measured by the equation, \( V = (0.52 \times d^2 \times D) \), where \( d \) and \( D \) are the small and large diameters in millimeters, respectively. Animals were sacrificed by cervical displacement when the tumor volume reached 3,000 mm^3. Using a treatment protocol, 1 × 10^5 B16F10-Nex2 cells were inoculated subcutaneously on the right flank of C57Bl/6 mice. After 10 days, tumor-bearing mice (average 100 mm^3) were daily treated intraperitoneally with 100 µL of peptides at 200 µmol/L until the 16th day. In the next day, all animals were sacrificed for histopathologic analysis of the tumor, lung, kidney, and liver tissues stained by hematoxylin–eosin (H&E).

**Statistic tests**

The Student *t* test was used in most cases. The Kaplan–Meier log-rank test was applied to survival data. *P* < 0.05 values were considered significant.

**Results**

**Selection of tumor-homing peptides and specificity of phage clones**

Detection of tumor-homing peptides was done after an *in vivo* biopannings against a subcutaneously developing A.

![Graph A](image1.png)

**Figure 1.** A, phage recovery from each tissue after acidic elution. An enrichment of 25-fold for phage peptide binding to the developing tumor was observed compared with the much lower phage binding to kidney or spleen. B, specificity of each individual phage peptide for the tumor. The specificity was measured by the ratio of tumor fold increase in binding compared with the kidney fold increase for each phage in relation to the original phage library. While peptide sequences A, B, and D were about 10 times more specific for the tumor, sequence C was more than 50 times tumor specific.

![Graph B](image2.png)

**Table 1.** Peptide sequence of 50 sequenced phages

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>No. of repetitions (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-CTALPRSLC-)</td>
<td>3 (6)</td>
</tr>
<tr>
<td>(-CWTRAPDLC-)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>(-CTTTSSWLC-)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>(-CNQESYARC-; A)</td>
<td>12 (24)</td>
</tr>
<tr>
<td>(-CHRPTLHQC-)</td>
<td>3 (6)</td>
</tr>
<tr>
<td>(-CSSFORNLCL-)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>(-CGNRPGPHC-; B)</td>
<td>4 (8)</td>
</tr>
<tr>
<td>(-CFPSKLPTC-)</td>
<td>3 (6)</td>
</tr>
<tr>
<td>(-CVNHPAFAC-; C)</td>
<td>7 (14)</td>
</tr>
<tr>
<td>(-CVPLCHVR-; D)</td>
<td>4 (8)</td>
</tr>
<tr>
<td>(-CTAGRNPPEC-)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>(-CNSGSTRAC-)</td>
<td>2 (4)</td>
</tr>
<tr>
<td>(-CHPRTRANAC-)</td>
<td>2 (4)</td>
</tr>
<tr>
<td>(-CPAHPSPWC-)</td>
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<tr>
<td>(-CNPGAAREC-)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>(-CLPTSLQSC-)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>(-CSQNKFSNC-)</td>
<td>1 (2)</td>
</tr>
</tbody>
</table>

**NOTE:** After the third round of panning, phages from random plaques were collected and sequenced. Four sequences (in bold) were prevalent.
murine melanoma. After 3 rounds of selection, we observed 25-fold enrichment for the tumor site compared with kidney or spleen (Fig. 1A). Fifty individual eluted phages from the third round were amplified for DNA sequencing and peptide determination. Among all sequenced phages, 4 of them repeated at least 4 times: phage A, sequence (-CNQESYARC-; 12/50); phage B, (-CQNRPGPHC-; 4/50), phage C, (-CVNHPAFAC-; 7/50), and phage D, (CVPLCTHVR-; 4/50; Table 1). Normally peptides from this library are flanked by cysteine residues; in phage D, one cysteine was replaced by arginine by some mutation.

To evaluate the specificity of phage clones for tumor homing, they were individually tested in vivo by the same biopanning protocol. All phage clones preferentially bound to the tumor rather than to the kidney. Phage clones A, B, C, and D showed increased binding of 2.6, 2.1, 1.6, and 4.2 times to kidney compared with 30, 12, 83, and 74 times, respectively, onto the tumor. Specificity was calculated by the ratio of detected phages in tumor and in the kidney (Fig. 1B). Phage clones A, B, and D showed a ratio lower than 20 times, whereas the ratio with phage C was 50-fold.

**In vivo competition of phage C with peptide C**

Because phage C rendered the best ratio, we selected it for further study. To confirm that the peptide (-CVNHPAFAC-) and not the peptide phage would be responsible for tumor homing, a competition assay was conducted. Mice with 1,000-mm³ tumor were injected with 10¹¹ phage C with 500 µg of the corresponding peptide. After 1 hour of circulation, phages were eluted from tumor and kidney. As shown in Fig. 2, peptide C was able to markedly displace phage C from tumor, 83 to 2.6 times, and it had no effect on kidney.

**Binding of phage C and peptide C to cells**

As the tumor itself comprises not only tumor cells but also endothelial cells from angiogenesis, we examined the binding of phage C either to B16F10-Nex2 or HUVECs. To test the binding of phage C to B16F10-Nex2 cells or HUVECs, 10⁴ cells were plated on milk-white 96-well plates and fixed with 0.5% of glutaraldehyde overnight at 4°C. After blocking with PBS-BSA 1%, cells were incubated with 10⁹ pfu of phage C or the phage library that was used as a normalizer. As evidenced in Fig. 3A, phage C preferentially bound to endothelial cells. A ratio of phage C to phage library readings was used to calculate the binding specificity.

As phage C binds to HUVECs, we evaluated the binding of the biotin-peptide C or the scramble peptide (-CAPFNHAVC-) to this cell lineage. In Fig. 3B, a dose-dependent increase in the luminescence only in cells incubated with the labeled peptide C. Thus, both peptide C and phage C are able to bind to HUVECs.

**Peptide C had no effect in vitro and in vivo against melanoma or endothelial cells**

We further investigated whether peptide C had any in vitro effect on B16F10-Nex2 or HUVECs. Cells were seeded at 5 × 10⁵ per well into 96-well plates 12 hours before the incubation with serially diluted peptides at 200 to 0 µmol/L in complete RPMI medium containing 10% FCS. After 24 hours, cells were detached with PBS-EDTA solution, and viable cells were determined in a Neubauer chamber with the exclusion dye Trypan Blue. No peptide effect was detected with both cell lines (data not shown). Because the peptide binds to endothelial cells, we investigated a possible in vivo activity of this peptide. Basically, 5 × 10⁴ B16F10-Nex2 cells were inoculated subcutaneously on

![Figure 2](image2.png)

**Figure 2.** Tumor specificity of peptide C. Phage C was coinjected in mice with 500 µg of the synthetic C-terminal amidated peptide C for binding competition. As shown, peptide C was able to displace phage C from the tumor tissue.

![Figure 3](image3.png)

**Figure 3.** A, cell ELISA of phage C binding to B16F10-Nex2 cells and HUVECs. Phage C bound with greater specificity to endothelial cells than to melanoma cells. B, cell ELISA of HUVECs and biotinylated peptide C or the scramble peptide at different concentrations.
The sequence of peptide C (-CVNHFAFAC-) was examined for similarity in a protein data bank (NCBI; Blast) selecting human proteins. The best match was the sequence -CINHPGTFAC- that belongs to the signal peptide CUB and epidermal growth factor–like domain from SCUBE2 protein. To examine the structural homology of these peptides, a 3-dimensional modeling was designed with the software ACD/ChemSketch (Fig. 4A). Because peptide C could mimic a domain of the SCUBE2 protein, we hypothesized that it could bind to sonic hedgehog protein, the ligand of SCUBE2. As shown in Fig. 4B, the biotin-peptide C greatly bonded to human sonic hedgehog protein in comparison with the biotin-scramble peptide.

To generate a bioactive peptide directed to tumor vasculature, we synthesized peptide C coupled to an antiangiogenic peptide sequence (HTMYYHYQHHHL), separated by a linker (GYG; Fig. 4C).

**In vitro antiangiogenic activity**

To check the antiangiogenic properties of the new peptide, we conducted an *in vitro* assay in which HUVECs were incubated with peptides and plated on Matrigel. After 18 hours at 37°C, the formation of proangiogenic structures was observed with an inverted microscope. Peptide C or the scramble peptide did not have any effect on angiogenesis, whereas both peptide C and the scramble peptide coupled to the antiangiogenic peptide showed were able to inhibit angiogenesis, reducing the proportion of proangiogenic structures by approximately 40% (Fig. 5).

**Antitumor effect of peptide C conjugated with antiangiogenic peptide**

We next examined the ability of peptide C as a specific delivery tool addressing the tumor vasculature. Treatment was conducted as described earlier, on the next day after cell inoculation, during 3 weeks in alternate days intraperitoneally with doses of 100 µL of the peptides at the concentration of 50 µmol/L. Peptide CAA was able to significantly delay tumor development (Fig. 6A) and increase mice survival (Fig. 6B; *, *P < 0.05), but no effect was observed with peptide ScrambleAA used as a control. The antiangiogenic peptide alone also had no effect (data not shown). Lower dosages, 10 µmol/L instead of 50 µmol/L, of peptide CAA did not have any effect, and higher doses up to 100 µmol/L also did not significantly improve the antitumor activity (data not shown).

In the treatment protocol in which mice bearing tumor (average 100 mm³) were treated with the CAA peptide, a significant delay in tumor progression (Fig. 6C) was observed. All other peptides tested were inactive. Treatment was conducted during 7 consecutive days at daily doses of 100 µL containing 200 µmol/L of the peptides intraperitoneally. All groups were sacrificed on the 17th day after tumor inoculation, and histopathologic slides stained with
H&E were obtained from tumor, liver, lung, and kidney. Large areas of necrosis (black arrows) and reduced number of vessels (red arrows) in the tumor mass were only observed in mice treated with CAA (Fig. 6D). No morphologic alterations were observed in the lungs, kidney, or liver of mice from other groups (data not shown).

Discussion

The use of bioactive peptides is a promising field in cancer therapy. Scientists can obtain peptides with necrotic or apoptotic activities, function-blocking peptides such as receptor-binding or cell-adhesion molecules, peptides with immunostimulatory activity, and tumor-targeting peptides by rational methods (19). Those molecules can be easily produced and modified for better efficacy and little degradation. To test the activity of bioactive peptides suitable responsive biological systems should be selected. In our laboratory, the B16F10 murine melanoma model has routinely been used in vitro and in vivo to evaluate the antitumor effects of bioactive peptides (20).

Phage display has been extensively used to rationally find specific ligands. This combinatorial technology is a powerful method to detect tumor-homing peptides by in vivo selection. This approach has the advantage to select specific peptides using the whole body for subtraction of unspecific phages (21). In 1998, Arap and colleagues (10) detected the peptide motif RGD for tumor vasculature by in vivo phage display. Association of the peptide CDCRGDCFC with doxorubicin was effective in the treatment of breast cancer and lowered the drug toxicity to liver and heart. Recently, the same cyclic peptide (CRGDGWC) was coupled to p53 and by intravenous administration in melanoma-bearing mice was able to induce apoptosis in tumor cells (22).

Using in vivo phage-display peptide screening, we characterized a new specific tumor-homing cyclic peptide sequence (-CVNHPAFAC-). This sequence showed great similarity with a region from the human SCUBE2 protein. Interestingly, this protein was first described in 2002 as a secreted glycoprotein produced by endothelial cells that maintain a stable association with the cell surface. In 2009, Tsai and collaborators showed that SCUBE2 plays a role in the hedgehog signaling pathway (24), which is implicated in the development of breast, brain, lung, prostate, pancreas, and skin, including melanoma (25–27). Immunohistochemical analysis showed that hedgehog was very abundant in the...
inhibited tumor angiogenesis

In the present work, the new tumor targeting peptide C (-CVNHPAFAC-) preferentially bound to developing tumors. In vitro, the peptide bound to HUVECs, as detected by cell ELISA, rather than to melanoma cells. It was then coupled to the antiangiogenic peptide HTMYHYHYQHHHL with a linker (GYG). The final peptide CVNHPAFACGYHTMYHYHYQHHHL was effective in delaying tumor progression when systemically injected even in mice bearing tumors. In comparison with the treatment used by Hetian and colleagues (11), we used 6 times less peptide that was systemically injected to achieve a similar inhibition of tumor progression. A significant increase in the survival of mice challenged with melanoma cells was observed. As shown, peptide C selected in vivo on murine melanoma was able to bind to human sonic hedgehog and HUVECs; therefore, it is expected also to be active in human cancer. Peptide C fused to the antiangiogenic peptide induced a delay in tumor growth, and the tumor mass showed numerous areas of necrosis due to the effective inhibition of angiogenesis. There are reports on the conjugation of bioactive peptides with cell-penetrating peptides or on tumor-homing peptides linked with proteins or toxins (35); however, ours is the first report on a bioactive peptide that was coupled to a tumor-homing peptide to achieve antitumor effect. This is a new approach that opens many perspectives, as peptides are easily synthesized and purified. Other bioactive peptides can be coupled to the same sequence-generating multivalent targeting drugs in a single synthesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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