A NEW PHAGE-DISPLAY TUMOR HOMING PEPTIDE FUSED TO ANTI-ANGIOGENIC PEPTIDE GENERATES A NOVEL BIOACTIVE MOLECULE WITH ANTI-MELANOMA ACTIVITY

Alisson L Matsuo\textsuperscript{a},* , Maria A Juliano\textsuperscript{b} , Carlos R Figueiredo\textsuperscript{a} , Wagner L Batista\textsuperscript{c} , Aparecida S Tanaka\textsuperscript{d} and Luiz R Travassos\textsuperscript{a}

\textsuperscript{a}Experimental Oncology Unit (UNONEX), Department of Microbiology, Immunology and Parasitology, \textsuperscript{b}Department of Biophysics, \textsuperscript{c}Department of Biological Sciences and \textsuperscript{d}Department of Biochemistry, Universidade Federal de São Paulo, São Paulo, SP 04023-062, Brazil

*Corresponding author at: UNONEX, Department of Microbiology, Immunology, Parasitology, UNIFESP, Rua Botucatu 862, 8th floor, São Paulo, SP, 04023-062 Brazil, Tel: 55-11-5576-4551. Fax: 55-11-5571-5877.
E-mail: almatsuo@unifesp.br

Running title: A NOVEL ANTI-TUMOR FUSION PEPTIDE

Keywords: Phage display, anti-tumor peptide, drug delivery, angiogenesis, melanoma
ABSTRACT

Phage display peptide libraries have been widely used to identify specific peptides targeting in vivo tumor cells and the tumor vasculature, 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 CVNHPAFACGYGHTMYYHYQHHL-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 anti-angiogenic peptide as a new anticancer therapeutics.
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 new methodologies to identify specific 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 over-expressing others, thus implying that specific delivery ligands are required for effective anti-tumor 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 et al. selected a novel peptide by phage display designated K237 (HTMYYHHYQHHL) that bound to VEGFR-2 and interfered with VEGF binding [11]. This peptide also exhibited in vivo activity when
injected at peri-tumor sites. Using an *in vivo* biopanning technique, Bussolati B et al. [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 [14] and a peptide targeting a desintegrin which 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 anti-tumor 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 with 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 lineages and human umbilical vein endothelial cells (HUVEC) were maintained in complete medium consisting of RPMI 1640, pH 7.2, supplemented with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), 24 mM sodium bicarbonate, 10% heat-inactivated fetal calf serum (FCS) from Gibco (Minneapolis, MN, USA) and 40 μg/ml gentamicin sulfate (Hipolabor Farmacêutica, Sabará, MG, Brazil) at 37°C in a humidified atmosphere with 5% CO2.

Phage library

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 performed as recommended by the manufacturer. The control bio-panning provided with the kit was used.
**In vivo bio-panning**

*In vivo* bio-panning 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 pfu (plaque forming units) of the random 7C7 library in the tail vein. After 60 min 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 M glycine, pH 2.2 for 10 min, neutralized with 1 M 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 but co-injecting 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 semi-preparative HPLC on an Econosil C-18 column. The molecular masses of synthesized peptides were confirmed by MALDI-TOF mass spectrometry, using a TofSpec-E from Micromass, Manchester, UK.

**Cell ELISA**
To test the binding of Phage-C, biotin-Peptide C or biotin-Scramble peptide to B16F10-Nex2 cells or HUVECs, 10⁴ cells were plated on milk-white 96-well plates by centrifugation (1,500 g for 10 min) and fixed with 0.5% of glutaraldehyde overnight at 4°C. After neutralization with 0.5 M glycine for 1 h at room temperature, plates were blocked with PBS containing 1% BSA for 3 h at 37°C followed by incubation with 10⁹ 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 (1:1000) or streptoavidin-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, Little Chalfont, UK) for 1 hour at 37°C, washed again and luminescence (Pierce, Rockford, USA) measured in a plate reader. Phage specificity was determined by the ratio of luminescence of Phage C and that of the phage library.

ELISA

A milk-white 96-well plate were coated with 1 µg/mL of human recombinant sonic hedgehog protein (R&D Systems, MN, USA) in carbonate-bicarbonate buffer (50 mM 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 µM 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 HUVEC were seeded at 5 x 10³/well into 96 well-plates (Corning Costar Co, NY, USA.) 12 h previously to the incubation with serial diluted peptides to a final volume of 100 µL in complete RPMI medium containing 10% FCS. After 24 h, cells were detached using PBS-EDTA solution and viable cells were determined in a Neubauer chamber using the exclusion dye Trypan Blue.

**In vitro angiogenesis assay**

The *in vitro* angiogenesis assay was performed as described elsewhere [17]. Briefly, HUVECs (5 x 10³ cells/well) were suspended in 100 µl of RPMI medium supplemented with 0.2% of FCS containing 10 µM of the peptides and plated on a BD Matrigel Matrix (BD Biosciences) and incubated at 37°C for 18 hours. Images were taken using a high-performance digital microscope camera (ProgRes C14 Plus - Jenoptik) coupled to a Nikon Inverted-Microscope. The percentage of angiogenic structures were determined from two independent experiments performed in triplicates.

**Treatment of subcutaneous murine melanoma**
The protocol used was the same described elsewhere [18]. Basically, 5 x 10⁴ 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 via i.p. with doses of 100 µM ranging from 10 to 100 µM of peptides. Tumor volume determination and animal sacrifice were performed as described above. The tumor volume in mm³ was measured by the equation, \( V = (0.52 \times d^2 \times D) \), where d and D are the small and great diameter in millimeters, respectively. Animals were sacrificed by cervical displacement when the tumor volume reached 3,000 mm³. Using a treatment protocol, 1 x 10⁵ B16F10-Nex2 cells were inoculated subcutaneously on the right flank of C57Bl/6 mice. After 10 days, tumor bearing mice (average 100 mm³) were daily treated i.p. with 100 µL of peptides at 200 µM until the 16th day. In the next day, all animals were sacrificed for histopathological analysis of the tumor, lung, kidney and liver stained by Hematoxylin-Eosin.

**Statistic tests**

The Student’s t test was used in most cases. Kaplan-Meier’s log rank test was applied to survival data. *p Values < 0.05 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 murine melanoma. After three rounds of selection, we observed 25-fold enrichment for the tumor site compared to kidney or spleen (Figure 1A). Fifty individual eluted phages from the third round were amplified for DNA sequencing and peptide determination. Among all sequenced phages, four of them repeated at least four 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 using the same biopanning protocol. All phage clones preferentially bound to the tumor rather than to the kidney. Phage A, B, C and D showed increased binding of 2.6, 2.1, 1.6 and 4.2 times to kidney compared to 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 (Figure 1B). Phage clones A, B and D showed a ratio lower than 20 times while the ratio with phage C was 50-fold.

In vivo competition of phage C with peptide C
Since 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 performed. Mice with 1,000-mm³ tumor were injected with $10^{11}$ phage C with 500 µg of the corresponding peptide. After 1 hour of circulation, phages were eluted from tumor and kidney. As shown in Figure 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 HUVEC, $10^4$ 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 $10^9$ pfu of phage C or the phage library that was used as a normalizer. As evidenced in Figure 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 biotinylated peptide C or the scramble peptide [-CAPFNHAVC-] to this cell lineage. In Figure 3B we observed 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 HUVEC.
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 HUVEC. Cells were seeded at 5 x 10³/well into 96 well-plates 12 h before the incubation with serial diluted peptides at 200 to 0 μM in complete RPMI medium containing 10% FCS. After 24 h, cells were detached using PBS-EDTA solution and viable cells were determined in a Neubauer chamber using the exclusion dye Trypan Blue. No peptide effect was detected with both cell lines (data not shown). Since the peptide binds to endothelial cells, we investigated a possible in vivo activity of this peptide. Basically, 5 x 10⁴ B16F10-Nex2 cells were inoculated subcutaneously on the right flank of C57Bl/6 mice and treated in the next day after inoculation, during 3 weeks in alternate days via i.p. with doses of 100 µL at the concentration of 50 μM. No antitumor effect was observed (data not shown).

Peptide C homology and binding to human sonic hedgehog

The sequence of peptide C [-CVNHPAFAC-] 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 EGF-like domain from SCUBE2 protein. To examine the structural homology of these peptides, a 3D modeling was designed using the software ACD/ChemSketch (Figure 4A). Since 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 Figure 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 anti-angiogenic peptide sequence (HTMYYHHYQHHL) separated by a linker (GYG) (Figure 4C).

**In vitro anti-angiogenic activity**

To check the anti-angiogenic properties of the new peptide, we performed 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 using 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 anti-angiogenic peptide showed were able to inhibit angiogenesis, reducing in approximately 40% the number of proangiogenic structures (Figure 5).

**Antitumor effect of peptide C conjugated with anti-angiogenic peptide**

We examined next the ability of peptide C as a specific delivery tool addressing the tumor vasculature. Treatment was performed as described above, in the next day after cell inoculation, during 3 weeks in alternate days via i.p. with doses of 100 µL of the peptides at the concentration of 50 µM. Peptide CAA was able to significantly delay tumor development (Fig 6A) and increased mice survival (Fig 6B) (*p<0.05) but no effect was observed with peptide ScrambleAA used as control. The anti-angiogenic peptide alone also had no effect (data not shown). Lower dosages, 10 µM instead of 50 µM of peptide CAA
did not have any effect, and higher doses up to 100 µM 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 performed during seven consecutive days at daily doses of 100 µL containing 200 µM of the peptides intraperitoneally. All groups were sacrificed on the 17th day after tumor inoculation and histopathological slides stained with HE 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 morphological 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 using rational methods [19]. Those molecules can be easily produced and modified for better efficacy and little degradation. In order 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 anti-tumor 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 using 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 SCUBE 2 protein. Interestingly, this protein was first described in 2002 [23] as a secreted glycoprotein produced by endothelial cells that maintain a stable association with the cell surface. In 2009, Tsai and collaborators
demonstrated that SCUBE2 plays a role in the Hedgehog (HH) 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 tumor microenvironment of B16F0 melanoma and was important for tumor development [28]. In the case of the new fused peptide, we demonstrated that peptide C binds to human sonic hedgehog and would be important to concentrate the peptide in the tumor vascular network thus increasing the reactivity of the antiangiogenic fused peptide.

Tumor vasculature differs from that of normal tissues in its architecture, genetic alterations, angiogenic signaling, protein expression and other features [29]. Some angiogenesis related genes such as those encoding cadherin 5, erythropoietin (as in hepatocellular carcinoma-A2), laminin 5 γ2-chain are up-regulated in aggressive tumors [30]. Angiogenesis is critical for tumor development, thus justifying the new methodologies and strategies to inhibit the process. Bevacizumab is a humanized anti-angiogenic monoclonal antibody approved by FDA (Food and Drug Administration) for cancer treatment which targets the vascular endothelial growth factor (VEGF). The efficacy of Bevacizumab is, however, tarnished by undesirable side-effects including hypertension, bleeding, proteinuria, thrombosis and increased risk of venous and arterial thromboembolic events [31]. In 2002, Hetian et al. selected a peptide, HTMYYHHYQHHL, by phage display that binds with high affinity to VEGFR-2 acting as antagonist [11]. This peptide inhibited solid tumor growth when injected peritumorally with 60 µL/dose containing 500 µM peptide every two days.

The VEGFR-2 is a transmembrane receptor mainly related with angiogenesis. The gene homozygous-deficient mice showed insufficient development of endothelial and
hematopoietic cells and die in the second week of gestation [32]. This receptor was detected in different types of cancer in high frequency, and is also over-expressed in melanoma [33]. In 2004, Kou et al. isolated and expressed a VEGFR-2 fragment in murine B16 melanoma cells that inhibited tumor angiogenesis in vivo [34].

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 HTMYYHYQHHL using a linker (GYG). The final peptide CVNHPAFACGYGHTMYYHYQHHL was effective in delaying tumor progression when systemically injected even in mice bearing tumors. In comparison with the treatment used by Hetian et al. [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 anti-tumor effect. This is a new approach that opens many perspectives since peptides are easily synthesized and purified. Other bioactive peptides can be coupled to the same sequence generating multivalent targeting drugs in a single synthesis.
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ACKNOWLEDGEMENTS

The present work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil.
FIGURE LEGENDS

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 to 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 to 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.

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

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.

Figure 4. A) Structural similarities between Peptide C and a domain in the SCUBE2 protein. B) Peptide C binds to human sonic hedgehog protein C) Schematic
representation of the homing peptide-(linker) fused to a bioactive peptide with anti-angiogenic activity.

Figure 5. Inhibition of proangiogenic structures. Peptide CAA and ScrambleAA at 10 µM were able to inhibit about 40% of the proangiogenic structures. No effect was observed with Peptide C or the scramble peptide.

Figure 6. *In vivo* effective delivery and antitumor effect of the anti-angiogenic peptide (AA) conjugated with peptide C. A) Subcutaneous tumor development in mice treated with the CAA peptide on day 1; B) Survival of mice subcutaneously challenged with tumor cells and treated, from (A); p<0.01; C) Tumor development in mice bearing tumor (100 mm3) and treated with the peptide-chimera on day 10; D) Tumor histopathology, stained by HE. Areas of necrosis (black arrows), blood vessels (red arrows) *p < 0.05.*
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Table 1. Peptide sequence of 50 sequenced phages. After the third round of panning, phages from random plaques were collected and sequenced. Four sequences (in bold) were prevalent.
Figure 1
Figure 2

- Phage C
- Phage C + peptide C

Fold Increase

Kidney

Tumor

Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
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Alisson Leonardo Matsuo, Maria Aparecida Juliano, Carlos Rogério Figueiredo, et al.

Mol Cancer Res Published OnlineFirst September 7, 2011.

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