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Increased Potency of the PHSCN Dendrimer as an Inhibitor of Human Prostate Cancer Cell Invasion, Extravasation, and Lung Colony Formation

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Abstract

Background—Activated $\alpha 5 \beta 1$ integrin occurs specifically on tumor cells and on endothelial cells of tumor-associated vasculature, and plays a key role in invasion and metastasis. The PHSCN peptide (Ac-PHSCN-NH₂) preferentially binds activated $\alpha 5 \beta 1$, to block invasion *in vitro*, and inhibit growth, metastasis and tumor recurrence in preclinical models of prostate cancer. In Phase I clinical trial, systemic Ac-PHSCN-NH₂ monotherapy was well tolerated, and metastatic disease progression was prevented for 4–14 months in one third of treated patients.

Results—We have developed a significantly more potent derivative, the PHSCN-polylysine dendrimer (Ac-PHSCNGGK-MAP). Using *in vitro* invasion assays with naturally serum-free basement membranes, we observed that the PHSCN dendrimer was 130– to 1900-fold more potent than the PHSCN peptide at blocking $\alpha 5 \beta 1$ -mediated invasion by DU 145 and PC-3 human prostate cancer cells, whether invasion was induced by serum, or by the Ac-PHSRN-NH₂ peptide, under serum-free conditions. The PHSCN dendrimer was also approximately 800 times more effective than PHSCN peptide at preventing DU 145 and PC-3 extravasation in the lungs of athymic mice. Chou-Talalay analysis suggested that inhibition of both invasion *in vitro* and extravasation *in vivo* by the PHSCN dendrimer are highly synergistic. We found that many extravasated DU 145 and PC-3 cells go on to develop into metastatic colonies, and that a single pretreatment with the PHSCN dendrimer was 100-fold more effective than the PHSCN peptide at reducing lung colony formation.

Conclusions—Since many patients newly diagnosed with prostate cancer already have locally advanced or metastatic disease, the availability of a well-tolerated, nontoxic systemic therapy, like the PHSCN dendrimer, which prevents metastatic progression by inhibiting invasion, could be very beneficial.

Keywords

Prostate cancer; invasion; extravasation; lung metastasis; integrin fibronectin receptor

Introduction

Invasion is key to prostate cancer metastasis because it promotes tumorigenesis by supporting endothelial cell invasion and neovascularization [1], as well as enabling tumor cells to invade the tissue surrounding a tumor, enter the circulatory system, and extravasate

at distant sites to form metastatic colonies [2,3]. Thus, unrestricted invasion is a very important, debilitating aspect of the metastatic phenotype [4]. Extravasation is a key step in hematogenous cancer metastasis [5], occurring after tumor cells arrest in microvasculature or sinusoids. Tumor cell arrest may occur by trapping without stable adhesion [6], or may involve preferential adhesion to the endothelial cells of specific sites [7]. After arrest and transit of the endothelial cell layer, invasion of the underlying basement membrane is required for completion of extravasation, and entry into interstitial connective tissue [5].

We have devised a peptide of 5 amino acids that is a potent inhibitor of $\alpha 5 \beta 1$ integrin-mediated invasion by prostate cancer cells. The acetylated, amidated PHSCN peptide, Ac-PHSCN-NH₂ [2], emerged as an invasion inhibitor during structure activity studies of the invasion-inducing PHSRN peptide [8]. The PHSRN sequence of the fibronectin cell binding domain [9,10] is uniquely an $\alpha 5 \beta 1$ ligand, interacting with a specific region of the $\alpha 5$ subunit. In addition to preventing prostate cancer cell invasion and metastasis [8], Ac-PHSCN-NH₂ is also a potent inhibitor of microvascular endothelial cell invasion and angiogenesis [1]. Systemic Ac-PHSCN-NH₂ prevented disease progression for prolonged periods in several preclinical models, and in phase 1 clinical trial [2,11–14]. Thus, Ac-PHSCN-NH₂ is a promising lead compound for targeted therapy of prostate cancer invasion and metastasis.

To increase its potency, we attached eight Ac-PHSCNGGK peptide moieties to the N-termini of the multiantigenic peptide (MAP) polylysine dendrimer. We report that this creates a significantly more potent inhibitor of $\alpha 5 \beta 1$ integrin-mediated DU 145 and PC-3 human prostate cancer cell invasion and matrix metalloproteinase 1 (MMP-1) induction *in vitro*, and of extravasation and lung colony formation *in vivo* in athymic nude mice. Furthermore, Chou-Talalay analysis of the *in vitro* invasion assay data suggests that inhibition by the PHSCN dendrimer is highly synergistic. We also report that the PHSCN dendrimer prevents DU 145 and PC-3 cell extravasation into the lungs of athymic, nude mice over 100-fold more effectively than the PHSCN peptide, and that a single pretreatment with the PHSCN dendrimer greatly reduces lung colony formation.

Materials and Methods

Cell lines and cell culture

DU 145 [15] and PC-3 [16] metastatic human prostate cancer cells were obtained from American Type Culture Collection (Manassas, VA). They were cultured as recommended, and frozen in liquid N₂ in aliquots within 2 months of receipt. Single aliquots were subsequently resuscitated as needed, and cultured as recommended. No aliquot of cells was cultured for more than 4 months, and the morphologies of all cultures were routinely checked by phase contrast microscopy. Growth curves of all cultures were always recorded, and checked for consistency. For all assays in serum-free (SF) medium, DU 145 and PC-3 cells were first serum-starved overnight.

Peptide and dendrimer synthesis

N-terminal acetylated, C-terminal amidated PHSRN, PHSCN, and HSPNC peptides (Ac-PHSRN-NH₂, Ac-PHSCN-NH₂, and Ac-HSPNC-NH₂) were synthesized, their structures confirmed, and their purities assessed as described [1–3,8,17]. Their purities were as follows: Ac-PHSRN-NH₂, 97%; Ac-PHSCN-NH₂, 98%; Ac-HSPNC-NH₂, 91%.

N-terminal acetylated PHSCN and HSPNC MAPs were synthesized by covalently attaching peptide C-termini to the N-termini of a polylysine dendrimer, 8 core MAP (Sigma-Aldrich, Saint Louis MO). MAPs were synthesized by Fmoc solid phase synthesis in a manual procedure with reaction monitoring by Ninhydrin test [18], to allow for complete coupling

of each amino acid. Quality control of the MAPs was performed by amino acid analysis [19], followed by Edman sequencing and preview analysis to reveal any deletions in the sequences [20]. By these analyses, all dendrimers appeared to be fully populated with PHSCNGGK or HSPNCGGK peptide moieties (not shown). Dendrimer purities were estimated to be as follows: Ac-PHSCNGGK-MAP, 94%; Ac-HSPNCGGK-MAP, 97% (not shown). The MAPs were also evaluated by MALDI for the expected mass of the fully populated dendrimer. The spectra showed the expected mass for the complete MAP and very little evidence of incomplete synthesis (not shown).

PHSCN and HSPNC peptides to be attached to polylysine dendrimers or ovalbumin were synthesized with two glycines and a lysine (GGK) on the C-terminal end (PHSCNGGK or HSPNCGGK) to provide a spacer and an attachment site for labeling. Ac-PHSCNGGK-NH₂ and Ac-HSPNCGGK-NH₂ had functional characteristics identical to Ac-PHSCN-NH₂ and Ac-HSPNC-NH₂, respectively (not shown). PHSCN-coupled ovalbumin (Ac-PHSCNGGK-Ova) was synthesized by coupling the PHSCNGGK C-terminus to ovalbumin with an attached EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) crosslinker (ThermoFisher Scientific, Waltham MA), according to established procedures [21]. Ac-PHSCNGGK-Ova purity was 90%.

***In vitro* invasion assays**

Naturally serum-free, selectively permeable basement membranes from sea urchin embryos were utilized as *in vitro* invasion substrates, as described [1–3,8,17]. All cells were serum starved prior to addition of 10% FBS or 0.1 µg/ml Ac-PHSRN-NH₂ to stimulate invasion. For assays evaluating the effects of blocking anti α5β1 MAb on invasion, serum starved DU 145 or PC-3 cells were incubated for 30 minutes on ice in serum-containing medium with 10 µg/ml or 50 µg/ml anti α5β1 MCA1187 function blocking monoclonal antibody (MAb) (Serotec, Oxford, England) [22], prior to placement on basement membranes. For assays evaluating the effects of inhibitors on PHSRN-induced invasion in SF medium, the concentration of Ac-PHSRN-NH₂ was 0.1 µg per ml/20,000 cells in an assay volume of 1 ml. Peptides or dendrimers were prebound to cells prior to placement on basement membranes. Data, mean invasion percentages, were analyzed using Graphpad Prism 5 software (San Diego, CA) as a function of log (inhibitor) vs. normalized data, variable slope.

Data analysis

Data were analyzed by the Combination Index (CI) method of Chou-Talalay [23], as described in [24]. The analysis was based on the multiple drug effect equation derived from the median effect principle of the mass-action law. The median-effect equation, $y = \log(f_a/f_u)$, with respect to $x = \log(\text{dose})$, defines the dose and effect relationship in the absence of reaction rate constants, where f_a is the fraction of cells affected (invasion-inhibited), and f_u is the fraction of cells unaffected (invaded). The $\times$ intercept represents the IC₅₀ value. CI and DRI values were determined assuming that the monomer represents the single dose, and the dendrimer (with 8 PHSCNGGK moieties) represents the combinatorial dose.

MMP-1 activity assays

Effects of Ac-PHSCNGGK-MAP, Ac-HSPNCGGK-MAP, or Ac-PHSCN-NH₂ on MMP-1 activities secreted by adherent cells were analyzed as described [17]. Adherent cells were serum-starved overnight, then pretreated with Ac-PHSCN-NH₂, Ac-PHSCNGGK-MAP, or Ac-HSPNCGGK-MAP at 250 µg per 1,000,000 cells for 1 hour. Treatment groups were then stimulated with 5 ml 10% FBS or SF medium containing 250 µg per 1,000,000 cells Ac-PHSRN-NH₂ for 24 hours prior to assay. Treatment groups were in triplicate. Supernatants from treated cells were collected for analysis. Human MMP-1 ELISA Kit (RayBiotech, Inc., Norcross GA) was used to quantitate MMP-1 activity, according to

manufacturer's instructions. Data are presented as means $\pm$ SD, and results were analyzed using Student's *t* test.

Fluorescent DiI labeling of cells

Confluent DU 145 or PC-3 cells were washed with Hanks Buffered Salt Solution (HBSS; Life Technologies, Grand Island NY), harvested with 0.25% trypsin/1% EDTA (Life Technologies), rewashed in HBSS, and red fluorescently labeled in 6 ml SF medium with 25 μ l of the lipophilic carbocyanine vital dye DiI, 1,1'-dilinoylel-3,3,3'-tetramethylindocarbocyanine perchlorate (Invitrogen), for 20 min in dark at 37 °C, as described [25,26]. DiI has been utilized as a vital dye in numerous studies, for example, to define neural crest invasive/migratory pathways in developing embryos [27]. Cells were pelleted at 1000 RPM for 2 minutes, prior to resuspension in 6 ml of medium, and recovery for 72 hours.

Extravasation in the lungs athymic mice

To compare the effects of blocking anti α 5 β 1 MAb on the accumulation of labeled DU 145 or PC-3 cells in lung tissue after intravenous injection, DiI labeled DU 145 or PC-3 cells were incubated for 30 minutes on ice with 10 μ g/ml or 50 μ g/ml anti α 5 β 1 MCA1187 function blocking monoclonal antibody (MAb) (Serotec, Oxford, England), as described [22]. A total of 10,000 pretreated cells in 0.1 ml HBSS were injected into the tail vein of each 8-week, nude athymic mouse (Jackson Laboratories, Bar Harbor ME). Each treatment group consisted of 10 mice. All mice were euthanized 24 hours later, and their lungs removed and thoroughly rinsed with phosphate buffered saline (PBS) prior to fixation with 4% paraformaldehyde in PBS overnight at 4 °C. Following fixation, lungs were rinsed at room temperature in PBS, placed in 20% sucrose overnight at 4 °C. The samples were cryoprotected by submerging in O.C.T. (Optimal Cutting Temperature): 20% sucrose mixture (VWR, Batavia IL) overnight at 4 °C, prior to freezing and storing at -80 °C. Frozen lungs were sectioned with a thickness of 10 μ m with a Zeiss Cryostat (M550). All slides were sealed with VECTASHIELD mounting medium with DAPI (VECTOR Laboratories, Burlingame CA). Sections were examined at 400-fold magnification with a Zeiss Scanning Laser Confocal microscope (LSM510), as previously described [28].

To compare Ac-PHSCNGGK-MAP, Ac-PHSCN-NH₂, and Ac-HSPNCGGK-MAP as inhibitors of cellular accumulation in the lung tissue, DiI-labeled DU 145 or PC-3 cells were prebound with appropriate concentrations of dendrimer or peptide in HBSS and incubated at 37 °C for 10 min. The concentrations used for prebinding were as follows: Ac-PHSCNGGK-MAP: 10, 1, 0.1, or 0.01 ng/ml; Ac-PHSCN-NH₂: 100, 10, 1, or 0.1 ng/ml; Ac-HSPNCGG-MAP: 100 ng/ml. Nude mice (Jackson Laboratories) received an equivalent intravenous dose of dendrimer or peptide (0.0005 to 5.0 μ g/kg) via tail vein, immediately prior to intravenous injection of 10,000 DiI labeled, dendrimer- or peptide-prebound DU 145 or PC-3 cells per mouse. Labeled, pretreated cells were intravenously injected in 0.1 ml HBSS, and mice euthanized 24 hours later, as described above. Each treatment group consisted of 10 mice. Lungs were removed, rinsed, fixed, treated, and frozen as described above. Lungs were sectioned and sections analyzed by confocal microscopy, as described above. Data are presented as means $\pm$ SD of 10 mice per group.

To determine precisely what fractions of DU 145 and PC-3 cells were intravascular or extravascular, cells and mice were treated and sections made as described above. Sections were immunostained with rat anti mouse PECAM-1 (platelet endothelial cell adhesion molecule-1) monoclonal antibody (Millipore, Temecula CA) [29], and FITC-conjugated secondary antibody (Jackson Laboratories, Bar Harbor, Maine). Sections were scored as described above, such that DU 145 and PC-3 cells surrounded by at least 75% of their

circumference with anti PECAM staining were judged to be intravascular. Cells surrounded by less anti PECAM staining were judged to be extravascular, and hence to have completed extravasation.

Lung Colony Formation Assays

To verify the enhanced potency of Ac-PHSCNGGK-MAP as an inhibitor of lung colony formation, suspended, DiI labeled DU 145 or PC-3 cells were briefly prebound with 10 ng/ml Ac-PHSCNGGK-MAP, 10 ng/ml and 100 ng/ml Ac-PHSCN-NH₂, or 100 ng/ml Ac-HSPNCGGK-MAP. Eight week-old, nude mice (Jackson Laboratories) received one systemic pretreatment with the appropriate peptide or dendrimer concentration by tail vein injection, as described above. Immediately after pretreatment, DU 145 or PC-3 cells, prebound with dendrimer or peptide, or with HBSS only, were injected into tail veins. Mice received no other systemic treatments. Each treatment group consisted of 10 mice. Six weeks after injection, mice were euthanized, and lungs were removed, fixed, prepared, and stored as above.

Sections of 10 μ m thickness were cut with a Zeiss cryostat as previously described: 20 sections were cut from each lung in all mice of each treatment group, at 200 μ m intervals. Thus, a total thickness of 4 mm was analyzed from each lung. Sections were stained with monoclonal, FITC-conjugated anti β -actin antibody (Chemicon, Temecula CA) and scored at 400-fold magnification, based on the retention of DiI red fluorescence by the prostate cancer cells, as described for other cancer types [25,26]. DU 145 and PC-3 colonies were scored only if they contained more than 50 cells, according to established criteria for colony formation [30–32]. Data are presented as mean numbers of colonies per lung $\pm$ SD of 10 mice per group.

Clonogenic assays

Clonogenic survival assays were performed as described [33].

Results

Inhibition of DU 145 and PC-3 accumulation in lungs by pretreatment with blocking anti α 5 β 1 MAb

We evaluated the potential role of α 5 β 1 integrin in extravasation by prebinding suspended DU 145 and PC-3 cells to blocking anti α 5 β 1 MCA1187 MAb prior to intravenous injection into mouse tail veins. As shown in Figure 1a and b, MCA1187 pretreatment reduced the numbers of DU 145 and PC 3 cells in lung tissue by six- to nine-fold, consistent with the hypothesis that α 5 β 1 plays a key role in the invasive stage of extravasation and encouraging the investigation of α 5 β 1 inhibitors.

Invasion inhibition by the PHSCN polylysine dendrimer, Ac-PHSCNGGK-MAP

To compare inhibitory potencies of Ac-PHSCNGGK-MAP and Ac-PHSCN-NH₂ for basement membrane invasion, serum-starved (SF) DU 145 and PC-3 cells were prebound to various concentrations of Ac-PHSCNGGK-MAP or Ac-PHSCN-NH₂, or to maximal concentrations of the appropriate specificity controls: Ac-HSPNCGGK-MAP or Ac-HSPNC-NH₂, prior to invasion induction and placement on basement membranes. We have identified plasma fibronectin (pFn) as the invasion-inducing protein in serum, and shown that the interaction of an acetylated, amidated peptide consisting of the PHSRN sequence of its cell binding domain, with the α 5 β 1 integrin fibronectin receptor is sufficient for invasion induction in human breast and prostate cancer cell lines [2,3,8,17], as well as in normal microvascular endothelial cells, epithelial cells, and fibroblasts [1,8]. Thus, we compared the inhibitory potencies of Ac-PHSCNGGK-MAP and Ac-PHSCN-NH₂ for both serum-induced

and PHSRN-induced, SF invasion by DU 145 and PC-3 cells. Since Ac-PHSCNGGK-MAP displays the PHSCN sequence on a significantly larger molecule (7575 vs. 598 Da), we also evaluated the effect on invasion inhibitory potency of a modified protein, resulting from coupling a single Ac-PHSCNGGK sequence to ovalbumin (45.0 kDa), to make Ac-PHSCNGGK-Ova (45.9 kDa).

To analyze the results of *in vitro* invasion assays, we constructed Hill-Slope plots, as shown in Figure 2. The nM concentrations of the PHSCN dendrimer and PHSCN peptide, and the HSPNC dendrimer and PHSCN-Ovalbumin negative controls were plotted vs. the mean percentages of DU 145 and PC-3 cells invaded, after stimulation with 10% FBS, or with 100 ng/ml Ac-PHSRN-NH₂, under serum-free conditions. Irrespective of whether $\alpha 5\beta 1$ mediated invasion was stimulated by the presence of serum or by the PHSRN peptide under serum-free conditions, the PHSCN dendrimer was two to three orders of magnitude more potent as an invasion inhibitor than the PHSCN peptide. High concentrations of HPSNC dendrimer failed to block invasion. Also, putting a single PHSCNGGK moiety on a larger molecule, ovalbumin, failed to increase its potency significantly. These data were also plotted according to the median-effect equation, $y = \log(f_a/f_u)$, with respect to $x = \log(\text{dose})$, where f_a is the fraction of cells affected (invasion-inhibited), and f_u is the fraction of cells unaffected (invaded). This analysis indicated a linear coefficient (r) value > 0.97 , suggesting conformity to the mass-action law principle (not shown).

The IC₅₀ values were calculated from the Hill-Slope plots (Figure 2) were similar to those derived from the median-affect equation, and are listed in units of nM in Table 1. As shown by the Dose Reduction Index (DRI) values for DU 145 and PC-3 cells, the PHSCN dendrimer was 131- or 475-fold more potent, and 1908- or 1048-fold more potent than the PHSCN peptide at blocking FBS-induced or SF, Ac-PHSRN-NH₂-induced invasion, respectively.

The Combination Index (CI) and the DRI values, shown in Table 1, allow determination of whether the 8 PHSCN moieties on the dendrimer result in an additive or a synergistic increase in invasion inhibitory potency. Synergism is demonstrated by $CI < 1$ and $DRI > 1$, and additive effects are indicated by $CI \geq 1$ and $DRI \geq 1$ [23]. Thus in combination, the CI and DRI values listed in Table 1 for both cell lines show the increased efficacy of the PHSCN dendrimer, and indicate that its 8 PHSCN moieties interact synergistically to block invasion for *in vitro* assays.

Inhibition of serum- or PHSRN-induced MMP-1 secretion by Ac-PHSCNGGK-MAP

The induction of MMP-1 secretion and MMP-1-dependent invasion by the PHSRN/ $\alpha 5\beta 1$ interaction [1–3,17], and the inhibition of $\alpha 5\beta 1$ -mediated induced MMP-1 secretion in DU 145 cells by Ac-PHSCN-NH₂ [3], suggested that Ac-PHSCNGGK-MAP would be a significantly more potent inhibitor of $\alpha 5\beta 1$ -mediated MMP-1 induction by serum, or by Ac-PHSRN-NH₂. Thus, adherent DU 145 and PC-3 cells were serum-starved overnight prior to stimulation with medium containing 10% FBS, or with SF medium containing Ac-PHSRN-NH₂ as in prior studies [3,17]. Inhibitors of serum- or Ac-PHSRN-NH₂-induced MMP-1 secretion included Ac-PHSCN-NH₂, Ac-PHSCNGGK-MAP, or the Ac-HSPNCGGK-MAP specificity control, all at the same mass concentration (250 $\mu\text{g}/1,000,000$ cells) as Ac-PHSRN-NH₂ and are shown in Figure 3.

Considering molar concentration instead of mass/volume, Ac-PHSCNGGK-MAP (at 33 μM) was a 65–650-fold more potent inhibitor of $\alpha 5\beta 1$ -mediated MMP-1 secretion by DU 145 and PC-3 cells than Ac-PHSCN-NH₂ (at 418 μM), whether induction was by 10% FBS or by Ac-PHSRN-NH₂.

Reduction of DU 145 or PC-3 cells in the lungs of nude mice after prebinding Ac-PHSCNGGK-MAP or Ac-PHSCN-NH₂

The enhanced potency of Ac-PHSCNGGK-MAP relative to Ac-PHSCN-NH₂ observed *in vitro* invasion assays with naturally occurring basement membranes suggested that Ac-PHSCNGGK-MAP should be a more potent inhibitor of intravenous DU 145 or PC-3 cell accumulation in mouse lungs. To compare efficacies of Ac-PHSCNGGK-MAP and Ac-PHSCN-NH₂ as inhibitors of prostate cancer cell accumulation in lungs, a constant number of DiI-labeled DU 145 or PC-3 cells, briefly prebound to varying concentrations of Ac-PHSCNGGK-MAP, Ac-HSPNCGGK-MAP, or Ac-PHSCN-NH₂, was injected into the tail veins of athymic, nude mice. A mean of 1484 (+/- 248, SD) DU 145 cells or 1324 (+/- 279, SD) PC-3 cells was observed in 4 mm of sectioned lung tissue 24 hours after untreated cells were injected. Pretreatment of both cell lines, with 0.01 to 10 ng/ml Ac-PHSCNGGK-MAP or 0.1 ng/ml to 100 ng/ml Ac-PHSCN-NH₂, resulted in dose dependent reductions of the numbers of cells in lung tissue, and are shown in Figure 4a and 4b as a function of concentration (nM). Pretreatment with the scrambled sequence control dendrimer (100 ng/ml Ac-HSPNCGGK-MAP) resulted in a mean of 1186 (+/- 148) or 890 (+/- 39) when the same number of suspended DU 145 cells or PC-3 cells were injected, respectively; indicating little or no effect. A typical example of the data used to obtain these results is shown in Figure 4c. Sections containing extravasated DU 145 cells had a very similar appearance (not shown).

The IC₅₀ values were determined from the extrapolated x-intercept (Figures 4a and b) and the DRI and CI values were calculated, as summarized in Table 2. The increased potency of Ac-PHSCNGGK-MAP over the Ac-PHSCN-NH₂ peptide (800-fold) for preventing extravasation was similar to that found for *in vitro* invasion assays with naturally occurring basement membranes. Furthermore, the DRI and CI values shown in Table 2 indicate that extravasation inhibition was highly synergistic.

The complete process of extravasation requires that blood-borne cells cross the endothelial cell layer and its basement membrane to enter the underlying connective tissue. To determine what fractions of DU 145 and PC-3 cells in the lungs were fully extravasated, and hence outside of the vasculature, a constant number of fluorescent DiI-labeled, Ac-PHSCNGGK-MAP-pretreated (10 ng/ml) or untreated cells were injected in the tail veins of nude mice. To delineate precisely the vascular walls, lung sections were immunofluorescently stained by reaction with rat anti-mouse PECAM-1 antibody [29], and fluorescent secondary antibody. The total numbers of DiI-labeled DU 145 and PC-3 cells were counted in 20 lung sections from each lung of each mouse injected. Their positions relative to the anti PECAM-stained endothelial cell layer were noted as described above, and the results of the analysis are shown in Figure 5a. Typical examples of sectioned anti PECAM-1-stained blood vessels, with DiI-labeled DU 145 or PC-3 cells, inside or outside of the lung vasculature, are shown in Figures 5b and 5c, respectively.

The results of this experiment indicate that PHSCNGGK-MAP pretreatment caused 78.5 percent (DU 145) or 82.8 percent (PC-3) of DiI-labeled prostate cancer cells to remain in the lung vasculature; while 21.5 percent or 17.2 percent, respectively, appeared to have extravasated. In contrast, only 14.2 percent (DU 145) or 16.4 percent (PC-3) of untreated prostate cancer cells appeared to remain inside the vasculature; whereas, 85.5 percent or 83.6 percent, respectively, appeared to have extravasated. Thus, a single, brief Ac-PHSCNGGK-MAP pretreatment of DU 145 or PC-3 cells at 10 ng/ml was able to reduce extravasation in the lungs of nude mice by 4- to 5-fold.

Inhibition of DU 145 or PC-3 lung colony formation in nude mice by prebinding Ac-PHSCNGGK-MAP or Ac-PHSCN-NH₂

Extravasation is a key step in metastasis formation [5,31]. Based on its increased anti-invasive potency, Ac-PHSCNGGK-MAP should be a more potent inhibitor of lung colony formation than Ac-PHSCN-NH₂, after intravenous injection of mice with pretreated DU 145 or PC-3 cells.

Because others have previously shown that both DU 145 and PC-3 cells, when intravenously injected into athymic mice, colonize the lungs and efficiently grow into macroscopic metastatic colonies [34,35], we utilized these cell lines to compare the effects of Ac-PHSCNGGK-MAP and Ac-PHSCN-NH₂ on lung colony formation. We found that pretreatment with 10 ng/ml (1.32 nM) Ac-PHSCNGGK-MAP reduced DU 145 lung colony formation by 5.8-fold, relative to untreated controls; whereas, pretreatment with 10 ng/ml (17 nM) Ac-PHSCN-NH₂ had a less inhibitory effect: reducing DU 145 lung colonies by 2.4-fold (Figure 6a). Pretreatment with a 10-fold higher concentration of Ac-PHSCN-NH₂ (100 ng/ml, 170 nM) reduced DU 145 lung colony formation by 5.9-fold, similar to the reduction observed after pretreatment with 10 ng/ml (1.32 nM) Ac-PHSCNGGK-MAP. No significant reduction in lung colony formation was obtained by pretreatment of DU 145 cells with Ac-HSPNCGGK-MAP, at 100 ng/ml (170 nM). As shown in Figure 6b, much the same results were obtained for PC-3 cells. The similar inhibitory responses of the doses 10 ng/ml (1.32 nM) Ac-PHSCNGGK-MAP and Ac-PHSCN-NH₂ (100 ng/ml, 170 nM) for both cell lines are separated by approximately 2 orders of magnitude in concentration (Figure 6a and b). This allows calculation of an approximate DRI of 128.

Since only growing micrometastases are functionally relevant, the total number of cells in each micrometastasis was determined for each treatment group shown in Figure 6. Each micrometastasis included in the totals contained from 50 to 200 cells (not shown), similar to criteria previously employed by others [30–32].

Because it was possible that inhibition of lung colony formation by the PHSCN dendrimer might have been due to reduction in survival of the extravasated DU 145 and PC-3 cells, the effects on clonogenic survival of several Ac-PHSCNGGK-MAP concentrations were compared to elevated concentrations of Ac-HSPNCGGK-MAP and Ac-PHSCN-NH₂. The Ac-PHSCNGGK-MAP and Ac-PHSCN-NH₂ concentrations tested (50 to 200 µg/ml) were 500- to 20,000-fold higher than the concentrations used in pretreatments to prevent extravasation and inhibit lung colony growth (0.01 to 0.1 µg per ml), and were present continuously throughout the period of clonogenic growth. As shown in Figure 7A, Ac-PHSCNGGK-MAP failed to reduce DU 145 clonogenic survival significantly, as compared with equal concentrations of the Ac-HSPNCGGK-MAP or Ac-PHSCN-NH₂. Thus, inhibition of DU 145 survival in lung colonies *in vivo* by Ac-PHSCNGGK-MAP pretreatment is a very unlikely explanation for the reduced lung colonies observed. As shown in Figure 7B, elevated concentrations of the PHSCN dendrimer (100 to 200 µg per ml) may have reduced PC-3 clonogenic survival by approximately 2-fold, when compared to the HSPNC dendrimer. However, the concentrations of PHSCN dendrimer required to achieve this effect were 2,000- to 20,000-fold higher than the concentrations used to prevent extravasation and limit lung colony growth. Thus, reduction of PC-3 lung colonies *in vivo* through inhibition of clonogenic survival by PHSCN dendrimer pretreatment is also unlikely.

Discussion

Prostate cancer is the most common non-skin malignancy, and the third leading cause of cancer deaths in men [36,37]. About 39% of men with prostate cancer will develop

metastatic disease at some point [38]. Metastatic prostate cancer is presently incurable, and most patients with this disease succumb over a period of months to years [39]. Lung metastasis is found in an average of 40% of metastatic prostate cancer patients at autopsy, with reports ranging from 16–56% [40]. Prostate cancer lung metastasis can consist of multiple nodules, diffuse lymphatic disease, or grossly normal lungs, exhibiting micrometastases only [41]. However, it can also have serious consequences, like malignant pleural effusion [42].

Because $\alpha 5\beta 1$ integrin fibronectin receptor-mediated invasion by microvascular endothelial cells to promote tumor growth, and by prostate cancer cells to cause widespread dissemination are key features of metastatic disease progression, we have pursued it as a therapeutic target in our previous studies [1–3]. Here, we have shown that attaching 8 PHSCNGGK peptide moieties to a polylysine dendrimer increases the invasion-inhibitory potency of the PHSCN sequence by 131- to 1908-fold for $\alpha 5\beta 1$ -mediated invasion *in vitro*, and Chou-Talalay analysis suggests that invasion inhibition is highly synergistic. Consistent with the requirement for MMP-1 in microvascular endothelial cell and prostate cancer cell invasion *in vitro* [1,3], and with recent reports that MMP-1 promotes prostate tumor growth and metastasis, and that overexpression of MMP-1 in prostate cancer cells increases invasion [43], we also observed that the PHSCN dendrimer was 65–650-fold more potent inhibitor of $\alpha 5\beta 1$ -mediated MMP-1 activity than the PHSCN peptide.

Moreover, we also found that the PHSCN dendrimer is about 800-fold fold more potent than the PHSCN peptide at blocking prostate cancer cell extravasation into lung tissue, and Chou-Talalay analysis also suggests that inhibition extravasation inhibition by the PHSCN dendrimer is highly synergistic. Furthermore, approximately 80% of the prostate cancer cells, found in lung tissue after prebinding to PHSCN dendrimer and intravenous injection, still appeared to be largely surrounded by anti PECAM-stained endothelial cells, suggesting that, while they may have adhered to the luminal surfaces of endothelial cells, only about 20% actually succeeded in completing the process of extravasation to enter the surrounding lung tissue. In contrast, less than 20% of untreated prostate cancer cells appeared to remain inside the lung vasculature; over 80% of them appeared to have completed extravasation.

Finally, a single pretreatment with PHSCN dendrimer, followed by a single, equivalent systemic dose, was 100-fold more effective than the PHSCN peptide at reducing lung colony formation, and no significant reduction in lung colony formation was obtained by pretreatment with HSPNC dendrimer. Moreover, a single PHSCN dendrimer treatment of 1 ng per mouse reduced lung colony formation by 6-fold, relative to untreated controls. In contrast, daily administration of 0.5 mg of the 8-mer YIGSR polylysine dendrimer was required to reduce B16F10 melanoma lung colony formation by a similar extent [44], suggesting that invasion may be a more sensitive therapeutic target for antimetastatic drugs than adhesion.

As indicated by clonogenic assays performed in the presence of greatly elevated (2 to 4 orders of magnitude higher) concentrations, there was no significant reduction of DU 145 clonogenic survival by either the PHSCN dendrimer or peptide, and only a slight reduction in PC-3 clonogenic survival. Thus, inhibition of DU 145 or PC-3 survival in lung colonies *in vivo* by PHSCN dendrimer pretreatment is a very unlikely explanation for the reduced lung colonies observed.

When unchecked by normal regulatory mechanisms, the regulatory pathways initiated by $\alpha 5\beta 1$ integrin may give rise to the progressive growth and invasion observed in metastatic prostate cancer. For example, the interaction of $\alpha 5\beta 1$ with the PHSRN sequence of the fibronectin cell binding domain is sufficient to induce interstitial collagenase expression and

invasion by microvascular endothelial cells [1] and metastatic prostate cancer cells [2,3], as well as breast cancer cells [17] and normal epithelial cells and fibroblasts [8]. Because fibronectin is found in all body fluids, proper regulation of $\alpha 5\beta 1$ -mediated invasion is very important. This is accomplished by another integrin fibronectin receptor, $\alpha 4\beta 1$. When fibronectin is intact, $\alpha 4\beta 1$ integrin interacts with the LDV sequence of the fibronectin connecting segment, LHGPEILDVPST, to repress $\alpha 5\beta 1$ -mediated interstitial collagenase expression [45], and invasion induction [17]. Fragmentation of fibronectin by urokinase plasminogen activator, which also functions in clot dissolution (reviewed in [46]), gives rise to $\alpha 5\beta 1$ -mediated invasion during wound healing [47,48]. Thus, an important attribute of $\alpha 5\beta 1$ -induced invasion in normal $\alpha 5\beta 1^+$ $\alpha 5\beta 1^+$ cells is its regulation by $\alpha 4\beta 1$ integrin. Although still expressing abundant surface $\alpha 5\beta 1$, many metastatic prostate and breast cancer cell lines have low levels of surface $\alpha 4\beta 1$, relative to mammary and prostate epithelial cells [49,50]. Loss of surface $\alpha 4\beta 1$, which can result from oncogene overexpression in transformed mammary epithelial cells [50], has been shown to cause constitutive invasiveness in the presence of the abundant pFn of blood, lymph, and interstitial fluid [51,52] in metastatic prostate cancer and breast cancer cell lines [2,17,50,53,54]. Thus, systemic therapy with an agent, like a PHSCN peptide derivative, that effectively targets the activated $\alpha 5\beta 1$ integrins of tumors, without affecting the $\alpha 5\beta 1$ receptors of healthy tissues, could represent potentially effective therapy for patients with metastatic prostate cancer.

The rationale for employing PHSCN-dendrimers as improved therapeutic agents is that their polyvalency will enhance the affinity of their interactions with their target protein, the $\alpha 5\beta 1$ integrin. There are many examples of the ability of multimeric peptides to enhance binding affinity. For example, a peptide complementary to a specific region of endothelin exhibited a binding affinity that was significantly greater when it was synthesized as a MAP peptide [55]. Peptide dendrimers are also used as inhibitors of increased potency. For example, peptide dendrimers have been used to inhibit the entry of malaria parasites into hepatocytes [56]. In another example, a peptide dendrimer based on an 8 amino acid sequence from gp120, the HIV-1 surface envelope glycoprotein, has been shown to inhibit HIV-1 infection of both CD4⁺ and CD4⁺ cells [57,58].

Lymph nodes, bone, lung, and liver are the most frequent sites of prostate cancer metastasis. Because lung metastasis occurs in over 40% of prostate cancer patients with hematogenous metastatic disease [38], this study focused on targeting the $\alpha 5\beta 1$ integrin fibronectin receptors of prostate cancer cells to reduce their extravasation into lung tissue, thereby limiting lung colony formation. However, bone is also an extremely important site for prostate cancer metastasis [59], and cooperation between $\alpha 5\beta 1$, $\alpha 3\beta 1$, and $\alpha v\beta 1$ integrins is thought to be necessary for the docking of PC-3 and DU 145 cells to stimulated human umbilical vein endothelial cells [60]. Thus, future studies will evaluate the effects of the PHSCN dendrimer on adhesion to endothelial cells, the initial step in extravasation, and will compare the effects of systemic PHSCN dendrimer with PHSCN peptide on prostate cancer cell bone metastasis in athymic mice.

Abbreviations

MAP	Multiantigenic peptide
SF	Serum-free
FBS	Fetal bovine serum
CI	Combination Index
DRI	Dose Reduction Index

Ova	Ovalbumin
EDC	1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
HBSS	Hanks buffered salt solution
MALDI	Matrix Assisted Laser Desorption/Ionization
MMP-1	Matrix metalloproteinase-1
ELISA	Enzyme-linked immunoabsorbant assay
DiI	1,1'-dilinoylel-3,3,3'-tetramethylindocarbocyanine perchlorate
MAb	Monoclonal antibody
SD	First standard deviation
SEM	Standard error of mean
PECAM-1	Platelet endothelial cell adhesion molecule-1
O.C.T.	Optimal cutting temperature
FITC	Fluorescein isothiocyanate

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Fig. 1. Inhibition of DU 145 and PC 3 cell extravasation into the lungs of nude mice by pretreatment with MCA1187 blocking anti $\alpha 5\beta 1$ MAb. X axes, treatments; Y axes, mean number of extravasated cells per lung ($\pm$ SD). Panel (a). Inhibition of DU 145 extravasation. Panel (b). Inhibition of PC-3 extravasation.

Fig. 2. Hill-Slope plots of the increased invasion-inhibitory potency of Ac-PHSCNGGK-MAP as compared with the Ac-PHSCN-NH₂ peptide with naturally serum-free, selectively permeable basement membranes from sea urchin embryos as invasion substrates. Panel (a). Inhibition of serum-induced DU 145 invasion. Panel (b). Inhibition of serum-induced PC-3 invasion. Panel (c). Inhibition of Ac-PHSRN-NH₂-induced, SF DU 145 invasion. Panel (d). Inhibition of Ac-PHSRN-NH₂-induced, SF PC-3 invasion. X axes, log peptide concentration in nM; Y axes, mean percentages of invaded cells, relative to uninhibited, FBS- or PHSRN-stimulated controls (+/- SD). Black circles, Ac-PHSCNGGK-MAP; black triangles, Ac-PHSCN-NH₂ peptide; black diamonds, Ac-HSPNCGGK-MAP; white circles, Ac-HSPNC-NH₂ peptide; white squares, Ac-PHSCNGGK-Ova.

Fig. 3.

Increased potency of Ac-PHSCNGGK-MAP as an inhibitor of $\alpha 5\beta 1$ -mediated MMP-1 secretion *in vitro* by ELISA detection. Panel (a). Inhibition of MMP-1 secretion by DU 145 cells. Panel (b). Inhibition of MMP-1 secretion by PC-3 cells. X axes, treatment groups in triplicate of Ac-PHSCN-NH₂, Ac-PHSCNGGK-MAP, and Ac-HSPNCGGK-MAP at 250 µg/1,000,000 cells; black bars, stimulation with 10% FBS; white bars, stimulation with 250 µg/1,000,000 cells Ac-PHSRN-NH₂. Y axes, mean % MMP-1 activity relative to stimulated control cells (+/- SD).

Fig. 4. Increased extravasation inhibition by Ac-PHSCNGGK-MAP prebinding, relative to the PHSCN peptide. Black circles, Ac-PHSCNGGK-MAP; black squares, Ac-PHSCN-NH₂ peptide. Panel (a). Median-affect plot for DU 145 cells. Panel (b). Median-affect plot for PC 3 cells. Panel (c). PC-3 cell extravasation, typical example of a scored section. Arrows indicate all extravasated cells visible in section.

Fig. 5. Reduced extravascular prostate cancer cells after PHSCNGGK-MAP treatment. Panel (a). Quantitation of intravascular and extravascular DU 145 and PC-3 cells in sectioned mouse lungs. X axis, cell lines; Y axis, mean percentages of cells. Blue bars, intravascular cells; gray bars, extravascular cells. Panels (b and c). Examples of DU 145 cells (b) and PC-3 cells (c) lung sections from untreated (Control) and Ac-PHSCNGGK-MAP treated mice. Arrows, extravascular cells. Stars, intravascular cells.

Fig. 6. Increased inhibition of lung colony formation by Ac-PHSCNGGK-MAP prebinding, relative to the PHSCN peptide. Panel (a). DU 145 colonies. Panel (b). PC 3 colonies. X axis, log (nM), Black bars, Ac-PHSCNGGK-MAP; white bars, Ac-PHSCN-NH₂; dark gray bars, Ac-HSPNCGGK-MAP. Y axis, % Colonies/section as compared to untreated tissue. Panels (c and d). Examples of sectioned lung tissue after DU 145 cell injection (c) or PC-3 cell injection (d) from untreated (Control), 10 ng/ml PHSCNGGK-MAP treated, and 100 ng/ml HSPNCGGK-MAP treated mice.

Fig. 7.

Effects of PHSCN dendrimer on clonogenic survival *in vitro* by DU 145 and PC-3 cells. Panel (a) Clonogenic survival of DU 145 cells. Panel (b) Clonogenic survival of PC-3 cells. X axis, µg per 2 ml well; Y axis, mean number of colonies per well (+/- SD). Black bars, Ac-PHSCNGGK-MAP; dark gray bars, Ac-HSPNCGGK-MAP, light gray bars, Ac-PHSCN-NH₂; white bar, untreated.

Table 1

Comparison of IC₅₀, CI, and DRI values for PHSCNGGK-MAP and PHSCN as inhibitors of FBS– or PHSRN–induced invasion by DU 145 or PC-3 cells. IC₅₀, inhibitory concentration for 50% invasion inhibition. PHSCN, Ac-PHSCN-NH₂; PHSCNGGK-MAP, Ac-PHSCNGGK-MAP. CI, Combination Index = Σ combinatorial/single dose; DRI, Dose Reduction Index = single/combinatorial dose.

	DU 145	PC-3
10% FBS		
IC₅₀ (nM)		
PHSCN	4.45	32.30
PHSCNGGK-MAP	0.034	0.068
CI*	0.008	0.002
DRI**	131	475
100 ng/ml Ac-PHSRN-NH₂		
IC₅₀ (nM)		
PHSCN	68.70	34.60
PHSCNGGK-MAP	0.036	0.033
CI*	0.0005	0.001
DRI**	1908	1048

* CI (Combination Index) = Σ combinatorial/single dose

** DRI (Dose Reduction Index) = single/combinatorial dose

Table 2

Comparison of IC_{50} , CI, and DRI values for PHSCNGGK-MAP and PHSCN as inhibitors of extravasation by DU 145 and PC-3 cells. IC_{50} , inhibitory concentration for 50% extravasation inhibition. PHSCN, Ac-PHSCN- NH_2 ; PHSCNGGK-MAP, Ac-PHSCNGGK-MAP. CI, Combination Index = Σ combinatorial/single dose; DRI, Dose Reduction Index = single/combinatorial dose.

	DU 145	PC 3
IC_{50} (pM)		
PHSCN	7.0	0.88
PHSCNGGK-MAP	0.009	0.001
CI	0.001	0.001
DRI	790	857