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Suppression of Tumor Recurrence and Metastasis by a Combination of the PHSCN Sequence and the Antiangiogenic Compound Tetrathiomolybdate in Prostate Carcinoma[1]

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Abstract

Plasma fibronectin–mediated invasion of human DU145 prostate cancer cell line was efficaciously inhibited in a rat tumor model by treatment with Ac-PHSCN-NH_2 peptide. Invasion of DU145 cells was stimulated by the PHSRN sequence of plasma fibronectin. However, PHSCN acts as a competitive inhibitor of PHSRN-mediated invasion. In the current study, we determined whether PHSCN could inhibit the recurrence and metastasis of DU145 tumors after excision of the primary tumor in an athymic nude mouse model. We demonstrated that mice treated thrice weekly with intravenous Ac-PHSCN-NH_2 peptide survived tumor-free for more than 30 weeks post-primary tumor excision, whereas their untreated counterparts succumbed to recurrence and/or metastatic disease in significantly less time. Because of the universal requirement for angiogenesis in solid tumor growth, we tested the efficacy of copper deficiency induced by tetrathiomolybdate (TM) to retard tumor growth in the Dunning prostate cancer model. Significant reduction in size of the primary tumor was observed in mice rendered copper deficient. We sought to reduce tumor growth at the primary and metastatic sites by combining the anti-invasion Ac-PHSCN-NH_2 peptide with TM. Improved survival, fewer metastatic lesions, and excellent tolerability were observed with the combination therapy.

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Keywords: prostate carcinoma, PHSCN, tetrathiomolybdate, metastasis, tumor recurrence.

Introduction

It is estimated that in the year 2001, approximately 198,100 men will be newly diagnosed with prostate cancer [1]. A large proportion of those diagnosed with localized disease will benefit from radical prostatectomy, which tends to be curative in early-stage disease [2]. Men who are diagnosed with locally advanced or metastatic disease will undergo androgen-ablation therapy, and the majority will progress locally or at distant sites within 18 months [3]. Hormone-refractory clones presumably cause relapse, which leads to 31,500 deaths from androgen-independent, metastatic prostate cancer in the US each year [1]. Therefore, novel strategies are being sought for the treatment of metastatic and high-risk prostate cancer.

Previously, Livant et al. [4,5] demonstrated the invasion-inducing role of plasma fibronectin using a naturally occurring, serum-free extracellular matrix (SU-ECM) *in vitro* system. Plasma fibronectin is a soluble extracellular matrix protein found in concentrations of 0.3 to 0.5 mg/ml in plasma, serum, lymph, and interstitial fluid [6]. DU145 prostate cancer cells were stimulated by the PHSRN peptide sequence located within the cell-binding domain of plasma fibronectin, which led to invasion through a process involving the $\alpha_5\beta_3$ integrin. Subsequently, it was established that the peptide analog PHSCN acts as a competitive inhibitor of the PHSRN sequence by efficiently blocking invasion and metastases of prostate cancer cells *in vitro* and *in vivo* [4]. The Ac-PHSCN-NH_2 peptide significantly blocked DU145 invasion in the naturally occurring serum-free SU-ECM system and inhibited tumor growth and metastasis of highly aggressive MAT-Ly-Lu prostate cancer cells in a rat model [4]. In contrast, the scrambled peptide control, Ac-HSPNC-NH_2, was completely ineffective in inhibiting invasion and had no antitumorigenic or antimetastatic activity in the same systems. These results suggest that Ac-PHSCH-NH_2 is a potent inhibitor of basement membrane invasion, particularly in the extravasation step of the metastatic cascade.

To further explore the ability of the Ac-PHSCN-NH_2 sequence to prevent recurrence and metastasis, we used the

Abbreviations: TM, tetrathiomolybdate; i.v., intravenous; SU-ECM, sea urchin serum-free extracellular matrix; MLL, Dunning Mat Ly-Lu; PHSCN, peptide–proline–histadine–serine–cysteine–asparagine peptide; Ac-PHSCN-NH_2, acetylated and aminated PHSCN peptide; Cu, copper

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DU145 prostate cancer cell model injected ectopically in the hind flank of male athymic nude mice. We found that tumor growth was retarded by thrice weekly intravenous (i.v.) peptide treatment. Moreover, after excision of the primary tumor, the tumor recurrence rate and appearance of metastases were completely inhibited in the PHSCN-treated mice compared with the incidence of aggressive recurrence and metastases in the control group. The untreated mice succumbed to either tumor burden from recurrence and/or metastatic disease by 15 weeks postsurgery. In contrast, Ac-PHSCN-NH_2–treated mice survived for more than 30 weeks disease free and were tumor and metastasis free at the time of necropsy. Seeking to test whether inhibition of the primary tumor could be enhanced by a global inhibitor of angiogenesis, combination therapy of the copper-lowering antiangiogenic compound, tetrathiomolybdate (TM) and PHSCN was administered and proved to be mildly more antitumorigenic, especially at the primary site than PHSCN alone. These data suggest that Ac-PHSCN-NH_2 may provide a well-tolerated, effective treatment for invasive and metastatic prostate cancer, alone or in combination with other modalities.

Materials and Methods

Cell Culture

DU145 cells (American Type Culture Collection, Rockville, MD) and Dunning MAT-Ly-Lu (MLL) cells were cultured according to established conditions. Cells were harvested before injection into mice by washing with 10 ml Hanks buffered salt solution (HBSS; Life Technologies, Grand Island, NY) and 0.25% trypsin/1% EDTA (Life Technologies), rewashed, and suspended in HBSS at a concentration of 1×10^6 cells/0.1 ml. The cell suspension was kept on ice and immediately used for injection.

To ensure that the cells were viable and invasive, a trypan blue (Sigma Chemical, St. Louis, MO) exclusion assay was performed before final suspension in HBSS. Cells were visualized and counted using a hemacytometer. Only cultures with more than 95% viable cells were used for injection. A Matrigel (BD Biosciences, Bedford, MA) invasion assay was performed as previously described to ensure that the DU145 cells were invasive [7]. Briefly, cells harvested for injection were diluted to a final concentration of 3.75×10^5 cells/ 0.3 ml in serum-free complete minimal essential medium (Life Technologies), and placed into the upper chamber of a Costar 6.5-mm Transwell (Corning, Corning, NY) plate coated with 10 μl of 10 mg/ml Matrigel (BD Biosciences) with either serum-free (as control) or complete minimal essential medium supplemented with 10% fetal bovine serum (Sigma) in the lower chamber. PHSCN inhibition of invasion was accomplished by adding 1 μg/ml PHSCN to the upper chamber of the Transwell with the cells. The cells were incubated in 5% CO_2 for 24 hours at 37°C. The filters were removed, fixed, and stained with hematoxylin and eosin (H&E). Ten random 40× fields were surveyed for invaded cells and the number of invaded cells in the serum-free controls was subtracted from the number of cells invaded in the 10% fetal bovine serum samples.

Peptide Synthesis

Large-scale peptide synthesis was performed using standard Fmoc/*t*-butyl protection strategies on a Perkin-Elmer/Applied Biosciences (Foster City, CA) model 433 peptide synthesizer as previously described [4]. Amidated peptides were synthesized on Rink resin. Completed peptides were cleaved from the resin support by anhydrous TFA. The peptides were precipitated with diethylether, purified by preparative high-performance liquid chromatography, and freeze-dried. To remove residual TFA, gel permeation chromatography was performed on a Sephadex G-10 column equilibrated with 1 N acetic acid. Peptide sequence was confirmed by mass spectroscopy and peptide purity evaluated by reverse phase high-performance liquid chromatography.

Athymic Nude Mice

One million DU145 cells in 0.1 ml HBSS (Life Technologies), were injected subcutaneously in the right hind flank of 8-week-old male nude mice. Treatment with 100 μl of 50 mg/kg Ac-PHSCN-NH_2, administered through tail vein injection, was begun 2 weeks after injection when tumors reached 0.5 mm^3. Ac-PHSCN-NH_2 treatment was given three times per week, continuously for the duration of the experiment. Control mice received 100 μl normal saline. In a separate experiment, Ac-PHSCN-NH_2 treatment was begun 24 hours after s.c. injection of tumor cells. Two groups of mice were treated daily by gavage with 0.7 mg/0.250 ml of the antiangiogenic compound TM. Daily treatment with this dose of TM has been previously shown to effectively render mice copper deficient to a level of 20% of normal. Mice were monitored four times weekly and tumor growth checked by measuring the length and the width of the tumor with a microcaliper. Tumor volumes were calculated by the formula: $(\text{length}\times\text{width}^2)/2$. Tumors were allowed to progress to approximately 900 mm^3 in size before excision. To perform excisions, mice were anesthetized by i.p. injection of 100 mg/kg ketamine and 10 mg/kg xylazine, tumors were then removed, and the mice kept for up to 30 weeks before sacrificing by CO_2 overdose. Tissues were collected and fixed in 10% buffered formalin. Gross metastases were counted on the lung surface under 100× magnification using a Nikon SMZ-U dissecting microscope. Lung tissue was paraffin embedded, 5-μm sections were cut and stained with H&E. Representative lung sections were surveyed for evidence of micrometastases under 400× magnification.

Copenhagen Rat Metastasis Model

One million MLL cells were injected into the hind limb of rats on day 0. Cells were implanted into three groups of 10 Copenhagen rats each,: the control group was untreated throughout, the copper-depleted group was treated with 1.5 mg/day of TM for 2 weeks before tumor cell injection, and the delayed treatment group began receiving TM on the

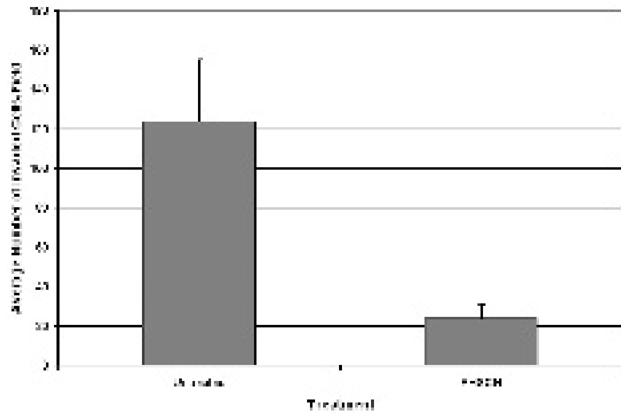

Figure 1. *Results of a Matrigel invasion assay comparing untreated with Ac-PHSCN-NH_2–treated DU145 prostate cancer cells. Ac-PHSCN-NH_2–treated cells were coincubated with 1 μg/ml Ac-PHSCN-NH_2 during the 24-hour invasion period. Addition of the Ac-PHSCN-NH_2 peptide significantly reduced the ability of the DU145 cells to invade.*

day of tumor implantation. After 4 weeks, the tumor-bearing limbs were removed, and the animals were sacrificed 2 weeks later to evaluate metastases.

Statistical Analysis

The University of Michigan Biostatistics Department performed statistical analysis of the data. Incidence of lung metastasis and tumor growth rates were compared using the Wilcoxon signed rank test and the Kruskal-Wallis test.

Results

The PHSCN Peptide Can Effectively Reduce DU145 Invasion In Vitro

It was previously demonstrated that DU145 prostate cancer cells were invasive in the SU-ECM assay and their invasive capabilities could be inhibited by the addition of the antagonist PHSCN peptide. To ensure that the DU145 cells used in our *in vivo* experiments were invasive *in vitro*, we performed a Matrigel (BD Biosciences) invasion assay to quantitate the invasive abilities of the cells before implantation. Figure 1 demonstrates that the cells were invasive through a Matrigel-coated filter in response to 10% serum containing medium. In the presence of the PHSCN peptide, the cells were six-fold less invasive than their untreated counterparts. These data demonstrate that DU145 prostate cancer cells are invasive *in vitro* and their ability to invade a Matrigel-coated filter can be significantly reduced by treatment with the PHSCN peptide.

PHSCN Treatment Can Effectively Block Tumor Recurrence, Metastasis, and Micrometastasis

The ability of the acylated and amidated PHSCN peptide to affect prostate tumor growth, recurrence, and metastasis was tested. Male nude mice were injected s.c. in the rear, right flank with DU145 cells. Approximately 2 weeks later mice began thrice weekly treatments with 50 mg/kg Ac-PHSCN-NH_2, administered through tail vein injection once the tumors reached palpable mass size (0.5 mm^3). Tumor growth was monitored by measuring tumor size with calipers and calculating approximate tumor volume. Tumor growth rate was not significantly effected by Ac-PHSCN-NH_2 treatment compared with saline controls. Tumors were excised from control and treated mice at the same time, approximately 8 to 9 weeks postinjection, when they reached a volume of approximately 900 mm^3. Interestingly, we noted that tumors from the Ac-PHSCN-NH_2–treated animals tended to be less invasive and appeared to be encapsulated compared with the controls. Treatment continued with the same dose schedule postsurgery.

Tumors began to recur in the control group between 2 and 3 weeks postexcision of the primary tumor. As demonstrated in Figure 2, all of the control mice (n=20) exhibited tumor recurrences between 3 weeks and 15 weeks postsurgery. Strikingly, none of the Ac-PHSCN-NH_2–treated mice (n=18) had tumor recurrences. Furthermore, the treated animals survived disease free, out to 30 weeks postsurgery, a time point arbitrarily chosen as an end-point for the evaluation of disease-free survival. In contrast, all of the untreated animals had evidence of recurrent disease by 17 weeks postsurgery. The volumes of the recurring tumors tended to be larger and grew at a faster rate than the primary tumors, with 5/15 tumors reaching 900 mm^3 within 5 weeks post-primary tumor excision. As shown in Table 1, the average tumor volume of the recurrences was 728 mm^3 at 15 weeks postsurgery. All of the untreated control mice eventually succumbed to large recurrences and/or metastatic disease, with a median number of lung metastases of 10 visible nodules (with a range of 0 to 22 visible nodules) in contrast to a median number of zero visible nodules (range of 0–0) in the Ac-PHSCN-NH_2–treated group. Upon examination of representative H&E-stained sections from the lungs of each of the animals, it was found that the Ac-PHSCN-NH_2 animals had significantly fewer lung

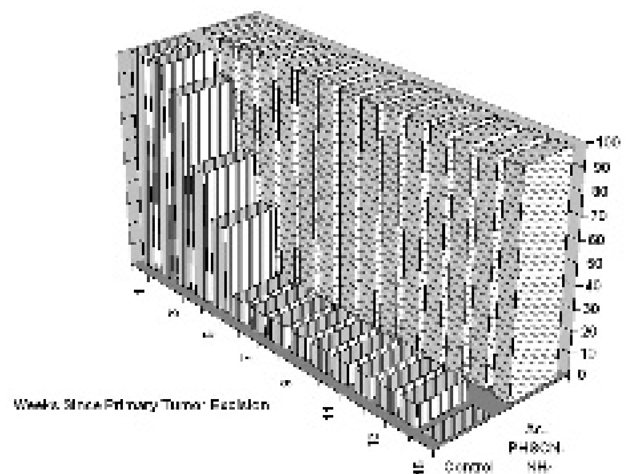

Figure 2. *Percentage of mice that were recurrence-free after primary DU145 tumor excision. Mice treated thrice weekly with 100 μl 50 mg/kg Ac-PHSCN-NH_2 i.v. remained recurrence-free up to 30 weeks post-primary tumor excision. In contrast, 100% of control mice treated with normal saline had recurrences out to 15 weeks post-primary tumor excision.*

Table 1. Comparison of DU145 Tumor–Bearing Mice Left Either Untreated or Treated with 50 mg/kg Ac-PHSCN-NH_2.

Treatment Group	Avg. Recurrence Volume (mm^3)	Median No. (Range) of Lung Metastases	Avg. No. of Lung Micrometastases
Untreated (*n*=20)	728	10 (0–22)*	308 (*n*=5)*
Ac-PHSCN-NH_3 (*n*=18)	0	0 (0–0)	17 (*n*=5)

Treated mice had no tumor recurrence and significantly less gross and micrometastases than untreated mice (*$P<.001$, as determined by a Wilcoxon signed rank test).

micrometastases ($P>.001$) than the untreated controls (Table 1).

Taken together, these data suggest that treatment of DU145 prostate tumors with Ac-PHSCN-NH_2 has minimal effect on primary tumor growth. However, this peptide can nearly abrogate tumor recurrences and the formation of macroscopic and microscopic metastases.

TM has Efficacy to Decrease Tumor Growth in the Dunning Prostate Cancer Tumor Model

We investigated the activity of TM in the Dunning rat model. The cell lines derived from the original rat tumor have diverse phenotypes such as nonmetastatic (AT.1) and highly aggressive (MAT-Ly-Lu; MLL). Arguably, the MLL model is a more aggressive prostate tumor in rodents than DU145. Previous experiments demonstrated efficacy of Ac-PHSCN-NH_2. Therefore, we wished to determine if treatment of MLL tumors with TM would lead to decreased primary tumor growth. They can be injected subcutaneously in the groin of syngeneic previously decoppered rats to test our hypotheses.

To test the hypothesis that copper (Cu) reduction to 20% of normal levels would be efficacious in controlling tumor growth in a prostate cancer implant model, we studied three groups of 10 Copenhagen rats each, implanted with the MLL prostatic tumorigenic cell line: the control group was untreated throughout, the copper-depleted group was treated with 1.5 mg/day of TM for 2 weeks before tumor cell injection, and the delayed-treatment group began receiving TM on the day of tumor implantation. After 4 weeks, the tumor-bearing limbs were removed, and the animals were sacrificed 2 weeks later to evaluate metastases. Figure 3, *A* and *B* depict the tumor weights and the average number of lung metastases, respectively, for the three groups. A statistically significant decrease in tumor weight was observed for the copper-depleted group in comparison to either the control or the delayed-treatment groups ($P=.0313$). Interestingly, both the copper-depleted and the delayed-treatment groups showed a trend toward lower numbers of metastases that did not reach statistical significance in this study. These results support the conclusion that TM interferes with the growth of implanted tumors, and does so most effectively after Cu deficiency is achieved. TM also appears to decrease metastatic growth under the same conditions. The safety, potency, and speed of action of TM as an anti-Cu agent in this model suggest that it may be synergistically efficacious in combination with anti-invasive therapies, such as the Ac-PHSCN-NH_2 peptide.

Inhibition of Tumor Growth, Recurrence and Metastasis by a Combination of Ac-PHSCN-NH_2 and TM

Because primary DU145 primary tumor growth was only moderately effected by Ac-PHSCN-NH_2 treatment, we sought to potentiate the effect on primary tumor growth by the peptide with TM. The effect of TM on prostate tumor growth in nude mice has not been previously determined. For these experiments, as before, treatment with peptide and TM began 24 hours after 1 million DU145 cells were implanted s.c. into the hind flank of male nude mice. Mice were treated with either thrice weekly Ac-PHSCN-NH_2 alone, daily TM alone, a combination of daily TM and thrice weekly Ac-PHSCN-NH_2, or normal saline as a control.

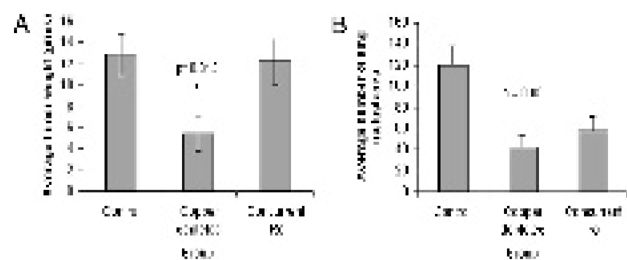

Figure 3. *Tumor development in Copenhagen rat following implantation with MLL cells in a limb. Control animals were treated with saline. Copper-depleted animals were treated with TM for 2 weeks before tumor cell injection. Concurrent treatment animals began TM treatment at the time of injection; these animals were not copper deficient until 5 to 7 days postinjection. (A) Tumor weight in grams at the time of necropsy. (B) Average number of lung metastases in each group.*

Table 2. Number of Weeks Postinjection to Tumor Excision (Tumor Volume ≈900 mm^3).

Treatment Group	Animal				
	1	2	3	4	5
Control	11	11	11	12	12
Ac-PHSCN-NH_2	11	11	12	12	15
TM	11	12	13	15	+22*
TM+Ac-PHSCN-NH_2	11	12	15	15	+22*

Number of weeks postinjection of DU145 tumor cells into nude mice until tumor excision. Although no significant differences were observed in the tumor growth rates, tumors in treated animals appeared to take longer to reach an excisable size. Furthermore, two treated animals had tumors that never grew larger than 500 mm^3.
*These animals never grew tumors greater than 500 mm^3.

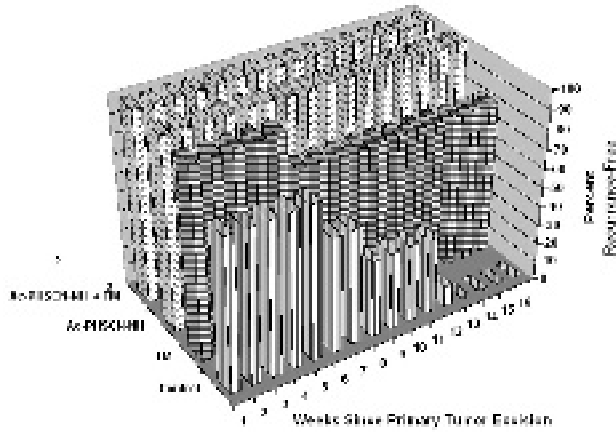

Figure 4. *Comparison of tumor recurrences after primary DU145 tumor excision. All mice treated with Ac-PHSCN-NH_2, either alone or in combination with the antiangiogenic compound tetrathiomolybdate (TM), remained recurrence free up to 30 weeks post-primary tumor excision, whereas 90% of mice treated with TM alone remained recurrence free up to 30 weeks postexcision. In contrast, 100% of control mice had sizeable tumor recurrences at 13 weeks post-primary tumor excision.*

As shown in Table 2, the time to tumor excision (900 mm^3) was faster for the untreated control animals compared with the animals in the treated groups. However, as described previously, there was not a significant difference in tumor growth rate between the untreated group and Ac-PHSCN-NH_2–only treated group (P=.18). The time to tumor excision in the TM-treated groups (TM alone and TM+Ac-PHSCN-NH_2) was significantly longer compared with the untreated controls. Interestingly, one mouse from each of the TM-treated groups never grew tumors greater than 500 mm^3. In fact, the tumor volumes for these mice fluctuated around a mean of only 200 mm^3. There was a slight difference in tumor growth between the TM and TM+Ac-PHSCN-NH_2 groups. These data indicate that TM is effective in significantly slowing primary tumor growth in nude mouse xenografts; in addition, its growth-retarding effect may be enhanced by cotreatment with the PHSCN peptide.

Tumor recurrences were measured in both the untreated and TM-only treated groups between 5 and 6 weeks post-primary tumor excision (Figure 4). All of the untreated animals had recurrences by 13 weeks postsurgery. In contrast, only one of the TM-alone treated mice had a recurrence and none of the Ac-PHSCN-NH_2-only or TM+Ac-PHSCN-NH_2 group had recurrences. The average volume of recurrence was, as expected, significantly higher (P=.01) in the untreated group compared with all other treatment groups (Table 3). All of the control mice were sacrificed 16 weeks after excision of the primary tumor due to recurrence and metastatic disease.

As previously observed, the untreated mice had significantly more gross lung metastases (P=.01) than all the treated animals with a median number of eight lung metastases (range of 0 to 13) for the controls and 0 lung metastases (ranges of 0–0, 0–1, and 0–1) for the treated animals. In each of the TM-treated groups, there was a single animal with one visible lung metastasis. Similarly, the average number of lung micrometastases was found to be highest in the untreated group with an average of 235 micrometastases. This was significantly higher (P<.001) than the treatment groups. The TM-treated mice had an average of 42 mircrometastases. Although this was higher than the Ac-PHSCN-NH_2– or TM+Ac-PHSCN-NH_2–treated groups (with an average of 27 and 19 micrometastases, respectively), the difference was not significant. These data suggest that TM and Ac-PHSCN-NH_2 can be combined *in vivo* to effectively retard DU145 prostate tumor growth, and to drastically inhibit tumor recurrence and metastasis after debulking surgery of the primary tumor.

Discussion

It has been demonstrated that invasive prostate cancer cells have a distinct integrin and cell adhesion molecule profile that allows for increased metastatic capabilities [8–10]. Expression of these integrins is thought to mediate cell adhesion with specific extracellular basement membranes such as laminin, vitronectin, and fibronectin and facilitate cell migration and invasion through specific pathways such as the focal adhesion kinase pathway [11]. Specific integrin pairs are also implicated in forming cell to cell (tumor cell to tumor cell or tumor cell to endothelial cell) interactions, required for tumor cell metastasis [12,13]. Integrins that recognize the canonical tripeptide sequence, RGD, found in a variety of extracellular matrix proteins, are thought to potentially mediate prostate cell interactions with endothelial cells [12]. The DU145 cell has specifically been shown to express the $\alpha_5\beta_1$ integrin pair, which recognizes the RGD sequence [9].

Table 3. Comparison of DU145 Tumor–Bearing Mice Left Untreated or Treated with Ac-PHSCN-NH_2 Alone, Tetrathiomolybdate (TM) Alone or TM+Ac-PHSCN-NH_2.

Treatment Group	Avg. Recurrence Volume (mm^3)	Median No. (Range) of Lung Metastases	Avg. Lung Micrometastases
Untreated (n=5)	882	8 (0–13)*	235**
TM (n=5)	540	0 (0–1)	42
Ac-PHSCN-NH_3 (n=5)	0	0 (0–0)	27
Ac-PHSCN-NH_3+TM (n=5)	0	0 (0–1)	19

Untreated and TM-treated mice had large tumor recurrences. However, only the untreated mice had significantly more gross lung and micrometastases (*P=.01 and **P<.001) than any of the treatment groups.

Plasma fibronectin is a soluble extracellular matrix protein found in plasma, lymph, and interstitial fluids [6]. DU145 and MAT-Ly-Lu cells in serum-free medium can be stimulated to invade through a naturally occurring serum-free basement membrane, SU-ECM, by the addition of plasma fibronectin [4]. Livant et al. demonstrated that the PHSRN sequence of plasma fibronectin, which maps to the cell-binding domain, was sufficient for inducing invasion of these cells [4]. Furthermore, they demonstrated that this is due to PHSRN binding to and stimulating the cell through the $\alpha_5\beta_1$ receptors on the cell surface. *In vivo*, prostate cells may also be induced to invade locally by liberation of fibronectin by prostate-specific antigen (PSA) [14].

Substitution of the arginine residue in the PHSRN sequence with cysteine forms a competitive inhibitor of basement membrane invasion. The PHSCN peptide can effectively block serum-free, PHSRN-induced invasion of human DU145 and rat MAT-Ly-Lu prostate carcinoma cells *in vitro*. Blocking the amino and carboxyl ends of the peptide by acetylation and amidation, respectively, increases its inhibitory activity by 30-fold in serum-containing medium. Taken together, these data led to the hypothesis that Ac-PHSCN-NH_2 may be useful in preventing prostate carcinoma metastasis *in vivo*, due to its anti-invasive properties. Rats that were systematically administered peptide beginning 24 hours after inoculation with MLL cells had significantly fewer lung metastases compared with rats treated with either saline alone or Ac-HSPNC-NH_2 scrambled peptide [4].

We, thus, surmised that administration of Ac-PHSCN-NH_2 after resecting a primary human prostate xenograft might reduce tumor recurrence and metastases by blocking invasion. Athymic nude mice that had tumors of approximately 900 mm^3 began Ac-PHSCN-NH_2 24 hours after tumor cell injection. None of the Ac-PHSCN-NH_2 had tumor at the site of surgery. All of the untreated animals succumbed to either tumor recurrence and/or metastasis within 17 weeks postsurgery. The peptide-treated animals remained disease free 30 weeks after surgery and had no evidence of disease at either the primary or secondary sites. Microscopic analysis of representative lung sections demonstrated 18-fold greater numbers of micrometastases in the untreated animals compared with the treated animals.

As suggested in previous experiments, the presence of micrometastasis suggest that metastasis could be occurring before surgery. However, metastases could also occur due to, or be enhanced by the "spilling" of tumor cells into the blood or lymph during surgical intervention. Given the observation that tumor recurrences reached a large volume much faster than the primary tumors, it is possible that tumor growth is enhanced by physical injury and proximity to a wound-healing process where brisk angiogenesis is occurring. Likewise, circulating factors could also stimulate tumor cell invasion [15–17]. Ac-PHSCN-NH_2 treatment is effective in controlling the growth of recurrences and distant metastases, even if the above processes are in effect.

Similar results were observed when Ac-PHSCN-NH_2 was combined with the antiangiogenic compound TM. TM exerts its antiangiogenic effects by sequestering dietary and circulating copper, forming a tripartite complex with copper and protein that is eliminated in the bile and urine. Because copper appears to be required for activation of the angiogenic switch in nascent tumors as well as for sustained angiogenesis in bulky tumors, TM-treated animals produced a significant difference in tumor growth rates relative to the untreated tumors reaching resectable size (900 mm^3), with the latter reaching the target size by 12 weeks whereas 1 of 5 of the TM-treated tumors never reached the target resectable size at all.

Most strikingly, the time to tumor resection was very different between the treatment groups and the control. The times to tumor resection increased in the following order: TM+Ac-PHSCN-NH_2>TM>Ac-PHSCN-NH_2>untreated. Interestingly, in each of the TM-treated groups there was one animal whose tumor never reached a resectable size. Also, the Ac-PHSCN-NH_2 tumors appeared to be more defined than the untreated or TM-treated tumors. The untreated tumors in particular had more diffuse contours and were visibly vascularized. TM alone was protective against tumor recurrence, albeit to a slightly lesser extent than Ac-PHSCN-NH_2 alone, with 80% of the TM-treated animals being recurrence free compared with 100% of the Ac-PHSCN-NH_2–treated animals. Most significantly, all of the treatment groups survived disease free for longer than 30 weeks, compared with the control group that succumbed to disease within 16 weeks postsurgery.

Taken together, these data strongly suggest that Ac-PHSCN-NH_2 alone, or in combination with TM, may provide a highly effective, tolerable, long-term treatment for the metastatic and recurrence events of prostate cancer in human xenografts postsurgery. Postsurgical administration of these compounds, each alone or in combination, can effectively prevent prostate cancer recurrence and metastasis. Because the Ac-PHSCN-NH_2 is so far available only in injectable form, it is very useful to consider combining or alternating therapy with an oral, nontoxic global inhibitor of angiogenesis such as TM, which alone has significant activity in the prevention of metastasis, has the added advantage of impairing primary tumor growth, and is amenable to long-term administration.

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