Identification of a small molecule class to enhance cell-cell adhesion and attenuate prostate tumor growth and metastasis

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Abstract

Expression of calcitonin (CT) and its receptor (CTR) is elevated in advanced prostate cancer, and activated CT-CTR autocrine axis plays a pivotal role in tumorigenicity and metastatic potential of multiple prostate cancer cell lines. Recent studies suggest that CT promotes prostate cancer metastasis by reducing cell-cell adhesion through the dis-assembly of tight and adherens junctions and activation of β-catenin signaling. We attempted to identify a class of molecules that enhances cell-cell adhesion of prostate cells and reverses the disruptive actions of CT on tight and adherens junctions. Screening several compounds led to the emergence of phenyl-methylene hydantoin (PMH) as a lead candidate that can augment cell-cell adhesion and abolish disruptive actions of CT on junctional complexes. PMH reduced invasiveness of PC-3M cells and abolished proinvasive actions of CT. Importantly, PMH did not display significant cytotoxicity on PC-3M cells at the tested doses. I.p. administered PMH and its S-ethyl derivative remarkably decreased orthotopic tumor growth and inhibited the formation of tumor micrometastases in distant organs of nude mice. PMH treatment also reduced the growth of spontaneous tumors in LPB-Tag mice to a significant extent without any obvious cytotoxic effects. By virtue of its ability to stabilize cell junctions, PMH could reverse the effect of CT on junctional disruption and metastasis, which strengthens the possibility of using PMH as a potential drug candidate for CT-positive androgen-independent prostate cancers. [Mol Cancer Ther 2009;8(3):509–20]

Introduction

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer deaths in men in America (1, 2). Although androgen ablation therapy is effective in prostate cancer patients for some time, the disease progresses to an androgen-independent stage (3) when the tumor becomes metastatic, chemoresistant, and life threatening (4, 5). The population of prostate cells expressing neuropeptides, such as calcitonin (CT) and its receptor (CTR), also increases during this progression (6, 7). Earlier results from this laboratory have shown that CT and CTR are exclusively localized in basal, but not secretory, compartment of normal prostate epithelium. This spatial specificity is lost during tumor progression as evidenced by a large increase in CT/CTR mRNA abundance in secretory epithelia of advanced prostate cancers (8). Moreover, exogenous CT or enforced activation of CT-CTR autocrine axis remarkably raises tumorigenicity, metastatic potential, and apoptosis resistance of several prostate cancer cell lines (8–14). Our recent studies suggest that the disruption of cell-cell adhesion may be a key mechanism associated with CT-stimulated prostate cancer progression and metastasis (15, 16). Because metastasis is the primary cause of morbidity and mortality associated with prostate cancer, we focused to identify a class of molecule(s) that augments cell-cell adhesion complexes and attenuates CT-stimulated prostate cancer growth and metastasis. We began by screening several structurally diverse marine natural products isolated from Red Sea sponges and identified phenyl-methylene hydantoins (PMH) as a lead class that strengthens cell-cell adhesion complexes of prostate cancer cell lines and attenuates CT-stimulated tumor growth and metastasis of prostate cancer cell lines. Moreover, PMH molecules can be easily, cost effectively, and regioselectively synthesized and display minimal side effects at the therapeutic doses in xenograft and transgenic mouse models.

Materials and Methods

Animals

Male BALB/c nu/nu mice (6–8 wk old) were purchased from Harlan and housed two per cage in microisolator units under controlled temperature/humidity. The animals were fed ad libitum on a standard sterilizable laboratory chow (Harlan Teklad) and quarantined for 1 wk before their use. LPB-Tag transgenic mice (12T-fast) were provided by Dr. Robert J. Matusik. The colony of these mice was established in our facility and newborn mice were identified by genotyping as previously described (17). Positive adult mice were used in the present study.
All animal procedures were conducted in accordance with the principles and procedures outlined by the NIH and Institutional Animal Care and Use Committee at University of Louisiana-Monroe.

**Surgical Orthotopic Implantation**

The surgical orthotopic implantation on nu/nu mice was done as described before (18). Briefly, the mice were anesthetized with ketamine (100 mg/kg)–xylazine (10 mg/kg) and placed in a supine position. A midline incision was made in the lower abdomen. Tumor cell suspensions and placed in a supine position. A midline incision was done as described before (18). Briefly, the mice were anesthetized with ketamine (100 mg/kg)–xylazine (10 mg/kg) and single-stitch sutures closed abdomen. The mice were periodically fluoroimaged with Kodak 4000 MM imaging station under anesthesia to monitor tumor growth and metastasis. The mice were sacrificed as described in Results and organs were collected for further analysis.

Because the goal of the present study was to identify novel molecules that inhibit CT-stimulated disruption of cell-cell adhesion and reduce invasive virulence of advanced prostate cancer, we chose PC-3M prostate cancer cells as a model cell line. This is because PC-3M cells are poorly differentiated, androgen-refractory, coexpress CT and CTR, and are highly metastatic (8, 19). The cells were maintained in the complete medium (RPMI 1640 supplemented with 10% FCS, 100 IU/mL penicillin G, and 100 mg/mL streptomycin) under standard culture conditions. Stable PC-3M-CT+ subline overexpresses CT and is more metastatic than PC-3M cells. This was generated by stable transfection of recombinant plasmid pcDNA 3.1 containing CT cDNA and selected in G418. The cell line was extensively characterized in recent studies (9).

Initially, phenylmethylene hydantoin was extracted from Hemimycyle arabica (a Red Sea sponge) and characterized by spectral methods, including full nuclear magnetic resonance analysis. Subsequently, PMH as well as its most potent analogue S-ethyl PMH (S-PMH) were synthesized (Supplementary Fig. S1). Extracted PMH and synthetic PMH showed identical nuclear magnetic resonance profiles. They were tested in multiple *in vitro* assays and were found to be equipotent. Synthetic PMH was used in the present studies.

**In vitro Assays: Cell-Cell Adhesion**

**Transepithelial Resistance.** Approximately 1 x 10^5 cells were plated and grown to confluency on 12-well transwell filters (0.4-µm pore size) in complete medium for the first 12 h and then in serum-free medium. Electrodes were placed at the upper and lower chambers and transepithelial resistance (TER) was measured in triplicate wells at multiple time points after CT/PMH addition with EVOM (World Precision Instruments). The TER values were normalized to the area of the monolayer filter and calculated by subtracting the blank values derived from the filters containing only bathing medium. The integrity and cell density of monolayers was carefully monitored during TER measurement studies.

**Paracellular Permeability.** This assay measured the diffusion of tetramethyl rhodamine–labeled dextran (TMR-dextran, 4 kDa) across a cell layer grown on a membrane of transwell insert (paracellular permeability). PC-3M cells were seeded (1 x 10^5 per insert) and cultured to form a monolayer for 5 d. The culture medium was then replaced with HBSS a few minutes before the addition of TMR-dextran (1 mg/mL in HBSS). TMR-dextran was added to the upper chamber and 100 µL of the samples were removed from the lower chamber after 1 h. Fluorescence was measured on a Bio-Tek ELISA plate reader (Ex530 nm/Em590 nm). The diffusion of TMR-dextran across the insert without cells was also measured to ascertain the integrity of cell monolayers. Each data point was in quadruplicates.

**Preparation of Cell Lysates: Triton X-100–Soluble and Triton X-100–insoluble Fractions.** Confluent 100-mm plates of PC-3M cells were serum-starved overnight and treated with diluent/CT/PMH as described in Results. Triton X-100–soluble extract (cytosolic) was obtained by incubating cells with 10 mmol/L Tris-HCl (pH 7.4; containing 150 mmol/L NaCl, 2 mmol/L CaCl_2, 1 mmol/L phenylmethylsulfonyl fluoride, 40 units/mL aprotinin, 15 µg/mL leupetin, 1% NP40, and 1% Triton X-100) for 30 min with occasional agitation. After washing the plates with TBS (containing protease inhibitors), the Triton X-100–insoluble fraction (plasma membrane-associated) was scraped out from the plates with TBS containing 0.5% SDS, 1% NP40, 40 units/mL aprotinin, and 15 µg/mL leupetin. The contents were homogenized and centrifuged at 14,000 x g for 5 min at 4°C to obtain the Triton X-100–insoluble fraction. Protein content of lysates was determined using Bio-Rad Reagent (Bio-Rad).

**Western Blotting.** The lysate fractions (100 µg protein per lane) were boiled for 5 min in 2 x Laemmli solution containing 20 mmol/L DTT, loaded on 10% SDS-polyacrylamide gel, and fractionated proteins were electrically transferred to a nitrocellulose membrane. The blots were incubated with appropriate antiserum as described in Results for 18 h at 4°C. Following three washes, the blots were incubated with appropriate horse radish peroxidase–conjugated secondary antiserum (either antirabbit or antimouse IgG). The immune complexes were visualized on chemiluminiscence radiography film using Western Blot Enhanced Chemiluminescence Detection System (Radiochemical Center, Amersham). The blots were then washed and reprobed for α-tubulin or β-actin. The same experiment was repeated two more times.

**In vitro Assays: Cell Growth and Invasion**

**Cell Proliferation Assay.** The proliferation of prostate cancer cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using the kit purchased from American Type Culture Collection. Exponentially growing PC-3M cells were plated in a 96-well
plate at a density of $8 \times 10^3$ per well and cultured for 24 h. The complete growth medium was then replaced with 100 μL of basal incubation medium (RPMI 1640 containing 0.1% bovine serum albumin, 10 mmol/L HEPES, 4 mmol/L L-glutamine, 100 IU/mL penicillin G, and 100 mg/mL streptomycin) and incubation was continued for additional 24 h. The cells were then treated with the MTT solution at 37°C for 4 h and then stop solution was added (100 μL per well). Absorbance of the samples was determined at 595 nm with an ELISA plate reader (Bio-Rad).

**In vitro Invasion Assay.** Invasion experiments were conducted in 24-well, two-compartment, Matrigel invasion chambers (Becton Dickinson). Exponentially growing PC-3M cells were serum-starved for 24 h with basal incubation medium and then seeded at a density of $25 \times 10^3$ cells per well in the upper insert of the Matrigel chamber. The lower chamber received the chemoattractant medium, which consisted of 90% basal RPMI and 10% conditioned medium from the cultures of PC-3M cells expressing constitutively active Gαs protein (20). The incubations were carried out for 24 h, after which Matrigel (along with noninvading cells) was scraped off with cotton swabs, and outer side of the insert was fixed and stained using Diff Quick kit (Dade-Behring Diagnostics). Invaded cells on the underside of the insert were counted in six or more randomly selected ×100 fields. The results were expressed as mean ± SE invaded cells per ×100 field. Each experiment was done in triplicates and the experiment was repeated twice.

**Growth Correction.** Because PC-3M cells exhibit a high proliferation rate, it is conceivable that the cells that migrated during the early part of the 24-h incubation period could proliferate during the remaining period of incubation, causing a slight overestimation of final results. To correct this, we determined the growth rate of PC-3M cells under identical conditions. Cells ($25 \times 10^3$) were plated at hourly intervals in six-well dishes and cultured for 1 to 24 h. Mean percentage increase in cell number in each well was determined. The relative CT-induced increase of the pooled results of all time points was found to be 1.19 (vehicle control = 1). This correction was applied to the results of invasion assays.

**Spheroid Disaggregation Assay.** Current evidence has shown that the cells of primary tumors in situ are released in clumps, which then attach to a favorable extracellular matrix (ECM), and are released gradually to migrate in all directions (21, 22). Based on this model, we have established a spheroid disaggregation assay where we first produce spheroids of prostate cancer cells, then place them on an ECM and monitor their disaggregation and the radial cell migration of tumors cells over a period of 48 h (23). In our experience, this model reliably predicts the relative metastatic potential of prostate cancer cell lines. We examined the effect of PMH and S-PMH on the metastatic potential of PC-3M cells in spheroid disaggregation assay. In brief, a 100-μL suspension of $5 \times 10^5$/mL PC-3M cells in RPMI 1640 serum-free medium was placed on 96-well low-attachment tissue culture plates. The plates were rocked on gyratory shaker in a CO₂ incubator at 37°C for 2 d, at the end of which the spheroids measuring 150 to 300 μm diameter (~4 × 10⁴ cells per spheroid) were formed. A single spheroid was then placed in the center of each well of ECM-coated 24-well microplate in 200 μL of serum-free medium, was allowed to attach to the ECM for 1 h, and digitally photographed ($t = 0$). The spheroids were then cultured at 37°C for 48 h, fixed, stained with Diff-Quik (Dade-Behring), and rephotographed. The diameter of the area covered with cells was measured in a microscope calibrated with a stage and ocular micrometer. The results are presented as μm migration ± SE, where migration = (diameter of the area covering migrated cells – diameter of the spheroid) / 2 from three separate experiments, totaling 5 to 10 spheroids.

**In vivo Assays: Orthotopic Tumor Growth and Metastasis**

**Stable Expression of Red Fluorescence Protein in PC-3M Cells.** To detect micrometastases of implanted tumor cells in mice, we stably transfected PC-3M cells with DsRed-MCherry-Hyg-N1, a mammalian expression vector that encodes DsRed-MCherry, a derivative of red fluorescent protein (Clontech). Hygromycin-resistant colonies were selected and observed over a period of 4 wk. All cell lines expressed strong red fluorescence at a steady level over the duration of the observation period. These cells were then used for orthotopic implantation in nu/nu mice.

**Administration of PMH/S-PMH in Mice**

**Nu/nu Mice.** PMH (or S-PMH) solution was prepared at 100× concentration in 10% DMSO and diluted to 1× with normal saline. The mice were administered i.p. to a group of six animals per molecule or a diluent as described in Results. The doses for PMH and S-PMH were 200 μg/100 μL per day or 5 μg/g body weight per day and 40 μg per day or 1 μg/g body weight per day, respectively. These were the maximal doses for each molecule without apparent cytotoxic effects as determined by initial studies. Tumor growth and metastases were monitored every 3 d beginning after day 25 postimplantation by fluoroimaging with Kodak 4000 MM image station. The scanned fluorescent images were quantitated with Molecular Imaging Software version 6.05f7 (Kodak).

The S-PMH solution was administered i.p. at the dose of 80 μg/wk or 2 μg/g of body weight per week. The treatment was started at the age of 30 d postnatal and continued until day 90. The mice were sacrificed on day 90 and their male reproductive organs were isolated, weighed, and fixed for histology.

At necropsy, the primary tumor and other organs were harvested and weighed. Wet sections of organs were examined for the presence of red fluorescent protein. Fluorescent images of tumor cells were acquired with a charge-coupled device Retiga 2000 RT digital camera connected to a microscope (Nikon Optiphot 2) and a computer. The images were then processed with IPLab Image Analysis Software (BD Biosciences). The remaining tissue portions were fixed in neutral buffered formalin and embedded in paraffin. The 5-μm-thick sections of the tumors were processed for H&E staining.
Statistical Analysis
The results were statistically evaluated by one-way ANOVA or other analyses as described in Results and significance was derived from Newman-Keuls test. The difference was considered statistically significant when \( P < 0.05 \).

Results
CT Disrupts Tight and Adherens Junctions in Polarized PC-3M Cells and PMH Abolishes This Action
A primary function of epithelia is to create a barrier that separates distinct tissue compartments by forming gap junctions, desmosomes, and tight and adherens junctions (TJ and AJ). TJs are located on most apical region of the cell membrane, whereas AJs are located immediately basal to TJs. AJs maintain cell-cell adhesion, whereas TJs function as a “fence” by sealing the spaces between adjacent cells and maintain apico-basal polarity and as a “barrier” by regulating paracellular permeability to control the diffusion of electrolytes and small molecules (24–26). We have recently reported that CT promotes prostate cancer metastasis by disrupting cell-cell adhesion through the disassembly of TJs and AJs (16). Therefore, we screened several molecules and identified PMH as a molecule that reverses the actions of CT on TJs and AJs.

PMH and TER of Polarized PC-3M Cells. The optimal concentrations of CT, PMH, and S-PMH were chosen by previous experimentation. We observed that 50 \( \mu \text{mol/L} \) PMH was most beneficial for the enhancement of TJ/AJ function, whereas 50 \( \text{nmol/L} \) CT had the most disruptive effect. These concentrations were then used in all in vitro studies. We first examined the effect of PMH on TER of diluent- and CT-treated polarized PC-3M cells. TER of PC-3M cultures steadily increased up to 48 hours and then it stabilized (Fig. 1A). CT significantly reduced TER. In contrast, PMH significantly increased TER of diluent- and CT-treated PC-3M cultures, suggesting that PMH has beneficial effects on TJs and it completely reverses the actions of CT on TER.

PMH and Paracellular Permeability of Polarized PC-3M Cells. Barrier function of TJs of PC-3M cultures can be examined by the ability of TMR-dextran to penetrate through the cell layer. Because PMH promoted TJ formation and reversed the action of CT on TER, we examined its effect on paracellular permeability of diluent- and CT-treated PC-3M cell layers. As expected, CT caused an almost 2-fold increase in paracellular permeability (Fig. 1B). In contrast, PMH decreased baseline paracellular permeability and abolished CT-induced increase.

PMH and Translocation of Key Junction Proteins from Triton X-100–Insoluble to Triton X-100–Soluble Compartment. Cell-cell adhesion is controlled by key AJ and TJ proteins that are located either on the membrane (such as claudins, occludins, or E-cadherin) or on the intracellular side of the membrane (such as zonula occludens-1 or \( \beta \)-catenin). Disruption of these junctions causes internalization of integral membrane proteins into the cytoplasm, which can be identified by the change in their solubility in Triton X-100 (membrane proteins are Triton X-100 insoluble and cytoplasmic ones are soluble; ref. 27). Because the results of Fig. 1A and B suggested that PMH attenuated CT-stimulated disassembly of junctions, we verified this by testing the effect of PMH on CT-stimulated changes in the solubility of key TJ proteins, such as zonula occludens-1 and occludin, and AJ proteins, such as E-cadherin and \( \beta \)-catenin. Equal amounts of cell lysate proteins of Triton X-100–insoluble or Triton X-100–soluble fractions were loaded onto a SDS-PAGE and immunoblotted for zonula occludens-1, occludin, E-cadherin, and \( \beta \)-catenin. Occludin and E-cadherin were predominantly present in the Triton X-100–insoluble fraction (Fig. 1C and D, lanes 1), whereas zonula occludens-1 and \( \beta \)-catenin were present in Triton X-100–insoluble as well as Triton X-100–soluble fractions (Fig. 1C and D, lanes 1 and 4). CT increased the solubility of all four proteins in Triton X-100 (Fig. 1C and D, lanes 2). Interestingly, PMH decreased the solubility of all four proteins in Triton X-100, under baseline as well as CT-stimulated conditions (Fig. 1C and D, lanes 3 and 4). These results confirm the results of Fig. 1A and B and show that PMH augments cell-cell adhesion by enhancing TJs and AJs and by abolishing the destabilizing actions of CT on these complexes.

Effect of PMH on Prostate Cancer Tumor Growth/Metastasis
Our recent studies have shown that CT significantly increases tumor growth and metastasis of prostate cancer cell lines and these effects may be mediated by the disruption of cell-cell adhesion (15, 16). Because PMH reversed the disruptive actions of CT on cell-cell adhesion, we tested the hypothesis that PMH attenuates CT-stimulated tumor growth/metastasis.

PMH and PC-3M Cell Proliferation. PMH caused a small but significant decline in the rate of baseline PC-3M cell proliferation (Fig. 2A). CT (50 \( \text{nmol/L} \), an optimal dose for stimulation of cell proliferation/invasion as determined in ref. 11, 28) caused a modest but significant increase in PC-3M proliferation, and PMH remarkably reduced the CT-stimulated increase. Moreover, PMH (50 \( \mu \text{mol/L} \) displayed a minimal cytotoxic effect on PC-3M cells as suggested by no decrease in cell viability in its presence.

PMH and PC-3M Cell Invasion. PC-3M cells displayed a high rate of invasion, and CT (50 nmol/L) caused almost a 2-fold increase in invaded cells (Fig. 2B). PMH (50 \( \mu \text{mol/L} \) had minimal effect on baseline invasion but almost attenuated the CT-stimulated increase.

Effect of PMH on Metastatic Potential: Spheroid Disaggregation Assay. To avoid repeated addition of exogenous CT during the long incubation period of 48 hours in a spheroid disaggregation assay, we used PC-3M-CT+ cells, which secrete large amounts of CT and have CTR (9). We first examined the migration of cells from diluent/PMH/S-PMH–treated PC-3M-CT+ spheroids on vitro-nectin, fibronectin, collagen, and laminin (Fig. 2C). This is because the migration of cancer cells depends on surface integrins and integrins have preferential affinity for different ECM proteins (29). PC-3M-CT+ cells displayed...
maximal migration on vitronectin and minimal migration on laminin. These results are consistent with our earlier observation that CT increases surface activity of αvβ3 integrins, which serve as vitronectin receptors (23). Considering the involvement of αvβ3 in bone metastasis (30, 31), we thought it was important to examine whether PMH and S-PMH affect CT-stimulated spheroid disaggregation on vitronectin. Interestingly, PMH, as well as S-PMH, maintained integrity of spheroids (Fig. 2E) and blocked spheroid disaggregation and attachment of the cells to vitronectin and, to a smaller extent, to other various ECM proteins (Fig. 2C). Therefore, all subsequent assays were done on vitronectin.

The experiment was then repeated at multiple doses of PMH and S-PMH. Both molecules inhibited spheroid disaggregation/cell migration in a dose-dependent manner with EC50 concentration of 150 μmol/L for PMH (Fig. 2D). S-PMH was approximately three times more potent than PMH in inhibiting PC-3M-CT+ spheroid dispersal/cell migration on vitronectin, suggesting that both drugs blocked CT-stimulated αvβ3 activity, a key factor in bone metastasis (23, 32).

Figure 2E depicts a typical example of untreated/S-PMH–treated PC-3M spheroids after 48 hours. The untreated spheroid is disaggregated as shown by the large circle of migrated cells around the spheroid. However, the presence of S-PMH (50 μmol/L) completely prevented the spheroidal disaggregation. Figure 2F provides the quantitative data of Fig. 2E.
Figure 2. Effect of PMH on the growth and invasion of PC cells. A, PC-3M cells were cultured for 3 d. After 2 d, complete growth medium was replaced with serum-free basal medium. Cells were then treated with diluent, 50 nmol/L CT, 50 μmol/L PMH, and 50 μmol/L PMH with 50 nmol/L CT for 24 h. Cell proliferation was assessed by the MTT assay. Absorbance at 595 nm was measured and error bars are mean ± SE of four independent experiments (n = 8). *, P < 0.05, significantly increased over the untreated PC-3M values (one-way ANOVA and Newman-Keul’s test). B, invasion of PC-3M cells treated with diluent, 50 nmol/L CT, 50 μmol/L PMH, and 50 μmol/L PMH with 50 nmol/L CT was examined as described in Materials and Methods. Triplicate experiments were done and six × 100 fields were counted for each data point. Results are mean ± SE for cells per ×100 field (n = 3 independent experiments × 6 fields). *, P < 0.01, significantly different from the control (diluent treated; one-way ANOVA and Newman-Keul’s test). C, disaggregation/migration of PC-3M-CT+spheroids on different ECMs in the presence or absence of PMH/S-PMH. Radial migration of spheroids under each condition [with and without the treatment of PMH or S-PMH on vitronectin (VN), fibrinogen, collagen, and laminin] was quantitated as described in Materials and Methods and graphed. The cell outgrowth was measured after 48 h of incubation. The results are presented as mean μm radial migration ± SE for n = 4. *, P <0.05, significantly different from their respective controls (one-way ANOVA and Newman-Keul’s test). D, effect of different concentrations of PMH/S-PMH on disaggregation/migration of PC-3M-CT+spheroids on vitronectin. Spheroids prepared from PC-3M-CT+ were treated with different concentrations of PMH and S-PMH and their disaggregation/migration was examined on vitronectin. The cell outgrowth was measured after 48 h of incubation. The results are presented as mean μm radial migration ± SE for n = 4. *, P <0.05, significantly different from their respective controls (one-way ANOVA and Newman-Keul’s test). E, typical photomicrographs of PC-3M-CT+spheroids at the end of the experiment. Cell migration of spheroids prepared with PC-3M-CT+ cells was examined in the absence or presence of 50 μmol/L S-PMH on vitronectin as described in Materials and Methods. F, the cell outgrowth was measured and photographed after 48 h of incubation. *, P <0.05, significantly different from vehicle-treated controls (one-way ANOVA and Newman-Keul’s test).
weighed 2.673 g ± 0.268 (n = 6). The corresponding tumor masses of PMH-treated and S-PMH–treated mice were significantly lower and were 1.597 g ± 0.138 (n = 12) and 1.405 g ± 0.305 (n = 8), respectively (Fig. 3C). Although PMH and S-PMH displayed a similar antitumor activity, that of S-PMH was more robust as it was given only at 20% of PMH dose. Survival curves of these mice suggest that the treatment with PMH extended the life span of mice from 50–57 to 56–65 and that with S-PMH to 65–70 (Fig. 3D).

PMH and S-PMH Reduce the Formation of Distant Micrometastases in Nu/Nu Mice. Because we implanted PC-3M-CT+ cells expressing red fluorescence protein, we could detect the presence of fluorescent micrometastases in wet sections of organs. We examined several organs for fluorescent tumor cell colonies and metastases were graded from 0 (no cells) to ++++ (large tumor mass) as depicted in Table 1A. Both PMH and S-PMH significantly reduced incidents of metastases in several organs (Table 1B). Figure 4 (A and B) provides representative fluromicro-

graphs of different organs of diluent/PMH/S-PMH–treated mice. Diluent-treated mice showed large tumor cell colonies in lymph node, seminal vesicles, bone, lungs, and smaller tumor cell numbers in other organs including brain. Both PMH and S-PMH remarkably decreased metastasis of PC-3M-CT+ cells in most organs and reduced the size of tumor cell colonies in organs where metastases were present. Comparatively, S-PMH seemed more antimetastatic than PMH. H&E staining of these organs confirmed the presence of micrometastases in these organs (Supplementary Fig. S2). 1

S-PMH and Tumor Growth/Metastasis in LPB-Tag Mice. Because S-PMH displayed potent antimetastatic effects in orthotopic xenograft model, we tested this molecule in LPB-Tag mice (33). This line of transgenic mice was established as a model for prostate cancer. The mice develop spontaneous prostate tumors around day 30 postnatal. The mice were given 2 μg S-PMH/g body weight per week i.p. in three equally divided doses per week beginning at day 30. The treatment was continued for the period of the next 60 days and was terminated on day 90.
Table 1.

A. Micrometastases of PC-3M cells in host organs

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<th>Diluent</th>
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<td>Testis</td>
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B. Frequency of micrometastases in host organs

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NOTE: –, no tumor cells; +, isolated tumor cells; ++, few tumor cell colonies; ++++, several tumor cell colonies spread throughout the organ. One-way ANOVA, level of significance: \( P < 0.001 \) (PMH versus diluent); \( P < 0.001 \) (S-PMH versus diluent). \( P > 0.05 \) (PMH versus S-PMH).

The reproductive organs of the mice were harvested and weighed. All LPB-Tag mice spontaneously produced prostate tumors as depicted in Fig. 5A. However, S-PMH treatment reduced the organ growth by 65% (Fig. 5B). H&E staining of prostate sections suggest the presence of large tumor mass in the prostate of diluent-treated mice. In contrast, S-PMH–treated mice displayed relatively normal prostate epithelium (Fig. 5C and D).

Discussion

Expression of CT and CTR is elevated in advanced prostate cancer, and activated CT-CTR autocrine axis increases tumorigenicity and metastatic potential of multiple prostate cancer cell lines (8–10). Recent results from this laboratory suggest that CT promotes prostate cancer metastasis by reducing cell-cell adhesion through the disassembly of TJs and AJs and activation of \( \beta \)-catenin signaling. The TJs are occluding junctions, which seal cells together in an epithelial sheet, and are critical for the determination of epithelial cell polarity. Either disassembly or remodeling of TJs can cause a loss of cell polarity and increase in motility (34, 35). Adherens junctions are adhesive contacts that hold cells together in a fixed position within the tissue. It has been shown that TJ along with AJ and desmosomes provide tissue integrity and promote cellular polarity, and AJ plays a pivotal role in regulating the entire activity of junctional complex (36–38). There is increasing evidence for the association between the loss cell-cell adhesion structures and metastasis of several other epithelial cancers (39, 40). Therefore, the primary objective of the present study was to identify a class of molecules that enhances cell-cell adhesion of prostate cells and abolishes disruptive actions of CT on TJs and AJs. PMH increased TER, decreased paracellular permeability, and prevented the translocation of key integral TJ and AJ membrane proteins into the cytoplasm, suggesting that PMH enhanced TJs and AJs. More importantly, PMH attenuated the disruptive effects of CT on TER, paracellular permeability, and internalization of key AJ and TJ proteins, such as zonula occludens-1, occludin, E-cadherin, and \( \beta \)-catenin.

Cell adhesion, proteolytic degradation, and cell migration are interrelated processes responsible for invasion and metastasis of cancer (41–43). Because PMH attenuated the disruptive actions of CT on cell-cell adhesion complexes, we tested a second hypothesis that PMH reduces invasiveness of highly metastatic PC-3M cells. We used a combination of \( \text{in vitro} \) and \( \text{in vivo} \) models to test this hypothesis. The results from cell proliferation assays revealed that PMH did not significantly alter baseline proliferation of PC-3M cells but abolished CT-stimulated cell proliferation. More importantly, PMH did not display significant cytotoxicity on PC-3M cells at the tested doses but reduced invasiveness of PC-3M cells and attenuated proinvasive actions of CT as assessed by Matrigel invasion assays and spheroid disaggregation assays. If cell-cell adhesion plays a key role in tumor metastasis, PMH should attenuate the metastatic potential of PC-3M sublines. We tested this possibility in two \( \text{in vivo} \) models. It has been shown that \( \text{in vitro} \) invasion assays are useful for studying early events of metastasis. However, the formation of distant metastases requires not only invasiveness of tumor cells but also additional characteristics like the capacity to survive during the process of intravasation into blood and lymphatic vasculature, extravasation into parenchyma of distant tissues, and ability to grow in ectopic environment (11, 44, 45). These characteristics can only be examined in animal models. In addition, \( \text{in vivo} \) assays provide other key information, such as the stability of the therapeutic molecule in the body and its ability to reach the target organ and produce intended beneficial effects. First, we tested the effect of PMH and S-PMH on tumor metastasis in an orthotopic xenograft model. As expected, PC-3M-CTR+ cells formed large orthotopic tumors and distant metastases in multiple organs of nude mice. I.p. administered PMH and S-PMH remarkably decreased orthotopic tumor growth and inhibited the formation of tumor micrometastases in distant organs. More importantly, the mice tolerated therapy well and did not display apparent cytotoxic effects of PMH and S-PMH at the tested doses. However, the mice produced remarkable beneficial responses, such as decreased morbidity and increased survival, reinforcing a possibility that the
disassembly of junctional complexes may be a key event in the progression of a localized prostate tumor to its metastatic form, and PMH can attenuate this process. Because S-PMH was more potent in attenuating PC-3M-CT+ xenograft growth and metastasis, it can potentially be useful for the treatment of patients with invasive or high CT prostate tumors. However, human tumors are not homogenous like PC-3M-CT+ xenografts. Therefore, we tested S-PMH therapy in LPB-Tag mice, a transgenic model developed to study prostate carcinogenesis (46).

![Figure 4. Metastasis of PC-3M-CT+ cells in organs of nu/nu mice.](image)

**A**

Dissemination of red fluorescent protein–expressing PC-3M-CT+ cells (1×10⁶) into the prostate of nude mice was examined. After 5 wk of treatment, the lungs, lymph nodes, kidneys, testes, and seminal vesicles were surgically removed, fixed, and stained for red fluorescent protein visualization. The images were captured with a fluorescent microscope at ×100 magnification.

**B**

Dissemination of red fluorescent protein–expressing PC-3M-CT+ cells into the lungs, lymph nodes, kidneys, testes, and seminal vesicles was examined. After 5 wk of treatment, the lungs, lymph nodes, kidneys, testes, and seminal vesicles were surgically removed, fixed, and stained for red fluorescent protein visualization. The images were captured with a fluorescent microscope at ×100 magnification.
tumors of LPB-Tag mice display significant similarities with human tumors, including tumor heterogeneity, neuroendocrine features, and relatively slower tumor growth (46). Our results show that S-PMH therapy was effective in LPB-Tag mice as assessed by a remarkably reduced growth of prostate tumors. These results strongly support S-PMH as a novel candidate therapeutic molecule for metastatic prostate cancer and a chemopreventive agent.

PMH has been tested for a possible anticonvulsant activity in experimental animals but no beneficial therapeutic effects have been reported. In contrast, S-PMH is a novel molecule. There have also been a few reports on pharmacologic activity of molecules that are structurally related to PMH/S-PMH. For example, cyclic imide hydantoins class of compounds has been investigated for anticonvulsant activity (47). Pharmacologic activity of hydantoins could be significantly modulated by substitution of active groups to produce fungicidal, herbicidal, anti-inflammatory, anti-HIV, antihypertensive, and hypolipidemic activities (47). Hydantoin analogues, such as substituted diisopropylbenzylidene hydantoins and 2-thiohydantoins, were patented for the inhibition of tyrosine kinase and anti-allergic activities (48). However, the compounds were potent only at a superpharmacologic dose of 100 mmol/L in guinea pigs (48). Propanoic acid, 2-[4-[(5-oxo-2-thioxo-4-imidazolidinylidene)methyl]phenoxyl, and related analogues were recently patented for their ability to suppress cell proliferation and inhibit binding between extracellular signal-regulated kinases and their substrates (49). Imidazol-2-amine, 5-chloro-N-(4-methylphenyl)-4-(phenylmethylene), was patented for in vitro anti-melanoma activity (50). However, the present report for the first time shows potent antimitastatic effects of PMH and S-PMH on prostate cancer in multiple in vitro and in vivo model systems through the enhancement of cell-cell adhesion.

Although additional studies are required to identify specific targets of PMH and precise molecular events through which PMH enhances junctional complexes, the present results suggest that S-PMH can potentially serve as a novel therapeutic molecule for treatment of advanced prostate cancer and cell-cell adhesion can be an important paradigm to screen or develop new class of therapeutic molecules for the treatment of advanced prostate cancer and possibly other epithelial tumors.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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Identification of a small molecule class to enhance cell-cell adhesion and attenuate prostate tumor growth and metastasis

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