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A prostate secretory protein94-derived synthetic peptide PCK3145 inhibits VEGF signalling in endothelial cells: Implication in tumor angiogenesis

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We have previously observed that the synthetic peptide corresponding to amino acids 31–45 (PCK3145) of PSP94 can reduce prostate tumor growth *in vivo*. Moreover, a recently concluded phase IIa clinical trial with patients with hormone refractory prostate cancer indicated that PCK3145 down-regulates the levels of plasma matrix metalloproteinase (MMP)-9, a MMP involved in metastasis and tumor angiogenesis. The purpose of our study was to investigate the molecular mechanisms of action of PCK3145 and whether this peptide could antagonize tumor neovascularization. We show that, in a syngeneic *in vivo* model of rat prostate cancer, the expression of endothelial cell (EC) specific CD31, a marker of tumor vessel density, was decreased by 43% in PCK3145-treated animals. *In vitro*, PCK3145 specifically antagonized in a dose-dependent manner the VEGF-induced ERK phosphorylation as well as the phosphorylation of the VEGFR-2 in cultured EC (HUVEC). These anti-VEGF effects were partly reproduced by pharmacological inhibitors such as PD98059 and PTK787, suggesting that PCK3145 inhibits the tyrosine kinase activity associated to VEGFR-2, which in turn prevents intracellular signalling through the MAPK cascade. Moreover, PCK3145 was also found to inhibit the PDGF-induced phosphorylation of PDGFR in smooth muscle cells. Finally, PCK3145 inhibited *in vitro* EC tubulogenesis and VEGF-induced MMP-2 secretion suggesting its potential implication as an antiangiogenic agent. Our study demonstrates that PCK3145 interferes with the tyrosine kinase activity associated with VEGF signalling axis in EC. The antiangiogenic properties of this peptide could be highly beneficial and exploited in novel antiangiogenic therapies, for patients with various cancers.

Key words: VEGF receptor; prostate cancer; tumor angiogenesis; ERK; tyrosine kinase inhibitor

Prostate secretory protein 94 (PSP94), also known as prostatic inhibin peptide or β-microseminoprotein, is one of the 3 predominant naturally occurring proteins secreted by the prostate gland along with prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP).[1,2] PSP94 has been shown to be differentially expressed depending upon the stage of prostate cancer progression and has been indicated as a potential prognostic marker.[3] In previous studies using the PC3 xenograft mouse model and the Dunning rat R-3327 MLL model, we have shown that PSP94 can reduce prostate cancer growth *in vivo*,[4] and that the amino acid 31–45 region of PSP94 (PCK3145) was sufficient to elicit PSP94-mediated antitumor effects.[5] More recently, the final phase IIa clinical trial results with PCK3145 confirmed its therapeutic safety and tolerability for metastatic hormone-refractory prostate cancer (HRPC).[6] This effect was in part correlated to a marked reduction in the high levels of plasma MMP-9 (values above 100 μg/ml), a gelatinase B enzyme involved in extracellular matrix (ECM) degradation and tumor invasion, of patients receiving PCK3145, suggesting a biological effect possibly related to control metastasis. Finally, further clinical evidence confirmed the pharmacokinetics, safety, tolerability and efficacy of PCK3145 in metastatic HRPC patients.[7] The exact molecular mechanisms of action of PCK3145 against prostate cancer tumor growth and metastasis, and tumor-associated angiogenesis, however, remain poorly understood.

The growth and spread of prostate cancer tumors are dependent on the formation of adequate vasculature, *i.e.*, angiogenesis regulated by angiogenic factors.[8–10] These factors stimulate endothelial cells (EC) through their receptors and the stimulated EC acquire specific characteristics to constitute new vessels. The major proangiogenic factors include vascular endothelial growth factor (VEGF), interleukin-8, fibroblast growth factor 2, epidermal growth factor and platelet-derived growth factor (PDGF). Tumor angiogenesis occurs through a series of steps, including proteolytic degradation of the basement membrane and surrounding extracellular matrix, EC migration toward angiogenic stimuli, EC proliferation to provide the necessary number of cells for making a new vessel and structural reorganization into a three-dimensionally tubular structure.[11,12] Several studies have revealed that increasing tumor-associated intratumoral microvessel density correlates with greater tumor aggressiveness, such as a higher frequency of metastases and decreased srvival in prostate cancer and other solid tumors.[13–15]

VEGF is one of the most potent angiogenic factors affecting EC proliferation, motility and vascular permeability. VEGF binds with high-affinity to the tyrosine kinase receptors *Flt-1* (VEGFR-1) and *Flk-1*/*KDR* (VEGFR-2) expressed by EC.[16] VEGF expression by prostate cancer specimens,[17] and LNCaP, PC-3 and DU 145 prostate cancer cell lines is far greater than that by stromal cells of the normal prostate.[18–20] These observations suggest that VEGF plays a role on tumor cell activation (autocrine regulation), in addition to paracrine actions, whereby it regulates EC functions and subsequent neovascular development.[17] Upon VEGF-mediated activation, VEGFR-2 undergoes dimerization and ligand-dependent phosphorylation, subsequently inducing the phosphorylation-mediated activation of several intracellular pathways, including Src, PI3K and Raf/MEK/ERK.[21] VEGFR-2 is considered to be the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF, and hence is a major target for antiangiogenic therapies. In addition, the levels of the VEGF receptor are correlated with a poorer grade of tumor differentiation and prognosis in prostate cancer.[22]

Because prostate cancer tumor-associated angiogenesis is a complex multifactorial process, one key molecular antiangiogenic strategy in the development of angiogenesis modulators is target-

Abbreviations: bFGF, basic fibroblast growth factor; EC, endothelial cell; ECM, extracellular matrix; ERKs, extracellular-signal-regulated protein kinases; HRPC, hormone-refractory prostate cancer; HUVEC, human umbilical vein EC; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; S1P, sphingosine-1-phosphate; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor-2.

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ing the transduction of the signal triggered by VEGF. Since the small PSP94-derived PCK3145 peptide was able to reduce tumor volume and vascularization *in vivo*, we sought to investigate the molecular basis involved in its potential to antagonize tumor-associated angiogenesis. In the present study, we specifically assessed the ability of PCK3145 to antagonize the VEGF-mediated intracellular signalling axis in EC.

Material and methods

Materials

PCK3145 was a gift from Procyon BioPharma, Inc. (Montreal, QC). Cell culture media were obtained from Life Technologies (Burlington, ON) and serum was purchased from Hyclone Laboratories (Logan, UT). Matrigel basement membrane matrix was from Becton Dickinson Labware (Bedford, MA). PTK787 was obtained from Novartis Pharmaceuticals (Basel, Switzerland). The MEK kinase inhibitor PD98059 was from Calbiochem (La Jolla, CA). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The anti-VEGFR-2, -PDGFR-β and -ERK pAb, and the anti-phospho-tyrosine PY99 mAb were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-ERK pAb was from Cell Signaling Technology (Beverly, MA). Anti-mouse and anti-rabbit horseradish peroxidase-linked secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and enhanced chemiluminescence (ECL) reagents were from PerkinElmer Life Sciences (Boston, MA). Human recombinant PDGF was obtained from R&D Systems (Minneapolis, MN). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). All other reagents were from Sigma-Aldrich Canada.

VEGF production

Vascular endothelial growth factor (VEGF, isoform 165) was PCR-amplified from a pBlast/VEGF plasmid (Invivogen, San Diego, CA) and cloned into the pTT vector.[23] VEGF was produced following large-scale transient tranfection of human 293SFE cells in serum-free medium, as described previously.[24] The recombinant protein was expressed by the transiently transfected cells and secreted into the medium. The culture medium was harvested 5 days after transfection, and clarified by centrifugation at 3,500*g* for 10 min and filtered through a 0.22-μm membrane. Clarified culture medium was loaded onto a Heparin–Sepharose column and the bound VEGF was then eluted using a NaCl gradient in phosphate-buffered saline (PBS) pH 7.4. A buffer exchange for PBS was performed by gel filtration and the final purified material was sterile-filtered, and stored in aliquots at −80°C.

Animal protocols

The Dunning R3327 Mat Ly Lu rat prostate cancer cells were maintained in culture. At 70% confluence, cells growing in serum-containing medium were washed with Hanks buffer, trypsinized and collected by centrifugation at 1,500*g* for 5 min. Cell pellets (5×10^5 cells) were resuspended in 100 μl saline and injected using 1-ml insulin syringes into the right flank of male Copenhagen rats, as previously described.[4,5] Starting on the day of tumor cell inoculation, experimental animals were treated with 100 μg/kg/day of PCK3145 *via* s.c. injections for 15 consecutive days. Control animals received PBS alone as vehicle control. Both control and experimental animals were killed on day 16 posttumor cell inoculation, and their tumors removed and used for histological analysis.

Histologic analysis

Paraffin embedded tumor samples were cut into 5-μm thick sections for immunohistochemical analysis. Immunohistochemical staining was performed as previously described, using the avidin-biotin-peroxidase complex method.[4,25] Briefly, the sections were dewaxed in xylene, and rehydrated through a series of ethanol to water gradients. The sections were incubated in 1% normal goat or rabbit serum (Vector Laboratories Inc., Burlingame, CA) for 30 min at room temperature before treatment with goat polyclonal antibody against CD31 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Biotinylated rabbit anti-goat IgG (Vector Laboratories Inc., Burlingame, CA) was used as the secondary antibody at 1:200 for 30 min at room temperature. The slides were treated with Vectastain ABC-AP kit (Vector Laboratories Inc., Burlingame, CA) for 30 min at room temperature, and subsequently developed with Fast Red TR/Naphthol AS-MX phosphate (Sigma-Aldrich, Oakville, ON) containing 1 mM levamisole for 10–15 min. The slides were then counterstained with hematoxylin (Fisher Scientific Ltd, Nepean, ON) and mounted with Kaiser's glycerol jelly. All sections were washed 3 times for 10 min each with Tris buffer (pH 7.6) after each step. For negative control sections, the primary antibody was omitted.

Computer-assisted image analysis

To quantify immunostaining, a computer-assisted image analysis system was used.[25] Briefly, images of stained sections were photographed with a Leica digital camera and processed using BioQuant image analysis software, version 6.50.10 (BioQuant Image Analysis Corporation, Nashville, TN). The threshold was set by determining the positive staining of control sections and was used to automatically analyze all recorded images of all samples that were stained in the same session under identical conditions. The area of immunohistochemical stained regions was calculated automatically by the software in each microscopic field. Pixel counts of the immunoreaction product were calculated automatically and were given as total density of the integrated immunostaining over a given area. This reflects the relative amount of the proteins detected by the antibodies.

Cell culture

Human umbilical vein endothelial cells (HUVEC) and pulmonary aortic smooth muscle cells (PASMC) were obtained from Clonetics and maintained in EC basal medium-2 (EBM-2; Clonetics) and smooth muscle medium-2 (SmGM-2; Clonetics), respectively. For experimental purposes, cells were plated in 100-mm plastic dishes at 5,000 cells/cm^2 and were grown to confluence before overnight serum starvation without supplements. Cells were treated with vehicle or with PCK3145 diluted in PBS and stimulated with 100 ng/ml VEGF, 10 ng/ml bFGF (basic fibroblast growth factor), 1 μM S1P (sphingosine-1-phosphate) or with 50 ng/ml PDGF. After treatment, cells were washed once with PBS/1 mM NaF/1 mM Na_3VO_4, and were incubated in the same medium for 1 h at 4°C. The cells were solubilized on ice in lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5% (v/v) Nonidet P-40, 1% (v/v) Triton X-100) containing NaF/Na_3VO_4. The cells were then scraped from the culture dishes and the resulting lysates clarified by centrifugation at 10,000*g* for 10 min.

Immunoprecipitation and immunoblotting procedures

Lysates were clarified by a 1 hr incubation at 4°C with a mixture of Protein A/Protein G Sepharose beads. After removal of the Sepharose beads by low-speed centrifugation, 200 μg of protein from each sample were transferred to fresh tubes and incubated in lysis buffer overnight at 4°C in the presence of 1 μg/ml of specific antibodies. Immunocomplexes were collected by incubating the mixture with 25 μl (50% suspension) of Protein A- (rabbit primary antibody) or Protein G- (mouse primary antibody) Sepharose beads, for 2 hr. Non-specific bound proteins were removed by washing the beads 3 times with lysis buffer and once with PBS containing 1 mM Na_3VO_4. The bound material was solubilized in 25 μl of 2-fold concentrated Laemmli sample buffer [125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 10% β-mercaptoethanol and 0.00125% bromphenol blue], boiled 4 min and resolved on 7.5% gels by SDS-PAGE. The proteins were transferred onto polyvinylidene difluoride membranes, blocked overnight at 4°C in

Tris-buffered saline/Tween 20 (147 mM NaCl, 20 mM Tris/HCl, pH 7.5 and 0.1% Tween 20) containing 2% (w/v) bovine serum albumin and probed with primary antibodies for 2 hr at room temperature. Immunoreactive bands were revealed after a 1 hr incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies, and the signals were visualized by ECL. The immunoreactive bands were quantified by scanning densitometry (Molecular Dynamics).

Zymography

Prior to stimulation, quiescent HUVEC were serum-starved for 16 hr in the presence or absence of PCK3145 or PD98059 and then stimulated with VEGF. The conditioned media were collected 24 hr after stimulation, and clarified by centrifugation. Identical volume of conditioned media were mixed with non reducing Laemmli sample buffer and subjected to 7.5% SDS-polyacrylamide gels containing 1 mg/ml gelatin. The gels were then incubated for 30 min at room temperature twice in 2.5% (v/v) Triton X-100 and rinsed 5 times in doubly distilled water. The gels were incubated at 37°C for a further 18 hr in 200 mM NaCl/5 mM $CaCl_2$/0.02% (v/v) Brij-35/50 mM Tris/HCl buffer (pH 7.6), then stained with 0.1% Coomassie Brilliant Blue R-250, followed by destaining in 10% (v/v) acetic acid/30% (v/v) methanol. Gelatinolytic activity was detected as unstained bands on a blue background.

Migration Assays

Transwells filters (8-μm pore size; Costar, Cambridge, MA) were precoated with 0.15% gelatin/PBS for 24 hr at 4°C. The transwells were then washed with PBS and assembled in 24-well plates. Cells were dislodged from the flasks by trypsinization, washed and resuspended in serum-free media at a concentration of 10^6 cells/ml. HUVEC (10^5 cells) were seeded onto gelatin-coated filters inserted in chambers and incubated at 37°C, 5% CO_2/95% air for 30 min to allow adequate cell anchoring to the filters. The monolayers were then exposed to serum-free media containing PCK3145 added within the upper and lower compartment of the chambers. After 2 hr, VEGF (50 ng/ml) was added in the lower chamber as a chemoattractant, and migration allowed for another 3 hr. Cells that had migrated to the lower surface of the filters were fixed with 10% formalin phosphate and stained with 0.1% Crystal Violet/20% (v/v) methanol. The migration was quantified using computer-assisted imaging, and data are expressed as the average density of migrated cells per 4 fields (magnification 50×).

Matrigel endothelial cell tube formation assay

Matrigel (12.5 mg/ml) was thawed at 4°C, and 50 μl were quickly added to each well of a 96-well plate and allowed to solidify for 10 min at 37°C. The wells were then incubated for 30 min at 37°C in 5% CO_2/95% air with 100 μl of HUVEC (20,000 cells/well) containing 1% fetal bovine serum to allow adequate adhesion to Matrigel. Cells were then treated for 18 hr by adding 100 μl of 2-fold concentrated PCK3145 prepared in serum-free medium, into the well. The formation of capillary-like structures was examined microscopically and pictures (50×) taken with a Retiga 1300 camera coupled to a Zeiss Axiovert S100 microscope. The extent of capillary-like structure formation was quantified by analysis of digitized images using a commercially available image analysis software (Northern Eclipse).[26]

Statistical data analysis

Data are representative of 3 or more independent experiments and are represented as means ± SEM. Statistical comparisons between groups were assessed using 1-way ANOVA followed by Student's unpaired *t*-test.

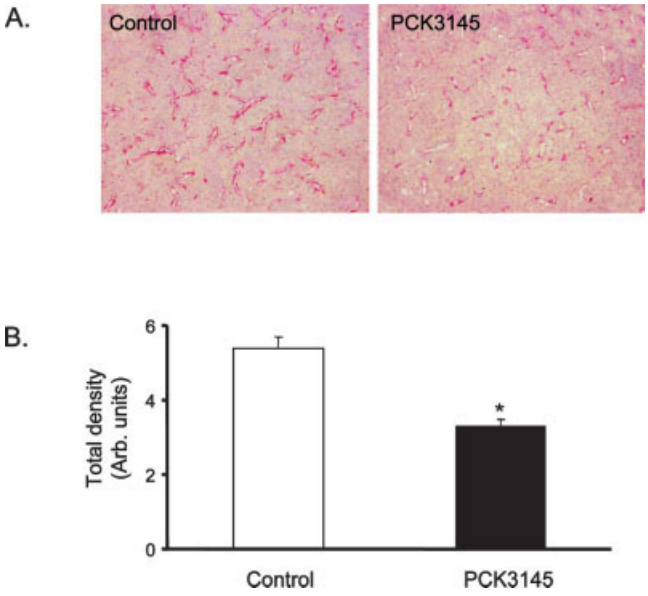

FIGURE 1 – PCK3145 inhibits tumor-associated vascularization. (*a*) The expression of CD31, as a marker of tumor vessel density, was determined on histological sections of Mat Ly Lu tumors from animals treated with vehicle alone as control or 100 μg/kg/day of PCK3145 as described in the Methods section. (*b*) Quantitative analysis was carried out in 10 high power fields (×400) in control (white bar) and experimental (black bar) sections. Results are representative of the mean ± SEM of 6 animals in each group from 2 separate experiments. Significant difference from control is shown by asterisks ($p < 0.05$).

Results

PCK3145 inhibits tumor-associated vascularization in vivo

In our well established syngeneic *in vivo* model of rat prostate cancer, both PSP94 and PCK3145 have shown significant reduction in tumor volume.[4,5] Whether this reduction was due to decreased tumor-associated angiogenesis was first evaluated. Following treatment of tumor bearing animals with vehicle alone or PCK3145, tumors were removed and evaluated for the ability of PCK3145 to alter tumor angiogenesis. Immunohistochemical analysis of primary tumors treated with vehicle alone or PCK3145 (100 μg/kg/day) with EC specific antibody CD31 (Fig. 1*a*) showed significantly decreased (43%) microvessel density following treatments with PCK3145 (Fig. 1*b*). Whether this *in vivo* observation resulted in the inhibition of the angiogenic growth factors response of EC was next investigated in cultured EC.

PCK3145 specifically inhibits VEGF-induced ERK phosphorylation in HUVEC

VEGF is a strong activator of ERK (Extracellular-signal-Regulated protein Kinases) *via* VEGFR-2. To test the ability of PCK3145 in antagonizing VEGF-mediated ERK phosphorylation, serum-starved quiescent HUVEC were incubated with vehicle (PBS pH 7.4) or with 300 μg/ml PCK3145 for 24 hr and then stimulated with VEGF, bFGF or S1P. While all 3 growth factors were found to significantly induce ERK phosphorylation, only that from VEGF was antagonized by PCK3145 (Fig. 2*a*). The total amount of ERK in each sample of cells was unaffected by PCK3145 (Fig. 2*a*). The results, thus, demonstrate a specific inhibitory effect of PCK3145 on ERK phosphorylation induced by VEGF but not on that induced by bFGF or S1P (Fig. 2*b*). Moreover, a dose-response to PCK3145 was found to gradually inhibit the extent of ERK phosphorylation by VEGF (Fig. 2*c*). Interest-

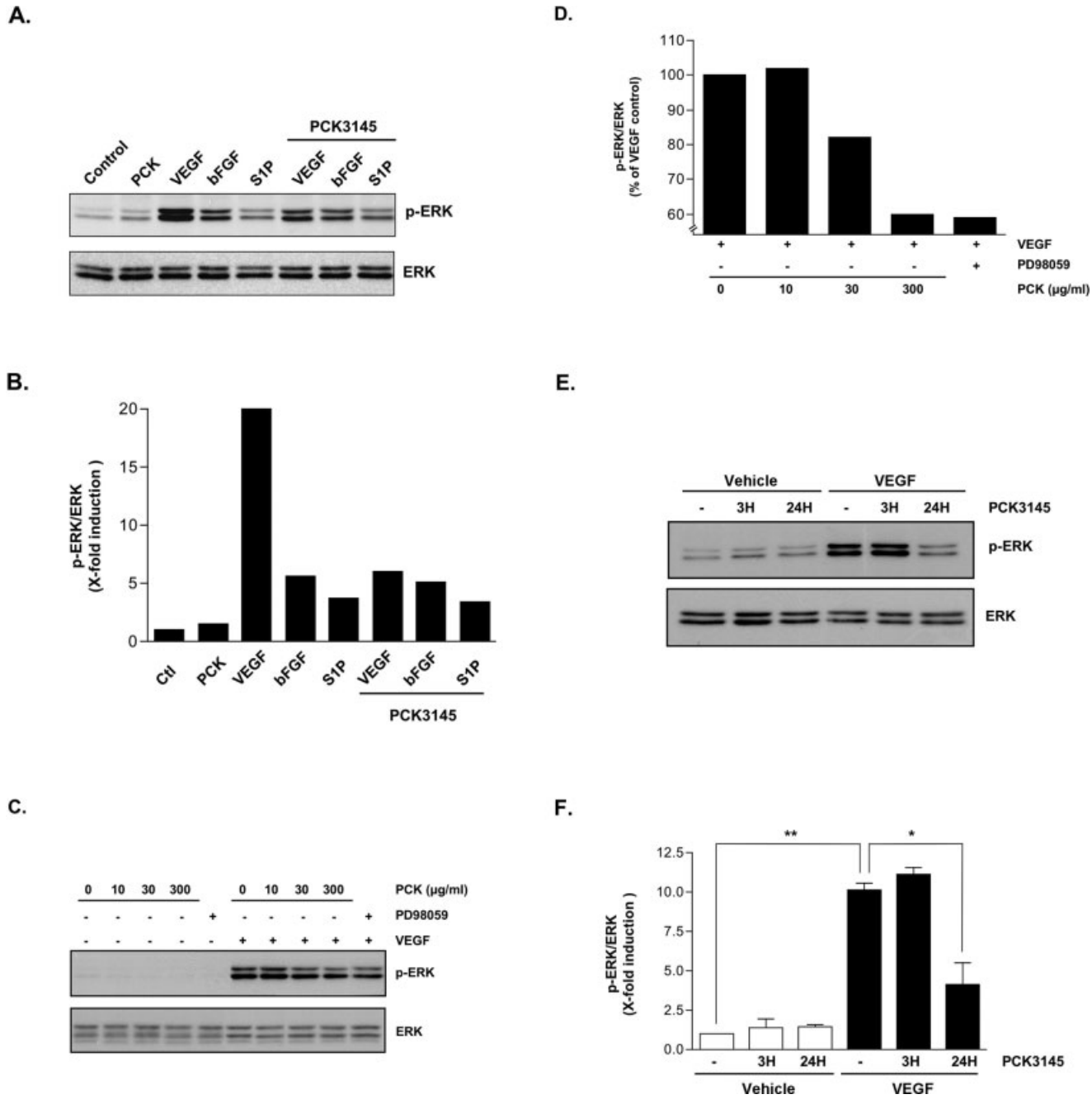

FIGURE 2 – PCK3145 specifically inhibits VEGF-induced ERK phosphorylation in HUVEC. (*a*) Serum-starved quiescent HUVEC were incubated with vehicle (PBS pH 7.4) or with 300 µg/ml PCK3145 for 24 hr and then stimulated with 100 ng/ml VEGF, 10 ng/ml bFGF or 1 µM S1P. (*c*) The effect of PCK3145 (0–300 µg/ml) or that of PD98059 (10 µM) was assessed on VEGF-induced ERK phosphorylation. (*e*) Time-course of 300 µg/ml PCK3145 inhibitory effect was assessed at 3 hr and 24 hr. At the end of all the experiments, cells were washed with PBS containing NaF/Na_3VO_4 and incubated in the same medium buffer for 1 hr at 4°C. The cells were scraped from the culture dishes and the resulting lysates clarified by centrifugation. Western blotting and immunodetection using anti-phospho-ERK and anti-ERK antibodies was then performed. The extent of ERK phosphorylation was quantified by densitometry and expressed as the ratio of p-ERK/ERK (*b*, *d*, *f*). * indicates statistically significant differences ($p < 0.05$) compared with VEGF control; ** indicates significant differences ($p < 0.01$) compared with that of the control untreated cells.

ingly, optimal inhibitory effect of PCK3145 on VEGF-induced ERK phosphorylation was found comparable to that of PD98059, an extremely potent pharmacological ERK1/2 MAPK-inhibitor (Fig. 2*d*).[27] Finally, a time-course of PCK3145 effect is shown at 3 and 24 hr (Fig. 2*e*) demonstrating the necessity of a long term action of PCK3145 with significant effect at 24 hr of cell treatment (Fig. 2*f*).

PCK3145 inhibits VEGF-induced phosphorylation of the VEGFR-2 in HUVEC

The multifunctionality of VEGF at the cellular level results from its ability to initiate a diverse, complex and integrated network of signalling pathways *via* its major receptor, VEGFR-2. Thus, we examined whether the inhibitory effect of PCK3145 on ERK phosphorylation induced by VEGF was a consequence of an

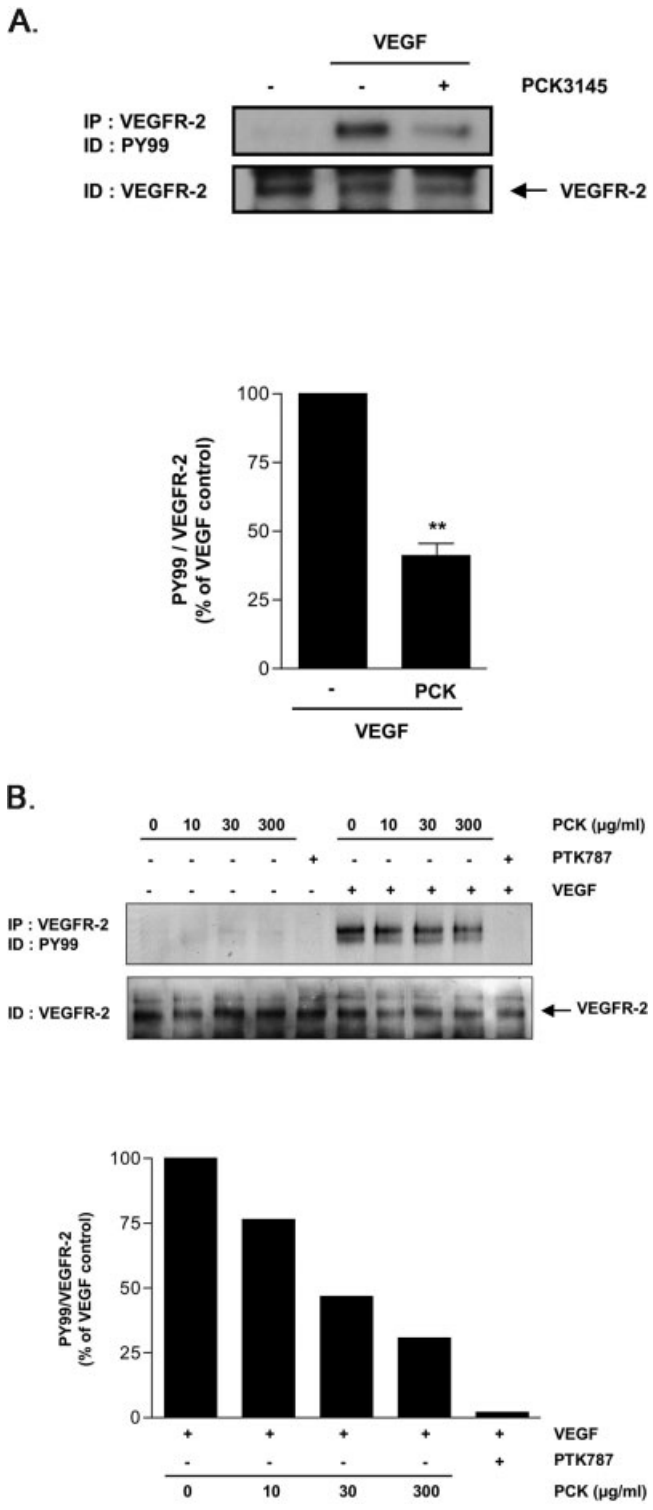

Figure 3 – PCK3145 inhibits VEGF-induced phosphorylation of VEGFR-2 in HUVEC. (*a*) HUVEC were grown, serum-starved, pretreated with PCK3145 (300 µg/ml; 24 hr) and stimulated with VEGF, as described previously by us.[53] ** indicates $p < 0.01$ versus VEGF alone. (*b*) A dose-response effect of PCK3145 was assessed in the presence or absence of 100 ng/ml VEGF, and compared to that of the effect of PTK787 (1 µM). After each treatment, equal amounts of protein were immunoprecipitated with anti-VEGFR-2 polyclonal antibodies and analyzed by Western blotting. The extent of VEGFR-2 phosphorylation was quantified by densitometry and expressed as PY99/VEGFR-2.

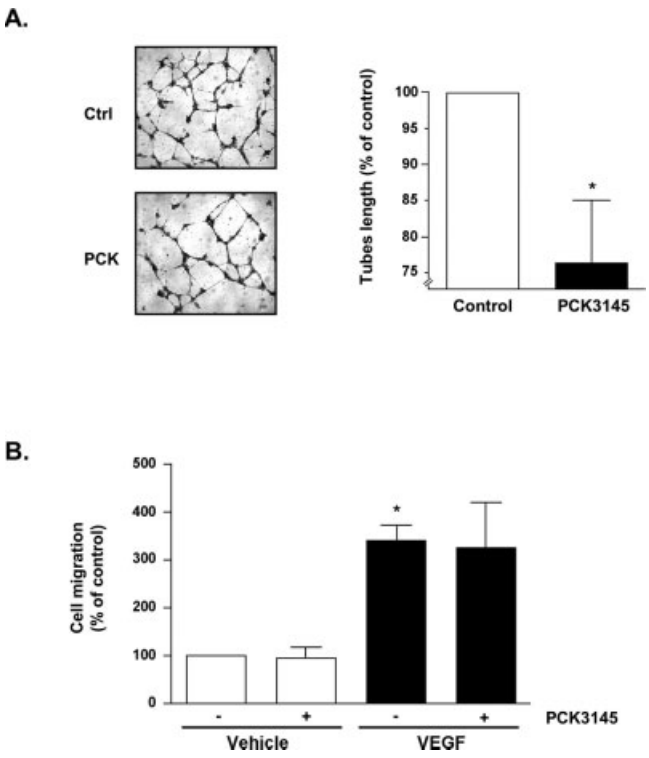

Figure 4 – PCK3145 inhibits tubulogenesis, but not VEGF-induced migration of HUVEC. (*a*) HUVEC were seeded on Matrigel and left to adhere for 30 min. PCK3145 (300 µg/ml) was then added in serum-free media, and incubated for 18 hr at 37°C, as described in the Methods section. For quantitation of tube formation, the total length of the tubes formed in a unit area was digitized and measured using the ECLIPSE software. For each test, 5 randomly chosen areas were measured and averaged. (*b*) HUVEC were seeded on gelatin-coated filters and allowed to migrate for 3 hr in the presence or not of 50 ng/ml VEGF and 300 µg/ml PCK3145. Cells that had migrated were counted, as described in the Methods section. * $p < 0.05$ versus the control untreated HUVEC.

inhibition of the tyrosine kinase autophosphorylation activity of the VEGFR-2 receptor. HUVEC were grown, serum-starved, pretreated with 300 µg/ml PCK3145 for 24 hr, and stimulated with VEGF. After each treatment, equal amounts of protein were immunoprecipitated with an anti-VEGFR-2 polyclonal antibody and analyzed by Western blotting. Our results show that PCK3145 inhibited the phosphorylation of VEGFR-2 induced by VEGF in HUVEC (Fig. 3*a*). This inhibitory effect of PCK3145 is also shown to be dose-dependent (Fig. 3*b*) and, although not as potent, be compared to the action of PTK787, a known pharmacological inhibitor of the tyrosine kinase activity associated to VEGFR-2. Finally, a 15-mer scrambled peptide was found inefficient in antagonizing VEGF-induced VEGFR-2 phosphorylation (not shown) and suggests that the primary sequence of PCK3145 is important and provides the peptide specificity of action.

PCK3145 inhibits in vitro tubulogenesis but not VEGF-induced migration in HUVEC

Matrigel assay is a two dimensional ECM model assay that provides physiologically relevant environment for studies of cell morphology, biochemical function and gene expression in EC. When plated on Matrigel, EC have the ability to form capillary-like structures. The extent of capillary-like structures formation (density and size of structures) can be quantified by analysis of digitized images to determine the relative size and area covered by the tube-like network, using an image-analysis software, as described in the Methods section. HUVEC were trypsinized,

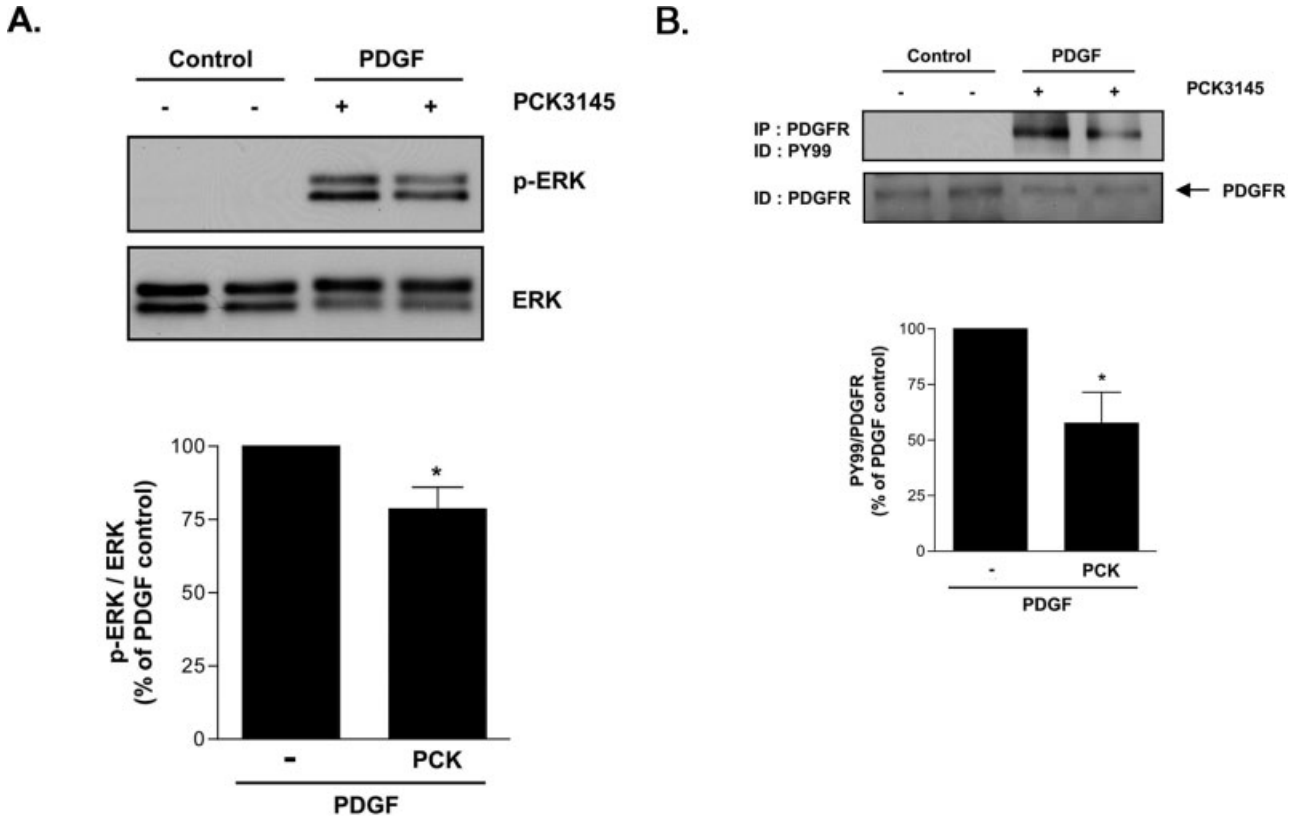

FIGURE 5 – PCK3145 inhibits PDGF-induced phosphorylation of ERK and of VEGFR-2 in PASMC. Serum-starved PASMC were incubated with PBS or with 300 μg/ml PCK3145 for 24 hr and then stimulated with 50 ng/ml PDGF. The effect of PCK3145 was assessed on (*a*) PDGF-induced ERK phosphorylation, and on (*b*) PDGF-induced PDGFR phosphorylation. At the end of all the experiments, cells were then washed with PBS containing NaF/Na_3VO_4 and incubated in the same medium buffer for 1 hr at 4°C. The cells were scraped from the culture dishes and the resulting lysates clarified by centrifugation. Western blotting and immunodetection using anti-phospho-ERK and anti-ERK antibodies was then performed. The extent of ERK and PDGFR phosphorylation was quantified by densitometry and expressed as the ratio of p-ERK/ERK (*a*) or PY99/PDGFR (*b*). * indicates $p < 0.05$ versus PDGF alone.

counted and seeded on Matrigel. Adhesion to Matrigel was left to proceed for 30 min. Treatment with PCK3145 (300 μg/ml) was then performed for 24 hr. The extent of capillary-like structure formation was assessed and the results show that PCK3145 inhibited tubulogenesis by 24% (Fig. 4*a*) compared to that of the vehicle-treated cells. This inhibitory effect is thought to partly involve cell cycle arrest as PCK3145-treated cells accumulated in sub-G1 stage (not shown).

Migration of EC is a key event in angiogenesis. *In vitro*, this process can be reconstituted by plating cells onto gelatin-coated filters inserted in modified Boyden chemotactic chambers. The effect of PCK3145 can be monitored by the number of cells that had migrated comparatively to untreated control cells. HUVEC were dislodged from the flasks by trypsinization, washed and resuspended in serum-free media. Cells were placed onto gelatin-coated filters inserted in chambers and incubated at 37°C, 5% CO_2 for 30 min to allow adequate anchoring to the filters. The monolayers were then exposed to serum-free media containing PCK3145 (300 μg/ml) added within the upper and lower compartment of the chambers. After 2 h, VEGF (50 ng/ml) was added in the lower chamber as a chemoattractant. Cell migration was allowed to proceed for another 3 h. Filters were then fixed, stained and the migrated cells quantified by microscopy, as described in the Methods section. The results show that PCK3145 treatment had no significant effect on basal cell migration or on VEGF-induced cell migration (Fig. 4*b*). The effect of PCK on S1P-induced HUVEC migration was also measured, but no inhibition was observed (not shown).

Effect of PCK3145 on the phosphorylation of PDGF receptors in smooth muscle cells

The potential inhibitory action of PCK3145 toward the tyrosine kinase activity associated to the VEGFR-2 prompted us to test whether it could inhibit tyrosine kinase activity associated with other cell surface receptors. We thus tested the effect of PCK3145 on the PDGF-mediated phosphorylation of the PDGF receptor (PDGFR) in PASMC (Pulmonary Aortic Smooth Muscle Cells). Serum-starved quiescent PASMC were incubated in the presence or absence of PCK3145 (300 μg/ml) for 24 hr and then stimulated with PDGF (50 ng/ml). The phosphorylation state of the PDGFR was assessed by immunoprecipitation of the receptor followed by phosphotyrosine immunodetection. Similarly to its effect on VEGF-mediated events in HUVEC, PCK3145 lead to the inhibition of the PDGF-induced ERK phosphorylation (Fig. 5*a*), as well as of the PDGFR phosphorylation induced by PDGF (Fig. 5*b*). This suggests that PCK3145 may potentially inhibit tyrosine kinase activities associated with alternate cell surface receptors.

PCK3145 effect on VEGF-induced proMMP-2 secretion

Matrix metalloproteinases (MMPs) secreted by EC are hypothesized to play a key role in the processes of matrix remodeling and EC sprouting during angiogenesis.[28,29] While proMMP-9 secretion is absent or at low levels in basal conditions, proMMP-2 secretion can, however, be increased by VEGF in HUVEC.[30] We have thus decided to assess the effect of PCK3145 on MMP extracellular levels by gelatin-zymography of the conditioned media of serum-starved HUVEC. After 16 hr of starvation,

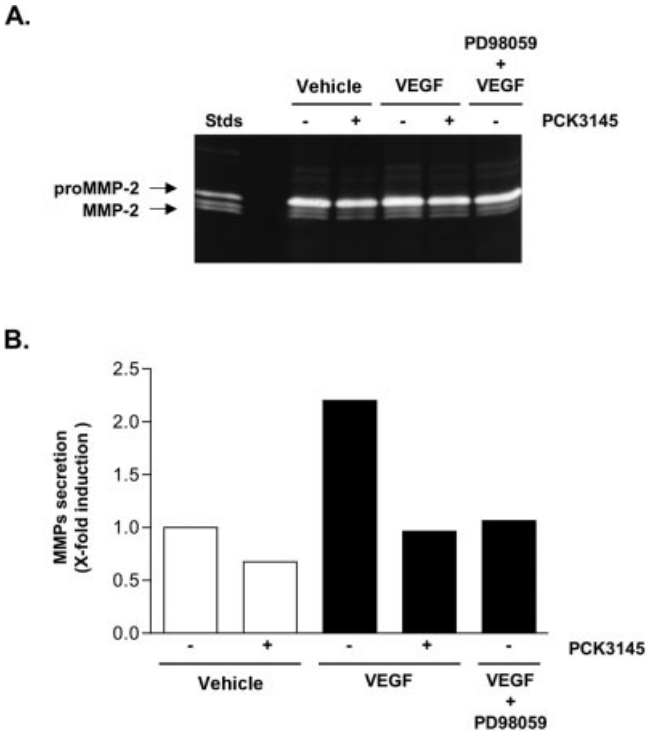

FIGURE 6 – PCK3145 inhibits VEGF-induced proMMP-2 secretion. (*a*) HUVEC were serum-starved and conditioned media isolated from control or VEGF-treated cells in the presence or not of 300 μg/ml PCK3145 or 10 μM PD98059. Zymography (15 μl/well) was performed by SDS-PAGE in gels containing gelatin, as described in the Methods section. Conditioned media from serum-starved HT-1080 fibrosarcoma cells was used as MMP standard (Stds). ProMMP-2 gelatinolytic activity was observed at ~72 kDa and that of active MMP-2 detected at 62 kDa. (*b*) Quantification of the extent of gelatin hydrolysis was performed by densitometry.

HUVEC were stimulated with VEGF in the absence of PCK3145. A subsequent 24 hr treatment shows that PCK3145 effectively downregulated by ~35% the basal proMMP-2 levels in the extracellular media (Fig. 6*a*). Most importantly, PCK3145 effect was also observed on VEGF-induced proMMP-2 secretion as the inhibition was of ~50%. When these experiments were performed in serum-free media, but in the presence of the MAPK inhibitor PD98059, VEGF-induced proMMP-2 extracellular levels were also significantly decreased (Fig. 6*b*). The effect of PCK3145 on MMP-9 secretion could not be assessed in HUVEC because only very low to undetectable levels of MMP-9 are secreted, as seen from Figure 6*a*. These results suggest that the effect of PCK3145 toward MMP secretion is indeed regulated through a MAPK pathway in EC.

Discussion

A major problem of therapy for advanced prostate cancer is disease that has progressed to a hormone resistant state (Hormone Refractory Prostate Cancer, HRPC). Therefore, there has been much interest in the therapeutic potential of inhibiting the effects of growth factors involved in prostate cancer progression.[31,32] As tumor-associated angiogenesis is a growth factors-mediated event and is a crucial step in the process of prostate cancer development, the currently observed *in vivo* inhibition of tumor neovascularization and destruction of tumor vasculature by PCK3145 (antiangiogenic therapy) may provide an additional novel prostate cancer treatment suitable for combination with standard therapies. Thus, therapeutic approaches targeting the receptors of proangiogenic molecules such as VEGF and its signal transduction cascade may result in small avascular prostate tumors and may potentiate their shrinkage.

Investigations into the molecular basis of tumor vascularization have demonstrated that tumors express a number of autocrine and paracrine factors that activate or otherwise facilitate angiogenesis. The proangiogenic factor VEGF is secreted by many tumors in high concentrations, and suppression of the VEGF-VEGFR signaling pathway is an intensively explored avenue for suppression of tumor growth.[33] Although prostate cells of normal, benign and of malignant phenotype have been shown to express VEGF, expression of the cognate receptors VEGFR-2 is generally believed to be restricted to EC. While the mechanism of VEGFR signal transduction is complex, it is thought that VEGFR-2 signals through both the MAPK cascade and the PI3K.[34,35] In light of our results, 2 main lines of evidence suggest and support the pleiotropic molecular effects of PCK3145 in EC. It antagonizes the VEGFR-2 tyrosine kinase-associated activity as well as the subsequent intracellular transduction through the MAPK pathway. Moreover, PCK3145 inhibited capillary-like structure formation by EC as well as MMP secretion, 2 cellular prerequisite for angiogenesis to occur. This inhibitory effect on MMP is interesting because there is a general correlation between the stage of tumor progression and level of MMP expression.[36] Moreover, a positive correlation was found between MMP-2 expression and Gleason score in prostate cancer patients.[37] Collectively, these properties reflect PCK3145 potential antiangiogenic action on EC. Whether PCK3145 effects occur directly on the VEGFR-2 receptor or are transduced through a yet to be identified receptor is under investigation.

In prostate cancer, as in other cancers, tumor associated angiogenesis is a crucial step in the process of tumor growth, invasion and metastasis.[38] Previous studies on prostate cancers have demonstrated a correlation between microvessel density, pathological stage and Gleason score.[39–41] Successful treatment of established human tumors might require not only prevention of further angiogenesis but also destruction of tumor blood vessels to reduce the already existing tumor mass.[42] Although interference with VEGF-mediated signalling events is effective in preventing the early growth of neovessels, mature vessels from more established tumors are largely resistant to inhibitors directed against either VEGF or its receptor VEGFR-2.[43] These mature vessels are surrounded by periendothelial cells, such as pericytes and smooth muscle cells (SMC), and the contact between these cells stabilizes new blood vessels, promotes endothelial survival and inhibits EC proliferation.[44,45] PDGF-B/PDGFR-β system is involved in vessel stabilization, and the interference with this signalling system resulting in disruption of already established endothelial/periendothelial associations and vessel destabilization.[46] Furthermore, the inhibition of both VEGF and PDGF receptors, by either simultaneous exposure to receptor-specific receptor tyrosine kinase inhibitors or by an inhibitor with broad kinase specificity (SU6668), blocks further growth of end-stage and well-vascularized tumors, eliciting detachment of pericytes and disruption of tumor vascularity.[43,47] Therefore, it is tempting to suggest that PCK3145 be used as a therapeutic agent in strategies devised either to interrupt or inhibit one or more of the pathogenic steps involved in the process of prostate tumor neovascularization or to directly target and destroy the prostate tumor vasculature. Our results show that PCK3145 may indeed possess the intrinsic capacity to interfere with the tyrosine kinase activity associated with either VEGF signalling axis in EC or PDGFR-associated tyrosine kinase activity in SMC. The inhibition of both receptors function by PCK3145 may still confer an intrinsic advantage to the use of this peptide to inhibit angiogenesis. In light of this, it will be extremely interesting to further investigate this inhibitory action on diverse tyrosine kinase activities, which will lead us to develop PCK3145 in alternate antiangiogenic therapeutic approaches. As such, the tyrosine kinase activity of VEGFR-1 may also potentially be considered as another alternate target for PCK3145 since a selective induction of MMP-9 was found to regulate lung metastasis by lung EC,[48] and that specific blockade of the

ERK pathway inhibits the invasiveness of tumor cells through MMP-9 downregulation.[48] In light of the recent clinical results demonstrating that MMP-9 levels were decreased in prostate cancer patients treated with PCK3145, it becomes tempting to suggest that PCK3145 may actually downregulate MMP-9 levels through its inhibitory action of ERK pathway.

Current cancer treatment strategies rely heavily on cytotoxic therapies, which have limited efficacy in terms of prolonging life and are associated with significant toxicity.[49,50] As a result, there is a demand for new therapies that more specifically target the cellular events involved in the development of malignancy and in normal host processes, such as angiogenesis, required for tumor progression.[51] In this context, we show that PCK3145 inhibits transduction of signals from the cell surface that may regulate prostate tumor cell growth, and survival and which may become subverted during the multistep processes of carcinogenesis and tumor progression. These signal transduction pathways depend on a vast array of proteins, many of which possess tyrosine kinase activity and which may become particularly attractive targets for PCK3145. These protein tyrosine kinases could either be transmembrane receptor tyrosine kinases or nonreceptor tyrosine kinases. Identification of these PCK3145 targeted proteins will lead to an understanding of their regulation at a molecular level.

In conclusion, a number of small molecule tyrosine kinase inhibitors, which show promise as a new generation of cancer therapies, are progressing through clinical development.[52] Similarly, PCK3145, which is derived from prostate secretory protein 94, may thus be viewed as not only a potential inhibitor of prostate tumor-associated angiogenesis, but also as an inhibitor of angiogenesis in highly vascularized tumors. We believe that these actions of PCK3145 are triggered through specific intracellular transduction pathways involving ERK, and whose subsequent mechanisms of action are to regulate cellular processes such as EC tubulogenesis, VEGFR-2-mediated signalling and secretion of MMP.

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