

A PSP94-derived peptide PCK3145 inhibits MMP-9 secretion and triggers CD44 cell surface shedding: Implication in tumor metastasis

Borhane Annabi^{1,*}, Mounia Bouzeghrane^{2,*}, Jean-Christophe Currie¹, Robert Hawkins³,
Hélène Dulude⁴, Luc Daigneault⁴, Marcia Ruiz⁴, Jan Wisniewski⁴, Seema Garde⁴,
Shafaat A. Rabbani⁵, Chandra Panchal⁴, Jinzi J. Wu⁴ & Richard Béliveau²

¹Laboratoire d'Oncologie Moléculaire, Département de Chimie-Biochimie, Université du Québec à Montréal, Montréal, Québec, Canada; ²Centre de Cancérologie Charles-Bruneau, Hôpital Sainte-Justine, Université du Québec à Montréal, Montréal, Québec, Canada; ³Christie Hospital NHS Trust, Manchester, UK; ⁴Procyon BioPharma, Inc., Montreal, Québec, Canada; ⁵Department of Medicine, Physiology, and Oncology, McGill University Health Centre, Montreal, Québec, Canada

Received 8 July 2005; accepted in revised form 1 September 2005

Key words: CD44, metastasis, MMP-9, MT1-MMP, prostate cancer, RhoA

Abstract

Purpose: PCK3145 is a synthetic peptide corresponding to amino acids 31–45 of prostate secretory protein 94, which can reduce experimental skeletal metastases and prostate tumor growth *in vivo*. Part of its biological action involves the reduction of circulating plasma matrix metalloproteinase (MMP)-9, a crucial mediator in extracellular matrix (ECM) degradation during tumor metastasis and cancer cell invasion. The antimetastatic mechanism of action of PCK3145 is however, not understood. **Experimental design:** HT-1080 fibrosarcoma cells were treated with PCK3145, and cell lysates used for immunoblot analysis of small GTPase RhoA and membrane type (MT)1-MMP protein expression. Conditioned media was used to monitor soluble MMP-9 gelatinolytic activity by zymography and protein expression by immunoblotting. RT-PCR was used to assess RhoA, MT1-MMP, MMP-9, RECK, and CD44 gene expression. Flow cytometry was used to monitor cell surface expression of CD44 and of membrane-bound MMP-9. Cell adhesion was performed on different purified ECM proteins, while cell migration was specifically performed on hyaluronic acid (HA). **Results:** We found that PCK3145 inhibited HT-1080 cell adhesion onto HA, laminin-1, and type-I collagen suggesting the common implication of the cell surface receptor CD44. In fact, PCK3145 triggered the shedding of CD44 from the cell surface into the conditioned media. PCK3145 also inhibited MMP-9 secretion and binding to the cell surface. This effect was correlated to increased RhoA and MT1-MMP gene and protein expression. **Conclusions:** Our data suggest that PCK3145 may antagonize tumor cell metastatic processes by inhibiting both MMP-9 secretion and its potential binding to its cell surface docking receptor CD44. Such mechanism may involve RhoA signaling and increase in MT1-MMP-mediated CD44 shedding. Together with its beneficial effects in clinical trials, this is the first demonstration of PCK3145 acting as a MMP secretion inhibitor.

Abbreviations: ECM – extracellular matrix; HRPC – hormone-refractory prostate cancer; HA – hyaluronan, hyaluronic acid; MMP-9 – matrix metalloproteinase-9; MT1-MMP – membrane type-1 MMP; PMA – phorbol 12-myristate 13-acetate; RECK – reversion inducing cysteine-rich protein with Kazal motifs; TGF- β – transforming growth factor- β ; TNF – tumor necrosis factor

Introduction

Prostate cancer is the most frequently diagnosed malignancy and the second leading cause of cancer-related

deaths in American males [1]. Androgen ablation as initial therapy for advanced prostate cancer provides high response rates but does not cure the disease, as nearly all men with metastases will eventually progress to hormone refractory prostate cancer (HRPC). It thus becomes crucial to develop new strategies to circumvent the progression of prostate cancer from localized growth to the invasion of surrounding tissues, and the development of distant bone and visceral organ metastasis. Prostate secretory protein 94 (PSP94), also known as prostatic

*These authors contributed equally to this work.

Correspondence to: Dr Richard Béliveau, PhD, Laboratoire de Médecine Moléculaire, Université du Québec à Montréal, C.P. 8888, Succ. Centre-ville, Montreal, Quebec, Canada, H3C 3P8. Tel: +1-514-987-3000, ext. 8551; Fax: +1-514-987-0246; E-mail: oncomol@nobel.si.uqam.ca

inhibin or β -microseminoprotein [2], is one of the three predominant naturally occurring proteins secreted by the prostate gland along with prostate-specific antigen and prostatic acid phosphatase [3]. In previous studies using the Dunning rat R-3327 MLL xenograft model, we have shown that PSP94 can reduce experimental skeletal metastases and prostate cancer growth *in vivo* [4], and that the amino acid 31–45 region of PSP94 (PCK3145) was sufficient to elicit PSP94-mediated anti-tumor effects [5]. More recently, a phase IIa clinical trial indicated that PCK3145 down-regulated the levels of plasma matrix metalloproteinase (MMP)-9 in patients with HRPC that had elevated levels ($> 100 \mu\text{g/l}$) at baseline, while those with levels below $100 \mu\text{g/l}$ remained low [6, 7]. Such efficacy in reducing the levels of plasma MMP-9 in patients receiving PCK3145 suggests a biological effect possibly targeting the control of tumor-related ECM degradation and metastasis processes.

Prostate cancer metastasis, especially to the bone, is a multistep process that occurs at high frequency in patients with advanced disease causing significant morbidity and mortality [8]. A key mediator of this metastatic process is the hydrolysis of the surrounding extracellular matrix (ECM) by secreted soluble MMPs [9]. Among these, MMP-9 has been associated with tumor cell invasion and metastasis and tumor-induced angiogenesis [10]. However, a major dilemma in our understanding of MMP-9 function is how the released protease is targeted to the right location at the pericellular space and how this regulates metastatic processes. Interestingly, recent functional characterization reveals that the cell surface CD44, a heavily glycosylated transmembrane protein that, as a consequence of extensive alternative splicing, exists in multiple variant forms, associates with MMP-9 in cultured murine mammary carcinoma and human melanoma cells [11, 12]. Such association of MMP-9 with CD44 has been suggested to link cell adhesion with pericellular proteolysis [11] and to promote tumor cell invasion in experimental metastasis assays [12]. CD44 cell surface function and regulation has been recently investigated by us in order to understand the highly infiltrative/metastatic phenotype of brain tumor-derived glioblastoma cells. We have shown that the caveolar localization of MT1-MMP, CD44, and RhoA at the leading edges of migrating glioma cells may initiate a functional crosstalk that would regulate the infiltrative phenotype of brain tumors [13]. Moreover, we have also provided evidence that cell surface shedding of CD44, in part, accounts for that infiltrative phenotype in U-87 glioblastoma cells [14].

In the present study, we address the possibility of PCK3145 targeting the above mentioned molecular pathways that regulate the metastatic phenotype of cancer cells. Our data provide new molecular evidence for the antimetastatic properties of PCK3145 as it triggers a RhoA/MT1-MMP-mediated CD44 cell surface shedding in HT-1080 fibrosarcoma cells. The use of such cellular model enabled us to simply ascertain the steps of the metastatic cascade that may further generate hypotheses that can be eventually evaluated in prostate

cancer. We also show that PCK3145 exhibits a dual molecular control on MMP-9 functions as it inhibits both MMP-9 secretion and subsequent MMP-9 cell surface docking. Altogether, our data suggest that PCK3145 may antagonize tumor cell invasion processes by inhibiting a CD44/MMP-9 interaction that would lead to a decrease in tumor-associated ECM degradation. The anti-cancer properties of this peptide will be highly beneficial and may potentially be exploited in targeting, not only the growth and spread of prostate cancer cells, but also in the metastasis processes of different other types of cancer.

Materials and methods

Materials

Agarose, sodium dodecylsulfate (SDS), gelatin, bovine serum albumin (BSA), phorbol-myristate acetate (PMA), tumor necrosis factor (TNF) and Triton X-100 were purchased from Sigma (Oakville, ON). TriZOL reagent was from Life Technologies (Gaithersburg, MD). FUGENE-6 transfection reagent was from Roche Diagnostics Canada (Laval, QC). Type I collagen was extracted from rat tail tendon according to classical protocols [15]. The anti-CD44 R-phycoerythrin-conjugated mouse anti-human monoclonal antibody (G44–26) and mouse IgG2bc (clone 27–35) were from BD Pharmingen (Franklin Lakes, NJ). The anti-MT1-MMP polyclonal antibody AB-815 was from Chemicon. Cell culture media was obtained from Life Technologies (Burlington, ON). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The polyclonal antibodies against RhoA and CD44 were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech (Baie d'Urfé, QC). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). All other reagents were from Sigma-Aldrich, Canada.

Cell culture and cDNA transfection method

The HT-1080 cell line was purchased from American Type Culture Collection and maintained in Dulbecco Minimum Essential Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 units/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and were cultured at 37 °C under a humidified atmosphere containing 5% CO₂. The MT1-MMP cDNA was generated and validated by us, and encoded the full length MT1-MMP protein (Met₁–Val₅₈₂) [16]. HT-1080 cells were transiently transfected with cDNA using the non-liposomal formulation FUGENE-6 transfection reagent. Transfection efficiency was confirmed by Western blotting. All experiments involving these cells were performed 36 h following transfection. Mock transfections of HT-1080 cultures

with pcDNA (3.1+) expression vector alone were used as controls.

Total RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from monolayers of cultured HT-1080 cells using the TriZOL reagent. One microgram of total RNA was used for first strand cDNA synthesis followed by specific gene product amplification with the One-Step RT-PCR Kit (Invitrogen, Burlington, ON). Primers for MMP-9 (forward: 5'-AAGATGCTGCTGTTTCAGCGGG-3', reverse: 5'-GT CCTCAGGGC-ACTGCAGGAT-3') [17], CD44s (forward: 5'-TTTG CCTCTTACAGTTGAGCCTG-3', reverse: 5'-GGTG CCATCACGGTTGACAATAG-3') [12], RECK (forward: 5'-CCTCAGTGAGCACAGTTCAGA-3', reverse: 5'-GCAGCACACACTGCTGT A-3') [18], MT1-MMP (forward: 5'-ATTGATGCTGCTCTCTTC TGG-3', reverse: 5'-GTGAAGACTTCATC GCTGCC-3') [19], and for RhoA (forward: 5'-CTGG TGATTGTT GGTGATGG-3', reverse: 5'-GCGATCATAATCTTCC TGCC-3') [20] were derived from human sequences and PCR conditions were optimized so that the gene products were at the exponential phase of the amplification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA amplification was used as an internal housekeeping gene control. PCR products were resolved on 1.5% agarose gels containing 1 µg/ml ethidium bromide.

Gelatin zymography

To assess the extent of PMA- and TNF-induced extracellular MMP-9 activity, gelatin zymography was used as described previously [21]. Briefly, an aliquot (20 µl) of the culture medium was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) in a gel containing 0.1 mg/ml gelatin. The gels were then incubated in 2.5% Triton X-100 and rinsed in ddH₂O. Gels were further incubated at 37 °C for 20 h in 20 mM NaCl, 5 mM CaCl₂, 0.02% Brij-35, 50 mM Tris-HCl buffer, pH 7.6, then stained with 0.1% Coomassie Brilliant blue R-250 and destained in 10% acetic acid, 30% methanol in H₂O. Gelatinolytic activity was detected as unstained bands on a blue background. All experiments were carried out with cells that had been serum-deprived by overnight incubation.

Immunoblotting procedures

Proteins from control and treated cells were separated by SDS-PAGE. After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked overnight at 4 °C with 5% non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris-HCl, pH 7.5) containing 0.3% Tween-20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies (1/1000 dilution) in TBST containing 3% bovine serum

albumin, followed by a 1 h incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1/10,000 dilution for MT1-MMP detection) or anti-mouse IgG (1/5000 dilution for RhoA, MMP-9 and CD44 detection) in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfée, QC).

TCA/acetone precipitation

Trichloroacetic acid (TCA)/acetone precipitation of secreted soluble MMP-9 or shed CD44 was performed as follows. Equal volumes (300 µl) of conditioned media was mixed with 20% TCA/80% ice-cold acetone and incubated at -20 °C for 1 h. Protein pellet was precipitated following centrifugation at 11,500 rpm (18,000 × g) for 15 min at 4 °C in a microcentrifuge. The supernatant was discarded, protein pellet washed with 1 ml ice-cold acetone and centrifuged as above. Acetone was driven-off by heating the dry pellet at 95 °C for 5–10 min.

Flow cytometry analysis

For assessment of cell surface CD44 and MMP-9 expression, cells were detached from plates, as previously described by us [13], and resuspended in 10% FBS/DMEM at a concentration of 10⁶ cells/ml, washed twice and blocked for 15 min at room temperature in PBS containing 5% inactivated fetal calf serum (FCS/PBS). The cells were then incubated in 0.5% FCS/PBS with 0.5 µg/ml of the CD44 mAb, MMP-9 mAb or mouse IgG2bκ at room temperature for 30 min, washed once and resuspended in 0.5% FCS/PBS. Flow cytometry data was analyzed on a FACS Calibur flow cytometer with CellQuestPro software (BD Biosciences, Mississauga, ON).

Cell migration assay

Migration/invasion of cancer cells is a key event in tumor metastasis. *In vitro*, this process can be reconstituted by plating cells onto ECM-coated filters inserted in modified Boyden chemotactic chambers. Cells were dislodged after brief trypsinization, washed extensively and resuspended in MEM at a concentration of 10⁶ cells/ml [13]. Cells (5 × 10⁴) were then dispersed onto 1 mg/ml HA/PBS-coated chemotaxis filters (Costar; 8-µm pore size) within Boyden chamber inserts. Migration proceeded for 3 h at 37 °C in 5% CO₂. Cells that had migrated to the lower surface of the filters were fixed with 10% formalin phosphate, colored with 0.1% crystal violet/20% methanol and counted by microscopic examination. The average number of migrating cells per field was assessed by counting at least four random fields per filter using Northern Eclipse software. Data points indicate the mean obtained from three separate chambers within one representative experiment. The migration was quantified using computer-assisted imaging and data are expressed

as the average density of migrated cells per four fields (magnification $\times 50$).

Cell adhesion assays

Adhesion assays were performed as previously described [22]. Briefly, adhesion wells were coated with 10 $\mu\text{g/ml}$ purified ECM protein solutions for 2 h at 37 °C, then blocked by adding a solution of PBS/BSA 0.5%. Cells were harvested as a single cell suspension by treatment with 0.53 mM EDTA in PBS pH 7.2, added to pre-coated wells and allowed to adhere to the substrata for 4 h at 37 °C. After washing, adherent cells were stained with a solution of 0.1% crystal violet/20% (v/v) methanol and lysed with 1% SDS. Spectrophotometric absorbance was then measured at 600 nm.

Statistical data analysis

Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired *t*-test and was used to compare the PCK3145 effect to vehicle treated cells. Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance in each figure.

Results

PCK3145 inhibits MMP-9 secretion from HT-1080 cells

In the recent phase IIa clinical trial in HRPC, PCK3145 was shown to reduce the levels of circulating MMP-9 plasma levels ranging from 34 to 90% in patients having elevated level of MMP-9 (above 100 $\mu\text{g/l}$) at study entry [6, 7]. In order to investigate the effect of PCK3145 on MMP-9 secretion *in vitro*, we treated serum-starved HT-1080 fibrosarcoma cells with increasing doses of PCK3145 for 24 and 48 h. Doses of PCK3145 were found not to be cytotoxic as assessed by the measurement of the pro-apoptotic caspase-3 activity (not shown). MMP-9 extracellular levels were then assessed by Western blotting and immunodetection. While MMP-9 extracellular levels continued to increase from 24 to 48 h in untreated cells, those from PCK3145-treatments decreased in a dose-dependent manner (Figure 1a). Interestingly, a 24 h treatment with PCK3145 decreased MMP-9 by 20%, while a treatment of 48 h was needed to inhibit by approximately 80% the extracellular levels of MMP-9 (Figure 1b). This result confirms those observed in the phase IIa clinical trial on the efficacy of PCK3145 to decrease MMP-9 levels up to 90%. Finally, no change in GAPDH and MMP-9 gene expression was observed upon treatment of the cells for 48 h with PCK3145 as assessed by RT-PCR (Figure 1c). Gene expression of two potential MMP-9 cell surface receptors, CD44 and RECK (reversion inducing cysteine-rich protein with

Kazal motifs), was also found not to vary upon PCK3145 treatment (Figure 1c). This suggests that PCK3145 inhibits intracellular signal transduction mechanisms that regulate MMP-9 secretion.

PCK3145 inhibits PMA- and TNF-induced MMP-9 secretion from HT-1080 cells

In order to evaluate the capacity of PCK3145 to inhibit signal transduction leading to MMP-9 secretion, we have treated HT-1080 cells with two extremely potent MMP-9 inducers in the presence of PCK3145. Gelatin zymography confirmed the inhibitory effect of PCK3145 on MMP-9 secretion (Figure 2a), and was used to monitor the effects of PCK3145 on phorbol-myristate-acetate (PMA)- and tumor necrosis factor (TNF)-induced MMP-9 secretion as described in the Methods Section. This resulted in low levels of detectable basal MMP-9 gelatinolytic activity (Figure 2b). In contrast, while PCK3145 was able to inhibit basal MMP-9 secretion (Figures 1 and 2a), we show that PMA as well as TNF induced high MMP-9 secretion (Figure 2b). Interestingly, this PMA- and cytokine-mediated effect on MMP-9 was also significantly inhibited by PCK3145 (Figure 2c). This result suggests that PCK3145 may efficiently inhibit intracellular transduction mediated by serine/threonine protein kinase (PKC, PMA being an analog of diacylglycerol) or that signaling triggered by cytokines.

PCK3145 specifically inhibits cell adhesion to laminin, type-I collagen and hyaluronic acid

ECM recognition is a crucial event in the cell adhesion process involved in tumor progression. This process is mediated and regulated through specialized cell surface receptors and integrins. Recent evidence suggests that a potential crosstalk between soluble MMP and cell surface integrins may regulate the cell's ability to recognize and adhere to its ECM environment [23]. We tested whether PCK3145 potentially downregulated HT-1080 cell adhesion. HT-1080 fibrosarcoma cells were treated with 300 $\mu\text{g/ml}$ PCK3145 for 48 h, then dislodged and seeded onto purified ECM proteins-coated dishes as described in the Methods Section. We observed that cells pre-treated with PCK3145 had their adhesion significantly diminished by 31% on type-I collagen, 23% on laminin, and by 46% on hyaluronic acid (hyaluronan, HA) (Figure 3). Cell adhesion was unaffected on the other ECM proteins tested (fibronectin, vitronectin, elastin, and fibrin) (Figure 3). These results suggest that the expression or the function of specific cell surface receptors either from the integrin or non-integrin family is regulated by PCK3145. One common cell surface receptor that may recognize all three ECM proteins, that are HA, laminin, and collagen, is CD44, which has recently been shown to also regulate prostate cell adhesion to extracellular HA [24–26].

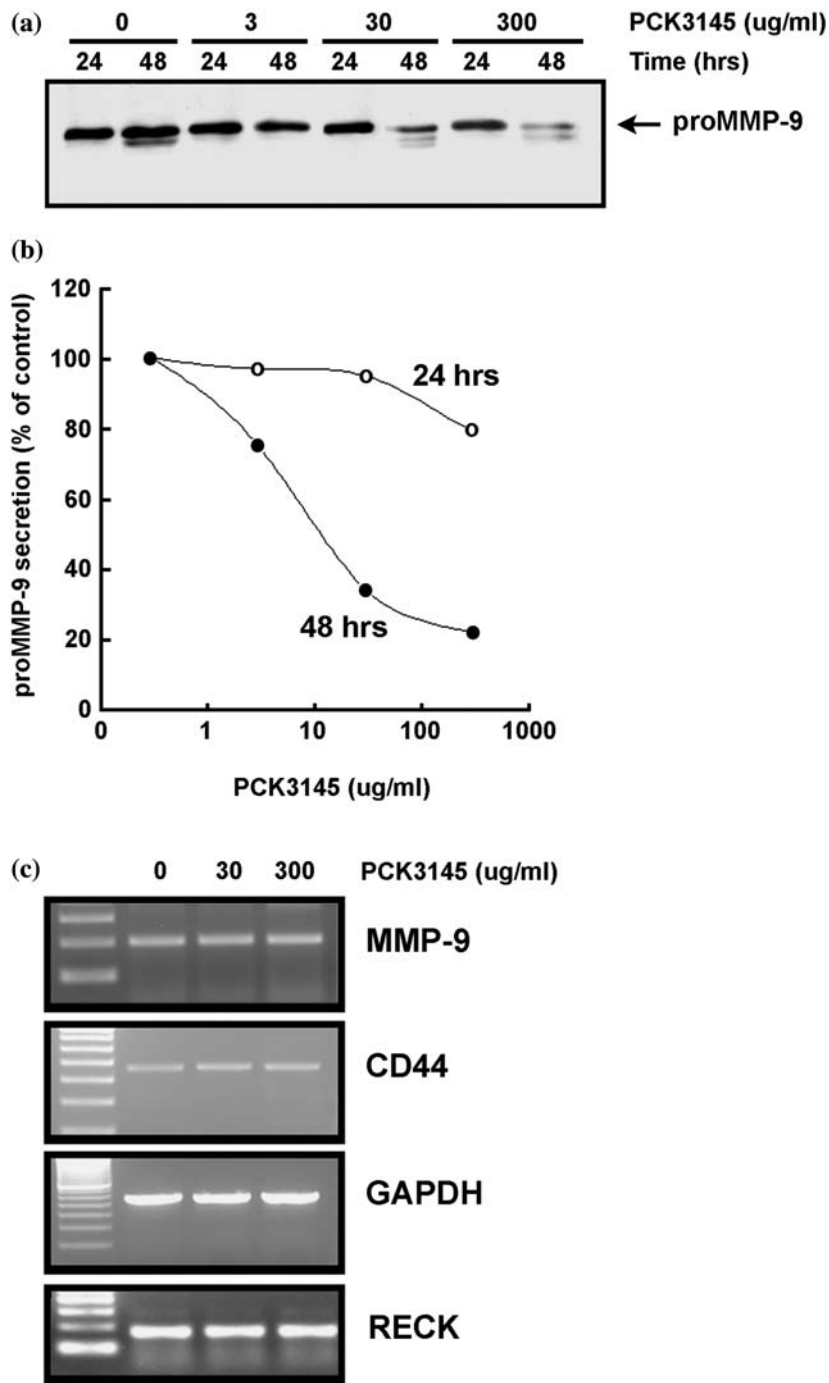


Figure 1. Extracellular MMP-9 levels are decreased in PCK3145-treated HT-1080 cells. (a) HT-1080 fibrosarcoma cells were treated with increasing PCK3145 concentrations for either 24 or 48 h in serum-free media. Equal volumes (300 μ l) of the conditioned media were TCA/acetone-precipitated and MMP-9 protein levels assessed by Western blotting and immunodetection with anti-MMP-9 antibody. (b) Quantification of the combined latent proMMP-9 and active MMP-9 immunoreactive bands was performed by scan densitometry from a representative experiment. (c) Total RNA was isolated from 48 h PCK3145-treated cells and RT-PCR performed. Amplified cDNA fragments of MMP-9, CD44, RECK, and GAPDH were run on 1.8% agarose gels containing ethidium bromide.

PCK3145 regulates the expression of CD44 functional regulators RhoA and MT1-MMP, and inhibits cell migration on hyaluronic acid

Since CD44 gene expression was not affected by PCK3145 (Figure 1c), we sought to evaluate whether CD44 functional regulation would be affected by PCK3145. Recent evidence suggests that GTPase RhoA and MT1-MMP, two of the main CD44 functional

regulators that also play an important role in number of processes related to metastasis, decreased cell migration and adhesion to HA [14]. Serum-starved HT-1080 cells were thus incubated with PCK3145 in conditions that significantly antagonize MMP-9 secretion (Figure 1a and b). Total RNA as well as cell lysates was isolated in order to specifically monitor RhoA and MT1-MMP respective gene and protein expression. We found that PCK3145-treated cells had their protein levels of RhoA

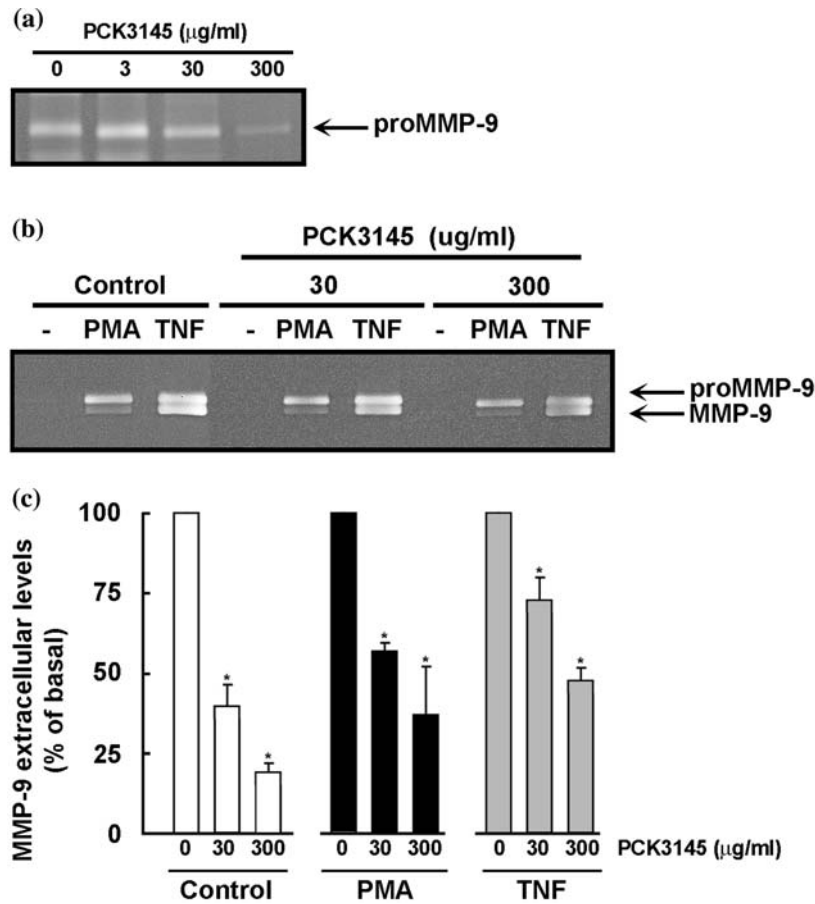


Figure 2. PCK3145 inhibits PMA- and TNF-induced MMP-9 secretion in HT-1080 cells. (a) Serum-starved HT-1080 fibrosarcoma cells were treated with increasing PCK3145 concentrations for 48 h in the absence or (b) presence of PMA (50 $\mu\text{g/ml}$) or TNF (10 $\mu\text{g/ml}$). Conditioned media was collected and 20 μl used to perform gelatin zymography as described in the Methods Section. (c) Densitometric quantification of the total extracellular levels of MMP-9 (combined latent proMMP-9 and active MMP-9) was performed from three independent experiments. Values are expressed as the percent of either untreated cells (white bars, values taken from Figure 2a), PMA-treated cells (black bars, values taken from Figure 2b), or TNF-treated cells (gray bars, values taken from Figure 2b).

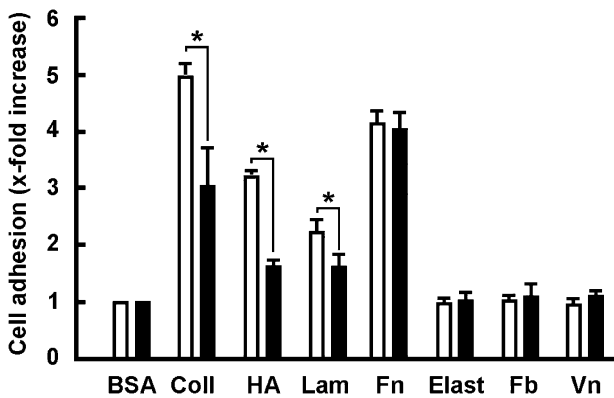


Figure 3. PCK3145 specifically inhibits HT-1080 cell adhesion to laminin, hyaluronic acid and to type-I collagen. HT-1080 fibrosarcoma cells were treated (black bars) or not (white bars) with 300 $\mu\text{g/ml}$ PCK3145 for 48 h. Cells were then trypsinized and seeded on 10 $\mu\text{g/ml}$ purified extracellular matrix proteins (BSA, bovine serum albumin; Coll, type-I collagen; Elast, elastin; Fb, fibrin; Fn, fibronectin; HA, hyaluronic acid; Lam, laminin-1; Vn, vitronectin) as described in the Methods Section. Cell adhesion was left to proceed for 4 h. Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance against the respective value of untreated cells that adhered to the specific ECM protein.

and MT1-MMP significantly increased (Figure 4a, left panel). This increase was correlated with increased gene expression when RT-PCR was performed with specific RhoA and MT1-MMP primers (Figure 4a, right panel). GAPDH protein and gene expression were used as internal control and found not to vary upon PCK3145 treatment. Densitometry quantification of both protein (black bars) and gene (gray bars) expression of RhoA and MT1-MMP is provided in Figure 4b. These results highlight the potential role of RhoA/MT1-MMP signaling axis as being critical for CD44 functions in binding HA and that would be inhibited by PCK3145. Next, we transfected cells with the MT1-MMP cDNA and subjected them to PCK3145 treatment. Conditioned media from the respective conditions was TCA/acetone-precipitated and Western blotting followed by anti-CD44 immunodetection performed. We show that cells submitted to PCK3145 treatment induced endogenous MT1-MMP expression (Figure 4c), and that transfection with MT1-MMP resulted in the appearance of a recombinant immunoreactive MT1-MMP protein (Figure 4c). Total extracellular signal-regulated kinase (ERK) protein expression is used as an internal loading

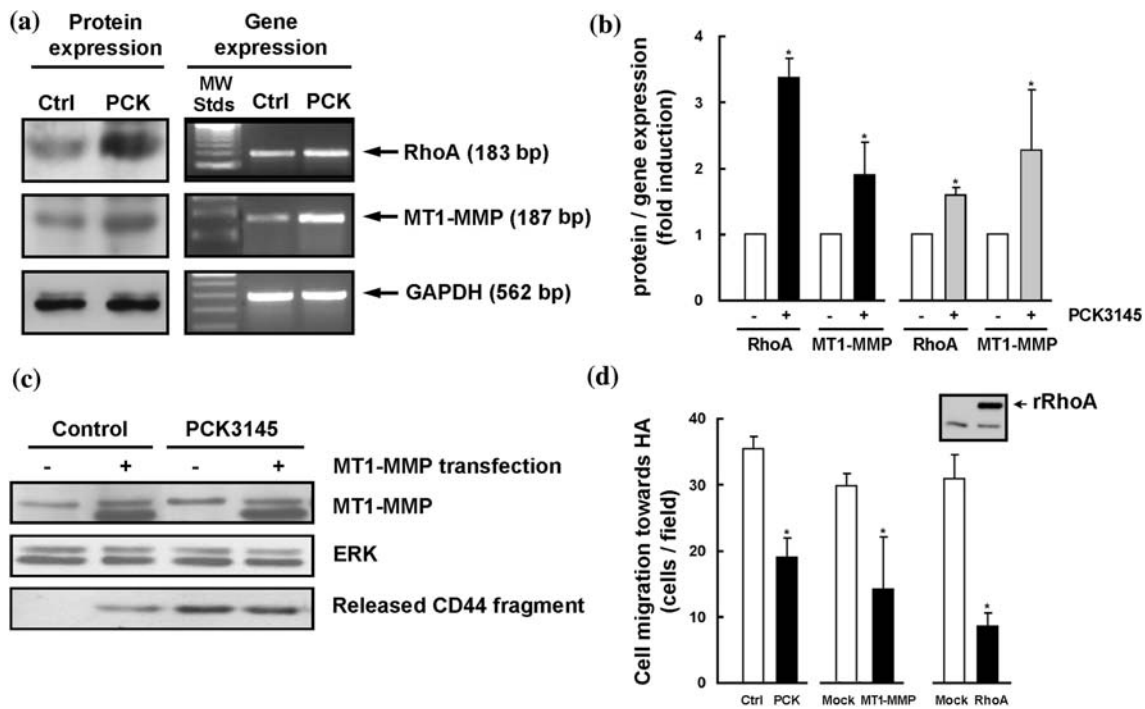


Figure 4. PCK3145 induces RhoA and MT1-MMP gene and protein expression that leads to CD44 cell surface shedding and decreased cell migration on hyaluronic acid. (a) HT-1080 fibrosarcoma cells were treated or not with 300 $\mu\text{g/ml}$ PCK3145 for 48 h. Cell lysates and total RNA was isolated in order to perform Western blotting or RT-PCR as described in the Methods Section. (b) Densitometry was performed in order to quantify the extent of protein (black bars) and gene expression (gray bars) induction by PCK3145 of RhoA and MT1-MMP. Data are expressed as the ratio of protein/gene expression over that of GAPDH. (c) HT-1080 cells were cultured on plastic dishes and subsequently transfected with empty vector (Mock) or with a cDNA plasmid encoding MT1-MMP. Thirty-six hours post-transfection, cells were starved in serum-free media containing or not 300 $\mu\text{g/ml}$ PCK3145 for 18 h. Cell lysates were used to monitor MT1-MMP endogenous and recombinant MT1-MMP. Conditioned media was then collected and centrifuged to eliminate any floating cells. Equal volumes (600 μl) of the conditioned media were TCA/acetone-precipitated and subjected to Western blotting and immunodetection of CD44. (d) Alternatively, 36 h post-transfection, cells were harvested by brief trypsinization and 5×10^4 cells seeded on hyaluronic acid-coated filters. Migration was allowed to proceed as described in the Methods Section. Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance against control (untreated or Mock cells). A representative immunodetection of the recombinant RhoA protein is shown.

control and did not show change in all conditions (Figure 4c). Interestingly, a proteolytic fragment of 75-kDa with CD44-immunoreactivity was detected in the conditioned media of both PCK3145-treated and MT1-MMP transfected cells (Figure 4c). This increase in CD44 shedding into the conditioned media was increased in PCK3145-treated MT1-MMP-transfected cells, but was not consistently induced suggesting a potential additive effect. Such effect has been already reported by many groups and is established as one of the MT1-MMP endpoint-mediated functions, together with the activation of proMMP-2, in the regulation of the ECM adhesion. The potential implication of decreased CD44 function thus prompted us to finally test the effect of PCK3145 on the migration of cells on top of HA-coated filters. The results show that PCK3145 pretreatment decreased basal HT-1080 cell migration by approximately 50% (Figure 4d). As expected, decreased cell migration on HA was similarly observed in RhoA- and MT1-MMP-transfected cells (Figure 4d). Collectively, the inhibitory action of PCK3145 on HA recognition and subsequent cell adhesion/migration processes suggests that the expression of HA cell surface receptors such as those from the CD44 family could be

targeted. Moreover, it is tempting to further suggest that this may also be a secondary regulation by PCK3145 of potential diminished cell surface docking of MMP-9 to CD44.

MMP-9 cell surface binding is downregulated in PCK3145-treated cells

In order to test the impact of the above forwarded hypothesis that PCK3145 triggers CD44 cell surface shedding, HT-1080 fibrosarcoma cells were treated with PCK3145. Cells were then subjected to immunophenotyping with either anti-CD44 or anti-MMP-9 antibodies as described in the Method Section. Flow cytometry was then performed to assess the respective cell surface levels of CD44 and membrane-bound MMP-9. In accordance with the capacity of PCK3145 to trigger the release of a CD44 immunoreactive fragment into the media, we show that the basal levels of CD44 and membrane-bound MMP-9 (Figure 5a, upper panels) were decreased in PCK3145-treated cells (Figure 5a, lower panels). This was quantified and shown to represent a 26% decrease in CD44 cell surface expression, and a 52% decrease in

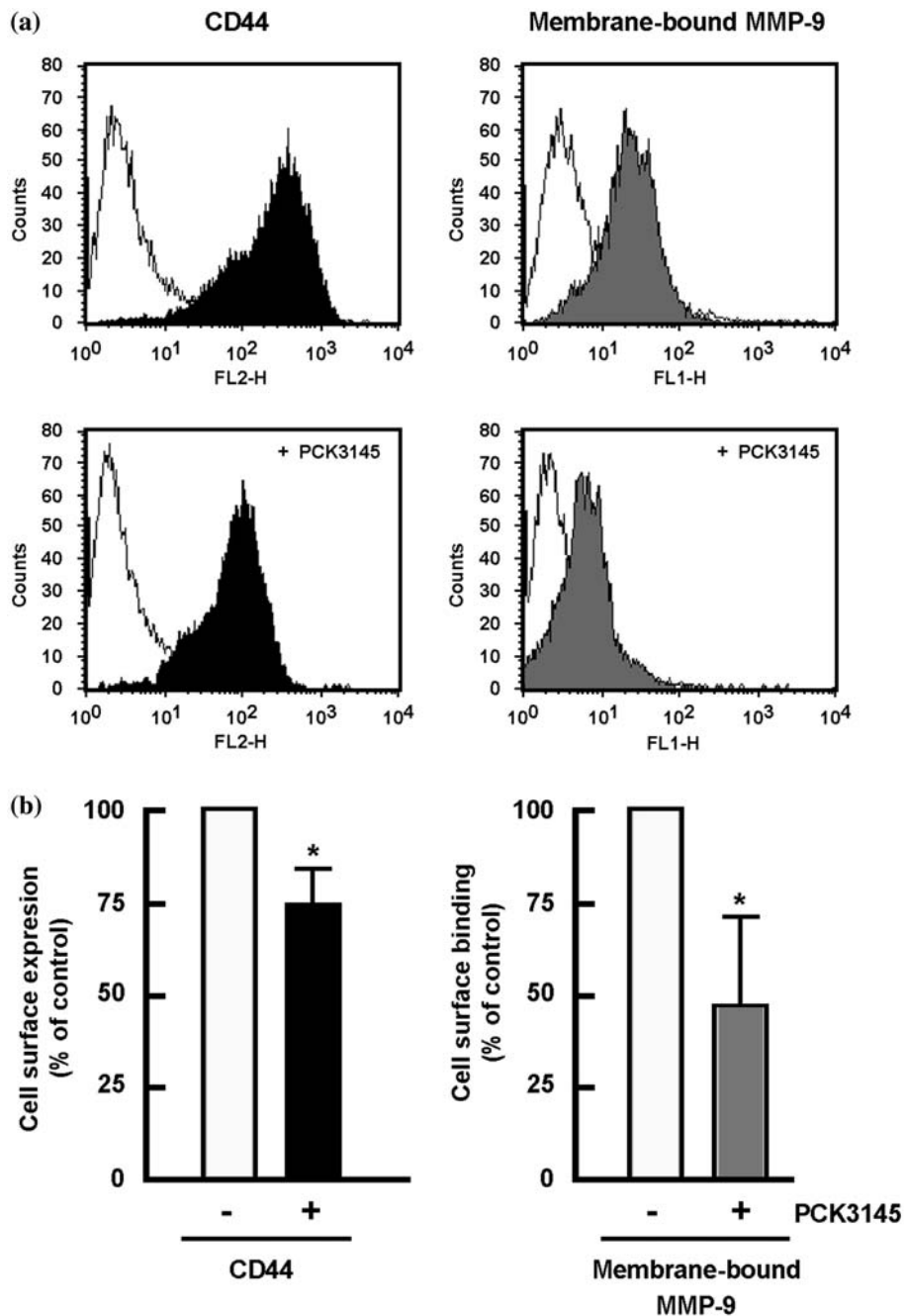


Figure 5. PCK3145-treated cells inhibits CD44 cell surface expression and MMP-9 cell surface binding. (a) HT-1080 fibrosarcoma cells were treated or not with 300 μ g/ml PCK3145. Cells were then harvested and immunophenotyping performed for cell surface expression of CD44 (left panels) and MMP-9 (right panels). Flow cytometry was used to monitor CD44 cell surface protein expression in untreated (upper panels) or PCK3145-treated cells (lower panels). White tracings represent immunofluorescence using the isotype control IgG. (b) Flow cytometric results were quantified and the ratio of relative geometric mean values calculated. The effect of PCK3145 (black bars for CD44, gray bars for MMP-9) on the relative expression of cell surface CD44 or MMP-9 is expressed in percent of untreated cells (white bars) and are representative of three independent experiments.

membrane-bound MMP-9. This effect of PCK3145 on CD44 cell surface expression may in part explain the decreased adhesion to HA, collagen, and laminin which all represent ligands of CD44 extracellular domain [27]. More importantly, this also suggests a potential secondary level of regulation of PCK3145 on membrane-bound MMP-9 functions that could complement its primary inhibition on MMP-9 secretion.

Discussion

Prostate cancer metastasis is a dynamic process involving adhesive interactions between cancer cells and ECM, and is an inevitable evolution in prostate carcinogenesis in HRPc patients [28]. New therapies that specifically target molecular entities regulating metastatic processes must be developed and mechanism of action under-

stood. The discovery of PCK3145 as a novel potential anti-metastatic agent against HRPC provides a viable alternative among traditional cytotoxic therapies. In fact, the recently conducted phase IIa dose escalating clinical trial demonstrated that PCK3145 was safe and well tolerated in all doses (from 5 to 80 mg/m²) in patients with metastatic HRPC. A downregulation in levels of circulating plasma MMP-9 ranging from 34 to 90% in patients having an elevated level of MMP-9 (above 100 µg/l) at study entry was also observed after the treatment with PCK3145 [6, 7]. This *in vivo* PCK3145 activity suggests a biological effect possibly related to the control of MMP-9 mediated metastasis and prompted us to further explore the PCK3145 mechanism of action.

In the present study, we provide the first molecular basis for the action of PCK3145 against tumor growth and metastasis. The use of HT-1080 fibrosarcoma cells enabled us to model certain steps of the metastatic cascade that may relate to MMP-9-mediated ECM hydrolysis. Since we have tested a prostate secretory protein (PSP)94-derived peptide, our current data, in turn, generate hypotheses that can be eventually evaluated in prostate cancer. PCK3145 can indeed inhibit cell–ECM interaction, and more specifically interaction towards laminin, type-I collagen, and HA. Among these ECM proteins, insoluble laminin-rich ECM is one of the major components of the surrounding stroma from which the primary tumor site-derived prostate cancer cells will have to escape [28, 29]. Once cancer cells metastasize and reach the bone, they must then utilize the bone microenvironment to survive and propagate. Since the main component of bone ECM is type I collagen [30], the PCK3145 inhibitory effect of cell adhesion to that particular ECM protein becomes extremely relevant and supports its use as a potential antimetastatic agent. Interestingly, while binding to both laminin and type-I collagen can be mediated through integrin cell surface receptors such as $\alpha 3 \beta 1$ [31], recent evidence also suggests that another transmembrane protein, CD44, which acts as a cell surface receptor for HA and as a docking receptor for MMP-9 [11], potentiated the adherence of metastatic prostate cancer cells to bone marrow endothelial cells [24]. Accordingly, we show that PCK3145, in conjunction with its inhibitory effect on cell adhesion to type-I collagen and to laminin, also inhibited cell adhesion and migration onto HA. Moreover, we show that PCK3145 triggers CD44 cell surface shedding as a potential secondary mechanism of regulation of MMP-9 functions. PCK3145 may thus efficiently inhibit MMP-9-mediated metastatic processes through both MMP-9 secretion and its subsequent binding to the cell surface.

Association of MMP-9 with the cell surface is mediated by a distinct array of surface proteins that serve to regulate multiple aspects of the enzyme function including localization, inhibition and internalization [32]. Together with its inhibitory effect on MMP-9 secretion, our study specifically provides molecular

evidence for an additional effect of PCK3145 on the cell surface association of MMP-9. Our observations suggest alternate new and unexpected conceptual considerations of this therapeutic peptide in targeting cancer cells metastasis processes. For instance, MMP-9 bound to the CD44 receptor is thought to process latent transforming growth factor (TGF)- β to its active form and to promote a degradative phenotype [11, 33, 34]. It becomes tempting to suggest that one of the PCK3145 biological consequences in inhibiting MMP-9 secretion and subsequent cell surface binding in metastatic processes may thus potentially impact on TGF- β ability to promote malignant progression and metastasis in inflammatory processes [35], bone metastatic tumor cells [36] and in prostate cancer cells [37].

The signal transducing events that lead to CD44 releasing process is also of particular interest in the PCK3145 action. Such proteolytic shedding from cells has been documented to involve intracellular signaling triggered by phorbol esters as well as cytokines [38, 39], two very potent inducers of MMP-9 in invading cells [40]. Interestingly, our results highlight the PCK3145 ability to efficiently inhibit PMA- and TNF-induced signaling. The latter pro-inflammatory cytokine being highly expressed in the serum of metastatic prostate cancer patients [41]. Moreover, while we show that PCK3145 was able to inhibit PMA- and TNF-induced MMP-9 secretion, PCK3145 also led to MT1-MMP activation and subsequent MT1-MMP-mediated CD44 proteolytic shedding. PCK3145-induced MT1-MMP-mediated CD44 shedding is supported by recent structure–function analysis demonstrating that the hemopexin-like domain of MT1-MMP was responsible for the binding and subsequent shedding of the standard haematopoietic form of CD44 [42]. This observation, however, suggests that several possible intracellular transduction pathways, besides that triggered by PMA and TNF, may be involved and regulated by PCK3145 action.

Interactions of prostate cancer cells within their microenvironment involve activation of cell surface receptors of integrin and non-integrin family, and this is reflected by the multiple downstream intracellular signaling triggered. Among these, intracellular Rho family GTPases play an important role in a number of processes related to metastasis, such as assembly of filopodia, lamellipodia and stress fibres [43]. Interestingly, RhoA-mediated signaling was also shown to regulate CD44 functions [44–46], and this mode of action is compatible with the induction of RhoA by PCK3145. Signaling that leads to the shedding of CD44 thus appears to regulate cell adhesion and migration involved in metastasis. Altogether, this published evidence further provides an interesting PCK3145-mediated intracellular crosstalk linking RhoA to the cell surface proteolytic activity and expression of MT1-MMP, which in turn regulates CD44 shedding and impairs MMP-9 cell surface docking. Accordingly, a crucial RhoA/MT1-MMP signaling axis that regulated the cell surface shedding of

CD44 was recently highlighted [14]. Noteworthy, cell fractionation data suggest that RhoA, CD44, MT1-MMP, and MMP-9 have the potential to co-localize at common cell surface Triton X-100-insoluble and cholesterol-enriched membrane domains termed caveolae [19, 47–49]. Interestingly, we show that PCK3145 targets all of these caveolae-associated proteins. Since caveolae also function as regulators of signal transduction in the pathogenesis of oncogenic cell transformation, tumorigenesis, and metastasis [50], it is tempting to suggest that PCK3145 acts as an inhibitor of caveolae-mediated intracellular transduction pathways that control MMP-9 secretion and cell–ECM interaction. As such, caveolin-1-mediated inhibition of invasion and metastasis was recently reported to occur partly through inhibition of MMP-2 and MMP-9 secretion [51], while genetic ablation of caveolin-1 delayed advanced prostate tumor development [52]. Whether PCK3145 regulates caveolin expression and/or functions is currently under investigation.

In conclusion, our current study shows that PCK3145 may target MMP-9-mediated cancer cells metastatic processes through the suppression of MMP-9 secretion and binding to the cell surface. This process, may in part involve the proteolytic shedding of CD44 through a RhoA/MT1-MMP-mediated mechanism. Moreover, we show that PCK3145 can potentially inhibit cytokines- and PMA-induced MMP-9 secretion suggesting a role as a signal transduction inhibitor. The implications of such pleiotropic mode of action of PCK3145 on MMP-9 functions may make an impact, not only in cancer, but also on pathological processes in which MMP-9 expression is upregulated such as inflammatory, degenerative, vascular, and infectious diseases.

Acknowledgements

B.A. holds a Canada Research Chair in Molecular Oncology from the Canadian Institutes of Health Research. This work is supported by a grant from the Canadian Institutes for Health Research to R.B.

References

- Fidler IJ. Modulation of the organ microenvironment for the treatment of cancer metastasis. *J Natl Cancer Inst* 1995; 87: 1588–92.
- Yang JP, Finkelman MA, Clarke MW. Detection of PSP94 and its specific binding sites in the prostate adenocarcinoma cell line LNCaP. *J Urol* 1998; 160: 2240–4.
- Sakai H, Tsurusaki T, Kanda S et al. Prognostic significance of β -microseminoprotein mRNA expression in prostate cancer. *Prostate* 1999; 38: 278–84.
- Shukeir N, Arakelian A, Kadhim S et al. Prostate secretory protein PSP94 decreases tumor growth and hypercalcemia of malignancy in a syngenic *in vivo* model of prostate cancer. *Cancer Res* 2003; 63: 2072–8.
- Shukeir N, Arakelian A, Chen G et al. A synthetic 15-mer peptide (PCK3145) derived from prostate secretory protein can reduce tumor growth, experimental skeletal metastases, and malignancy-associated hypercalcemia. *Cancer Res* 2004; 64: 5370–7.
- Hawkins RE, Daigneault L, Cowan R et al. Safety and tolerability of PCK3145, a synthetic peptide derived from prostate secretory protein 94 (PSP94) in metastatic hormone-refractory prostate cancer. *Clin Prostate Cancer* 2005; 4: 91–9.
- Daigneault L, Panchal C, Scher HI et al. A synthetic peptide, PCK3145, for the treatment of hormone refractory prostate cancer. *ASCO Prostate Cancer Symposium 2005, Annual Meeting Proc* 2005; 164: 260.
- Jemal A, Ward E, Wu X et al. Geographic patterns of prostate cancer mortality and variations in access to medical care in the United States. *Cancer Epidemiol Biomarkers Prev* 2005; 14: 590–5.
- Kleiner DE, Stetler-Stevenson WG. Matrix metalloproteinases and metastasis. *Cancer Chemother Pharmacol* 1999; 43(Suppl): S42–S51.
- Dong Z, Bonfil RD, Chinni S et al. Matrix metalloproteinase activity and osteoclasts in experimental prostate cancer bone metastasis tissue. *Am J Pathol* 2005; 166: 1173–86.
- Yu Q, Stamenkovic I. Localization of matrix metalloproteinase-9 to the cell surface provides a mechanism for CD44-mediated tumor invasion. *Genes Dev* 1999; 13: 35–48.
- Bourguignon LY, Gunja-Smith Z, Iida N et al. CD44v(3, 8–10) is involved in cytoskeleton-mediated tumor cell migration and matrix metalloproteinase (MMP-9) association in metastatic breast cancer cells. *J Cell Physiol* 1998; 176: 206–15.
- Annabi B, Thibeault S, Moudjian R et al. Hyaluronan cell surface binding is induced by type I collagen and regulated by caveolae in glioma cells. *J Biol Chem* 2004; 279: 21888–96.
- Annabi B, Bouzeghrane M, Moudjian R et al. Probing the infiltrating character of brain tumors: Inhibition of RhoA/ROK-mediated CD44 cell surface shedding from glioma cells by the green tea catechin EGCG. *J Neurochem* 2005; 94: 906–16.
- Silver FH, Trelstad RL. Type I collagen in solution. Structure and properties of fibril fragments. *J Biol Chem* 1980; 255: 9427–33.
- Annabi B, Pilorget A, Bousquet-Gagnon N et al. Calmodulin inhibitors trigger the proteolytic processing of membrane type-1 matrix metalloproteinase, but not its shedding in glioblastoma cells. *Biochem J* 2001; 359: 325–33.
- Mukhopadhyay S, Munshi HG, Kambhampati S et al. Calcium-induced matrix metalloproteinase-9 gene expression is differentially regulated by ERK1/2 and p38 MAPK in oral keratinocytes and oral squamous cell carcinoma. *J Biol Chem* 2004; 279: 33139–46.
- Liu LT, Chang HC, Chiang LC et al. Histone deacetylase inhibitor up-regulates RECK to inhibit MMP-2 activation and cancer cell invasion. *Cancer Res* 2003; 63: 3069–72.
- Annabi B, Lachambre MP, Bousquet-Gagnon N et al. Localization of membrane-type 1 matrix metalloproteinase in caveolae membrane domains. *Biochem J* 2001; 353: 547–53.
- Turcotte S, Desrosiers RR, Béliveau R. HIF-1 α mRNA and protein upregulation involves Rho GTPase expression during hypoxia in renal cell carcinoma. *J Cell Sci* 2003; 116: 2247–60.
- Beaulieu E, Kachra Z, Mousseau N et al. Matrix metalloproteinases and their inhibitors in human pituitary tumors. *Neurosurgery* 1999; 45: 1432–40.
- Rigot V, Lehmann M, Andre F et al. Integrin ligation and PKC activation are required for migration of colon carcinoma cells. *J Cell Sci* 1998; 111: 3119–27.
- Hornebeck W, Maquart FX. Proteolyzed matrix as a template for the regulation of tumor progression. *Biomed Pharmacother* 2003; 57: 223–30.
- Draffin JE, McFarlane S, Hill A et al. CD44 potentiates the adherence of metastatic prostate and breast cancer cells to bone marrow endothelial cells. *Cancer Res* 2004; 64: 5702–11.
- Aaltomaa S, Lipponen P, Tammi R et al. Strong stromal hyaluronan expression is associated with PSA recurrence in local prostate cancer. *Urol Int* 2002; 69: 266–72.
- Miyake H, Hara I, Okamoto I et al. Interaction between CD44 and hyaluronic acid regulates human prostate cancer development. *J Urol* 1998; 160: 1562–6.
- Naor D, Sionov RV, Ish-Shalom D. CD44: Structure, function, and association with the malignant process. *Adv Cancer Res* 1997; 71: 241–319.

28. Sikes RA, Nicholson BE, Koeneman KS et al. Cellular interactions in the tropism of prostate cancer to bone. *Int J Cancer* 2004; 110: 497–503.
29. Tantivejkul K, Kalikin LM, Pienta KJ. Dynamic process of prostate cancer metastasis to bone. *J Cell Biochem* 2004; 91: 706–17.
30. Hauschka PV, Mavrakos AE, Iafrati MD et al. Growth factors in bone matrix. Isolation of multiple types by affinity chromatography on heparin-Sepharose. *J Biol Chem* 1986; 261: 12665–74.
31. Iyer V, Pumiglia K, DiPersio CM. Alpha3beta1 integrin regulates MMP-9 mRNA stability in immortalized keratinocytes: A novel mechanism of integrin-mediated MMP gene expression. *J Cell Sci* 2005; 118: 1185–95.
32. Fridman R, Toth M, Chvyrkova I et al. Cell surface association of matrix metalloproteinase-9 (gelatinase B). *Cancer Metast Rev* 2003; 22: 153–66.
33. Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* 2000; 14: 163–76.
34. Danielpour D. Functions and regulation of transforming growth factor-beta (TGF-beta) in the prostate. *Eur J Cancer* 2005; 41: 846–57.
35. Wahl SM, Allen JB, Weeks BS et al. Transforming growth factor beta enhances integrin expression and type IV collagenase secretion in human monocyte. *Proc Natl Acad Sci USA* 1993; 90: 4577–81.
36. Duivenvoorden WC, Hirte HW, Singh G. Transforming growth factor beta1 acts as an inducer of matrix metalloproteinase expression and activity in human bone-metastasizing cancer cells. *Clin Exp Metast* 1999; 17: 27–34.
37. Sehgal I, Thompson TC. Novel regulation of type IV collagenase (matrix metalloproteinase-9 and -2) activities by transforming growth factor-beta1 in human prostate cancer cell lines. *Mol Biol Cell* 1999; 10: 407–16.
38. DeGrendele HC, Estess P, Siegelman MH. Requirement for CD44 in activated T cell extravasation into an inflammatory site. *Science* 1997; 278: 672–5.
39. Ristamaki R, Joensuu H, Jalkanen S. Does soluble CD44 reflect the clinical behavior of human cancer? *Curr Top Microbiol Immunol* 1996; 213: 155–66.
40. Lohi J, Keski-Oja J. Calcium ionophores decrease pericellular gelatinolytic activity via inhibition of 92-kDa gelatinase expression and decrease of 72-kDa gelatinase activation. *J Biol Chem* 1995; 270: 17602–9.
41. Michalaki V, Syrigos K, Charles P et al. Serum levels of IL-6 and TNF-alpha correlate with clinicopathological features and patient survival in patients with prostate cancer. *Br J Cancer* 2004; 90: 2312–6.
42. Suenaga N, Mori H, Itoh Y, Seiki M. CD44 binding through the hemopexin-like domain is critical for its shedding by membrane-type 1 matrix metalloproteinase. *Oncogene* 2005; 24: 859–68.
43. Sahai E, Marshall CJ. RHO-GTPases and cancer. *Nat Rev Cancer* 2002; 2: 133–42.
44. Bourguignon LY. CD44-mediated oncogenic signaling and cytoskeleton activation during mammary tumor progression. *J Mammary Gland Biol Neoplasia* 2001; 6: 287–97.
45. Shi M, Dennis K, Peschon JJ, Chandrasekaran R, Mikecz K. Antibody-induced shedding of CD44 from adherent cells is linked to the assembly of the cytoskeleton. *J Immunol* 2001; 167: 123–31.
46. Kawano Y, Okamoto I, Murakami D, Itoh H, Yoshida M, Ueda S, Saya H. Ras oncoprotein induces CD44 cleavage through phosphoinositide 3-OH kinase and the rho family of small G proteins. *J Biol Chem* 2000; 275: 29628–35.
47. Abecassis I, Olofsson B, Schmid M et al. RhoA induces MMP-9 expression at CD44 lamellipodial focal complexes and promotes HMEC-1 cell invasion. *Exp Cell Res* 2003; 291: 363–76.
48. Perschl A, Lesley J, English N et al. Transmembrane domain of CD44 is required for its detergent insolubility in fibroblasts. *J Cell Sci* 1995; 108: 1033–41.
49. Gingras D, Gauthier F, Lamy S et al. Localization of RhoA GTPase to endothelial caveolae-enriched membrane domains. *Biochem Biophys Res Commun* 1998; 247: 888–93.
50. Williams TM, Lisanti MP. Caveolin-1 in oncogenic transformation, cancer, and metastasis. *Am J Physiol Cell Physiol* 2005; 288: C494–C506.
51. Williams TM, Medina F, Badano I, Hazan RB, Hutchinson J, Muller WJ, Chopra NG, Scherer PE, Pestell RG, Lisanti MP. Caveolin-1 gene disruption promotes mammary tumorigenesis and dramatically enhances lung metastasis *in vivo*. Role of Cav-1 in cell invasiveness and matrix metalloproteinase (MMP-2/9) secretion. *J Biol Chem* 2004; 279: 51630–46.
52. Williams TM, Hassan GS, Li J, Cohen AW, Medina F, Frank PG, Pestell RG, Di Vizio D, Loda M, Lisanti MP. Caveolin-1 promotes tumor progression in an autochthonous mouse model of prostate cancer: Genetic ablation of Cav-1 delays advanced prostate tumor development in TRAMP mice. *J Biol Chem* 2005; 280: 25134–45.