

# Inhibition of MMP-9 secretion by the anti-metastatic PSP94-derived peptide PCK3145 requires cell surface laminin receptor signaling

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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. These anti-metastatic and anti-tumoral effects of PCK3145 are partially explained by the in-vivo and in-vitro decrease in matrix metalloproteinase (MMP)-9 extracellular levels through as yet unidentified molecular mechanisms of action. Gelatin zymography and immunoblots were used to monitor the levels of secreted MMP-9 from HT-1080 cells. Flow cytometry was used to monitor HT-1080 cell surface binding of FITC-labeled PCK3145 and biotin-labeled laminin. PCK3145-coated cell culture dishes were used to monitor cell adhesion. HT-1080 cell lysates were used for immunoblotting of HuR, extracellular signal-regulated protein kinase (ERK) and phospho-ERK. Total RNA was isolated and RT-PCR used to monitor HuR gene expression. We found that PCK3145 bound to the HT-1080 cell surface and that this binding rapidly triggered ERK phosphorylation that, ultimately, led to a reduction of secreted MMP-9. Laminin inhibited both cell surface binding and ERK phosphorylation by PCK3145. Overexpression of the 67-kDa laminin receptor led to an increased binding of the cells to PCK3145. HuR, a protein that can bind to and stabilize MMP-9 mRNA, was found to be downregulated by PCK3145. The mitogen-activated protein kinase/ERK (MEK) inhibitor PD98059 as well as

native laminin and SIKVAV laminin-derived peptide prevented that downregulation. Our data suggest that PCK3145 rapidly triggers intracellular signaling through cell surface laminin receptors. This leads to decreased HuR expression and subsequent destabilization of MMP-9 transcripts. This is the first molecular evidence demonstrating the intracellular signaling and anti-metastatic mechanism of action of PCK3145 that leads to the inhibition of MMP-9 secretion. *Anti-Cancer Drugs* 17:429–438 © 2006 Lippincott Williams & Wilkins.

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## Introduction

Prostate cancer is frequently associated with bone metastases, which are in fact the main cause of morbidity and mortality for this tumor. It is also 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 disease, as nearly all men with metastases will eventually progress to hormone-refractory prostate cancer (HRPC). Currently, no effective treatments exist for patients where hormone treatment has failed and the management of HRPC is solely palliative. 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 inhibin or  $\beta$ -microseminoprotein [2], is a naturally occurring protein synthesized primarily in the prostate and found in large quantities in the seminal fluid [3]. Although the complete physiological role of PSP94 is not known, it is believed to be involved as a growth inhibitor and a promoter of cell death in the natural control of excessive and/or abnormal proliferation of epithelial cells, both in normal prostate tissue as well as in malignant prostate tissues.

In previous studies, we have shown that PSP94 can reduce experimental skeletal metastases and prostate cancer growth *in vivo* [4], and that the amino acid 31–45

The first two authors contributed equally to this work.

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 (above 100 µg/l) at baseline [6]. Such therapeutic efficacy in reducing the levels of plasma MMP-9 in HRPC patients receiving PCK3145 suggests a biological effect possibly targeting the control of tumor-related extracellular matrix (ECM) degradation and metastasis without safety concerns or adverse effects.

We have recently reported several molecular processes involved in tumor progression and which are targeted by PCK3145. Indeed, we demonstrated the potential anti-angiogenic effect of PCK3145 *in vivo* and *in vitro* in endothelial cells as it inhibited vascular endothelial (VEGF) signaling through the tyrosine kinase activity associated with the VEGF receptor [7]. Moreover, we also have provided evidence for an anti-metastatic effect of PCK3145 targeting both MMP-9 secretion and subsequent cell surface docking to CD44 [8]. The complete mechanism of action of PCK3145, including the elucidation of the cell surface receptors and intracellular pathways that lead to reduced MMP-9, remained elusive however. Our study shows for the first time that cell surface laminin receptors transduce PCK3145 intracellular signaling which involves a rapid and transient phosphorylation of extracellular signal-regulated protein kinase (ERK). These effects of PCK3145 lead to the inhibition of the MMP-9 mRNA-binding and -stabilizing protein HuR gene and protein expression. The identification of the mechanism of action of PCK3145 as well as the potential involvement of cell surface laminin receptors allows us to extend PCK3145 ability to target other types of cancer such as those expressing high levels of laminin receptors, as observed in leukemia cells within bone marrow.

## Materials and methods

### Materials

Agarose, SDS, gelatin, BSA and Triton X-100 were purchased from Sigma (Oakville, Ontario, Canada). TRIzol reagent was from Life Technologies (Gaithersburg, Maryland, USA). FUGENE-6 transfection reagent was from Roche Diagnostics (Laval, Quebec, Canada). Type I collagen was extracted from rat tail tendon according to classical protocols [9]. Cell culture media was obtained from Life Technologies (Burlington, Ontario, Canada). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, Ontario, Canada). The polyclonal antibodies against the 67-kDa laminin receptor (67LR) and HuR were purchased from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania, USA). The anti-phospho-ERK was from Cell Signaling Technology (Beverly, Maryland, USA), while the polyclonal anti-MMP-9 and ERK antibodies were from Santa

Cruz Biotechnology (Santa Cruz, California, USA). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, Illinois, USA). FITC N-terminal-labeled PCK3145 and the laminin-1-derived peptide SIKVAV were synthesized by EZBiolab (Westfield, Indiana, USA). Epigallocatechin-3-gallate (EGCg) and all other reagents were from Sigma-Aldrich Canada (Oakville, Ontario, Canada).

### Cell culture and cDNA transfection method

The HT-1080 cell line was purchased from ATCC (Manassas, Virginia, USA), maintained in DMEM containing 10% (v/v) FBS (HyClone, Logan, Utah, USA), 2 mmol/l glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, and cultured at 37°C under a humidified atmosphere containing 5% CO<sub>2</sub>. The human 67LR full-length cDNA was from OriGene Technologies (Rockville, Maryland, USA). 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<sup>+</sup>) expression vector alone were used as controls.

### Total RNA isolation and 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, Ontario, Canada). Primers for HuR (forward: 5'-TCGCAGCTGTACCCTCGCCAG-3'; reverse: 5'-CCAAACATCTGC-CAGAGGATC-3') [10] were derived from human sequences and PCR conditions were optimized so that the gene products were at the exponential phase of amplification. PCR products were resolved on 1.5% agarose gels containing 1 µg/ml ethidium bromide.

### Immunoblotting procedures

Proteins from control and treated cells were separated by SDS-PAGE. After electrophoresis, proteins were electrotransferred to PVDF membranes which were then blocked overnight at 4°C with 5% non-fat dry milk in Tris-buffered saline (150 mmol/l NaCl, 20 mmol/l 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% BSA, followed by a 1-h incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1/10 000 dilution for MMP-9, HuR, ERK and phospho-ERK detection) or anti-mouse IgG (1/5000 dilution) in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Ur-fée, Quebec, Canada).

### Gelatin zymography

To assess the extent of MMP-9 activity, gelatin zymography was used as described previously [11]. Briefly, an aliquot (20  $\mu$ l) of the culture medium was subjected to SDS-PAGE in a gel containing 0.1 mg/ml gelatin. The gels were then incubated in 2.5% Triton X-100 and rinsed in nanopure distilled H<sub>2</sub>O. Gels were further incubated at 37°C for 20 h in 20 mmol/l NaCl, 5 mmol/l CaCl<sub>2</sub>, 0.02% Brij-35, 50 mmol/l 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 H<sub>2</sub>O. Gelatinolytic activity was detected as unstained bands on a blue background. All experiments were carried out with cells that had been deprived of serum by overnight incubation.

### Cell adhesion assays

Adhesion assays were performed as previously described [12]. Briefly, adhesion wells were coated with increasing concentrations of PCK3145 for 2 h at 37°C and then blocked by adding a solution of PBS/BSA 0.5%. Cells were harvested as a single-cell suspension by treatment with 0.53 mmol/l 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 fixed and 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.

### Flow cytometry analysis and FITC-labeled PCK3145-binding assay

FITC is currently the most commonly used fluorescent dye for FACS analysis and was conjugated to the N-terminus of PCK3145. This enables us to follow cell binding of FITC-PCK3145 through the shift in fluorescence associated with cells that bind to it. HT-1080 cells (80–90%) confluent were dislodged after brief PBS-citrate treatment, washed extensively, resuspended in 10% FBS/DMEM at a concentration of 10<sup>6</sup> cells/ml, washed once with 0.1% PBS/0.1% BSA and then incubated with 10  $\mu$ g/ml FITC-PCK3145 for 1 h on ice (4°C). After washing with PBS/BSA, the cells were suspended in 1 ml PBS/BSA and analyzed on a FACSCalibur flow cytometer with CellQuestPro software (BD Biosciences, Mississauga, Ontario, Canada). Results are expressed as the ratio of relative geometric mean values from the PCK3145-treated cells to their untreated controls and are representative of three independent experiments.

### PCK3145 biotinylation and HPLC analyses

PCK3145 was biotin labeled according to Pierce and purified by FPLC chromatography using Akta-explorer with a resource RPC-30ml column (Amersham Bioscience, Quebec, Canada). The biotin-labeled peptide was eluted with a gradient from 20% CH<sub>3</sub>CN to 80% CH<sub>3</sub>CN (+ 0.05% TFA) at a flow rate of 4 ml/min. Products were monitored at different wavelengths (205, 229 and 254 nm) with a UV-900 cell-10.

### Gene reporter assay

The Great EscAPe SEAP reporter system (Clontech, Palo Alto, CA, USA), in which a secretory alkaline phosphatase (SEAP) form is fused to promoters activated by different responsive elements (described in the legend to Fig. 5), was used to monitor the effect of PCK3145 on different response elements. Cells were transfected with the various constructs and aliquots of the conditioned media were collected at different times. SEAP activity was measured by the hydrolysis of *p*-nitrophenylphosphate [13].

### Statistical 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

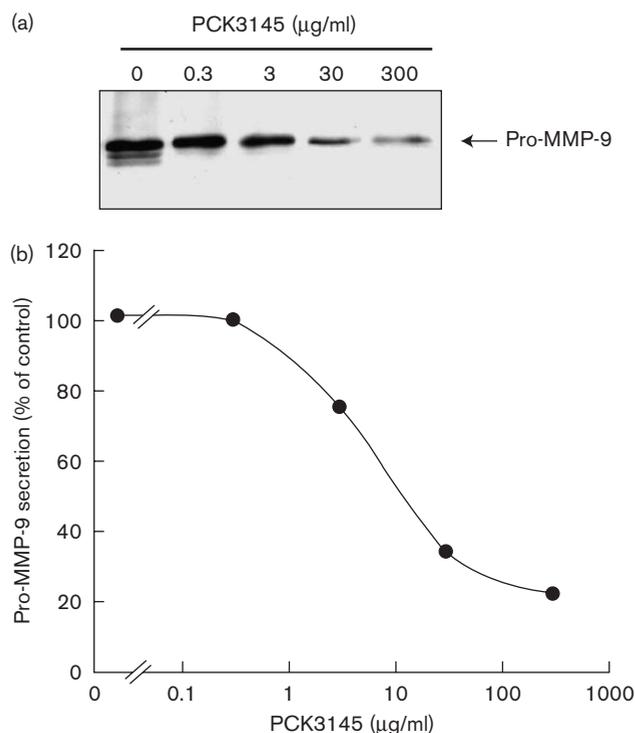
### PCK3145 inhibits MMP-9 secretion from HT-1080 cells

PCK3145 was shown to reduce the levels of circulating MMP-9 plasma levels in a phase IIa clinical trial [6]. In order to investigate and confirm the effect of PCK3145 on MMP-9 secretion *in vitro*, we treated serum-starved HT-1080 fibrosarcoma cells with increasing doses of PCK3145 for 48 h [8]. 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. As expected from the clinical data, MMP-9 extracellular levels decreased significantly in a dose-dependent manner in PCK3145-treated cells (Fig. 1a). A maximum inhibition of approximately 80% of extracellular levels of MMP-9 was observed at 300  $\mu$ g/ml (Fig. 1b). This *in vitro* result confirms those observed in the phase IIa clinical trial on the efficacy of PCK3145 to decrease MMP-9 levels.

### PCK3145 binding to the cell surface of HT-1080 cells is inhibited by laminin

In order to assess whether the inhibitory action of PCK3145 on MMP-9 secretion involves any cell surface receptor-mediated signaling, we first need to know if PCK3145 binds to the cell surface of HT-1080 fibrosarcoma cells. N-terminal FITC-labeled PCK3145 was synthesized and incubated with HT-1080 cells for 30 min at 4°C in order to minimize intracellular uptake of the labeled peptide [14] and cell-associated fluorescence measured by flow cytometry as described previously [15]. As shown by the significant shift in fluorescence, we demonstrate that FITC-PCK3145 effectively bound to the HT-1080 cell surface in a dose-dependent manner with a plateau reached around 10  $\mu$ g/ml (Fig. 2a). Furthermore, because we had previously shown that PCK3145 inhibited cell adhesion to

Fig. 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 48 h in serum-free media. Equal volumes (300 µl) of the conditioned media were precipitated with TCA, and MMP-9 levels assessed by Western blotting and immunodetection with anti-MMP-9 antibody. (b) Quantification of a representative experiment is presented and was performed by scan densitometry.

laminin [8] and that this may inhibit metastasis processes, we also performed a competition experiment by incubating the cells with FITC-labeled PCK3145 (Fig. 2b, upper panel, shaded plot) or with both laminin (30 µg/ml) and FITC-labeled PCK3145 (Fig. 2b, lower panel). Interestingly, our results show a complete inhibition of PCK3145 cell surface binding in the presence of excess laminin (Fig. 2b). This latter observation is the first evidence that cell surface receptors with laminin-binding activities might trigger the subsequent intracellular signaling by PCK3145.

#### PCK3145 inhibits cell surface binding of laminin

In an attempt to characterize the potential laminin receptor involved in PCK3145 binding to the cells, we decided to label laminin with biotin and to analyze cell surface binding by flow cytometry. Moreover, the effect of EGCg, a green tea-derived molecule which shares the ability with PCK3145 to inhibit MMP secretion [16,17] and for which the receptor was identified as the non-integrin 67LR [18], was also tested. We show that labeled laminin bound to the cell surface of HT-1080 cells by the

shift in fluorescence (Fig. 3a, control). Interestingly, while excess non-labeled laminin (Fig. 3a, Laminin) and PCK3145 (Fig. 3a, PCK3145) effectively competed for, respectively, 30 and 45% inhibition (Fig. 3b) with that binding of labeled-laminin, we found that EGCg was a very potent agent, inhibiting biotin-labeled laminin cell surface binding by 80% (Fig. 3a, EGCg). The observed partial inhibitory effect of PCK3145 may be due to the fact that laminin can bind to multiple cell surface receptors from the integrin and non-integrin family, while PCK3145 may rather bind to a more specific cell surface laminin receptor. Whether 67LR is involved in recognizing PCK3145 was next investigated.

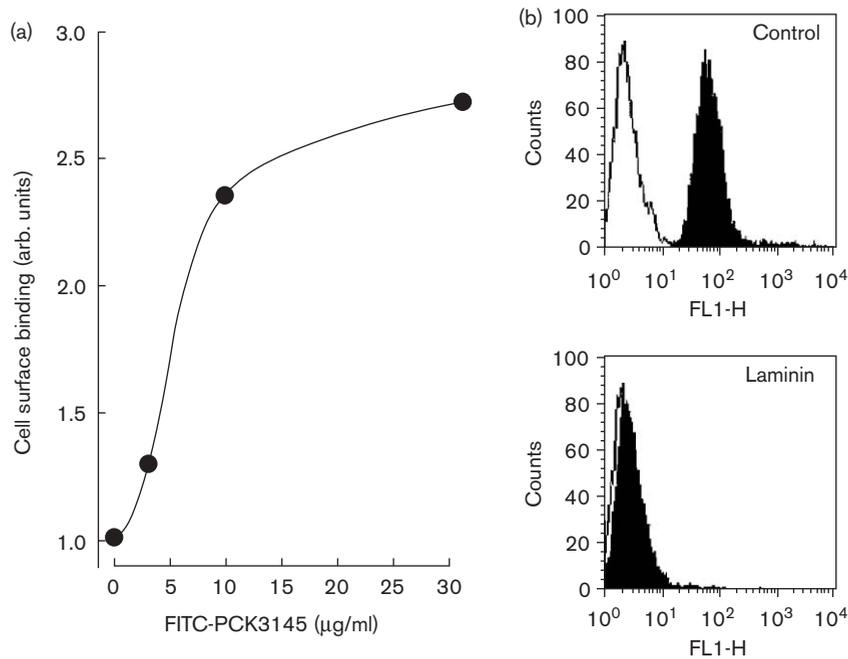
#### Cell adhesion to PCK3145 is induced in 67LR-transfected cells

In order to characterize the potential involvement of the 67LR protein in interacting with PCK3145, we designed an adhesion assay to PCK3145-coated dishes. Native HT-1080 cells were dislodged by trypsinization and seeded on top of culture dishes that were coated with increasing concentrations of PCK3145. We found that HT-1080 cells recognized and adhered to PCK3145-coated dishes with a maximal adhesion found at 10 µg/ml of PCK3145 (Fig. 4a). Interestingly, although higher concentrations of PCK3145 still triggered significant cell adhesion, it was gradually decreasing. Whether this binding to PCK3145 involved 67LR was next investigated by transiently transfecting HT-1080 cells with 67LR plasmid cDNA. Using mock- versus 67LR-transfected cells, we observed that while basal cell adhesion remained unaffected, an increase in adhesion to PCK3145 occurred when cells overexpressed the recombinant 67LR protein (Fig. 4b). Immunoblotting was used to show that efficient expression of the recombinant 67LR protein was generated (Fig. 4c, insert) and an increase in cell adhesion monitored over time (Fig. 4c).

#### PCK3145 induces a rapid, but transient, phosphorylation of ERK

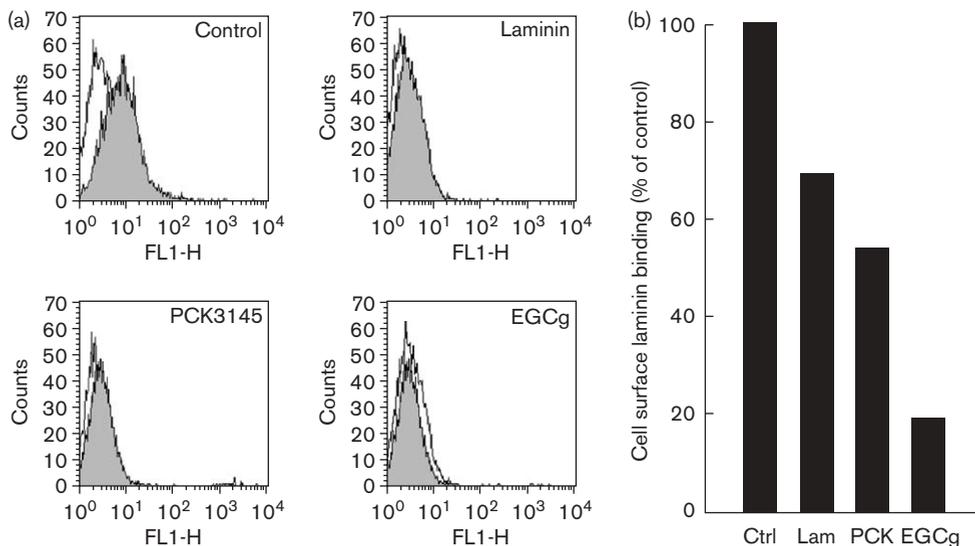
Ligand binding to laminin receptors induces the activation of many intracellular pathways [19]. We next tested which potential intracellular pathways are activated by PCK3145 potential binding to the cell surface. We performed a gene reporter assay as described in Materials and methods in order to monitor the activity of eukaryotic promoters and enhancers that are triggered by PCK3145. We show that PCK3145 significantly triggers two pathways: the mitogen-activated protein kinase (MAPK)/ERK pathway (MEK/SRE) and the NF-κB pathway (Fig. 5a, black bars). The 6.6-fold increase in the MAPK/ERK pathway is extremely strong as compared to that of the NF-κB pathway (3.5-fold). The latter, however, potentially suggests the involvement of pro-apoptotic pathways that would be triggered by PCK3145 and that we previously reported in our in-vivo model [5]. PCK3145 induction of the MAPK pathway is further confirmed by

Fig. 2



Cell surface binding of PCK3145 is inhibited by laminin. (a) HT-1080 fibrosarcoma cells were trypsinized and  $10^6$  cells incubated with increasing concentrations of N-terminal FITC-labeled PCK3145 for 1 h at 4°C. Cell-associated fluorescence was measured by flow cytometry. (b) HT-1080 cells were incubated with 10 µg/ml of FITC-labeled PCK3145 alone (Control) or in the presence of 30 µg/ml laminin (Laminin) and cell-associated fluorescence measured under the same conditions as above.

Fig. 3

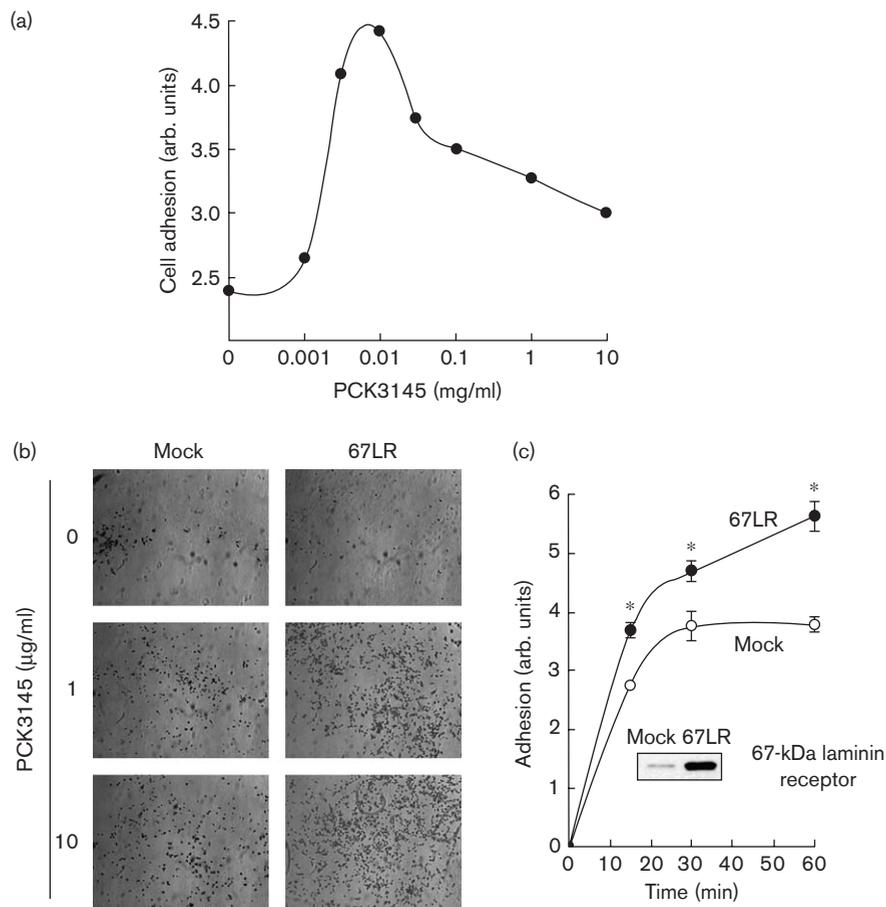


Cell surface binding of laminin is inhibited by PCK and by the green tea catechin EGCg. (a) HT-1080 fibrosarcoma cells were trypsinized and  $10^6$  cells incubated with 1 µg/ml biotin-labeled laminin (shaded plots) for 1 h at 4°C. Cell-associated fluorescence was measured by flow cytometry as described in Materials and methods in the presence of 30 µg/ml laminin, 30 µg/ml PCK3145 or 30 µM EGCg. A representative experiment is presented and (b) Quantification performed as described in Materials and methods.

the rapid and transient induction of ERK phosphorylation (Fig. 5b), peaking at 1 min, whereas total ERK levels remained unaffected (Fig. 5b and c). Finally, the effects

of PCK3145 were also compared with those of a scrambled peptide which was unable to induce ERK phosphorylation as PCK3145 did (not shown). This rapid

Fig. 4



Cell adhesion to PCK3145 is increased in 67LR-transfected cells. (a) HT-1080 fibrosarcoma ( $25 \times 10^4$  cells/well) were seeded on top of  $10 \mu\text{g/ml}$  PCK3145-coated dishes. Cell adhesion was left to proceed for 1 h, and was quantified as described in Materials and methods. (b) Control (Mock) or 67LR-transfected HT-1080 cells were seeded on PCK3145-coated dishes and left to adhere. Representative pictures are taken and show increased adhesion to PCK3145 when 67LR is overexpressed. (c) A time course of cell adhesion to  $10 \mu\text{g/ml}$  PCK3145 shows a rapid and significant increase in 67LR-transfected cells. A representative immunodetection of the 67LR precursor is shown (insert).

and transient effect of PCK3145 suggests that a 'hit-and-run' mechanism of action be envisioned.

#### Laminin and laminin-derived peptide SIKVAV antagonize PCK3145-induced ERK phosphorylation and inhibitory action on MMP-9 secretion

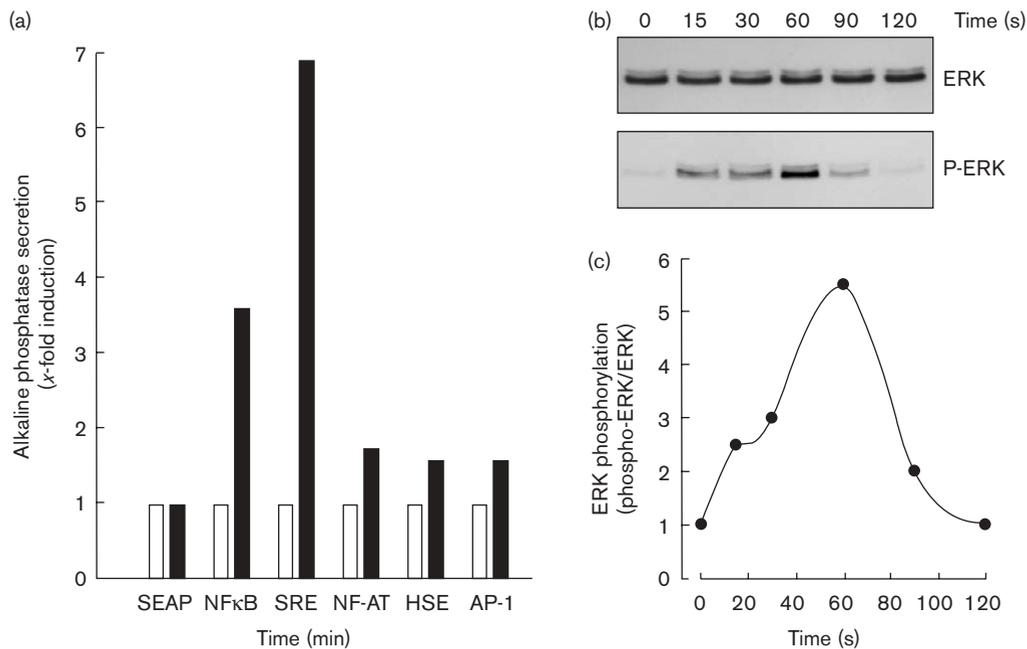
In order to confirm the transduction mechanism involved in PCK3145 inhibitory action on MMP-9 secretion, we treated HT-1080 cells with either the native laminin-1 protein or with a laminin-1-derived peptide SIKVAV. The latter is known for its capacity to induce MMP-9 secretion [20]. Confluent serum-starved cells were treated with these agents in the presence or not of  $300 \mu\text{g/ml}$  PCK3145 for 24 h. Conditioned media was isolated and gelatin zymography performed to monitor MMP-9 extracellular levels. Results show that PCK3145 was indeed able to inhibit MMP-9 extracellular levels by 50% (Fig. 6a, control). Interestingly, SIKVAV-induced MMP-9 secretion was also antagonized by PCK3145 (Fig. 6b), while native laminin-1 inhibited PCK3145

inhibitory action on MMP-9 secretion (Fig. 6b). Finally, PCK3145-induced ERK phosphorylation was also monitored in the presence of laminin-1 and laminin-1-derived peptide SIKVAV. We show that ERK phosphorylation is completely inhibited by the presence of native laminin or SIKVAV (Fig. 6c). Collectively, these results present strong evidence that laminin and laminin-derived peptides interfere with PCK3145 intracellular signaling and subsequent MMP-9 secretion inhibitory actions.

#### PCK3145 inhibits gene and protein expression of the MMP-9 mRNA stabilizing factor HuR

A missing link between laminin and MMP-9 expression still hampers any elucidation of the potential mechanism of action of PCK3145. Recent studies suggested that MMP-9 expression is induced through a stabilizing nuclear factor HuR [21] and that  $\alpha_3\beta_1$  integrin, an integrin known to bind laminin, regulated MMP-9 mRNA [22]. This published evidence links HuR, a mRNA-stabilizing factor that is ubiquitously expressed, and that

Fig. 5



PCK3145 transduces its intracellular effects through the MAPK pathway. (a) HT-1080 cells were transfected with cDNA plasmids encoding the following transcription factor binding elements: Constitutively secreted alkaline phosphatase (SEAP), nuclear factor of  $\kappa$ B cells (NF $\kappa$ B), Serum responsive element (SRE), nuclear factor of activated T-cells (NFAT), heat shock element (HSE) and activator protein-1 (AP-1). Cells were then treated with 300  $\mu$ g/ml of PCK3145 for 24 h, and secreted alkaline phosphatase assessed in the media as described in Materials and methods. (b) Overnight serum-starved quiescent HT-1080 cells were incubated with vehicle (PBS) or 300  $\mu$ g/ml PCK3145 for up to 2 min at 37°C. The cells were scraped from the culture dishes in PBS containing NaF/Na<sub>3</sub>VO<sub>4</sub> and incubated in the same medium buffer for 1 h at 4°C. The resulting lysates were further clarified by centrifugation. Western blotting and immunodetection using anti-phospho-ERK and anti-ERK antibodies was then performed. (c) The extent of ERK phosphorylation in a representative experiment was assessed by scanning densitometry.

has the ability to bind to AU-rich elements (AREs) and prevent mRNA degradation. Interestingly, AREs are expressed in the 3'-untranslated region of MMP-9 [21,23]. Whether PCK3145 regulates HuR expression was analyzed. Serum-starved cells were treated with PCK3145 in the presence or not of laminin or SIKVAV and cells harvested for either RNA extraction or cell homogenates. RT-PCR revealed that HuR gene expression was indeed downregulated by PCK3145, and that both laminin and laminin-derived peptide SIKVAV antagonized PCK3145's inhibitory effect (Fig. 7a). This was further confirmed independently with a specific anti-HuR antibody and immunodetection (Fig. 7b). Furthermore, treatment of the cells with PD98059, a MEK inhibitor, completely reversed the inhibition of HuR expression by PCK3145 (Fig. 7c). This effect of PCK3145 on HuR may partly explain the inhibition of MMP-9 expression and subsequently diminished extracellular levels.

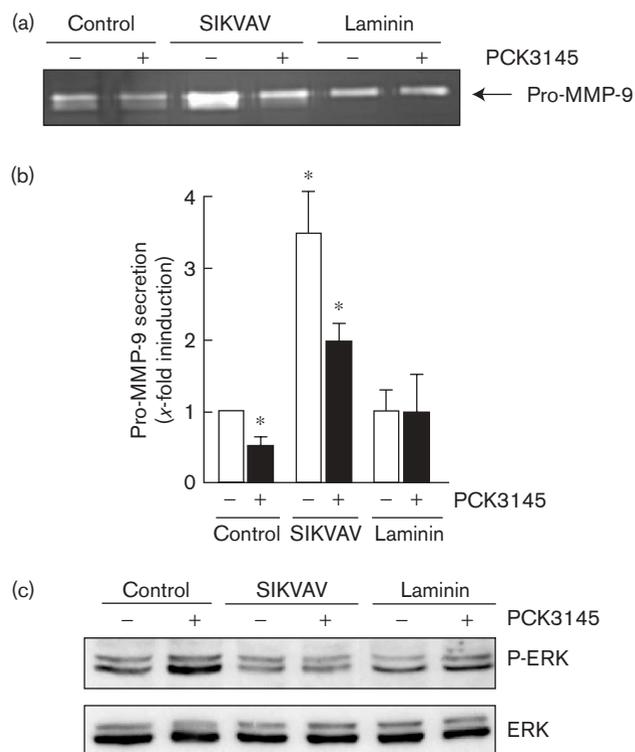
## Discussion

MMP-9 is a MMP involved in prostate cancer progression and for which expression can be regulated at several levels. Although most published studies have focused on transcriptional control of MMP-9, there is increasing

evidence that its expression can also be regulated at the steps of mRNA stability, translation and protein secretion. The ability to modulate MMP-9 expression at multiple steps through distinct signaling pathways may be particularly important during malignant conversion and metastasis, when tumor cells need to induce or maintain MMP-9 levels in response to changing environmental cues. While PCK3145, a 15-mer peptide derived from PSP94, does not affect MMP-9 enzymatic activity *per se*, we show that it significantly reduces its gene expression, which consequently leads to decreased extracellular secreted MMP-9 levels ([24] and this study). We believe that we are now able to integrate the recently obtained experimental data on the mechanism of action of PCK3145 into a cohesive model linking cell surface laminin-binding activities, CD44 cell surface expression modulation and MMP-9 secretion to the metastatic process (Fig. 8).

The novel laminin receptor-mediated regulation of the MMP-9 expression mechanism of action of PCK3145 provides a solid foundation to our hypothesis that PCK3145 acts through receptor-mediated signaling. Further investigations into the molecular basis of laminin

Fig. 6



Laminin and laminin-derived peptide SIKVAV antagonize PCK3145-induced ERK phosphorylation and inhibitory action on MMP-9 secretion. (a) Serum-starved HT-1080 cells were treated with either 300  $\mu$ g/ml PCK3145, 30  $\mu$ g/ml SIKVAV, 30  $\mu$ g/ml laminin or a combination of SIKVAV or laminin with PCK3145. Pro-MMP-9 levels in the conditioned media were assessed by gelatin zymography as described in Materials and methods. (b) A mean of three experiments was quantified by scanning densitometry in non-treated (white bars) and PCK3145-treated (black bars) cells. (c) The extent of ERK phosphorylation was monitored at 1 min of incubation as described in the legend to Fig. 5.

receptor-mediated tumor progression may help unravel new targets for PCK3145. For instance, increased expression of 67LR has been reported in a variety of human carcinomas (colon, breast, stomach, liver and ovary), and directly correlates with a higher proliferation rate of malignant cells and tendency to metastasize [25]. In addition, 67LR is detectable in anaplastic large cell lymphomas and in small subsets of high-grade B cell non-Hodgkin's or Hodgkin's lymphomas [26], and the detection of 67LR in prostate cancer biopsies has been proposed to act as a predictor of recurrence after radical prostatectomy [27]. Moreover, expression of 67LR has been found to mediate acute myeloid leukemia cell adhesion to laminin and to be frequently associated with monocytic differentiation [28]. In light of these documented expression profiles of 67LR, it is thus tempting to suggest that alternate cancers, or cancers at a specific stage of development, such as monocytic-oriented acute myeloid leukemia, could potentially be efficiently targeted by PCK3145.

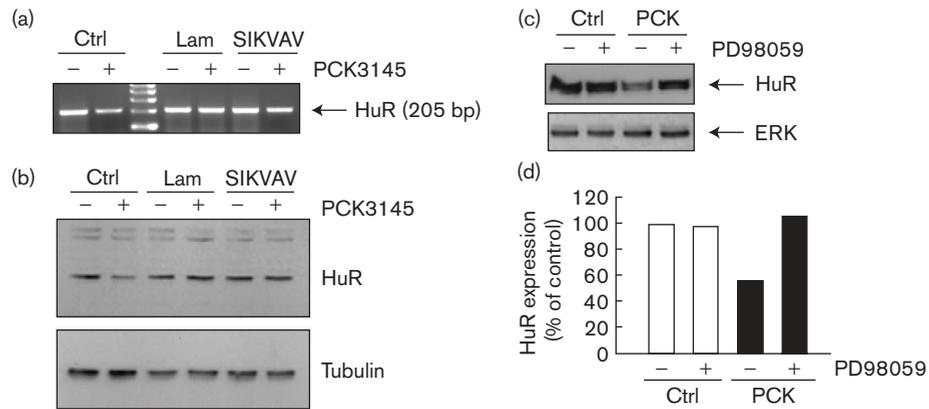
Our data suggest that intracellular signaling by PCK3145, which leads to the inhibition of MMP-9 extracellular levels, is mediated through cell surface laminin receptor-like activity such as that transduced by 67LR. This is reinforced by the unexpected observation that PCK3145 shares, to some extent, homology with different structural chain precursors of laminin including  $\alpha_2$ ,  $\alpha_5$  and  $\beta_1$  chains (not shown), whose characteristic suggests that PCK3145 may also share the potential to interact with common cell surface receptors. Interestingly, EGCg, a green tea catechin that similar to PCK3145 inhibits MMP-9 secretion, is also a 67LR ligand that antagonized cell binding to PCK3145. This confirms that such cell surface receptors may regulate PCK3145 effects. The partial inhibition of the cell surface laminin binding by PCK3145, however, suggests the potential involvement of other cell surface laminin receptors. Undoubtedly, our data support that PCK3145 cell signaling, at least through ERK phosphorylation, involves laminin receptors.

We have identified HuR, a MMP-9 mRNA stabilizing factor, to be targeted by PCK3145. Indeed, both its gene and protein expression were downregulated by PCK3145, and this downregulation was reversed by laminin receptor ligands. PCK3145 can thus be viewed as a laminin receptor-mediated signal transduction inhibitor. Whether alternate intracellular signaling is involved in PCK3145 actions still remains to be investigated although RhoA- and ERK-induced pathways have been highlighted ([8] and this study). As HuR also binds to the AU-rich elements of RNAs encoding genes for cytokines, growth factors, tumor-suppressor genes, protooncogene and cell cycle regulators, one can envision that downregulation of HuR by PCK3145 may also inhibit cell proliferation or induce apoptosis. These alternate cellular processes are currently under investigation.

PCK3145 may transduce its intracellular HuR-inhibitory effects through the binding of specific cell surface receptors of non-integrin origin such as 67LR. Such PCK3145 intracellular transducing events involve ERK, and previous studies have interestingly demonstrated similar cooperativity between integrins and growth factor receptors in the regulation of MAPK signaling pathways. For example, growth factor-dependent induction of ERK signaling in NIH 3T3 cells is strongly dependent on integrin-mediated cell adhesion where the activation of MEK/ERK signaling was identified as an adhesion-dependent event. Our model (Fig. 8) suggests a distinct novel mechanism, whereby cell surface laminin receptor-like activities can cooperate with ECM protein stimuli to induce MEK/ERK expression that could potentially be targeted by PCK3145.

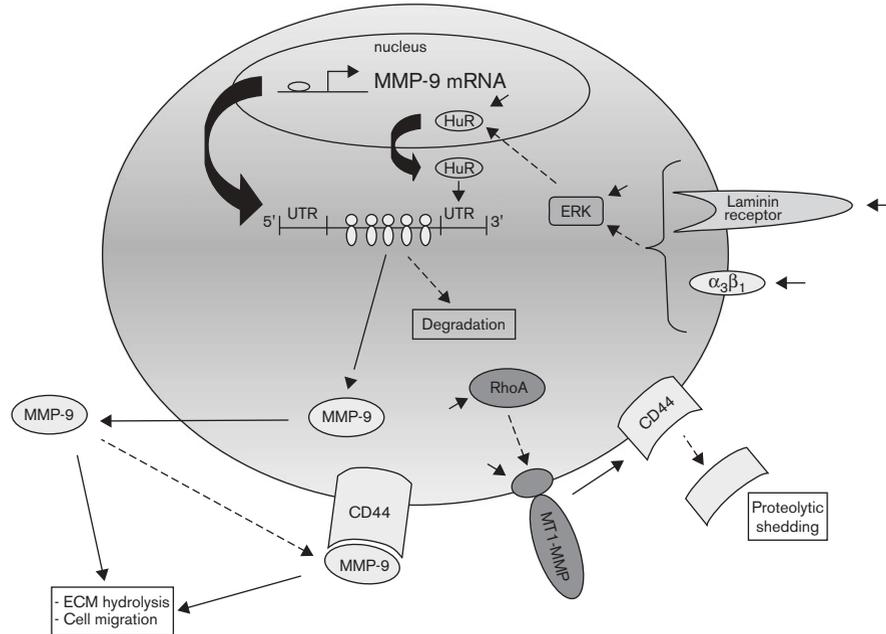
The recently conducted phase IIa clinical trial with PCK3145 confirmed its therapeutic safety and tolerability for HRPC [6]. Interestingly, this effect was in part

Fig. 7



Laminin and laminin-derived peptide SIKVAV antagonize the PCK3145 effect on HuR expression. (a) HT-1080 cells were treated with 300  $\mu$ g/ml PCK3145 in the presence or not of laminin or laminin-derived peptide SIKVAV for 24 h. Total RNA was extracted and RT-PCR performed in order to assess HuR gene expression as described in Materials and methods. (b) Cell lysates from treated cells were used to monitor HuR protein expression by Western blotting. Tubulin expression was used as an internal control. (c) Cells were treated with PCK3145 in the presence or not of PD98059, a MEK kinase pharmacological inhibitor. HuR and ERK protein expression was assessed by Western blotting as above. (d) Densitometric quantification of HuR protein expression was performed in order to assess the extent of PCK3145 effect (black bars) in the presence or not of PD98059.

Fig. 8



Integrative model of the combined anti-metastatic effects of PCK3145 on cancer cells. In light of our results, we propose that PCK3145 transduces its inhibitory effect on MMP-9 secretion, and subsequent downregulation of HuR gene and protein expression, through some laminin cell surface binding activity. We have characterized 67LR as one of the potential cell surface receptors for PCK3145. However, we cannot exclude the implication of alternate laminin-binding activities such as that of  $\alpha_3\beta_1$  integrin, which was recently also reported to regulate HuR expression [22]. HuR downregulation by PCK3145 will favor the degradation of MMP-9 mRNA levels. We also provide evidence for a rapid and transient activation of ERK by PCK3145 that relays the signaling regulating HuR expression and subsequent MMP-9 extracellular levels. A secondary regulation mechanism of MMP-9 functions was also recently reported by us [8], whereas CD44, the docking receptor for extracellular MMP-9, was proteolytically shed from the cell surface through a RhoA/MT1-MMP mechanism [8]. Collectively, as indicated by the arrows, we have highlighted the multilevel molecular mechanism of action of PCK3145 that we believe is involved in its anti-metastatic effects.

correlated to a marked reduction in the high levels of plasma MMP-9 (values above 100 µg/ml), suggesting a biological effect possibly related to control metastasis. Although we have already characterized some of the potential anti-angiogenic and anti-metastatic properties of PCK3145 [7,8], the present study provides the first molecular clue for the potential mechanism of action of PCK3145 against prostate cancer tumor growth and metastasis. The identification of the cell surface laminin receptor activities involved in the transduction of PCK3145 intracellular actions may now help extend the future applications of that therapeutic peptide against other types of cancer in which high levels of MMP-9 and/or laminin receptor expression are associated.

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