Published in final edited form as:

Mol Carcinog. 2017 March ; 56(3): 1182–1188. doi:10.1002/mc.22550.

Targeting Proprotein Convertases in Furin-Rich Lung Cancer Cells Results in Decreased *In vitro* and *In vivo* Growth

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Abstract

Proprotein convertases (PCs) are serine proteases with an active role in the post-translational processing of numerous inactive proteins to active proteins including many substrates of paramount importance in cancer development and progression. Furin (PCSK3), a well-studied member of this family, is overexpressed in numerous human and experimental malignancies. In the present communication we treated two furin-overexpressing non-small cell carcinoma (NSCLC) cell lines (Calu-6 and HOP-62) with the PC inhibitor CMK (Decanoyl-Arg-Val-Lys-Argchloromethylketone). This resulted in a diminished IGF-1R processing and a simultaneous decrease in cell proliferation of two NSCLC lines. Similarly, growth and proliferation of subcutaneous xenografts of both cell lines, were partially inhibited by an *in vivo* treatment with the same drug. These observations point to a potential role of PC inhibitors in cancer therapy.

Keywords

Proprotein convertases; furin; PCSK3; IGF-1R; tumor development; lung cancer

INTRODUCTION

Proprotein convertases (PCs) are serine proteases with an active role in the post-translational activation of numerous inactive proteins to active proteins of great biological relevance (1–2). The PC family comprises several members, PC1/3 (PSC1), PC2 (PCSK2), Furin (PCSK3), PC4 (PCSK4), PC5/6 (PCSK5), PACE4 (PCSK6), PC7/8 (PCSK7), SKI-1 (PCSK8), and NARC-1 (PCSK9). PCs recognize and cleave substrates mostly within the general motif (K/R)-(X)_n(K/R)_↓, where $n = 0, 2, 4$ or 6 and X any amino acid, yet they are not completely redundant, as shown by their different substrates preferences, pattern of inhibition, and phenotypes of the different PC's knockout animals (2–3). Many of the PCs protein substrates have a direct role in tumor progression, as matrix-degrading enzymes (4), growth factors and their receptors, adhesion proteins, and others (5, 6). Furthermore, PCs are overexpressed in numerous malignancies from different sites such as skin, head and neck, ovary, prostate, colon and others (7–12). Amongst all PCs, furin has been the most studied in the context of tumor growth and progression. Furin processes into its mature form and

activates stromelysin 3, a MMP involved in tumor invasion, (13). Similarly, furin activates a family of MMPs, known as membrane type metalloproteinases (MT- MMPs) also involved in tumor invasion and degradation of the extracellular matrix, and several other biomolecules involved in tumor development and progression such as TGF beta, IGF-1R, VEGF-C (1,14–18). Thus, furin is an attractive candidate as a therapeutic target in human cancer because it is overexpressed in human cancer cell lines and primary squamous cell carcinomas from lung, head and neck among many others (8). In addition, previous reports on PCs and cancer describe the increase expression of PCs in primary lung cancers and the possible use of furin as a tumor marker, i.e., furin is overexpressed in non-small cell lung carcinoma and not in small cell carcinomas and has been proposed as a marker to differentiate between these two tumor types (19–21). Given furin's role in processing cancer relevant substrates, its inhibition could open a new therapeutic alternative, either used alone or in combination with other drugs in the treatment of lung cancer. The use of PC inhibitors, alpha 1-PDX (PDX) and CMK (decanoyl-Arg-Val-Lys-chloromethylketone), have been successfully used in pilot studies (reviewed in 22). Herein, we propose to evaluate a panel of lung cancer cell lines for furin levels and treat a selected group of cancer cell lines endogenously producing high levels of furin with CMK. The hypothesis behind this approach is that inhibition of PCs will abolish or significantly reduce the growth and proliferation of lung cancer cells.

MATERIAL & METHODS

Cell lines

A series of human non-small cell lung cancer cell lines were used for this study. The cell lines were obtained from ATCC: Calu-1, Calu-6, A 549, NCI-H226, NCI-H460, NCI-H520, NCI-H522, HOP-92 and HOP-62.

Western Blot Analyses and Immunohistochemistry

Proteins (50 µg cell lysates per experimental condition as described previously (7) were separated using an 8% SDS–PAGE. Antibodies used for immunoblot analysis included antiIGF-1R β-subunit (C-20, sc-713, Santa Cruz, CA.) and furin monoclonal antibody MON-152 (ALX-803–017; ALEXIS). The quantification of Western blot results was performed by using ImageJ developed by NIH. Furin IHC was performed with the same antibody using paraffin embedded normal lung and tumors. P-Histone 3 immunohistochemistry was used to detect cells in the G2-M phases of mitosis. The primary anti phospho-Histo 3 (ser10) rabbit antibody, catalog number 9701, from Cell Signaling (Danvers, MA) was applied to paraffin sections as recommended by the supplier. Once stained, the mitotic index was calculated by counting positively stained p-Histone 3 cells per 200 X field (each field equivalent to 0.32 mm²) in ten to fifteen fields per tumor. A total of 5 control and 5 CMK treated xenotransplants were used per cell line xenografts. All paraffin sections were subjected to a previously published immunostaining protocol using positive and negative controls (8–9). All specimens were analyzed and counted using a Nikon Optiphot with a Plan/Apo objective 20X, NA, 0.75, Nikon eyepiece X10, final magnification X200.

CMK Treatments

Cells were treated with a general convertase inhibitor, CMK (decanoyl-Arg-Val-Lys-chloromethylketone), ALX-260-022, Enzo Life Sciences, Plymouth Meeting, PA) at different concentrations (50 and 100 μ M). Twenty-four hours after CMK treatment, cells were lysed and subjected to Western blot analysis for IGF-1R β .

Cell Growth Assay

[³H]-thymidine incorporation was evaluated after plating in 2.5×10^5 cells/well in 6-well culture plates. Twenty-four hours after plating, cells were incubated in a growth arrest medium (0.5% FBS-MEM, L-Glu, Pen-Strep) for 24 hours. Then, cells were incubated an additional 24 hours either in the presence of serum. When indicated, the furin inhibitor, CMK, 50 or 100 μ mol/L was included in the incubation mixture. For the last 4 hours of incubation, 3 μ Ci/well [³H]methylthymidine (Moravek, Biochemicals, Brea, CA) was added. After this period of time, cells were washed three times with phosphate-buffered saline (PBS) and treated with 10% trichloroacetic acid at 4°C for 30 minutes and washed three times with water. Cells were then rinsed with 70% ethanol and air-dried. Labeled DNA was dissolved with 200 μ l 10 mmol/L NaOH, 1% SDS and counted with Scintiverse (Fisher, Pittsburgh, PA).

In Vivo Tumorigenicity

A total of 2.5 million Calu-6 or HOP-62 cells per mouse were inoculated sc into 10 nude mice per cell line. Tumors were detected seven days after cell inoculation, and the initial volume was measured (200–300 mm³). At this stage, CMK, dissolved in DMSO:PBS (1:10) to a final concentration of 200 μ M, was injected intratumorally. Control animals (n=5) were injected with the vehicle only. Tumors were measured every other day using a Vernier caliper. Volumes (V) of the tumors were obtained by using the following equation: $V = [(L1 + L2)/2] \times L1 \times L2 \times 0.526$, where L1 and L2 are the length and width of the s.c. tumor. The statistical significance of the differences of tumor development and mitotic indices were calculated using a Wilcoxon two sample tests and/or two tailed t-test.

RESULTS

Expression levels of furin in tumor cell lines and primary tumors

After the detection of proprotein convertase (PC) expression in human cell lines was completed using RT-PCR we confirmed by Western analysis that most human non-small cell lung cancer (NSCLC) cell lines (5/9) expressed furin protein (Figure 1A). Two cell lines expressed higher levels of furin expression (Calu-6 and HOP-62) than any of the other cell lines, whereas high to moderate expression was seen in A549, NCI-H460 and H522 cell lines and was minimal to absent in Calu-1, HOP-92, NCI H-520 and NCI H-526 cells. Immunohistochemical analysis showed that normal bronchial epithelium was negative for furin (Figure 1B) and 17 intraepithelial neoplastic lesions of the bronchi (dysplastic lesions and carcinoma in situ) showed moderate furin expression in approximately 50% of the lesions, especially in the basal and parabasal cells (Figure 1C). Human primary tumors (58 lung adenocarcinomas and 39 squamous cell carcinomas) showed moderate to strong furin

stain in the cytoplasm of 50 and 75 % of these tumors, respectively (Figure 1D to H). Only 16% of all tumors were negative. No significant differences were seen in tumors of different grade, stage, or survival. Thus, furin expression gradually increases during the process of tumorigenesis but reaches its peak in full-fledged invasive carcinomas.

Effects of Furin Inhibition of Lung Cancer Cells In Vitro

In order to evaluate the effects of inhibiting furin expression, Calu-6 (a high furin expressor), was transfected with furin siRNA. Knocking down furin with siRNA would thus establish a proof of principle for inhibition of this PC. As expected siRNA transfection decreased markedly the expression of furin as compared to the respective control scrambled RNA-transfected counterparts (Figure 2A). The most effective consequence of furin inhibition was a decrease in the IGF-1R processing (Figure 2B) and in the proliferative ability of the knocked-down cells (reduction of up to 60% in Calu-6 cells) (Fig. 2C). The decreased processing of the furin substrate IGF-1R (seen as an increase in the intensity of the band corresponding to the Pro IGF-1R in figure 2B) was detected by Western analysis. A similar result was obtained when treating the cells with the PC inhibitor CMK (figure 3). Treating Calu-6 (figure 3 A-B) with 100 μ M CMK reduced markedly cell proliferation (approximately 50%). This was accompanied as well by an increased accumulation of the IGF-1R pro-form, indicating a reduction in processing after CMK treatment (Figure 3 A). The decreased activation of IGF-1R in Calu-6 cells was dosedependent as seen in Figure 3 A. This figure shows that CMK processing was inhibited using two different concentrations of CMK, with the higher dose having a marked effect on the accumulation of the pro-form. A similar gradient of inhibition of cell proliferation was seen after a short term CMK treatment (overnight) using the same two concentrations of CMK (Figure 3 B). A long term treatment of these cells (one week, new dose of CMK every 2 days) showed the same effect as the short term treatment (data not shown).

This experiment was repeated with a different NSCLC cell line, namely HOP-62, also a high furin expressor, with very similar results (Figure 3 C-D). This cell line also showed a remarkable accumulation of the IGF-1R pro-form after exposure to different concentrations of CMK (Figure 3 C). We also attempted to compare a single short-term exposure to CMK (24hs) versus multiple exposures (3 treatments, every second day the medium containing fresh CMK was replaced and proliferation was evaluated 24hs after the third treatment) (not shown). No major difference in proliferation between the two modalities was demonstrated. In both cases the proliferation rate was reduced in approximately 33%.

Effects of Furin Inhibition on In Vivo Growth and Proliferation of Xenotransplanted Human Lung Cancer Cells

We selected the Calu-6 cell line for *in vivo* analysis because these cells were more sensitive to the *in vitro* effects of CMK than the other cell lines tested, e.g., a concentration of 100 μ M of CMK decreased about 50% the proliferation rate in this aggressive cell line. As can be seen in Figure 4 panel A, CMK delayed *in vivo* growth, as noticed by the reduced slope in the growth rate corresponding to the treated animals. This difference in the growth rate reflected in a reduced tumor volume of approximately 45% ($P=0.05-0.09$ in the last four time points). A similar experiment using HOP-62 xenografts was performed. The results

show the same tendency (Figure 4B), with a decrease of tumor volume in the last time points of approximately 30% ($P=0.04$).

The proliferation changes in xenografts at the end of three weeks after cell inoculation was evaluated in paraffin tissue sections using immunohistochemical detection of p-histone 3, a marker of the G2-M cell cycle stages, i.e., a labeling index was calculated that is basically equivalent to the classical mitotic index used in pathology. This parameter showed a decrease in mitotic index after CMK treatment in both cell lines. Although the difference was more marked in CMK treated versus untreated Calu-6 cells ($P=0.001$) the difference was also significant when comparing treated and control HOP-62 cells ($P=0.01$) (Figure 4C-G).

DISCUSSION

Furin is an ubiquitous PC that has been detected in numerous carcinomas of different types and sites. Its ability to activate a large number of cancer-associated substrates that are bonafide enhancers of the malignant phenotype and result in increased aggressiveness through tumor cell hyperproliferation and invasiveness makes this PC a promising target for cancer therapeutics. General inhibitors of PCs such as PDX and CMK have been used with success in *in vitro* and *in vivo* experimental settings (7, 16, 22–23). Several putative furin-specific inhibitors that have promising properties have been recently proposed (24–26). Therapies using an autologous tumor-based product containing a plasmid encoding granulocyte-macrophage colony-stimulating factor and a bifunctional short hairpin RNAi that targets furin called FANG or *Vigil* have been or are being evaluated in clinical trials with patients suffering from advanced cancer of the ovary, liver and sarcomas (27–29). The mechanism of action of *Vigil* includes the blockage by shRNA of furin protein production. This reduction results in a decrease in conversion of transforming growth factor TGF- β into active TGF- β 1 and TGF- β 2 protein isoforms (15,30) that results, among several other cancer cell changes, in inhibition of immunosuppression (31). In our laboratory we have found that PDX inhibition decreases processing of TGF-beta in head and neck squamous cell carcinoma cells and astrocytoma cell lines together with decreased growth and invasiveness of these cells (32–33). Other furin substrates such as MT-MMPs, stromelysin-3, and IGFR-1 were equally affected (1, 22, 32–33). In the present report we have demonstrated that PC inhibition with CMK of furin-rich lung cancer cell lines results in a diminished IGF-1R processing resulting in a decreased cell proliferation of two non-small cell lung carcinoma cell lines. In addition to IGFR-1, other PC substrates including IGF-1 itself and PDGF-B (34) may be involved in the PC regulation of cell proliferation. Nevertheless, IGFR-1 has been shown to be one of the most frequently involved growth factor receptor in studies investigating the role of PCs in tumor cell proliferation (1,7,12,16). Furthermore, together with decreased cell proliferation we have demonstrated that growth of *subcutaneous* xenografts of two NSCLC cell lines, was also partially halted by an *in vivo* treatment with CMK.

Taken together these results constitute “a proof of principle” showing that furin inhibition of NSCLC growth can be accomplished *in vitro* as well as *in vivo*. In both cases, we have found that PC inhibition was accompanied by a decrease in cell proliferation. These results

open a new possibility, i.e., that therapies with PC inhibitors alone or combined with traditional and novel therapeutic agents might improve the dismal prognosis of lung cancer.

Acknowledgements

This work was supported in part by grants from the National Institutes of Health, R01CA133001 and P30CA06927, and by an appropriation from the Commonwealth of Pennsylvania. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health.

Abbreviations:

CMK	decanoyl-Arg-Val-Lys-chloromethylketone
IHC	Immunohistochemistry
NSCLC	Non-small cell lung carcinoma
PDX	alpha 1-PDX (alpha-1 antitrypsin Portland variant)
PCs	Proprotein convertases

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Figure 1:

A: Western analysis of human lung cancer cell lines. MON-152 detects the expression of furin, GAPDH represents the loading control. Densitometric evaluation of the blots are expressed as ratios under each lane. This panel illustrates that Calu-6 and HOP-62 cells have very high levels of furin expression, especially when compared with A459, NCI-H460 and H-522 cells that show relatively less furin expression and especially with lines Calu-1 HOP-92, H-520 and H-526 that show minimal expression.

Immunohistochemistry of furin innormal (B), and a preneoplastic lesion(C), from bronchial specimens show negative immunohistochemical stain in the normal bronchial epithelium and positive stain in the precursor lesion. The bar graph (D) shows the distribution of furin staining intensity, i.e., negative, 1 plus, 2 plus and 3 plus in IHC stained lung tumors (y axis: % tumors; white columns: squamous cell carcinomas (SCC), grey columns: adenocarcinomas (AdCA) and black columns: all non-small cell lung carcinomas (NSCLC). Example of scoring scale are depicted in panels E to H. Panel E: negative, Panel F: 1 plus, Panel G: 2 plus and Panel H: 3 plus. Furin IHC & hematoxylin counterstain.

Figure 2:

Furin expression was knocked down with siRNA (A) using Calu-6 cells, levels of furin RNA were determined by real time PCR (Histogram) and Western analysis (lower panel A).

IGF-1R processing was detected by Western blotting (B) and the effects on cell proliferation by [3 H] thymidine incorporation (C).

Figure 3: Calu-6 (A and B) or HOP-62 cells (C and D) cells were treated with two concentrations of the furin inhibitor CMK. The inhibition of IGF-1R processing was dosedependent (A, C). Also the proliferation rates decreased after overnight treatment with CMK (B, D).

Figure 4:

Calu-6 and HOP-62 (xenografts were injected intratumorally daily either with 200 μ M CMK or with vehicle alone. Tumors were measured every two or three days. Volume changes of both types of xenografts showed a reduction of volume after CMK treatment (A & B) ($P=0.04$ and 0.06). Solid line (control), dotted line (CMK treatment). Better levels of significance ($P=0.001$ and 0.01 for Calu-6 and HOP-62 respectively) were seen when the mitotic index (evaluated as labeling index of p-Histone 3) in paraffin sections at the last time point of treatment (control versus CMK treated tumors) were compared. Values are expressed in the y axis as mean number of positively stained nuclei/microscopic field $\pm$ SEM (C). Panels D to E show the immunohistochemical detection of p-Histone 3 in Calu-6 control xenografts (D), Calu-6 xenografts treated with CMK (E), HOP-62 xenografts control

(F) and HOP-62 xenograft treated with CMK (G). The latter four panels were stained with p-Histone 3 IHC, counterstained with hematoxylin and digitally photographed at X200.

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