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„Small Cell Lung Cancer: Chemosensitivity to Fascaplysin and Significance of secreted Proteases."

verfasst von / submitted by

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1 Preamble and Concluding Discussion

The topic of both manuscripts is the characterization of Small Cell Lung Cancer (SCLC) cells and the evaluation of a cytotoxic drug as well as the assessment of proteolytic enzymes as possible drug targets to prevent metastasis. SCLC is an aggressive neuroendocrine tumor which is caused by extensive tobacco consumption. It is generally detected at a disseminated stage in most patients and although first-line chemotherapy with platinum-based combination chemotherapy is highly efficient, the tumor relapses invariably after approximately one year as chemoresistant disease with a dismal prognosis.

In the first study, a marine drug, namely fascaplysin, was investigated for antitumor activity *in vitro* against SCLC cell lines, SCLC circulating tumor cell (CTC) lines and Non-Small Lung Cancer (NSCLC) lines (Chapter 2.1.). A panel of SCLC CTC lines was established in our lab for the first time world-wide. CTCs are released by primary tumors and function in established metastases in distal organs, including the brain. Global chemoresistance of SCLC seems to be associated with the spontaneous formation of large spheroids, termed tumorospheres, which exhibit increased resistance to chemotherapeutics due to decreased drug perfusion and presencence und hypoxic cells. Fascaplysin is a Cyclin-dependent kinase 4 (CDK4) inhibitor and intercalates in DNA which resulted in marked toxicity against SCLC and SCLC CTC cell lines. Investigation of the alterations in signal transduction indicated a DNA damage response. In contrast to the conventional chemotherapeutics, fascaplysin revealed high activity against the large spheroids and showed an additive effect in combination with cisplatin.

In the second work dealing with proteolytic enzymes expressed by SCLC and SCLC CTC cells, protein expression of the secreted enzymes was assayed using Western blot arrays which detect 35 proteases (Chapter 2.2.). For these experiments, SCLC cell lines GLC14, GLC16, SCLC26A and NCI-H526 and SCLC CTC lines BHGc7 and BHGc10 were employed. Expression of matrix metalloproteinases (MMPs), ADAM/TS, cathepsins, kallikreins and others exhibited characteristic differences between relapsed/invasive and local SCLC lines. In detail, MMP9 and cathepsin S seem to be associated with an invasive SCLC phenotype. Whereas inhibition of MMPs were not successful in clinical trials, specific inhibition of cathepsin S which has been reported to be involved in brain metastasis may constitute an interesting way to target this fatal SCLC dissemination.

In conclusion, both manuscripts by Barbara Rath et al. describe new methods to either eliminate invasive and resistant SCLC cells or to impair spread of SCLCs to brain tissue. Since clinical care of SCLC has not been improved for the last decades such new data is expected to contribute to new therapeutic modalities.

2 Synopsis of the Publication

2.1 Anticancer Activity of Fascaplysin against Lung Cancer Cell and Small Cell Lung Cancer Circulating Tumor Cell Lines

[1] Rath B, Hochmair M, Plangger A, Hamilton G. Anticancer Activity of Fascaplysin against Lung Cancer Cell and Small Cell Lung Cancer Circulating Tumor Cell Lines. Mar Drugs. 2018 Oct 14;16(10). pii: E383. doi: 10.3390/md16100383

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Contribution of the applicant to the work; Barbara Rath cultivated the respective cell lines in tissue culture, performed the cytotoxicity tests (MTT assay), including data evaluation (OriginLab, Northhampton, MA, USA) and filing. She carried out the Western blot arrays for phosphoproteins including data evaluation (Image J, OriginLab). Furthermore, she made all tables and figures for the publications and was involved in discussion of the results, writing of the manuscripts as well as scientific literature research and referencing.

Mar. Drugs **2018**, *16*(10), 383; doi:10.3390/md16100383

Article

Anticancer Activity of Fascaplysin against Lung Cancer Cell and Small Cell Lung Cancer Circulating Tumor Cell Lines

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Abstract

Lung cancer is a leading cause of tumor-associated mortality. Fascaplysin, a bis-indole of a marine sponge, exhibit broad anticancer activity as specific CDK4 inhibitor among several other mechanisms, and is investigated as a drug to overcome chemoresistance after the failure of targeted agents or immunotherapy. The cytotoxic activity of fascaplysin was studied using lung cancer cell lines, primary Non-Small Cell Lung Cancer (NSCLC) and Small Cell Lung Cancer (SCLC) cells, as well as SCLC circulating tumor cell lines (CTCs). This compound exhibited high activity against SCLC cell lines (mean IC_{50} 0.89 µM), as well as SCLC CTCs as single cells and in the form of tumorospheres (mean IC_{50} 0.57 µM). NSCLC lines showed a mean IC_{50} of 1.15 µM for fascaplysin. Analysis of signal transduction mediators point to an ATM-triggered signaling cascade provoked by drug-induced DNA damage. Fascaplysin reveals at least an additive cytotoxic effect with cisplatin, which is the mainstay of lung cancer chemotherapy. In conclusion, fascaplysin shows high activity against lung cancer cell lines and spheroids of SCLC CTCs which are linked to the dismal prognosis of this tumor type. Derivatives of fascaplysin may constitute valuable new agents for the treatment of lung cancer.

Keywords: fascaplysin; lung cancer; circulating tumor cells; signal transduction; cytotoxicity; cisplatin

1. Introduction

Among malignant diseases, lung cancer is the leading cause of mortality [1]. NSCLC constitutes the most common subtype with approximately 85% of cases and a 5-year survival rate ranging from 50–17%, depending on the stage of the disease [2]. SCLC accounts for the rest of the cases; it is associated with smoking and has a poor prognosis upon dissemination [3]. NSCLC tumors feature a similar poor prognosis, except for those variants amenable to specific therapies directed to mutated epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), and other kinases [4,5]. Targeted therapies in the form of tyrosine kinase inhibitors (TKIs) and immunotherapy directed to checkpoint proteins have successfully changed the treatment of NSCLC; however, patients lacking markers for precision medicine or eventually progressing after specific regimens are nevertheless referred to classical chemotherapy consisting of platinum-drug-based combinations [6]. Cisplatin/carboplatin combined with either etoposide, docetaxel, or pemetrexed have limited clinical activity, and new agents may lead to increased responses and survival. The dismal prognosis of SCLC seems to be linked to the formation of large spheroidal aggregates, termed tumorospheres, which are difficult to eliminate due to poor drug perfusion and to the existence of quiescent and hypoxic tumor cells in the interior layers of the 3D-structures [7]. A host of diverse drugs have failed to provide clinical improvements for SCLC in recent decades [3].

The marine drug fascaplysin (12,13-Dihydro-13-oxopyrido[1,2-a:3,4-b′] diindol-5-ium chloride) is a red bis-indole alkaloid of the Fascaplysinopsis Bergquist sp. sponge which was isolated by Roll et al. in 1988 [8]. The structure of fascaplysin is shown in Figure 1. Novel derivatives comprise 3-bromo-fascaplysin, 4-chloro-fascaplysin, and 7-phenyl-fascaplysin, among others. Fascaplysin possesses antibacterial, antifungal, and antiviral properties as well as antiangiogenic and antiproliferative activity against a range of cancer cell lines [9,10,11]. Cyclin-dependent kinase 4 (CDK4) was reported as the main target of fascaplysin (IC_{50} of 0.35 µM), and accordingly, drug-treated cancer cell lines arrested

preferentially in the G0/1 cell cycle phase [12,13,14]. Minor activity of fascaplysin was observed against other CDKs with IC_{50} of >100 µM for CDK1, >50 µM for CDK2 as well as 20 µM for CDK5 [14]. In addition, fascaplysin was demonstrated to exhibit DNA-intercalating capability with an affinity similar to those of other typical DNA intercalators [15]. Non-planar derivatives of fascaplysin have been developed in order to possibly reduce non-CD4-mediated cytotoxic effects [16].

Figure 1. Structure of fascaplysin.

Cytotoxicity tests showed broad activity of fascaplysin towards a panel of 36 cancer cell lines (IC_{50} values 0.6–4 µM) [9]. Anticancer activities of fascaplysin in cell lines in vitro resulted in reduced expression of CDK4, cyclin D1 and downregulation of CDK4-specific Ser795 retinoblastoma (Rb) phosphorylation in HeLa cells [17]. Fascaplysin-induced apoptosis was characterized by the activation of effector caspases, relocalization of cytochrome c into cytosol, and reduced expression of Bcl-2. Cytotoxicity of fascaplysin in chemosensitive promyelocytic HL-60 cancer cells activated both pro-apoptotic events like PARP-1 cleavage/caspase activation and triggered autophagy, as shown by the increased expression of LC3-II, ATG7 and beclin [17]. In experimental animal models, fascaplysin suppressed tumor growth in a murine sarcoma S180 through apoptosis as well as antiangiogenesis, and HCT-116 colon tumors showed reduced size in the absence of drug toxicity [18]. Angiogenesis was blocked by fascaplysin by the inhibition of vascular endothelial growth factor (VEGF) and apoptosis of endothelial cells [19].

SCLC responds to first-line chemotherapy with platinum-based drugs/etoposide, but relapses early with topotecan remaining as the single approved therapeutic agent [3]. We have previously assessed cytotoxic activity of fascaplysin against SCLC cell lines, not covered by the NCI60 cell line panel, a tumor entity that accounts for a significant fraction of lung cancer deaths [20]. Fascaplysin was found to show high cytotoxicity against SCLC cells and to induce cell cycle arrest in G1/0 at lower and S-phase at higher concentrations, respectively. The compound generated reactive oxygen species (ROS) and induced apoptotic cell death in the chemoresistant NCI-H417 SCLC cell line. Furthermore, fascaplysin revealed marked synergism with camptothecines [21,22]. Fascaplysin IC_{50} values measured in SCLC cell lines were found to be similar to the two chemoresistant NSCLC cell lines H1299 and A549 and the chemosensitive H23 cell line, respectively.

In the present work, the investigation of the cytotoxic effects of fascaplysin is extended to include single cell suspensions and spheroids of SCLC circulating tumor cells (CTCs) and several NSCLC cell lines. Our lab has established a panel of 6 CTC SCLC cell lines derived from the blood samples of distinct patients with extended disease SCLC [7]. Furthermore, the effects of fascaplysin on the main pathways of cellular signal transduction and stress response were assessed employing phosphoprotein Western blot arrays and the NCI-H526 SCLC and the A549 NSCLC cell line, respectively.

2. Results

2.1. Fascaplysin Cytotoxicity against SCLC, NSCLC and Non-lung Cancer Cell Lines

The chemosensitivity of a range of cancer cell line to fascaplysin was measured in MTT cytotoxicity assays. Figure 2 shows the IC_{50} values of breast cancer and ovarian cell lines (range: 0.48–1.21 µM), SCLC cell lines (range: 0.2–1.48 µM) and NSCLC cell lines (range: 0.63–2.04 µM). Whereas SCLC and breast/ovarian cancer cell lines exhibited similar mean IC_{50} values (0.96 ± 0.5 versus 0.89 ± 0.45 µM), NSCLC cell lines proved to be less sensitive (1.15 ± 0.59 µM). SCLC26A and S457 are primary SCLC cell lines derived from pleural effusions of patients before and after therapy failure, respectively. The nonmalignant HEK293 cell line showed an IC_{50} value of 1.6 ± 0.42 µM. BH295 and IVIC-A are primary NSCLC cell lines derived from pleural effusions of patients with ALK and EGFR TKI resistance. Numerical values of the IC50 data are presented in Supplementary Table S1.

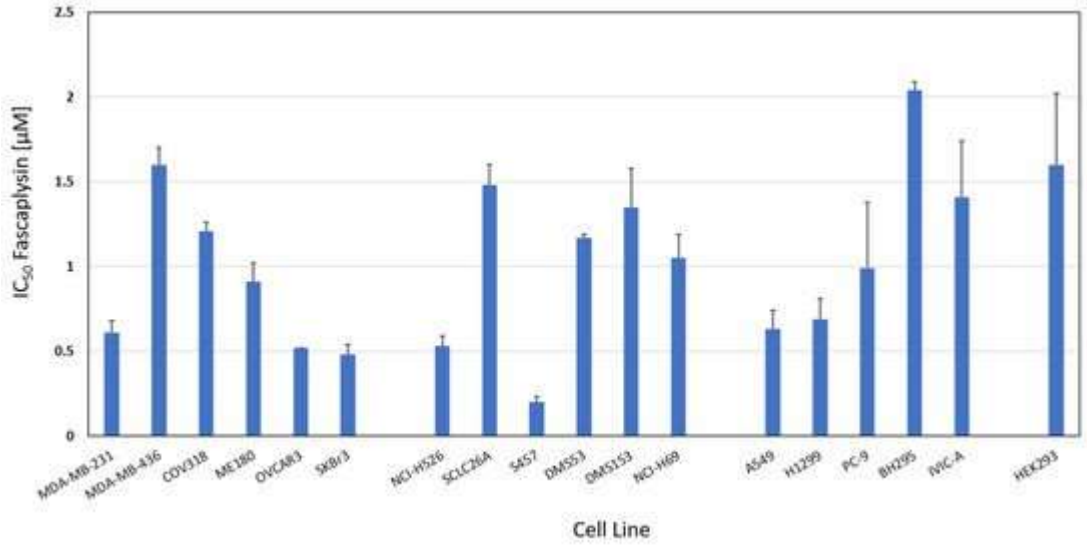

Figure 2. Fascaplysin chemosensitivity of a panel of SCLC, NSCLC and a panel of non-lung cancer lines. IC_{50} values are presented as mean values ± SD. Non-lung cancer cells used for comparison are breast and ovarian cancer cell lines and nonmalignant HEK293 cells are shown as normal tissue control.

2.2. Fascaplysin Cytotoxicity against SCLC CTC Single Cells and Tumorospheres

The SCLC CTCs form spontaneously large spheroids which are markedly chemoresistant to cisplatin and other drugs used for the treatment of SCLC patients in comparison to CTCs in form of single cell suspensions. The chemosensitivity of such single cell suspensions and tumorospheres against fascaplysin were compared in MTT tests (Figure 3). With exception of BHGc26 and 27 CTC lines, fascaplysin IC_{50} values of the other lines were equal or below 0.5 µM. A comparison of the ratios of IC_{50} values of single cell suspensions and tumorospheres for cisplatin and fascaplysin demonstrates that for fascaplysin, the differences in chemosensitivities between these 2D- and 3D-cultures are much less than for the platinum drug (Table 1) indicating superior anticancer activity for spheroids.

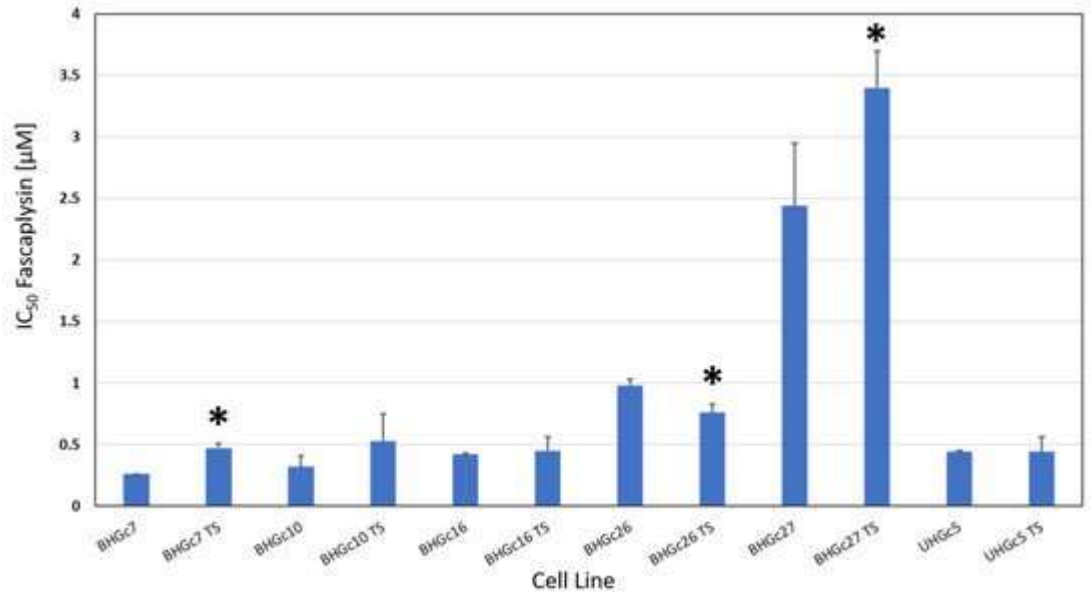

Figure 3. Chemosensitivity of SCLC CTC lines for fascaplysin. The CTC lines were tested in form of single cell suspensions and as tumorospheres. IC_{50} values are presented as mean values ± SD and significant differences between 2D- and 3D-cultures are indicated by an asterisk.

Table 1. Mean values of IC_{50} ratios for SCLC CTC tumorospheres versus single cell suspensions for fascaplysin and cisplatin, respectively (mean values ± SD). All ratios for fascaplysin, cisplatin, and the CTC lines are significantly different.

CTC Cell Line	Fascaplysin		Cisplatin	
	Mean Ratio (TS/SC)	SD	Mean Ratio (TS/SC)	SD
BHGc7	1.83	0.1	4.31	0.2
BHGc10	1.63	0.2	2.32	0.4
BHGc16	1.06	0.1	7.22	0.3
BHGc26	0.77	0.1	5.20	0.3
BHGc27	1.39	0.5	2.17	0.2
UHGc5	0.99	0.1	4.8	1.0

Fascaplysin versus cisplatin showed a 1.5 fold increased cytotoxic activity for tumorospheres for BHGc10 and BHGc27, 2.5 fold for BHGc7 and UHGc5, and 6.7 fold for BHGc16 and 26, respectively (Table 1). The mean cytotoxicity ratios between fascaplysin and cisplatin are significantly different for all SCLC CTC cell lines.

2.3. Alterations of Selected Phosphoproteins of NCI-H526 and A549 in Response to Fascaplysin

Figure 4 shows the first part of the phosphoproteins assayed with the ARY003 human proteome profiler kit for fascaplysin-treated NCI-H526 and A549, respectively. In contrast to the cytotoxicity assays, incubation time for phosphoprotein analysis was reduced to 72 h to prevent cell death. In NCI-H526 SCLC cells fascaplysin induced significantly increased phosphorylation of src kinases (Hck, Fyn, Yes and Fgr), CHK-2 and FAK, whereas phosphorylation of mTOR, CREB and p38α was significantly decreased compared to untreated controls. In contrast, A549 NSCLC cells revealed increased phosphorylation of CHK-2 in combination with CREB, HSP27, and STAT5b, with decreased phosphorylation of src kinases (except Fgr) and FAK.

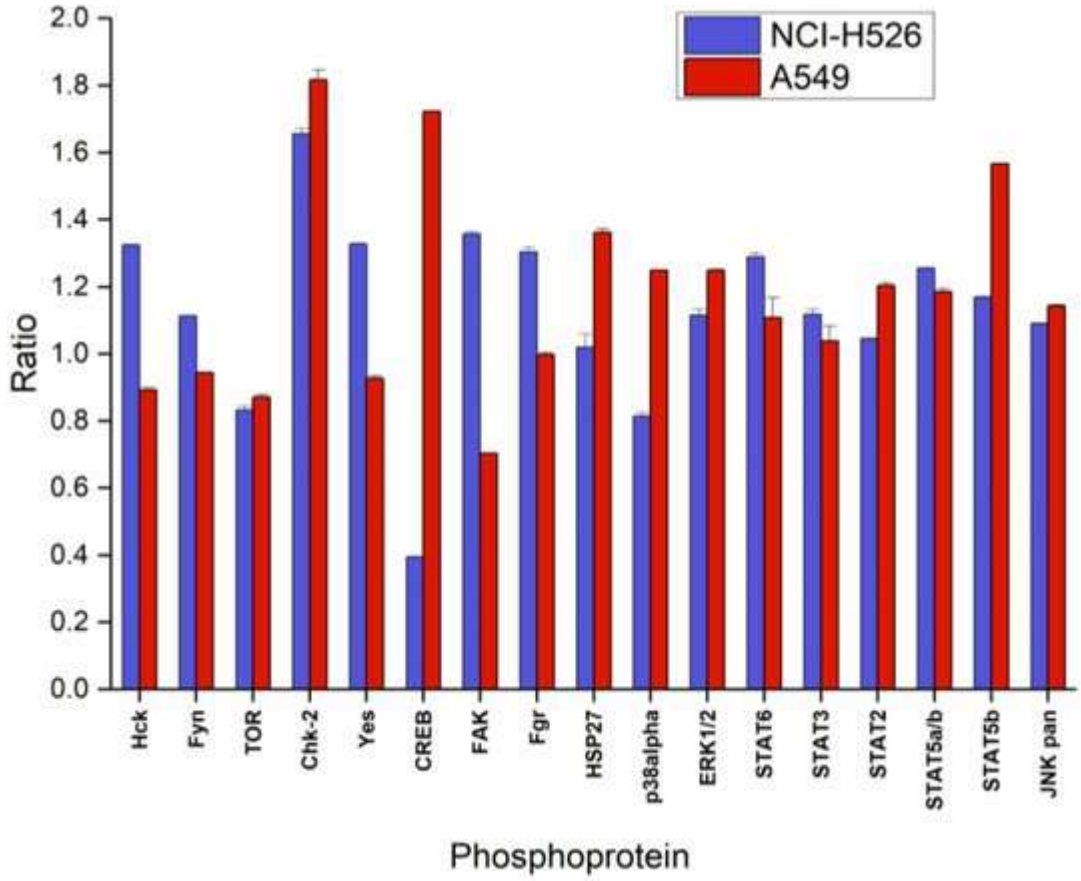

Figure 4. Relative phosphorylation (ratio of treatment:control) of selected components (part A of the array) of the signal transduction system (mean ± SD) of NCI-H526 and A549 cells treated with 0.5 µM fascaplysin for 72 h (NCI-H526: significantly different to controls, except for HSP27 and STAT2; A549 significantly different to controls, except Fgr and STAT3).

Analysis of the second part of phosphoproteins of the ARY003 kit yielded decreased phosphorylation of Akt, p53(S46/S392) and increased phosphorylation of STAT4, eNOS, c-Jun, and p27(T157) in the case of fascaplysin-pretreated NCI-H526, and numerous increases of phosphorylation in A549 cells, with the exception of decreases in p70 S6 kinase, STAT4, and p53(S392) (Figure 5). Phosphoproteins of the ARY003 blots which exhibited no significant changes for NCI-H526 or A549 cells in response to treatment with fascaplysin were not included in these figures.

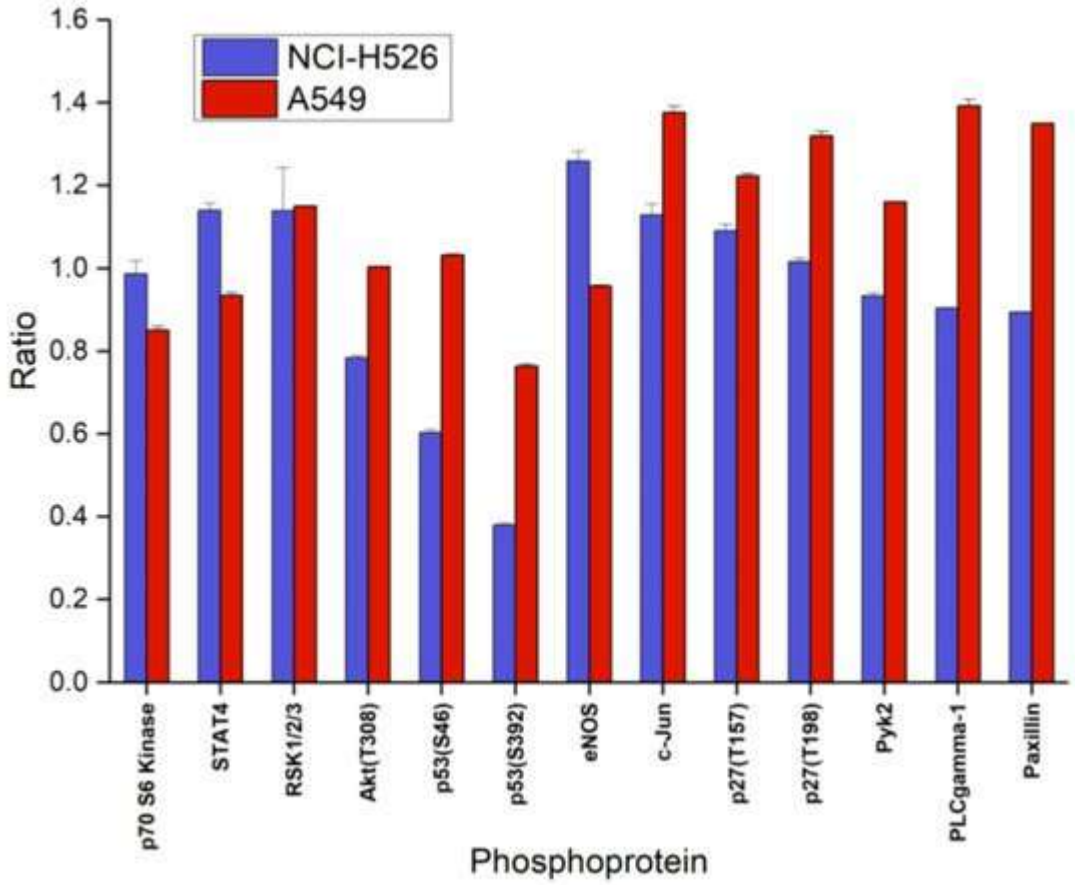

Figure 5. Relative phosphorylation (ratio treatment: control) of selected components (part B of the array) of the signal transduction system (mean ± SD) of NCI-H526 and A549 cells treated with 0.5 µM fascaplysin for 72 h (NCI-H526: significantly different to controls, except forp70 S6 kinase and p27; A549 significantly different to controls, except Akt, p53/S46, and eNOS).

2.4. Signaling Pathways Affected by Fascaplysin in NCI-H526 and A549 Lung Cancer Cells

The signal transduction mediators related to fascaplysin-induced alterations in NCI-H526 and A549 cells are depicted schematically in Figure 6. The schemes start with fascaplysin-induced DNA damage (left) and receptors/src kinases (right), respectively. Chk2 is activated by upstream DNA damage-sensing ATM and modulate functions of CREB, p53, CDC25, and stress kinases (left). Src kinases are activated by a number of connected membrane receptors (X) or oncogenic mutation, and regulate the activities of Stat5, FAK, and the Akt–mTOR axis (right).

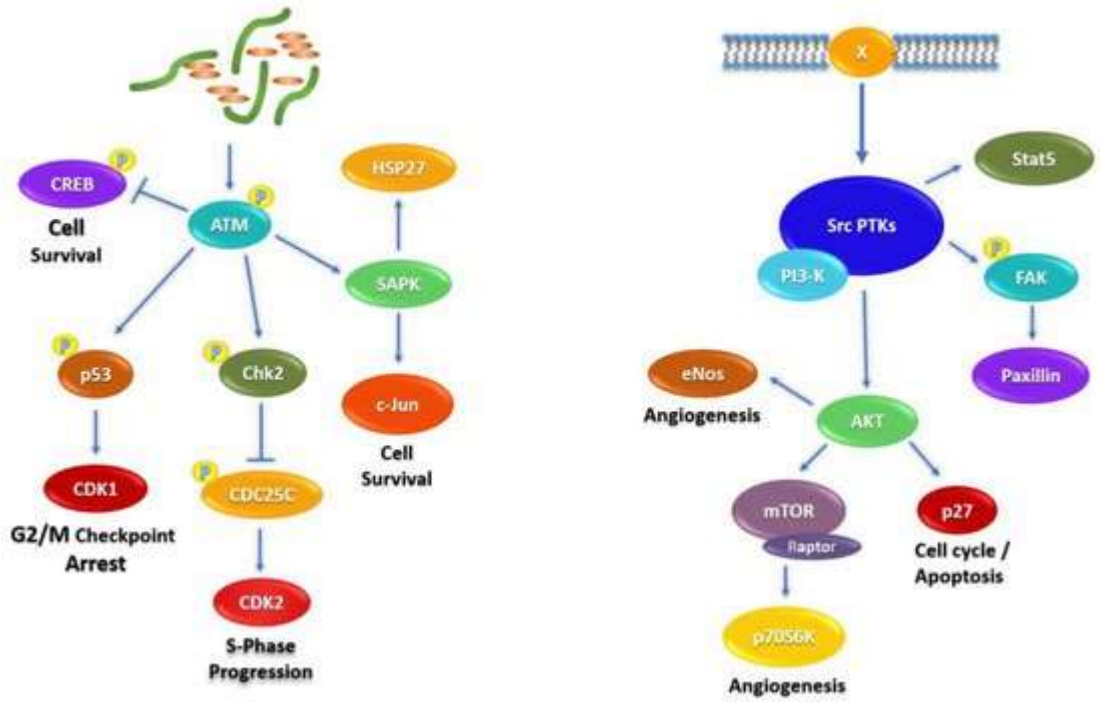

Figure 6. Schematic presentation of the signaling pathways involved in fascaplysin treatment of NCI-H526 and A549 lung cancer cells.

2.5. Combination of Cisplatin/Etoposide with Fascaplysin in Cytotoxicity Assays for NCI-H526 and A549 Cell Lines

Combination indices (CI) were calculated using the Chou-Talaly method, indicating synergy at values <1 [23]. For NCI-H526, CIs < 0.49 were found for cisplatin concentrations 0.625–5 µg/mL and 0.125–1 µM fascaplysin (fixed ratio of 2.5:1), similar to A549 with CI < 0.62 for cisplatin concentrations 1.25–10 µg/mL and 0.25–2 µM fascaplysin (fixed ratio of 5:1). For the NSCLC lines PC-9 and A549, the synergistic effects of fascaplysin with cisplatin or etoposide are shown in Figure 7.

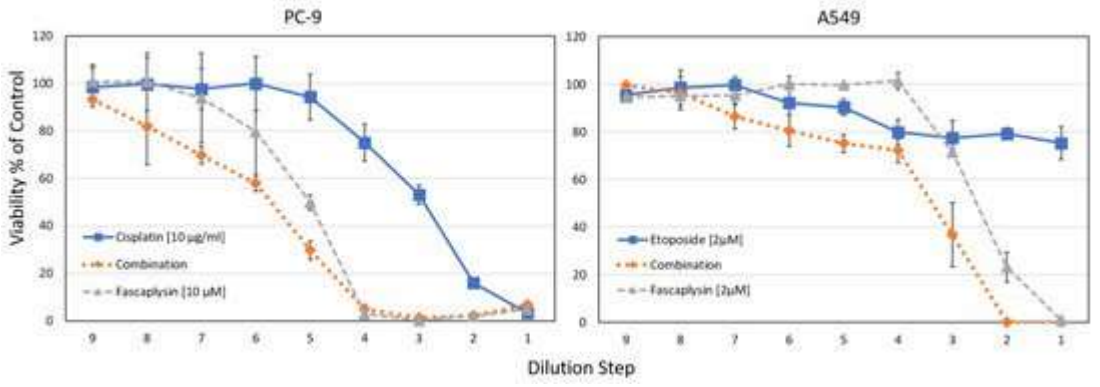

Figure 7. Cytotoxicity tests investigating fascaplysin combinations with chemotherapeutics.
For the NSCLC cell lines PC-9 and A549, combinations of fascaplysin and cisplatin or etoposide were tested in cytotoxicity tests. For both tests, IC_{50} values for the combinations using the concentrations as indicated revealed synergistic interactions. The CI values ranged from 0.26–0.76 for PC-9 fascaplysin-cisplatin and from 0.1–0.94 for A549 fascaplysin-etoposide, respectively. Data are shown as mean ± SD, the initial concentrations were titrated in 9 two-fold dilution steps.

3. Discussion

Lung cancer is the leading cause of cancer-related mortality in both men and women worldwide [1]. Targeted therapy is applicable to a minor fraction of NSCLC patients [4]. Patients with advanced lung cancer exhibit low survival rates and novel modes of chemotherapy need to be developed [2]. Deregulated proliferation of tumor cells is accomplished by alterations of the cell cycle and checkpoint controls amenable to inhibition by targeting of cell cycle and checkpoint kinases (CDKs) [24]. In particular, CDK4/6 inhibitors seem to present suitable targets in a majority of patients with advanced cancer [25,26]. Besides CDK4/6 inhibitors palbociclib and LY2835219, which have shown high activity in breast cancer, a host similar drugs are under development, and fascaplysin and derivatives share the same target [27]. Proteins in this cell proliferative pathway include p16, an endogenous suppressor of CDK4/6, cyclin D1, the regulatory subunit of CDK4/6, and retinoblastoma (Rb) protein, a tumor suppressor [28]. Both CDK4 and CDK6 encode cyclin-dependent kinases which complexed with cyclines of the D-type phosphorylate the Rb protein. Rb in turn triggers the expression of gene products for G1-S phase cell cycle progression. Rb inactivation is a common event in lung cancer, and is more frequent in SCLC than in NSCLC [29]. In SCLCs, Rb alterations can be found in a high percentage of cases, i.e., from 88% to 100% of the biopsy samples [30]. Therefore, in the present study we compared the effects of fascaplysin in the A549 Rb-wildtype NSCLC cell line to the Rb-mutated NCI-H526 SCLC cell line. Although, both cell lines have a similar chemosensitivity to fascaplysin, analysis of the intracellular signal transduction by Western blotting of selected phosphoproteins revealed marked differences in response to this drug.

DNA damage response is triggered when sensor proteins ATM (ataxia telangiectasia mutated) and ATR (also called ataxia telangiectasia and Rad3-related protein) detect structural distortions or breaks [31]. After DNA damage, CHK2 is phosphorylated by ATM on the priming site T68, and in turn, phosphorylates more than 24 proteins to induce apoptosis, DNA repair, or tolerance of the damage [32]. In wildtype cells, CHK2 phosphorylates Rb which enhances the formation of the transcriptionally-inactive pRb/E2F-1 complex causing G1/S arrest and suppression of apoptosis. Pronounced activation of CHK-2 in NCI-H526 and A549 cells indicates direct damage of DNA by fascaplysin and activation of the corresponding cellular responses in both cell lines. The cyclic AMP response element-binding protein (CREB) initiates transcriptional responses associated with cell survival to a wide variety of stimuli following its phosphorylation on Ser-133. Whereas fascaplysin treatment resulted in decreased phosphorylation of CREB in NCI-H526 cells, this transcription factor is hyperphosphorylated in A549 cells, possibly indicating anti- and pro-survival signaling, respectively [33,34]. Furthermore, cisplatin-induced activation of FAK has been linked to increased chemoresistance in ovarian cancer cells and FAK inhibitors induce tumor cell apoptosis [35]. Activated FAK forms a complex with Src family kinases and seems to provide a prosurvival signal in NCI-H526 cells, in contrast to fascaplysin-treated A549 cells [36]. In addition, overexpression of Src in cancer accelerates metastasis and is responsible for chemoresistance via multiple downstream signaling pathways, concerning Akt, MAPKs, STAT3, cytokines, etc. [37]. Therefore, activation of a number of Src kinases in NCI-H526 cells (Hck, Fyn, Yes and Fgr) may counteract fascaplysin toxicity and retard cell death; possibly contributing to the observed slower rate of loss of viability in the presence of increasing doses of fascaplysin in these cells. The stress kinases p38 and JNK are generally activated by inflammatory cytokines and different stressors, including DNA-damaging compounds [38]. p38 MAPK signaling results in the phosphorylation of CREB at Ser133, which seem to occur in A549 cells, contrary to NCI-H526 which shoes decreased p38 activity and phosphorylation of CREB [39]. Clearly, fascaplysin is an inhibitor of CDC25, and this pathway is expected to be inhibited in NCI-H526 cells [40].

In conclusion, fascaplysin shows marked anticancer activity in NSCLC and SCLC cells independently of the function of the CDK4 pathway, thus pointing to direct effects on DNA and the transcription of various proteins. The mechanisms of the antitumor effect of fascaplysin demonstrated on several carcinoma models indicate that fascaplysin is close to some drug groups such as intercalating agents, inhibitors of serine-threonine, and tyrosine kinases. Additionally, fascaplysin increases phosphorylation of AKT/PKB and adenosine monophosphate-activated protein kinase (AMPK), which feature anti-apoptotic or pro-survival functions in cancer [41]. In detail, fascaplysin abolishes the phosphorylation of mTOR, 4EBP1, and p70S6K1, which trigger the cap-dependent translation machinery and affect the expression of oncoproteins, such as survivin, c-myc, cyclin D1, VEGF, and HIF-1α. Similarly, 7-chloro-fascaplysin inhibited cell survival through interference with the PI3K/Akt/mTOR pathway, which in turn modulates HIF-1α, eNOS and MMP-2/9 in a breast cancer cell line [42]. The cytotoxicity of 4-chlorofascaplysin (4-CF) was reversed by co-treatment with the VEGF and Akt inhibitors or in response to neutralizing VEGF antibodies. Fascaplysin has stronger anti-cancer effects than other CDK4 inhibitors on lung cancer cells that are wild-type or null for Rb, indicating that unknown target molecules might

be involved in the antitumor activity of fascaplysin [43]. In good accordance with the results of Oh et al. and Sharma et al., our results show alterations of phosphoproteins altering the Akt-mTor pathway which are triggered mainly by upstream stress and src kinases.

Relapsed SCLC is resistant to a wide range of drugs and clinical trials have not led to improvements in survival rates over recent decades. Chemoresistance of SCLC seems to be related to the formation of large spheroids, termed tumorospheres, which limit drug access and contain quiescent and hypoxic tumor cells which are less sensitive to chemotherapeutics. Such 3D-structures were demonstrated to show increased resistance to cisplatin, etoposide, topotecan, and epirubicin when compared to the same SCLC CTC cells in form of single cell suspensions. In particular, fascaplysin is cytotoxic against SCLC CTC tumorospheres which exhibit high chemoresistance against a range of commonly-administered chemotherapeutics. Fascaplysin-induced cell death of outer SCLC CTC cell layers seems to trigger the elimination of the whole spheroid. Especially in SCLC cells, the induction of ROS by fascaplysin is expected to exert increased damage due to the small volume of the cytoplasmic fraction [7]. It should be noted that spheroids are similarly observed in pleural effusions of NSCLC patients. Although the parent drug fascaplysin seems too toxic for clinical application, derivatives such as 3-bromofascaplysin and 7-phenylfascaplysin were demonstrated to possess higher cytotoxic efficiency and different profiles [44,45,46]. Furthermore, the alkaloid derivative 4-CF exhibits five times higher cytotoxic IC_{50} value in normal cells, as well as no apparent toxicities in murine xenograft models at therapeutic doses [42].

4. Cell Culture and Methods

4.1. Chemicals

Unless otherwise noted, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's phosphate buffered saline (PBS) was purchased from Gibco/Invitrogen (Carlsbad, CA, USA). Compounds were prepared as stock solutions of 2 mg/mL in either DMSO or in 0.9% NaCl solution (cisplatin), sterilized by filtration in case of cisplatin, and aliquots stored at −20 °C.

4.2. Cell Culture

The A549 NSCLC A549 (Rb/p53 wild-type) and NCI-H526 SCLC A549 (Rb protein not expressed/p53 wild-type) cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA), as well as the other cell lines except primary lines and all SCLC CTCs established in our lab [7]. Cells were grown in RPMI-1640 bicarbonate medium (Seromed, Berlin, Germany), supplemented with 10% FBS (Seromed), 4 mM glutamine, and antibiotics (final concentrations: 50 U/mL of penicillin, 50 µg/mL of streptomycin, and 100 µg/mL neomycin; Sigma-Aldrich, St. Louis, MO, USA), and subcultivated twice a week. A549 is p53 wildtype and DNA profiling by short tandem repeat analysis of the NCI-H526 cells proved their identity to the American Type Culture Collection specifications, and the yeast p53 functional assay revealed expression of fully active p53 (functional assay of separated alleles in yeast FASAY; data not shown).

4.3. Phosphokinase Array

Relative protein phosphorylation levels of 38 selected proteins were obtained by analysis of 46 specific phosphorylation sites using the Proteome Profiler Human Phospho-Kinase Array Kit ARY003 (R&D Systems, Minneapolis, MN, USA) in duplicate tests according to the manufacturer's instructions. Briefly, cells were rinsed with PBS, 1×10^7 cells/mL lysis buffer were solubilized under permanent shaking at 4 °C for 30 min, and aliquots of the lysates were stored frozen at −80 °C. After blocking, membranes with spotted catcher antibodies were incubated with diluted cell lysates at 4 °C overnight. Thereafter, cocktails of biotinylated detection antibodies were added at room temperature for 2 h. Phosphorylated proteins were revealed using streptavidin-HRP/chemiluminescence substrate (SuperSignal West Pico, Thermo Fisher Scientific, Rockford, IL, USA) and detection with a Molecular Imager VersaDoc MP imaging system (Bio-Rad, Hercules, CA, USA). Images were quantified using the ImageQuant TL v2005 software (Amersham Biosciences, Buckinghamshire, UK) and Microsoft Excel software (Microsoft, Redmond, WA, USA). The different Western blot

membranes were normalized using the 3 calibration spots included. Signaling pathways affected by fascaplysin in NCI-H526 and A549 lung cancer cells were produced using Power Point software (Microsoft, Redmond, WA, USA).

4.4. Cytotoxicity Assay

Aliquots of 1×10^4 cells in 200 µL medium were treated for four days with twofold dilutions of fascaplysin or cisplatin, respectively in 96-well microtiter plates in quadruplicate (Greiner, Kremsmuenster, Austria). For SCLC CTC tumorospheres, an equivalent number of cells in form of spheroids were tested as described [7]. The plates were incubated under tissue culture conditions, and cell viability was measured using a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (EZ4U, Biomedica, Vienna, Austria). Optical density was measured using a microplate reader at 450 nm with an empty well as reference. Values obtained from control wells containing cells and media alone were set to 100% proliferation. For the assessment of the interaction of fascaplysin with cisplatin, tests were performed comprising the individual drugs alone and in combination, followed by analysis using the Chou-Talalay method with help of the Compusyn software (ComboSyn, Inc. Paramus, NJ, USA).

4.5. Statistics

Statistical analysis was performed using Student's t test for normally distributed samples (* $p < 0.05$ was regarded as statistically significant). Values are shown as mean ± SD.

5. Conclusions

CDKs are a group of serine/threonine kinases which are critical in the regulation of the cell cycle. A major role of CDK-4 is the phosphorylation of Rb, which is inhibited by fascaplysin and a range of other compounds. Mutations in Rb, along with those of cyclin D and p16(INK4a), has been seen frequently during tumorigenesis of cancers. Investigation of a part of the kinome of NCI-H526 SCLC and A549 NSCLC cell lines reveals different responses to treatment with fascaplysin, most likely to be connected to the Rb phenotype. In NCI-H526 cells, fascaplysin sensitivity is determined by the absence of the CDK4–Rb pathway and DNA damage in combination with putative CDC25 inhibition, whereas in A549, inhibition of CDK4 seems to be the major effect with distinct and small effects on phosphoproteins. Fascaplysin exhibits marked anticancer activity against permanent and primary SCLC and NSCLC cells, with cytotoxic effects against SCLC CTC tumorospheres that are far superior to those of other therapeutics. Therefore, fascaplysin and derivatives with a better clinical profile may constitute valuable agents for lung cancer therapy.

Supplementary Materials

The following are available online at https://www.mdpi.com/1660-3397/16/10/383/s1, Table S1: Fascaplysin activity against a panel of cell lines.

Author Contributions

B.R. and A.P. were involved in experimental work and preparation of the manuscript, M.H. in design of the experiments and G.H. in all aspects of this project.

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Conflicts of Interest

The authors declare no conflict of interest.

References

1. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J. Clin. **2018**, in press. [Google Scholar] [CrossRef] [PubMed]
2. Herbst, R.S.; Morgensztern, D.; Boshoff, C. The biology and management of non-small cell lung cancer. Nature **2018**, 553, 446–454. [Google Scholar] [PubMed]
3. Kalemkerian, G.P. Small Cell Lung Cancer. Semin. Respir. Crit. Care Med. **2016**, 37, 783–796. [Google Scholar] [CrossRef] [PubMed]
4. Parums, D.V. Current status of targeted therapy in non-small cell lung cancer. Drugs Today (Barc) **2014**, 50, 503–525. [Google Scholar] [CrossRef] [PubMed]
5. Nguyen, K.S.; Neal, J.W.; Wakelee, H. Review of the current targeted therapies for non-small-cell lung cancer. World J. Clin. Oncol. **2014**, 5, 576–587. [Google Scholar] [CrossRef] [PubMed]
6. Fennell, D.A.; Summers, Y.; Cadranel, J.; Benepal, T.; Christoph, D.C.; Lal, R.; Das, M.; Maxwell, F.; Visseren-Grul, C.; Ferry, D. Cisplatin in the modern era: The backbone of first-line chemotherapy for non-small cell lung cancer. Cancer Treat. Rev. **2016**, 44, 42–50. [Google Scholar] [CrossRef] [PubMed]
7. Klameth, L.; Rath, B.; Hochmaier, M.; Moser, D.; Redl, M.; Mungenast, F.; Gelles, K.; Ulsperger, E.; Zeillinger, R.; Hamilton, G. Small cell lung cancer: model of circulating tumor cell tumorospheres in chemoresistance. Sci. Rep. **2017**, 7, 5337. [Google Scholar] [CrossRef] [PubMed]
8. Roll, D.M.; Ireland, C.M.; Lu, H.S.M.; Clardy, J. Fascaplysin, an unusual antimicrobial pigment from the marine sponge Fascaplysinopsis sp. J. Org. Chem. **1988**, 53, 3276–3278. [Google Scholar] [CrossRef]
9. Segraves, N.L.; Robinson, S.J.; Garcia, D.; Said, S.A.; Fu, X.; Schmitz, F.J.; Pietraszkiewicz, H.; Valeriote, F.A.; Crews, P. Comparison of fascaplysin and related alkaloids: A study of structures, cytotoxicities, and sources. J. Nat. Prod. **2004**, 67, 783–792. [Google Scholar] [CrossRef] [PubMed]
10. Segraves, N.L.; Lopez, S.; Johnson, T.A.; Said, S.A.; Fu, X.; Schmitz, F.J.; Pietraszkiewicz, H.; Valeriote, F.A.; Crews, P. Structures and cytotoxicities of fascaplysin and related alkaloids from two marine phyla—Fascaplysinopsis sponges and Didemnum tunicates. Tetrahedron Lett. **2003**, 44, 3471–3475. [Google Scholar] [CrossRef]
11. Bharate, S.B.; Manda, S.; Mupparapu, N.; Battini, N.; Vishwakarma, R.A. Chemistry and biology of fascaplysin, a potent marine-derived CDK-4 inhibitor. Mini Rev. Med. Chem. **2012**, 12, 650–664. [Google Scholar] [CrossRef] [PubMed]
12. Soni, R.; Muller, L.; Furet, P.; Schoepfer, J.; Stephan, C.; Zumstein-Mecker, S.; Fretz, H.; Chaudhuri, B. Inhibition of cyclindependentkinase 4 (Cdk4) by fascaplysin, a marine natural product. Biochem. Biophys. Res. Commun. **2000**, 275, 877–884. [Google Scholar] [CrossRef] [PubMed]
13. Soni, R.; O'Reilly, T.; Furet, P.; Muller, L.; Stephan, C.; Zumstein-Mecker, S.; Fretz, H.; Fabbro, D.; Chaudhuri, B. Selective in vivo and in vitro effects of a small molecule inhibitor of cyclindependent kinase-4. J. Natl. Cancer Inst. **2001**, 93, 436–446. [Google Scholar] [CrossRef] [PubMed]
14. Shafiq, M.I.; Steinbrecher, T.; Schmid, R. Fascaplysin as a specific inhibitor for CDK4: Insights from molecular modelling. PLoS ONE **2012**, 7, e42612. [Google Scholar] [CrossRef] [PubMed]
15. Hormann, A.; Chaudhari, B.; Fretz, H. DNA binding properties of marine sponge pigment fascaplysin. Bioorg. Med. Chem. **2001**, 9, 917–921. [Google Scholar] [CrossRef]
16. Mahale, S.; Bharate, S.B.; Manda, S.; Joshi, P.; Bharate, S.S.; Jenkins, P.R.; Vishwakarma, R.A.; Chaudhuri, B. Biphenyl-4-carboxylic Acid [2-(1H-Indol-3-yl)-ethyl]-methylamide

(CA224), a nonplanar analogue of Fascaplysin, inhibits Cdk4 and tubulin polymerization: evaluation of in vitro and in vivo anticancer activity. J. Med. Chem. **2014**, 57, 9658–9672. [Google Scholar] [CrossRef] [PubMed]
17. Kumar, S.; Kumar Guru, S.; Pathania, A.S.; Manda, S.; Kumar, A.; Bharate, S.B.; Vishwakarma, R.A.; Malik, F.; Bhushan, S. Fascaplysin induces caspase mediated crosstalk between apoptosis and autophagy through the inhibition of PI3K/AKT/mTOR signaling cascade in human leukemia HL-60 cells. J. Cell Biochem. **2015**, in press. [Google Scholar] [CrossRef] [PubMed]
18. Yan, X.; Chen, H.; Lu, X.; Wang, F.; Xu, W.; Jin, H.; Zhu, P. Fascaplysin exert anti-tumor effects through apoptotic and anti-angiogenesis pathways in sarcoma mice model. Eur. J. Pharm. Sci. **2011**, 43, 251–259. [Google Scholar] [CrossRef] [PubMed]
19. Zheng, Y.L.; Lu, X.L.; Lin, J.; Chen, H.M.; Yan, X.J.; Wang, F.; Xu, W.F. Direct effects of fascaplysin on human umbilical vein endothelial cells attributing the anti-angiogenesis activity. Biomed. Pharmacother. **2010**, 64, 527–533. [Google Scholar] [CrossRef] [PubMed]
20. Hamilton, G. Cytotoxic effects of fascaplysin against small cell lung cancer cell lines. Mar Drugs **2014**, 12, 1377–1389. [Google Scholar] [CrossRef] [PubMed]
21. Hamilton, G.; Olszewski, U.; Klameth, L.; Ulsperger, E.; Geissler, K. Synergistic anticancer activity of topotecan—Cyclin-dependent kinase inhibitor combinations against drug-resistant small cell lung cancer (SCLC) cell lines. J. Cancer Ther. **2013**, 4, 47–53. [Google Scholar] [CrossRef]
22. Hamilton, G.; Klameth, L.; Rath, B.; Thalhammer, T. Synergism of cyclin-dependent kinase inhibitors with camptothecin derivatives in small cell lung cancer cell lines. Molecules **2014**, 19, 2077–2088. [Google Scholar] [CrossRef] [PubMed]
23. Chou, T.C. Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res. **2010**, 70, 440–446. [Google Scholar] [CrossRef] [PubMed]
24. Aarts, M.; Linardopoulos, S.; Turner, N.C. Tumor selective targeting of cell cycle kinases for cancer treatment. Curr. Opin. Pharmacol. **2013**, 13, 529–535. [Google Scholar] [CrossRef] [PubMed]
25. Whittaker, S.R.; Mallinger, A.; Workman, P.; Clarke, P.A. Inhibitors of cyclin-dependent kinases as cancer therapeutics. Pharmacol. Ther. **2017**, 173, 83–105. [Google Scholar] [CrossRef] [PubMed]
26. Graf, F.; Mosch, B.; Koehler, L.; Bergmann, R.; Wuest, F.; Pietzsch, J. Cyclin-Dependent Kinase 4/6 (Cdk4/6) Inhibitors: Perspectives in Cancer Therapy and Imaging. Mini Rev. Med. Chem. **2015**, 15. in press. [Google Scholar] [CrossRef]
27. Jaganathan, H.; Overstreet, K.; Reed, E. Improving breast cancer therapy with CDK4/6 inhibitors. Clin. Breast Cancer **2014**, 14, 379–380. [Google Scholar] [CrossRef] [PubMed]
28. Beasley, M.B.; Lantuejoul, S.; Abbondanzo, S.; Chu, W.S.; Hasleton, P.S.; Travis, W.D.; Brambilla, E. The P16/cyclin D1/Rb pathway in neuroendocrine tumors of the lung. Hum. Pathol. **2003**, 34, 136–142. [Google Scholar] [CrossRef] [PubMed]
29. Shapiro, G.I.; Edwards, C.D.; Kobzik, L.; Godleski, J.; Richards, W.; Sugarbaker, D.J.; Rollins, B.J. Reciprocal Rb inactivation and p16INK4 expression in primary lung cancers and cell lines. Cancer Res. **1995**, 55, 505–509. [Google Scholar] [PubMed]
30. Coe, BP.; Lockwood, W.W.; Girard, L.; Chari, R.; Macaulay, C.; Lam, S.; Gazdar, A.F.; Minna, J.D.; Lam, W.L. Differential disruption of cell cycle pathways in small cell and non-small cell lung cancer. Br. J. Cancer **2006**, 94, 1927–1935. [Google Scholar] [CrossRef] [PubMed]
31. Ciccia, A.; Elledge, S.J. The DNA damage response: making it safe to play with knives. Mol. Cell **2010**, 40, 179–204. [Google Scholar] [CrossRef] [PubMed]
32. Zannini, L.; Delia, D.; Buscemi, G. CHK2 kinase in the DNA damage response and beyond. J. Mol. Cell Biol. **2014**, 6, 442–457. [Google Scholar] [CrossRef] [PubMed]
33. Trinh, A.T.; Kim, S.H.; Chang, H.Y.; Mastrocola, A.S.; Tibbetts, R.S. Cyclin-dependent kinase 1-dependent phosphorylation of cAMP response element-binding protein decreases

chromatin occupancy. J. Biol. Chem. **2013**, 288, 23765–23775. [Google Scholar] [CrossRef] [PubMed]
34. Rolli, M.; Kotlyarov, A.; Sakamoto, K.M.; Gaestel, M.; Neininger, A. Stress-induced stimulation of early growth response gene-1 by p38/stress-activated protein kinase 2 is mediated by a cAMP-responsive promoter element in a MAPKAP kinase 2-independent manner. J. Biol. Chem. **1999**, 274, 19559–19564. [Google Scholar] [CrossRef] [PubMed]
35. Villedieu, M.; Deslandes, E.; Duval, M.; Héron, J.F.; Gauduchon, P.; Poulain, L. Acquisition of chemoresistance following discontinuous exposures to cisplatin is associated in ovarian carcinoma cells with progressive alteration of FAK, ERK and p38 activation in response to treatment. Gynecol. Oncol. **2006**, 101, 507–519. [Google Scholar] [CrossRef] [PubMed]
36. Warmuth, M.; Damoiseaux, R.; Liu, Y.; Fabbro, D.; Gray, N. SRC family kinases: potential targets for the treatment of human cancer and leukemia. Curr. Pharm. Des. **2003**, 9, 2043–2059. [Google Scholar] [CrossRef] [PubMed]
37. Lieu, C.; Kopetz, S. The SRC family of protein tyrosine kinases: a new and promising target for colorectal cancer therapy. Clin. Colorectal Cancer **2010**, 9, 89–94. [Google Scholar] [CrossRef] [PubMed]
38. Silvers, A.L.; Bachelor, M.A.; Bowden, G.T. The role of JNK and p38 MAPK activities in UVA-induced signaling pathways leading to AP-1 activation and c-Fos expression. Neoplasia **2003**, 5, 319–329. [Google Scholar] [CrossRef]
39. Gao, J.; Wagnon, J.L.; Protacio, R.M.; Glazko, G.V.; Beggs, M.; Raj, V.; Davidson, M.K.; Wahls, W.P. A stress-activated, p38 mitogen-activated protein kinase-ATF/CREB pathway regulates posttranscriptional, sequence-dependent decay of target RNAs. Mol. Cell Biol. **2013**, 33, 3026–3035. [Google Scholar] [CrossRef] [PubMed]
40. Lazo, J.S.; Wipf, P. Is Cdc25 a druggable target? Anticancer Agents Med. Chem. **2008**, 8, 837–842. [Google Scholar] [CrossRef] [PubMed]
41. Oh, T.I.; Lee, Y.M.; Nam, T.J.; Ko, Y.S.; Mah, S.; Kim, J.; Kim, Y.; Reddy, R.H.; Kim, Y.J.; Hong, S.; et al. Fascaplysin Exerts Anti-Cancer Effects through the Downregulation of Survivin and HIF-1α and Inhibition of VEGFR2 and TRKA. Int. J. Mol. Sci. **2017**, 18, E2074. [Google Scholar] [CrossRef] [PubMed]
42. Sharma, S.; Guru, S.K.; Manda, S.; Kumar, A.; Mintoo, M.J.; Prasad, V.D.; Sharma, P.R.; Mondhe, D.M.; Bharate, S.B.; Bhushan, S. A marine sponge alkaloid derivative 4-chloro fascaplysin inhibits tumor growth and VEGF mediated angiogenesis by disrupting PI3K/Akt/mTOR signaling cascade. Chem. Biol. Interact. **2017**, 275, 47–60. [Google Scholar] [CrossRef] [PubMed]
43. Oh, T.I.; Lee, J.H.; Kim, S.; Nam, T.J.; Kim, Y.S.; Kim, B.M.; Yim, W.J.; Lim, J.H. Fascaplysin Sensitizes Anti-Cancer Effects of Drugs Targeting AKT and AMPK. Molecules **2017**, 23, E42. [Google Scholar] [CrossRef] [PubMed]
44. Calcabrini, C.; Catanzaro, E.; Bishayee, A.; Turrini, E.; Fimognari, C. Marine Sponge Natural Products with Anticancer Potential: An Updated Review. Mar. Drugs **2017**, 15, E310. [Google Scholar] [CrossRef] [PubMed]
45. Mahale, S.; Bharate, S.B.; Manda, S.; Joshi, P.; Jenkins, P.R.; Vishwakarma, R.A.; Chaudhuri, B. Antitumour potential of BPT: A dual inhibitor of cdk4 and tubulin polymerization. Cell Death Dis. **2015**, 6, e1743. [Google Scholar] [CrossRef] [PubMed]
46. Lyakhova, I.A.; Bryukhovetsky, I.S.; Kudryavtsev, I.V.; Khotimchenko, Y.S.; Zhidkov, M.E.; Kantemirov, A.V. Antitumor Activity of Fascaplysin Derivatives on Glioblastoma Model In Vitro. Bull. Exp. Biol. Med. **2018**, 164, 666–672. [Google Scholar] [CrossRef] [PubMed]

2.2 Expression of Proteolytic Enzymes by Small Cell Lung Cancer Circulating Tumor Cell Lines

[2] Rath B, Klameth L, Plangger A, Hochmair M, Ulsperger E, Huk I, Zeillinger R, Hamilton G. Expression of Proteolytic Enzymes by Small Cell Lung Cancer Circulating Tumor Cell Lines. Cancers (Basel). 2019 Jan 19;11(1). pii: E114. doi: 10.3390/cancers11010114

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Contribution of the applicant to the work; Barbara Rath cultivated the respective cell lines in tissue culture, performed the cytotoxicity tests (MTT assay), including data evaluation (OriginLab) and filing. She carried out the Western blot arrays for proteases including data evaluation (Image J, OriginLab). Furthermore she made all figures for the publications and was involved in discussion of the results, writing of the manuscripts as well as scientific literature research and referencing.

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Article

Expression of Proteolytic Enzymes by Small Cell Lung Cancer Circulating Tumor Cell Lines

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Abstract

Small cell lung cancer (SCLC) is an aggressive type of lung cancer which disseminates vigorously and has a dismal prognosis. Metastasis of SCLC is linked to an extremely high number of circulating tumor cells (CTCs), which form chemoresistant spheroids, termed tumorospheres. Intravasation and extravasation during tumor spread requires the activity of a number of proteases to disintegrate the stroma and vascular tissue. Generation of several permanent SCLC CTC lines allowed us to screen for the expression of 35 proteases using Western blot arrays. Cell culture supernatants of two CTC lines, namely BHGc7 and 10, were analyzed for secreted proteases, including matrix metalloproteinases (MMPs), ADAM/TS, cathepsins, kallikreins, and others, and compared to proteases expressed by SCLC cell lines (GLC14, GLC16, NCI-H526 and SCLC26A). In contrast to NCI-H526 and SCLC26A, MMP-9 was highly expressed in the two CTC lines and in GLC16 derived of a relapse. Furthermore, cathepsins (S, V, X/Z/P, A and D) were highly expressed in the CTC lines, whereas ADAM/TS and kallikreins were not detectable. In conclusion, SCLC CTCs express MMP-9 and a range of cathepsins for proteolysis and, aside from tissue degradation, these enzymes are involved in cell signaling, survival, and the chemoresistance of tumor cells.

Keywords: small cell lung cancer; circulating tumor cells; proteases; MMP-9; cathepsin S; metastasis

1. Introduction

Metastasis describes the dissemination of cancer cells from the primary tumor to adjacent normal tissue, and further to distal organs where the secondary lesions are a major cause of mortality [1,2]. Metastasis progresses in a series of discrete and interrelated steps: cancer cells are released from the primary tumor, intravasate into the blood and lymphatic systems, survive in the circulatory system, and finally extravasate at distal microvasculature and invade distant organs [3]. Metastatic cells also manipulate the microenvironment to promote the proliferation, angiogenesis, and protumor activities of normal stromal cells. The metastatic process is inherently of low efficacy, but eventually renders the cancer incurable. Cancer dissemination can start early in tumorigenesis, preceding the clinical manifestation of tumors for years [4]. In solid tumors, this implies cellular migration, movement, degradation of the extracellular matrix (ECM), and the dissolution of cell–cell contacts to neighboring epithelial cells [5,6]. Furthermore, ECM remodeling contributes to cancer progression through activation of signaling pathways, which results in invasion of single cells or clusters [7].

Circulating tumor cells (CTCs) are the primary effectors of metastatic relapse in patients with cancer, as shown by their correlation to prognosis and drug response [3,8]. Although large numbers of CTCs enter the circulatory system, only a small fraction of these cells survive successfully and extravasate into distant sites [9]. Extravasation seems to require factors altering vascular permeability and vascular endothelial barriers, including vascular endothelial growth factor VEGF, disintegrin, and metalloproteinase domain-containing proteins (ADAMs), matrix metalloproteinases (MMPs), as well as other enzymes and growth factors [10]. The cellular biological characteristics of CTCs are difficult to study due to their heterogeneity, occurrence in low numbers, and due to the inability to select for the actual metastasis-inducing subpopulation which will ultimately survive and generate secondary tumors [3,4]. Therefore, random isolation of single cells and tests for markers, secreted enzymes and cytokines are not suitable for characterizing the properties of truly effective CTCs. However, the blood of small cell lung cancer (SCLC) patients can exhibit extreme numbers of CTCs and the circulation of a sufficient number of CTCs with metastasis-initiating potential allowed us to establish several permanent CTC lines ex vivo [11,12,13].

Lung cancer remains one of the most prevalent and malignant cancers worldwide, with SCLC representing its most aggressive variant [14,15]. The majority of cases are diagnosed at late stages, when local invasion and placement of distal metastases has already occurred. SCLC dissemination is known to occur via three major routes, namely blood, lymphatic vessels, and transcoelomic spread into the pleural, pericardial, and abdominal cavities [14,15]. Involved steps comprise angiogenesis, degradation of ECM by proteases, increases in cellular motility and resistance, as well as protection from immune surveillance [16,17]. The process of cancer metastasis and the mechanisms dictating cancer dissemination and setup of secondary lesions is still poorly understood [17].

SCLC represents a suitable model for studying early tumor spread and the development of drug resistance. This tumor entity is distinguished by an extremely high count of CTCs, which was reported to be linked to prognosis and response to therapy [13]. SCLC CTCs enriched from blood samples of patients with more than 400 CTCs/7.5 mL blood could be used to establish xenotransplants in immunocompromised mice [18,19]. However, an investigation of the proteases of CTCs employed to dissolute ECM would depend on the availability of a larger number of a homogenous and pure population of relevant tumor-initiating cells. So far, cultures of CTCs, except our SCLC CTC lines, were only reported for one colon cancer and several breast cancer CTC lines [20,21]. Cultures of patient-derived CTCs may allow the study of mechanisms of tumorigenesis, invasion, and metastasis and novel therapeutic strategies [22]. Our CTC lines were established from patients with metastatic disease and showed similar characteristics and formation of highly chemoresistant spheroids, termed tumorospheres [23]. In the present work, we used two CTC cell lines, namely BHGc7 and BHGc10, which are tumorigenic in NOD-SCID mice, to screen for the expression of 35 proteases using Western blot arrays. Proteases are involved in tumor angiogenesis, invasion and metastasis during malignant progression and this group comprises the largest family of enzymes in the human genome [5,6]. They are a part of a system of proteolytic interactions between factors of the tumor microenvironment and proteases—such as cathepsins, urokinase-type plasminogen activator (uPA), and several matrix metalloproteinases (MMPs), among others. Besides the first attempt to identify proteases expressed by SCLC CTCs lines, we included a cell line pair, GLC14 and GLC16, established before and after chemotherapy, as well as two cell lines derived from a bone metastasis, NCI-H526, and from a pleural effusion, SCLC26A, as representatives of local metastatic lesions [24,25]. Additionally, proteases were screened in a conditioned medium of a coculture of SCLC CTC and macrophages, which are recruited and educated by such tumor cells [26]. Functionally, the invasion of the SCLC CTC cells into ECM was tested.

2. Results

2.1. Protease Expression of GLC14 and GLC16 Cell Lines

For all of the following experiments, only the significantly expressed proteases out of the 35 enzymes assayed are shown. GLC14 and GLC16 represent lines established from metastases of the same patient before and after failure of chemoradiotherapy, respectively. Results demonstrate that during the progressive disease, GLC16 showed elevated expression of most proteases, with marked overexpression of MMP-9, cathepsins S, X/Z/P and D, as well as partial downregulation of cathepsin V (Figure 1).

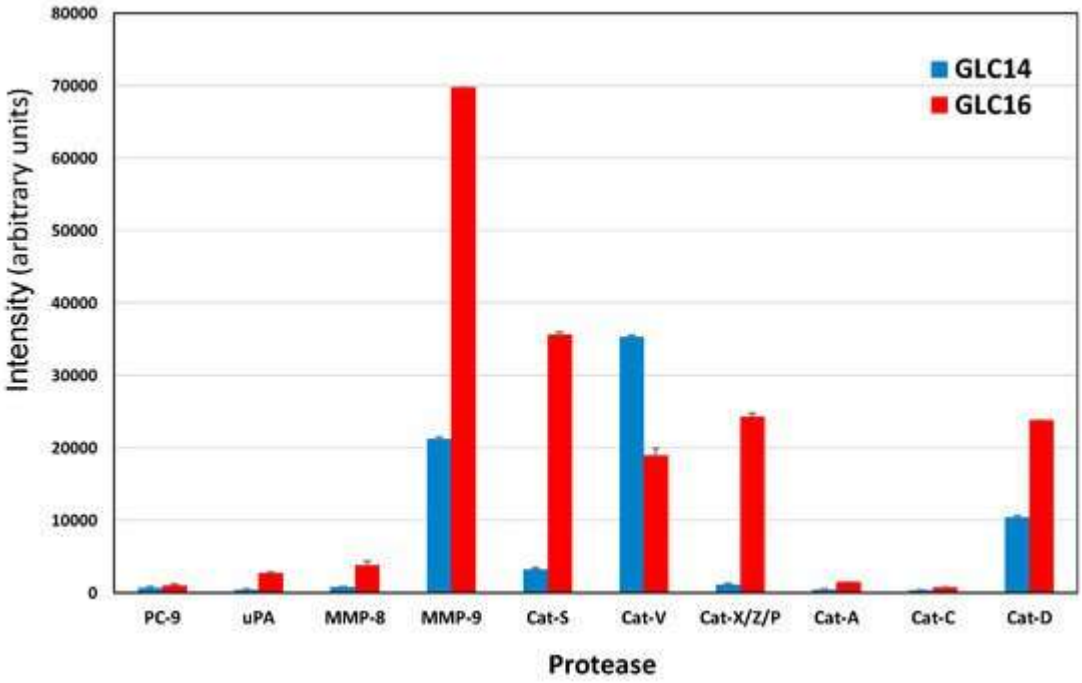

Figure 1. Selected proteases expressed by GLC14 and GLC16 small cell lung cancer (SCLC) cell lines, established from the same patient before therapy and following relapse, respectively. Values represent mean ± SD (arbitrary intensity units) and all differences are statistically significant, except for PC-9 and Cat-C.

2.2. Protease Expression of NCI-H526 and SCLC26A Cell Lines

NCI-H526 and SCLC26A represent local metastases to bone and pleural fluid, respectively. NCI-H526 exhibited high expression of proprotein convertase-9 (PC-9) and cathepsins V and D, with weaker occurrence of cathepsin C and low levels of other cathepsins, urokinase-type plasminogen activator (uPA), and MMP-8/9 (Figure 2, left side). The SCLC26A cell line again highly expressed PC-9 and cathepsins V, X/Z/P, A, C, and D (Figure 2, right side). Secreted MMP-8, uPA, and cathepsin S were present at very low concentrations.

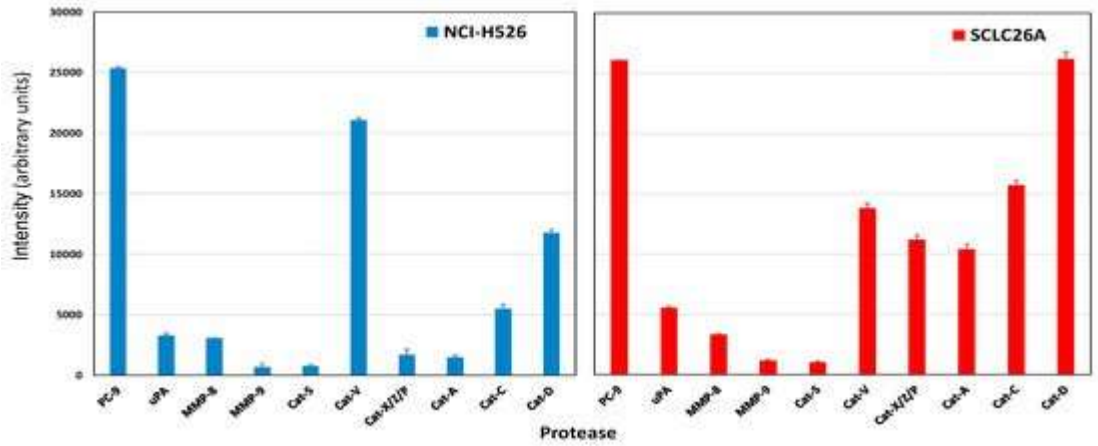

Figure 2. The figure shows selected proteases expressed by NCI-H526 (left side) and SCLC26A (right side) SCLC cell lines, established from a bone metastasis and from pleural effusion, respectively. Values represent mean ± SD.

2.3. Protease Expression of CTC Cell Lines BHGc7 and BHGc10

The two CTC cell lines derived from SCLC shared expression of MMP-9 and cathepsin S, the latter not found in the other cell lines tested, except in metastatic GLC16. Furthermore, cathepsins V, X/Z/P, A, and D were maintained in BHGc10, and all cathepsins tested, namely V, X/Z/P, A, C, and D in BHGc7 (Figure 3). PC-9 was not expressed in significant concentrations in both CTC cell lines, as well as uPA, MMP-8 and all other of the 35 proteases included in the test panel.

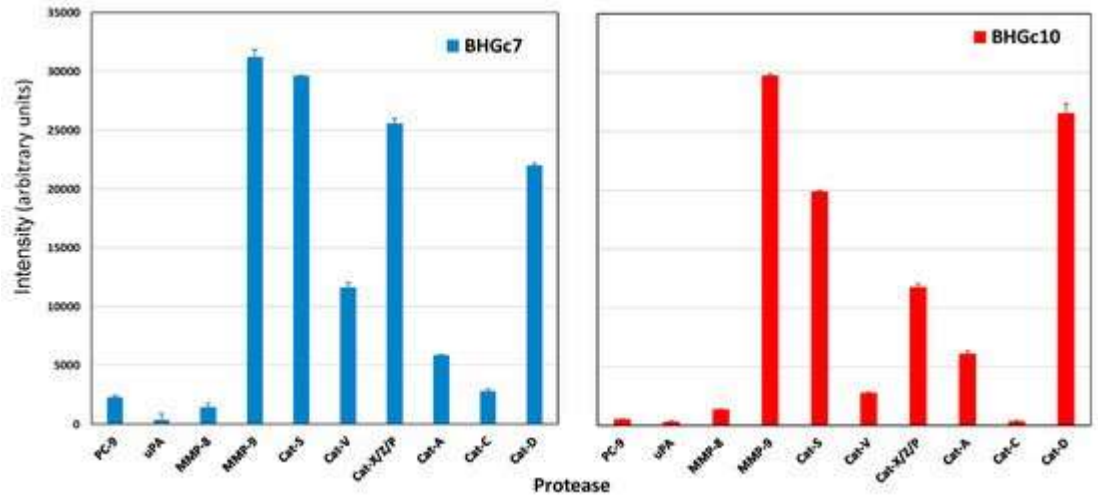

Figure 3. Selected proteases expressed by BHGc7 (left side) and BHGc10 (right side) SCLC CTC lines, established from blood samples of two refractory SCLC patients, respectively. Values represent mean ± SD.

2.4. Protease Expression of a Macrophage CTC Coculture Supernatant

Macrophages which developed in coculture with the BHGc10 CTC line were supplemented with fresh medium, and following incubation tested for the presence of proteases using the Western blot arrays. The result showed that this type of macrophage expressed all proteases found in SCLC CTCs in abundance, with the addition of high amounts of uPA and MMP-8 (Figure 4).

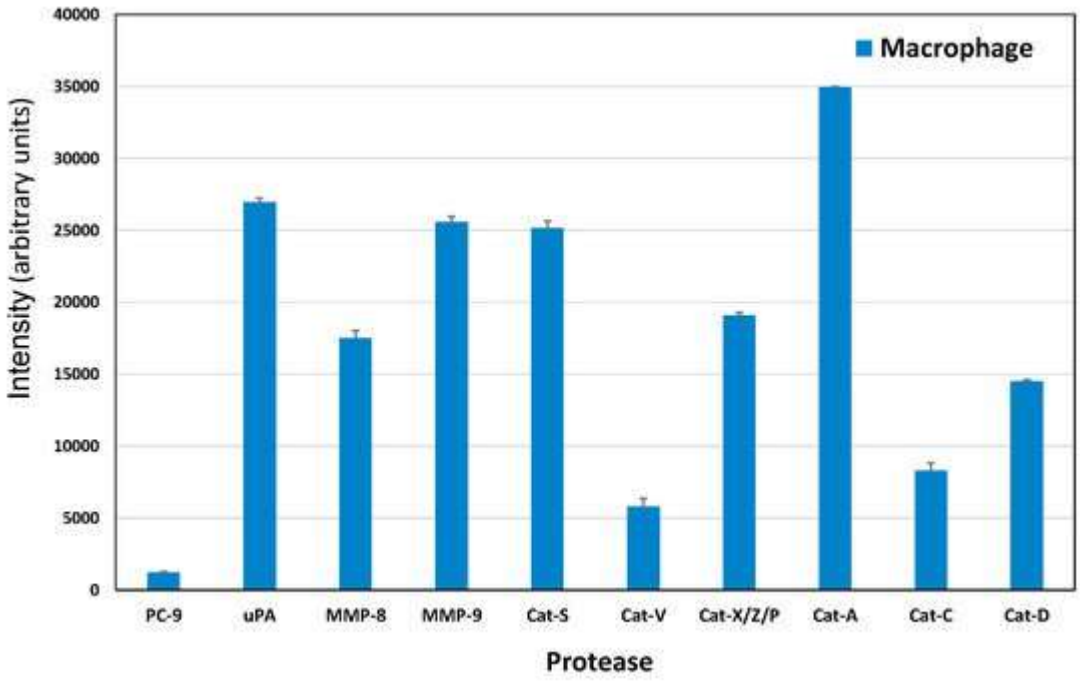

Figure 4. Proteases expressed by macrophages corresponding to the enzymes detected in SCLC lines. Values represent mean ± SD.

2.5. ECM Invasion of SCLC CTC Cells

SCLC CTC lines grow as large spheroids, called tumorospheres, which continuously increase in diameter in regular tissue cultures. Upon embedding in Matrigel, cells of the tumorosphere started to invade the surrounding matrix (Figure 5). According to the light microscopic appearance, the ECM was dissolved during this invasive growth. Quantitation of the areas covered by the original cluster (60,070 square pixels) and the invaded cells (142,460 square pixels) revealed an approximately 2.4-fold extension of the initial tumorosphere within 12 days. The pan-cathepsin inhibitor E-64 did not inhibit invasion into ECM (data not shown), pointing to MMP-9 as major effector.

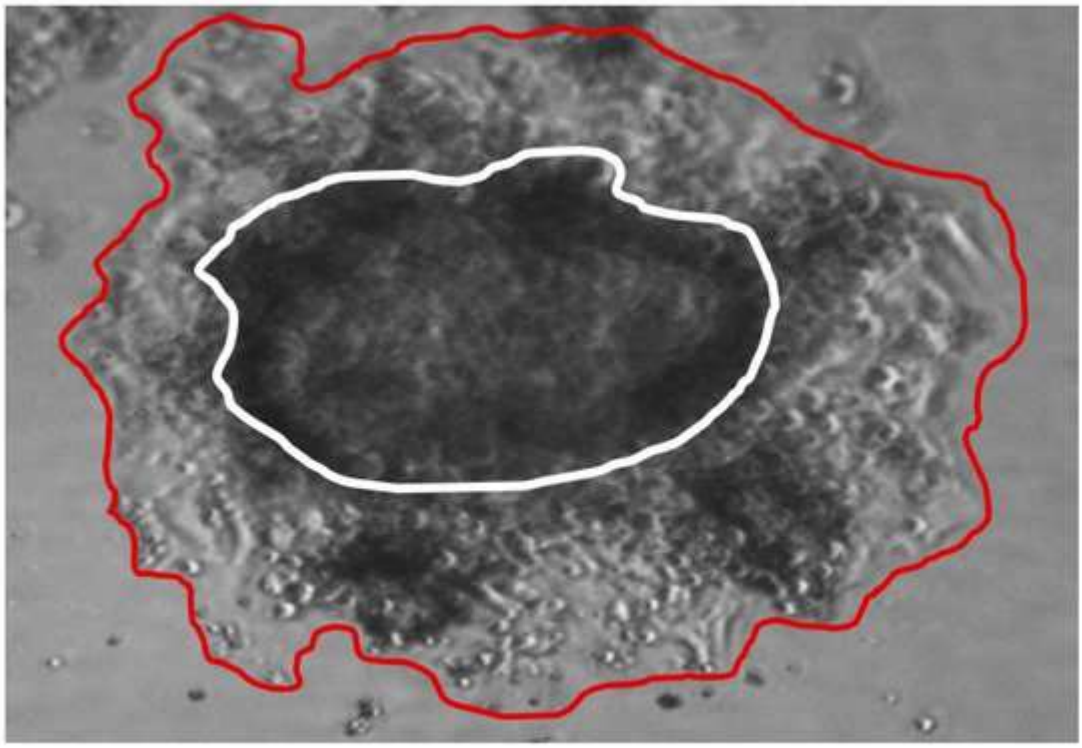

Figure 5. Light microscopic picture of a SCLC tumorosphere embedded in extracellular matrix (ECM) showing invasive outgrowth of cancer cells. The white line indicates the contour of the original spheroid at beginning (magnification 40×).

2.6. Scheme of Tumor Cell and CTC—Normal Cells Protease Interaction

Figure 6 depicts the MMP and cathepsin proteases involved in tumor cell and CTC interactions with normal cell types. Proteases occurring in tumor cell interactions are shown in green color, and those in CTC interactions in red color. For CTCs, the specific proteases comprise MMP9, Cathepsin S, and cathepsin X/Z/P. Cathepsin C is involved in tumor interactions with mast cells, lymphocytes, fibroblasts and macrophages, and uPA with fibroblasts and endothelial cells, respectively. Additionally, MMP-8 functions in relation to neutrophils. In the case of CTCs, the cells interact with all normal cell types, except mesenchymal cells via one or more of the proteases specifically expressed.

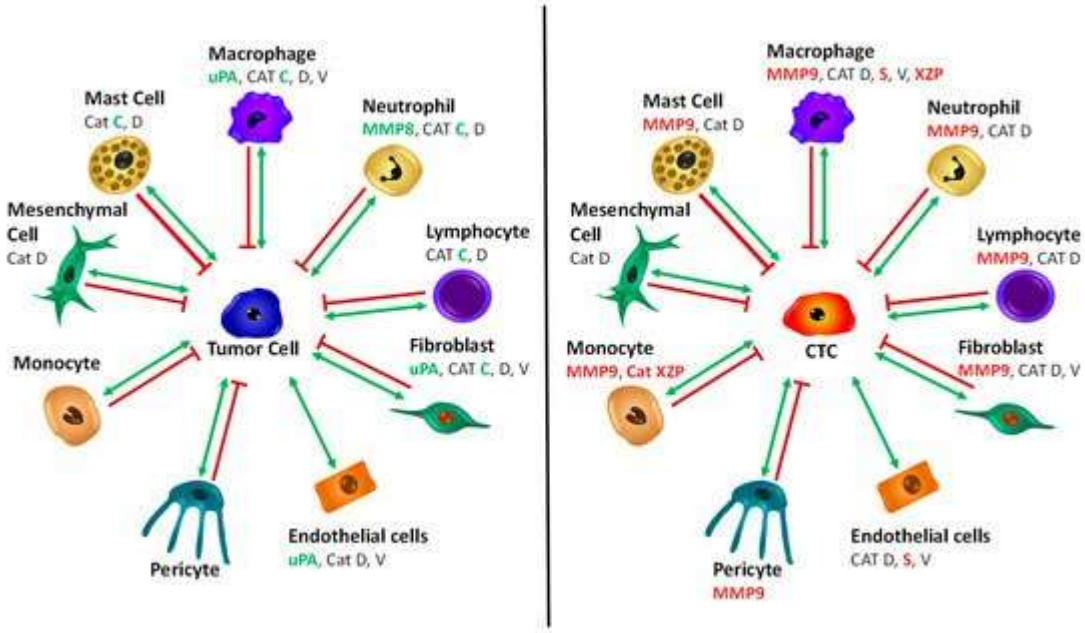

Figure 6. Scheme of involvement of distinct proteases in tumor cell-normal cell and circulating tumor cells (CTC)-normal cell interactions.

3. Discussion

Tumor cell spread to distant sites is a complex process involving multiple cell types, soluble growth factors, adhesion receptors, and tissue remodeling [1,27]. Pericellular proteases are involved in cancer invasion and metastasis due to their ability to degrade ECM constituents [11,12]. Furthermore, proteases regulate progression and dissemination through processing of cell adhesion molecules, cytokines, growth factors, and kinases [1]. SCLC is surrounded by an extensive stroma of ECM, protecting cancer cells by prosurvival signaling [28,29,30]. Although several enzymes of the proteolytic tumor network are associated with invasion and metastasis, the proteases responsible for the migration and invasion of CTCs have not been identified so far. CTCs are highly heterogeneous and only a small fraction of these cells is capable of inducing metastases [9,15]. Availability of two CTC cell lines established from SCLC enabled us to screen the proteases secreted by these tumor cells in vitro. Both CTC lines used were in tissue culture for several months after initiation of the lines. However, according to their transcriptomic and proteomic profile as well as biomarkers and morphology (formation of spheroids), these lines exhibit a stable phenotype. This Western blot screen comprised 35 proteases including ADAMs 8, 9, S1, and S13; cathepsins A, B, C, D, E, L, S, V, and X/Z/P; MMPs 1, 2, 3, 7, 8, 9, 10, 12, 13; kallikreins 5, 6, 7, 10, 11, 13; neprilysin/CD10, presenilin-1, PC-9, proteinase 3, and uPA. Of all these enzymes, uPA, MMP-8 and -9 as well as several cathepsins were expressed in SCLC tumor lines, the two SCLC CTCs and conditioned macrophages in our screening experiments.

Longitudinal biopsies are rarely available for SCLC patients. However, a series of three cell lines, namely GLC14, GLC16, and GLC19, were established from the biopsies of a single SCLC patient [24]. In detail, the GLC14 cell line was from a right supraclavicular node metastasis of the patient and, following treatment with several cycles of cyclophosphamide, doxorubicin, and etoposide, the chemoresistant GLC16 cell line was established from a biopsy of the relapsing tumor [31,32]. Our results demonstrate that progression to this chemoresistant relapse is characterized by increased expression of MMP-9, as well as cathepsins S, X/Z/P, D and decreased expression of cathepsin V. In order to study protease expression by locally invasive tumor cells, we employed cell lines NCI-H526 and SCLC26A, representing a bone metastasis and a local pleural metastasis, respectively. Both cell lines exhibited high expression of PC-9. The PCs are secretory proteolytic enzymes that activate precursor proteins into biologically active forms by limited proteolysis at internal sites [33]. Many PC substrates are well known cancer-associated proteins such as growth factors, growth factor receptors, integrins, and MMPs [34]. For example, insulin-like growth factor 1 (IGF-1) and its receptor, transforming growth factor beta (TGF-beta), VEGF-C, and MMPs have direct roles in tumor progression and metastasis [35]. Additionally, IGF-1 and platelet-derived growth factor (PDGF) were found to mediate a mitogenic/antiapoptotic function through Akt activation [36]. SCLC26A was found to rely on EGF, IGF-1, and insulin for proliferation, thus, PC-9 seems to be required to process growth factors in these two metastatic cell lines (results not shown).

Protease secretion of two CTC cell lines BHGc7 and BHGc10, derived from SCLC, is largely confined to MMP-9 and several members of the cathepsin family, with cathepsin S exclusively found in the CTCs and the metastatic and chemoresistant GLC16 cell line, but not in lines established from local metastases. Cathepsins V, X/Z/P, A, and D are expressed in BHGc10, and all cathepsins tested, namely V, X/Z/P, A, C, and D in BHGc7. PC-9 is not expressed in significant concentrations in both CTC cell lines, as well as uPA, MMP-8 and all other of the 35 proteases included in the Western blot array panel. In most cancers, there are increased levels of one or several members of the MMPs and, in particular, MMP-9 is closely associated with the invasive and metastatic potential of most types of solid cancers [37,38,39]. MMP-9 is expressed by neutrophils, macrophages, fibroblasts, and endothelial cells, among others, and can cleave many ECM proteins, soluble mediators and release cell surface proteins. The most important substrates of this enzyme are gelatin, collagen, elastin, and type-IV collagen of basement membranes [37,38,39]. In lung cancer, both non-small cell lung cancer NSCLC and SCLC tumor samples showed significantly higher MMP-9 expression compared to normal tissues as well as elevated MMP-9 in serum samples [40,41]. MMPs and tissue inhibitors of metalloproteinase (TIMPs) are widely expressed in SCLC [42,43]. MMP-9 was found to be elevated in the serum of NSCLC patients compared to healthy controls and to potentiates formation of pulmonary metastasis [44]. Furthermore, the MMP-9 serum level was higher in chemoresistant prostate cancer patients upon disease progression [45]. Among normal cells, inflammatory cell-derived MMP-9 promotes extravasation in combination with tumor-derived MMP-9 and endothelial cell clusters at metastatic sites are stimulated to produce MMP-9 by circulating VEGF [39]. Furthermore, MMP-9 from inflammatory cells, particularly neutrophils and tumor-associated macrophages (TAMs), codetermines prognosis and outcome [46]. In chronic obstructive pulmonary disease (COPD), increased expression of MMP-9 by inflammatory cells e.g., neutrophils

and macrophages, is correlated with a variety of processes that cause lung damage [47]. However, the development of MMP broad-range inhibitors failed to result in a clinical benefit for patients [48].

Secretion of cathepsin S seems to be a specific characteristic of the SCLC CTCs tested. A large study showed a significant correlation between elevated serum cathepsin S levels and increased mortality risk in older adults [49]. Although cysteine cathepsins have been identified as key regulators of cancer growth, their specific role in tumor development remains unclear [50]. Cysteine cathepsin proteases are frequently dysregulated during transformation and participate in cancer progression, invasion, metastasis, and drug resistance [6,51]. The human cysteine cathepsin family comprises 11 endopeptidases which are synthesized as inactive zymogens and are activated in acidic tumor regions [52,53]. Intracellular cathepsins are acid hydrolases involved in protein catabolism, autophagy, and signal transduction. Secreted cathepsins adapt the tumor microenvironment through degradation of ECM and processing of growth factors, cytokines, and chemokines. Cathepsins contribute to tissue invasion and metastasis by cleavage of cell-cell adhesion molecules. Besides cancer cells, various other cell types express cathepsins with exceptional abundance of cathepsins B, H and S in TAMs. Furthermore, cathepsin Z is essential for the activation of focal adhesion kinase (FAK) and SRC and, furthermore, cathepsins regulate tumor angiogenesis [6,54,55,56]. For example, coadministration of the pan-cathepsin inhibitor E-64 with gemcitabine doubled the median survival in a murine model of pancreatic cancer [57]. However, the clinical failure of broad-spectrum MMP inhibitors has disapproved therapeutic strategies targeting protease families in general.

Cathepsin S is involved in presentation on major histocompatibility complex (MHC) class II molecules, and in contrast to other lysosomal proteases, it retains stability outside the lysosome and cleaves ECM proteins including laminin, fibronectin, elastin, osteocalcin, and some collagens [58]. Immune cells, including macrophages and microglia, secrete cathepsin S in response to inflammatory mediators derived from tumor cells [59]. Investigations on breast, lung, brain and head and neck tumors, as well as in body fluids of ovarian, uterine, melanoma, and colorectal carcinoma bearing patients, have shown that cathepsins are highly predictive for survival [60,61,62,63]. Cysteine cathepsins upregulation has been demonstrated in many human tumors, including breast, lung, brain, gastrointestinal, head and neck cancer, and melanoma [64]. Cathepsin S plays an active role in angiogenesis by generation of proangiogenic peptides, promotes tumor growth, and has been shown to be a significant prognostic factor for patients with glioblastoma [58,65]. High cathepsin S expression at the primary site correlated with decreased brain metastasis-free survival in breast cancer patients [61]. Both macrophages and tumor cells produce cathepsin S, and only the combined depletion significantly reduced brain metastasis in vivo. Sevenich et al. described a role for cathepsin S in brain-specific metastasis and identified JAM-B, a blood–brain barrier component, as a cathepsin S substrate [61]. A cathepsin S inhibitor reduced MC38 and MCF7 tumor cell invasion and furthermore, significantly reduced vascular endothelial tubule formation in vitro [62]. The inhibitor reduced the tumor growth of both cell lines in an in vivo xenograft model. The initial development of cathepsin S inhibitors targeted irreversible, covalent inhibitors, but more recently, the focus has been on reversible inhibitors [66]. The specific expression of cathepsin S by SCLC CTCs seems to be of high significance, since SCLC frequently leads to development of brain metastases, which continue to be associated with short median survival of 4.9 months [67]. Cathepsin D is a protease involved in the metastasis and angiogenesis of mammary carcinomas [68]. Procathepsin D (pCD) is overexpressed and secreted by cells of various tumor types, including breast and lung carcinomas, affecting multiple features of tumor cells including proliferation, invasion, metastasis, and apoptosis [69]. Studies have demonstrated that enzymatic function of cathepsin D is not restricted solely to acidic milieu of lysosomes, with important consequences in regulation of apoptosis [70]. Apoptosis is also regulated by catalytically inactive mutants of cathepsin D, which suggests that it interacts with other important molecules and influences cell signaling. Moreover, procathepsin D (pCatD), secreted from cancer cells, acts as a mitogen on both cancer and stromal cells, and stimulates their pro-invasive and pro-metastatic properties.

Despite the role of uPA and its receptor uPAR/CD87 as major regulators of ECM degradation, and their involvement in cell migration and invasion under physiological and pathological conditions, they were not found in the CTCs tested here [71]. Otherwise, this system is involved in the development of most invasive cancer phenotypes and is a strong predictor of poor patient survival [72]. Furthermore, high serum uPAR(I) levels are associated with short overall survival in SCLC patients and identify chemoresistant cells [71,73]. Endopeptidase CD10 hydrolyzes bioactive peptides, including neuropeptides, but was not found in our assays, in accordance with previous findings demonstrating the absence of CD10 in most SCLCs [74,75]. DPPIV/CD26 is expressed in almost all cases of adenocarcinoma, whereas all cases of squamous cell carcinoma, SCLC, large cell carcinoma and carcinoid were negative [76,77]. ADAM/TSs are involved in the regulation of growth factor activities and integrin functions, leading to promotion of cell growth and invasion [78,79,80]. ADAM8 is overexpressed in the vast majority of lung cancers and can be a diagnostic marker of

lung cancer [81,82]. Extensive cytoplasmic expression of tissue and plasma kallikrein was observed in SCLC and NSCLC, but these enzymes as well as ADAM/TS were not detected in BHGc7/10 [83].

The functional activity of CTC-derived proteases has been studied with help of a microfluidics system [84]. This system concentrates rare cancer cells by size, flushes the CTCs to remove contaminants, and encapsulates the CTCs into microdroplets containing a fluorescent MMP substrate. CTCs from prostate cancer patients showed increased MMP activity (1.7- to 200-fold) over those of leukocytes from the same patient (average ratio 2.6 ± 1.5). Samples from 6/7 metastatic castration-resistant prostate cancer patients contained CTCs, and 87% of these CTCs secreted MMPs. However, this contradicts the finding that only a very small fraction of CTCs are actual metastasis-initiating cells. Nevertheless, our results are in agreement with this microfluidic study which proved increased MMP-9 activity. Embedding of the SCLC CTC clusters and subsequent cellular outgrowth demonstrates invasion, and is expected to provide a suitable model for studying the participation of individual proteases. In a suspension tissue culture, the same clusters increase in size continuously but show no release of viable cells [23]. In conclusion, assessment of the pattern of secreted proteases of SCLC CTCs revealed for the first time cathepsin S as specific enzyme associated with this class of unique cells, executing tumor dissemination to distal sites. Cathepsin S has been discussed as putative cancer target, but not in relation to CTCs [85]. Specific cathepsin S inhibitors like LY3000328 have been developed for application in nonmalignant diseases, and may be checked for their effects on tumor spread [86,87].

4. Materials and Methods

4.1. Cell Lines and Tissue Culture

GLC14 and GLC16 were obtained from Department of Radiation Biology, the Finsen Centre, National University Hospital, Copenhagen, Denmark and NCI-H526 was obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). GLC14, GLC16, and GLC19 constitute a series of three cell lines which have been established from biopsies of a single SCLC patient [24]. In detail, the GLC14 cell line was from a right supraclavicular node metastasis of the patient and, following treatment with several cycles of cyclophosphamide, doxorubicin, and etoposide, the chemoresistant GLC16 cell line was established from a biopsy of the relapsing tumor [31,32]. SCLC26A was established in our laboratory from pleural effusion of an SCLC patient before treatment and the two CTC cell lines, BHGc7 and BHGc10, were grown from peripheral blood samples of two refractory SCLC patients [10]. Cell lines were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) medium supplemented with 10% fetal bovine serum (Seromed, Berlin, Germany) and antibiotics (Sigma-Aldrich, penicillin-streptomycin-neomycin solution). All cell lines were grown in suspension or loosely attached and were subcultivated by replacing part of the medium. All other reagents were from Sigma-Aldrich.

4.2. Western Blot Protease Screening Array

For assessment of the proteases expressed, cell culture supernatants were processed using a Human Proteome Profiler Protease Kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). In brief, this Western blot array comprised reagents to detect 35 proteases, including ADAM/TS, kallikreins, MMPs, cathepsins, uPA, neprilysin (CD10), presenilin-1, DPPIV (CD26), and proprotein convertase 9 (PC-9). Assays were performed in duplicate. The different arrays contain several control spots to calibrate for protein content of the samples applied. Conditioned medium of the respective cell lines (500 µL) were used for performing the assay and the spots detected by chemoluminescence were analyzed using Origin 9.0 software (OriginLab, Northampton, MA, USA).

4.3. ECM Invasion Assay

Tumorospheres of BHGc10 cell line were isolated by sedimentation and resuspended in Matrigel (Sigma-Aldrich). Matrigel was thawed overnight at 2–8 °C before use, mixed with medium containing the spheroids (1:1) and dispensed to 18-well plates (Greiner, Kremsmuenster, Austria) using pre-cooled pipettes. Outgrowth of tumor cells was observed by light microscopy and areas covered by the original cluster and the invaded cells quantitated using Image J.

4.4. Statistics

Results were evaluated using unpaired t tests, using Origin 9.0 software. $p < 0.05$ was regarded as statistically significant.

5. Conclusions

Due to the high heterogeneity of the CTCs, detection of the expression of proteases has been limited to demonstration of increased levels of MMP-9 compared to normal blood cell types [84]. Our screens have shown the expression of MMP-9 and cathepsins by pure populations of CTC lines for the first time. This analysis has allowed for the differentiation of the protease expression of tumor and normal cells, respectively [88,89]. Detection of Cathepsin S in SCLC CTCs may be of special importance for this tumor in respect to frequent occurrence of brain metastases. Invasion of ECM by SCLC CTCs may constitute an important model for studying the participating cellular factors.

Author Contributions

B.R., experimentation and writing of the manuscript; L.K., experimentation; A.P., tissue culture; M.H. and E.U., discussion of the results; I.H. and R.Z., designing of the project; G.H. interpretation of the data and discussion

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Conflicts of Interest

The authors declare no conflict of interest.

References

1. Seyfried, T.N.; Huysentruyt, L.C. On the origin of cancer metastasis. Crit. Rev. Oncog. **2013**, 18, 43–73. [Google Scholar] [CrossRef] [PubMed]
2. Chaffer, C.L.; Weinberg, R.A. A perspective on cancer cell metastasis. Science **2011**, 33, 1559–1564. [Google Scholar] [CrossRef] [PubMed]
3. Micalizzi, D.S.; Maheswaran, S.; Haber, D.A. A conduit to metastasis: Circulating tumor cell biology. Genes Dev. **2017**, 31, 1827–1840. [Google Scholar] [CrossRef] [PubMed]
4. Hamilton, G.; Rath, B. Circulating Tumor Cells in the Parallel Invasion Model Supporting Early Metastasis. Oncomedicine **2018**, 3, 15–27. [Google Scholar] [CrossRef][Green Version]
5. Mason, S.D.; Joyce, J.A. Proteolytic networks in cancer. Trends Cell Biol. **2011**, 21, 228–237. [Google Scholar] [CrossRef] [PubMed][Green Version]
6. Sevenich, L.; Joyce, J.A. Pericellular proteolysis in cancer. Genes Dev. **2014**, 28, 2331–2347. [Google Scholar] [CrossRef] [PubMed]
7. Belli, C.; Trapani, D.; Viale, G.; D'Amico, P.; Duso, B.A.; Della Vigna, P.; Orsi, F.; Curigliano, G. Targeting the microenvironment in solid tumors. Cancer Treat. Rev. **2018**, 65, 22–32. [Google Scholar] [CrossRef]

8. Paterlini-Bréchot, P. Circulating tumor cells: Who is the killer? Cancer Microenviron. **2014**, 7, 161–176. [Google Scholar] [CrossRef]
9. Andree, K.C.; van Dalum, G.; Terstappen, L.W. Challenges in circulating tumor cell detection by the CellSearch system. Mol. Oncol. **2016**, 10, 395–407. [Google Scholar] [CrossRef]
10. Katt, M.E.; Wong, A.D.; Searson, P.C. Dissemination from a Solid Tumor: Examining the Multiple Parallel Pathways. Trends Cancer **2018**, 4, 20–37. [Google Scholar] [CrossRef]
11. Hamilton, G.; Burghuber, O.; Zeillinger, R. Circulating tumor cells in small cell lung cancer: Ex vivo expansion. Lung **2015**, 193, 451–452. [Google Scholar] [CrossRef] [PubMed]
12. Hamilton, G.; Hochmair, M.; Rath, B.; Klameth, L.; Zeillinger, R. Small cell lung cancer: Circulating tumor cells of extended stage patients express a mesenchymal-epithelial transition phenotype. Cell Adhes. Migr. **2016**, 10, 360–367. [Google Scholar] [CrossRef] [PubMed][Green Version]
13. Hou, J.M.; Krebs, M.; Ward, T.; Sloane, R.; Priest, L.; Hughes, A.; Clack, G.; Ranson, M.; Blackhall, F.; Dive, C. Circulating tumor cells as a window on metastasis biology in lung cancer. Am. J. Pathol. **2011**, 178, 989–996. [Google Scholar] [CrossRef] [PubMed]
14. Byers, L.A.; Rudin, C.M. Small cell lung cancer: Where do we go from here? Cancer **2015**, 121, 664–672. [Google Scholar] [CrossRef] [PubMed]
15. Semenova, E.A.; Nagel, R.; Berns, A. Origins, genetic landscape, and emerging therapies of small cell lung cancer. Genes Dev. **2015**, 29, 1447–1462. [Google Scholar] [CrossRef] [PubMed][Green Version]
16. Perlikos, F.; Harrington, K.J.; Syrigos, K.N. Key molecular mechanisms in lung cancer invasion and metastasis: A comprehensive review. Crit. Rev. Oncol. Hematol. **2013**, 87, 1–11. [Google Scholar] [CrossRef] [PubMed]
17. Jiang, W.G.; Sanders, A.J.; Katoh, M.; Ungefroren, H.; Gieseler, F.; Prince, M.; Thompson, S.K.; Zollo, M.; Spano, D.; Dhawan, P.; et al. Tissue invasion and metastasis: Molecular, biological and clinical perspectives. Semin. Cancer Biol. **2015**, 35, S244–S275. [Google Scholar] [CrossRef] [PubMed][Green Version]
18. Hodgkinson, C.L.; Morrow, C.J.; Li, Y.; Metcalf, R.L.; Rothwell, D.G.; Trapani, F.; Polanski, R.; Burt, D.J.; Simpson, K.L.; Morris, K.; et al. Tumorigenicity and genetic profiling of circulating tumor cells in small-cell lung cancer. Nat. Med. **2014**, 20, 897–903. [Google Scholar] [CrossRef]
19. Yu, N.; Zhou, J.; Cui, F.; Tang, X. Circulating tumor cells in lung cancer: Detection methods and clinical applications. Lung **2015**, 193, 157–171. [Google Scholar] [CrossRef]
20. Cayrefourcq, L.; Mazard, T.; Joosse, S.; Solassol, J.; Ramos, J.; Assenat, E.; Schumacher, U.; Costes, V.; Maudelonde, T.; Pantel, K.; et al. Establishment and characterization of a cell line from human circulating colon cancer cells. Cancer Res. **2015**, 75, 892–901. [Google Scholar] [CrossRef]
21. Yu, M.; Bardia, A.; Aceto, N.; Bersani, F.; Madden, M.W.; Donaldson, M.C.; Desai, R.; Zhu, H.; Comaills, V.; Zheng, Z.; et al. Cancer therapy. Ex vivo culture of circulating breast tumor cells for individualized testing of drug susceptibility. Science **2014**, 345, 216–220. [Google Scholar] [CrossRef] [PubMed]
22. Friedlander, T.W.; Premasekharan, G.; Paris, P.L. Looking back, to the future of circulating tumor cells. Pharmacol. Ther. **2014**, 142, 271–280. [Google Scholar] [CrossRef] [PubMed]
23. Klameth, L.; Rath, B.; Hochmaier, M.; Moser, D.; Redl, M.; Mungenast, F.; Gelles, K.; Ulsperger, E.; Zeillinger, R.; Hamilton, G. Small cell lung cancer: Model of circulating tumor cell tumorospheres in chemoresistance. Sci. Rep. **2017**, 7, 5337. [Google Scholar] [CrossRef] [PubMed]
24. Berendsen, H.H.; de Leij, L.; de Vries, E.G.; Mesander, G.; Mulder, N.H.; de Jong, B.; Buys, C.H.; Postmus, P.E.; Poppema, S.; Sluiter, H.J.; et al. Characterization of three small cell lung cancer cell lines established from one patient during longitudinal follow-up. Cancer Res. **1988**, 48, 6891–6899. [Google Scholar] [PubMed]

25. Hamilton, G.; Klameth, L.; Rath, B.; Thalhammer, T. Synergism of cyclin-dependent kinase inhibitors with camptothecin derivatives in small cell lung cancer cell lines. Molecules **2014**, 19, 2077–2088. [Google Scholar] [CrossRef] [PubMed]
26. Hamilton, G.; Rath, B. Circulating tumor cell interactions with macrophages: Implications for biology and treatment. Transl. Lung Cancer Res. **2017**, 6, 418–430. [Google Scholar] [CrossRef] [PubMed]
27. Quail, D.F.; Joyce, J.A. Microenvironmental regulation of tumor progression and metastasis. Nat. Med. **2013**, 19, 1423–1437. [Google Scholar] [CrossRef][Green Version]
28. Rintoul, R.C.; Sethi, T. The role of extracellular matrix in small-cell lung cancer. Lancet Oncol. **2001**, 2, 437–442. [Google Scholar] [CrossRef]
29. Buttery, R.C.; Rintoul, R.C.; Sethi, T. Small cell lung cancer: The importance of the extracellular matrix. Int. J. Biochem. Cell Biol. **2004**, 36, 1154–1160. [Google Scholar] [CrossRef]
30. Hodkinson, P.S.; Mackinnon, A.C.; Sethi, T. Extracellular matrix regulation of drug resistance in small-cell lung cancer. Int. J. Radiat. Biol. **2007**, 83, 733–741. [Google Scholar] [CrossRef]
31. De Vries, E.G.; Meijer, C.; Timmer-Bosscha, H.; Berendsen, H.H.; de Leij, L.; Scheper, R.J.; Mulder, N.H. Resistance mechanisms in three human small cell lung cancer cell lines established from one patient during clinical follow-up. Cancer Res. **1989**, 49, 4175–4178. [Google Scholar] [PubMed]
32. Hamilton, G.; Olszewski, U. Chemotherapy-induced Enrichment of Cancer Stem Cells in Lung Cancer. J. Bioanal. Biomed. **2013**, S9, 3. [Google Scholar] [CrossRef]
33. Klein-Szanto, A.J.; Bassi, D.E. Proprotein convertase inhibition: Paralyzing the cell's master switches. Biochem. Pharmacol. **2017**, 140, 8–15. [Google Scholar] [CrossRef] [PubMed]
34. Bassi, D.E.; Fu, J.; Lopez de Cicco, R.; Klein-Szanto, A.J. Proprotein convertases: "master switches" in the regulation of tumor growth and progression. Mol. Carcinogenes. **2005**, 44, 151–161. [Google Scholar] [CrossRef] [PubMed]
35. Artenstein, A.W.; Opal, S.M. Proprotein convertases in health and disease. N. Engl. J. Med. **2011**, 365, 2507–2518. [Google Scholar] [CrossRef] [PubMed]
36. Scamuffa, N.; Calvo, F.; Chrétien, M.; Seidah, N.G.; Khatib, A.M. Proprotein convertases: Lessons from knockouts. FASEB J. **2006**, 20, 1954–1963. [Google Scholar] [CrossRef] [PubMed]
37. Hadler-Olsen, E.; Winberg, J.O.; Uhlin-Hansen, L. Matrix metalloproteinases in cancer: Their value as diagnostic and prognostic markers and therapeutic targets. Tumour Biol. **2013**, 34, 2041–2051. [Google Scholar] [CrossRef]
38. Huang, H. Matrix metalloproteinase-9 (MMP-9) as a cancer biomarker and MMP-9 biosensors: Recent advances. Sensors (Basel) **2018**, 18, 3249. [Google Scholar] [CrossRef]
39. Farina, A.R.; Mackay, A.R. Gelatinase B/MMP-9 in tumour pathogenesis and progression. Cancers (Basel) **2014**, 6, 240–296. [Google Scholar] [CrossRef]
40. El-Badrawy, M.K.; Yousef, A.M.; Shaalan, D.; Elsamanoudy, A.Z. Matrix metalloproteinase-9 expression in lung cancer patients and its relation to serum MMP-9 activity, pathologic type, and prognosis. J. Bronchol. Interv. Pulmonol. **2014**, 21, 327–334. [Google Scholar] [CrossRef]
41. Jumper, C.; Cobos, E.; Lox, C. Determination of the serum matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in patients with either advanced small-cell lung cancer or non-small-cell lung cancer prior to treatment. Respir. Med. **2004**, 98, 173–177. [Google Scholar] [CrossRef] [PubMed][Green Version]
42. Michael, M.; Babic, B.; Khokha, R.; Tsao, M.; Ho, J.; Pintilie, M.; Leco, K.; Chamberlain, D.; Shepherd, F.A. Expression and prognostic significance of metalloproteinases and their tissue inhibitors in patients with small-cell lung cancer. J. Clin. Oncol. **1999**, 17, 1802–1808. [Google Scholar] [CrossRef] [PubMed]

43. Li, Z.; Guo, Y.; Jiang, H.; Zhang, T.; Jin, C.; Young, C.Y.; Yuan, H. Differential regulation of MMPs by E2F1, Sp1 and NF-kappa B controls the small cell lung cancer invasive phenotype. BMC Cancer **2014**, 14, 276. [Google Scholar] [CrossRef] [PubMed]
44. Van Kempen, L.C.; Coussens, L.M. MMP-9 potentiates pulmonary metastasis formation. Cancer Cell **2002**, 2, 251–252. [Google Scholar] [CrossRef]
45. Skerenova, M.; Mikulova, V.; Capoun, O.; Zima, T.; Tesarova, P. Circulating tumor cells and serum levels of MMP-2.; MMP-9 and VEGF as markers of the metastatic process in patients with high risk of metastatic progression. Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech Repub. **2017**, 161, 272–280. [Google Scholar] [CrossRef] [PubMed]
46. Vandooren, J.; Van den Steen, P.E.; Opdenakker, G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9): The next decade. Crit. Rev. Biochem. Mol. Biol. **2013**, 48, 222–272. [Google Scholar] [CrossRef] [PubMed]
47. Muroski, M.E.; Roycik, M.D.; Newcomer, R.G.; Van den Steen, P.E.; Opdenakker, G.; Monroe, H.R.; Sahab, Z.J.; Sang, Q.X. Matrix metalloproteinase-9/gelatinase B is a putative therapeutic target of chronic obstructive pulmonary disease and multiple sclerosis. Curr. Pharm. Biotechnol. **2008**, 9, 34–46. [Google Scholar]
48. Fields, G.B. New strategies for targeting matrix metalloproteinases. Matrix Biol. **2015**, 44–46, 239–246. [Google Scholar] [CrossRef]
49. Jobs, E.; Ingelsson, E.; Risérus, U.; Nerpin, E.; Jobs, M.; Sundström, J.; Basu, S.; Larsson, A.; Lind, L.; Ärnlöv, J. Association between serum cathepsin S and mortality in older adults. JAMA **2011**, 306, 1113–1121. [Google Scholar] [CrossRef]
50. Olson, O.C.; Joyce, J.A. Cysteine cathepsin proteases: Regulators of cancer progression and therapeutic response. Nat. Rev. Cancer **2015**, 15, 712–729. [Google Scholar] [CrossRef]
51. Shree, T.; Olson, O.C.; Elie, B.T.; Kester, J.C.; Garfall, A.L.; Simpson, K.; Bell-McGuinn, K.M.; Zabor, E.C.; Brogi, E.; Joyce, J.A. Macrophages and cathepsin proteases blunt chemotherapeutic response in breast cancer. Genes Dev. **2011**, 25, 2465–2479. [Google Scholar] [CrossRef] [PubMed][Green Version]
52. Kos, J.; Lah, T.T. Cysteine proteinases and their endogenous inhibitors: Target proteins for prognosis, diagnosis and therapy in cancer. Oncol. Rep. **1998**, 5, 1349–1361. [Google Scholar] [CrossRef] [PubMed]
53. Estrella, V.; Chen, T.; Lloyd, M.; Wojtkowiak, J.; Cornnell, H.H.; Ibrahim-Hashim, A.; Bailey, K.; Balagurunathan, Y.; Rothberg, J.M.; Sloane, B.F.; et al. Acidity generated by the tumor microenvironment drives local invasion. Cancer Res. **2013**, 73, 1524–1535. [Google Scholar] [CrossRef] [PubMed]
54. Gocheva, V.; Wang, H.W.; Gadea, B.B.; Shree, T.; Hunter, K.E.; Garfall, A.L.; Berman, T.; Joyce, J.A. IL4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. Genes Dev. **2010**, 24, 241–255. [Google Scholar] [CrossRef] [PubMed]
55. Small, D.M.; Burden, R.E.; Jaworski, J.; Hegarty, S.M.; Spence, S.; Burrows, J.F.; McFarlane, C.; Kissenpfennig, A.; McCarthy, H.O.; Johnston, J.A.; et al. Cathepsin S from both tumor and tumor-associated cells promote cancer growth and neovascularization. Int. J. Cancer **2013**, 133, 2102–2112. [Google Scholar] [CrossRef] [PubMed]
56. Wang, B.; Sun, J.; Kitamoto, S.; Yang, M.; Grubb, A.; Chapman, H.A.; Kalluri, R.; Shi, G.P. Cathepsin S controls angiogenesis and tumor growth via matrix-derived angiogenic factors. J. Biol. Chem. **2006**, 281, 6020–6029. [Google Scholar] [CrossRef] [PubMed]
57. Gopinathan, A.; Denicola, G.M.; Frese, K.K.; Cook, N.; Karreth, F.A.; Mayerle, J.; Lerch, M.M.; Reinheckel, T.; Tuveson, D.A. Cathepsin B promotes the progression of pancreatic ductal adenocarcinoma in mice. Gut **2012**, 61, 877–884. [Google Scholar] [CrossRef] [PubMed]
58. Kos, J.; Sekirnik, A.; Kopitar, G.; Cimerman, N.; Kayser, K.; Stremmer, A.; Fiehn, W.; Werle, B. Cathepsin S in tumours, regional lymph nodes and sera of patients with lung

cancer: Relation to prognosis. Br. J. Cancer **2001**, 85, 1193–1200. [Google Scholar] [CrossRef]
59. Ward, C.; Kuehn, D.; Burden, R.E.; Gormley, J.A.; Jaquin, T.J.; Gazdoiu, M.; Small, D.; Bicknell, R.; Johnston, J.A.; Scott, C.J.; et al. Antibody targeting of cathepsin S inhibits angiogenesis and synergistically enhances anti-VEGF. PLoS ONE **2010**, 5. [Google Scholar] [CrossRef]
60. Mohamed, M.M.; Sloane, B.F. Cysteine cathepsins: Multifunctional enzymes in cancer. Nat. Rev. Cancer **2006**, 6, 764–775. [Google Scholar] [CrossRef]
61. Sevenich, L.; Bowman, R.L.; Mason, S.D.; Quail, D.F.; Rapaport, F.; Elie, B.T.; Brogi, E.; Brastianos, P.K.; Hahn, W.C.; Holsinger, L.J.; et al. Analysis of tumour- and stroma-supplied proteolytic networks reveals a brain-metastasis-promoting role for cathepsin S. Nat. Cell Biol. **2014**, 16, 876–888. [Google Scholar] [CrossRef] [PubMed][Green Version]
62. Shi, G.P.; Munger, J.S.; Meara, J.P.; Rich, D.H.; Chapman, H.A. Molecular cloning and expression of human alveolar macrophage cathepsin S, an elastinolytic cysteine protease. J. Biol. Chem. **1992**, 267, 7258–7262. [Google Scholar] [PubMed]
63. Bunatova, K.; Obermajer, N.; Kotyza, J.; Pesek, M.; Kos, J. Levels of cathepsins S and H in pleural fluids of inflammatory and neoplastic origin. Int. J. Biol. Mark. **2009**, 24, 47–51. [Google Scholar] [CrossRef]
64. Berdowska, I. Cysteine proteases as disease markers. Clin. Chim. Acta **2004**, 342, 41–69. [Google Scholar] [CrossRef] [PubMed]
65. Wilkinson, R.D.; Williams, R.; Scott, C.J.; Burden, R.E. Cathepsin S: Therapeutic, diagnostic and prognostic potential. Biol. Chem. **2015**, 396, 867–882. [Google Scholar] [CrossRef] [PubMed]
66. Wiener, J.J.; Sun, S.; Thurmond, R.L. Recent advances in the design of cathepsin S inhibitors. Curr. Top. Med. Chem. **2010**, 10, 717–732. [Google Scholar] [CrossRef] [PubMed]
67. Lukas, R.V.; Gondi, V.; Kamson, D.O.; Kumthekar, P.; Salgia, R. State-of-the-art considerations in small cell lung cancer brain metastases. Oncotarget **2017**, 8, 71223–71233. [Google Scholar] [CrossRef]
68. Dian, D.; Heublein, S.; Wiest, I.; Barthell, L.; Friese, K.; Jeschke, U. Significance of the tumor protease cathepsin D for the biology of breast cancer. Histol. Histopathol. **2014**, 29, 433–438. [Google Scholar]
69. Vetvicka, V.; Fusek, M. Procathepsin D as a tumor marker, anti-cancer drug or screening agent. Anticancer Agents Med. Chem. **2012**, 12, 172–175. [Google Scholar] [CrossRef]
70. Benes, P.; Vetvicka, V.; Fusek, M. Cathepsin D—Many functions of one aspartic protease. Crit. Rev. Oncol. Hematol. **2008**, 68, 12–28. [Google Scholar] [CrossRef][Green Version]
71. Gutova, M.; Najbauer, J.; Gevorgyan, A.; Metz, M.Z.; Weng, Y.; Shih, C.C.; Aboody, K.S. Identification of uPAR-positive chemoresistant cells in small cell lung cancer. PLoS ONE **2007**, 2, e243. [Google Scholar] [CrossRef] [PubMed]
72. Maksimowicz, T.; Gacko, M.; Chyczewska, E.; Chyczewski, L.; Myćko, G.; Worowski, K. Plasminogen activators and plasmin in lung cancer. Rocz. Akad. Med. Bialymst. **1997**, 42, 72–78. [Google Scholar] [PubMed]
73. Almasi, C.E.; Drivsholm, L.; Pappot, H.; Høyer-Hansen, G.; Christensen, I.J. The liberated domain I of urokinase plasminogen activator receptor—A new tumour marker in small cell lung cancer. APMIS **2013**, 121, 189–196. [Google Scholar] [CrossRef] [PubMed]
74. Maguer-Satta, V.; Besançon, R.; Bachelard-Cascales, E. Concise review: Neutral endopeptidase (CD10): A multifaceted environment actor in stem cells, physiological mechanisms, and cancer. Stem Cells **2011**, 29, 389–396. [Google Scholar] [CrossRef] [PubMed]
75. Cohen, A.J.; Bunn, P.A.; Franklin, W.; Magill-Solc, C.; Hartmann, C.; Helfrich, B.; Gilman, L.; Folkvord, J.; Helm, K.; Miller, Y.E. Neutral endopeptidase: Variable expression in human lung, inactivation in lung cancer, and modulation of peptide-induced calcium flux. Cancer Res. **1996**, 56, 831–839. [Google Scholar] [PubMed]

76. Asada, Y.; Aratake, Y.; Kotani, T.; Marutsuka, K.; Araki, Y.; Ohtaki, S.; Sumiyoshi, A. Expression of dipeptidyl aminopeptidase IV activity in human lung carcinoma. Histopathology **1993**, 23, 265–270. [Google Scholar] [CrossRef] [PubMed]
77. Dimitrova, M.; Ivanov, I.; Todorova, R.; Stefanova, N.; Moskova-Doumanova, V.; Topouzova-Hristova, T.; Saynova, V.; Stephanova, E. Comparison of the activity levels and localization of dipeptidyl peptidase IV in normal and tumor human lung cells. Tissue Cell **2012**, 44, 74–79. [Google Scholar] [CrossRef]
78. Cal, S.; López-Otín, C. ADAMTS proteases and cancer. Matrix Biol. **2015**, 44–46, 77–85. [Google Scholar] [CrossRef]
79. Kumar, S.; Rao, N.; Ge, R.A. Emerging Roles of ADAMTSs in Angiogenesis and Cancer. Cancers (Basel) **2012**, 4, 1252–1299. [Google Scholar] [CrossRef][Green Version]
80. Mochizuki, S.; Okada, Y. ADAMs in cancer cell proliferation and progression. Cancer Sci. **2007**, 98, 621–628. [Google Scholar] [CrossRef][Green Version]
81. Zhang, W.; Wan, M.; Ma, L.; Liu, X.; He, J. Protective effects of ADAM8 against cisplatin-mediated apoptosis in non-small-cell lung cancer. Cell Biol. Int. **2013**, 37, 47–53. [Google Scholar] [CrossRef] [PubMed]
82. Ishikawa, N.; Daigo, Y.; Yasui, W.; Inai, K.; Nishimura, H.; Tsuchiya, E.; Kohno, N.; Nakamura, Y. ADAM8 as a novel serological and histochemical marker for lung cancer. Clin. Cancer Res. **2004**, 10, 8363–8370. [Google Scholar] [CrossRef] [PubMed]
83. Chee, J.; Naran, A.; Misso, N.L.; Thompson, P.J.; Bhoola, K.D. Expression of tissue and plasma kallikreins and kinin B1 and B2 receptors in lung cancer. Biol. Chem. **2008**, 389, 1225–1233. [Google Scholar] [CrossRef] [PubMed]
84. Dhar, M.; Lam, J.N.; Walser, T.; Dubinett, S.M.; Rettig, M.B.; Di Carlo, D. Functional profiling of circulating tumor cells with an integrated vortex capture and single-cell protease activity assay. Proc. Natl. Acad. Sci. USA **2018**, 115, 9986–9991. [Google Scholar] [CrossRef] [PubMed]
85. Zhang, L.; Wang, H.; Xu, J. Cathepsin S as a cancer target. Neoplasma **2015**, 62, 16–26. [Google Scholar] [CrossRef] [PubMed]
86. Jadhav, P.K.; Schiffler, M.A.; Gavardinas, K.; Kim, E.J.; Matthews, D.P.; Staszak, M.A.; Coffey, D.S.; Shaw, B.W.; Cassidy, K.C.; Brier, R.A.; et al. Discovery of Cathepsin S Inhibitor LY3000328 for the Treatment of Abdominal Aortic Aneurysm. ACS Med. Chem. Lett. **2014**, 5, 1138–1142. [Google Scholar] [CrossRef][Green Version]
87. Wilkinson, R.D.; Young, A.; Burden, R.E.; Williams, R.; Scott, C.J. A bioavailable cathepsin S nitrile inhibitor abrogates tumor development. Mol. Cancer **2016**, 15, 29. [Google Scholar] [CrossRef]
88. Shay, G.; Lynch, C.C.; Fingleton, B. Moving targets: Emerging roles for MMPs in cancer progression and metastasis. Matrix Biol. **2015**, 44–46, 200–206. [Google Scholar] [CrossRef]

89. Madsen, D.H.; Bugge, T.H. The source of matrix-degrading enzymes in human cancer: Problems of research reproducibility and possible solutions. J. Cell Biol. **2015**, 209, 195–198. [Google Scholar] [CrossRef][Green Version]

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3 Abstract

Small Cell Lung Cancer (SCLC) is an aggressive neuroendocrine tumor with low survival rates at advanced stage mainly because therapy has not improved for the past decades. Rapid tumor spread is linked to spheroids of circulating tumor cells (CTC), termed tumorospheres, which are markedly chemoresistant due to limited drug perfusion and presence of quiescent/hypoxic cells. The marine drug fascaplysin, a CDK4 inhibitor and DNA intercalator, was demonstrated to exert higher cytotoxic activity against SCLC CTC lines than the conventional chemotherapeutics and to trigger a DNA repair response via signal transduction kinases. Fascaplysin showed an additive effect with cisplatin. Furthermore, metastasis of SCLC CTCs involves the activity of proteolytic enzymes to intravasate into the circulation and eventually, extravasate to establish metastases. The first assessment of a panel of 35 secreted proteases of two CTCs and several SCLC cell lines revealed a higher expression of matrix metalloproteinase 9 (MMP9) and cathepsin S in invasive SCLC and the CTC cell lines. MMP9 inhibitors failed in clinics but cathepsin S may constitute a suitable target to prevent the frequent brain metastases in SCLC. In conclusion, these studies demonstrated high antitumor activity of the marine drug fascaplysin against SCLC and revealed selective expression of cathepsin S which is known to be involved in intracranial tumor spread.

Das kleinzellige Lungenkarzinom (SCLC) ist ein aggressiver, neuroendokriner Tumor mit sehr niedrigen Überlebensraten im fortgeschrittenen Stadium, die in erster Linie durch fehlende Fortschritte in der Therapie während der letzten Jahrzehnte zustande kommen. Die rasche Tumorausbreitung steht in Verbindung mit Sphäroiden der zirkulierenden Tumorzellen (CTC), welche als „Tumorospheres“ bezeichnet werden, die durch verringerte Wirkstoffperfusion und des Gehalts an ruhenden/hypoxischen Zellen charakterisiert sind und dadurch eine hohe Chemoresistenz aufweisen. Der aus einem Meeresorganismus gewonnene Wirkstoff Fascaplysin ist ein CDK4 Inhibitor und DNS-Interkalator, der eine höhere Toxizität gegen die SCLC CTC Linien aufwies als die konventionellen Chemotherapeutika und über Kinasen der Signaltransduktion einen DNS-Reparatur-Response auslöste. Fascaplysin wirkt additiv mit Cisplatin. Zusätzlich involviert die Metastasierung durch SCLC CTCs die Wirkung von proteolytischen Enzymen um in die Zirkulation zu intravasieren und schließlich nach Extravasation Metastasen zu bilden. Die erstmalige Untersuchung der Expression von 35 sekretierten Proteasen zweier CTC und mehrerer SCLC Linien ergab eine höhere Expression von Matrix-Metalloprotease 9 (MMP9) und Cathepsin

S in invasiven SCLC und den CTC Linien. MMP9 Inhibitoren waren in klinischen Studien nicht erfolgreich, aber Cathepsin S kann ein geeignetes Zielprotein darstellen um die häufig auftretenden Gehirnmetastasen bei SCLC zu verhindern. Zusammenfassend zeigen die zwei Studien eine hohe Antitumorwirksamkeit des Fascaplysins gegen SCLC Zellen und wiesen erstmals die selektive Expression des Cathepsin S nach, dessen Rolle bei der Bildung von intrakranialen Metastasen bereits bekannt war.