CD133+ cancer stem-like cells in small cell lung cancer are highly tumorigenic and chemoresistant but sensitive to a novel neuropeptide antagonist

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Running title:- CD133+ SCLC cells are sensitive to neuropeptides

Precis: Small cell lung cancer has neuroendocrine features that suggest its targeting by neuropeptide antagonists, an idea that is strongly reinforced by the findings of this study for targeting cancer stem-like cells in this deadly disease.

Keywords: SCLC, stem cell, neuropeptide, chemoresistance

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Abstract

Small cell lung cancer (SCLC) is a highly aggressive malignancy with poor survival rates, with initial responses nearly invariably followed by rapid recurrence of therapy-resistant disease. Drug resistance in SCLC may be attributable to the persistence of a sub-population of cancer stem-like cells (CSC) that exhibit multiple drug resistance. In this study, we characterised the expression of CD133, one important marker of CSC in other cancers, in SCLC cancer cells. CD133 expression correlated with chemoresistance and increased tumorigenicity in vitro and in vivo accompanied by increased expression of Akt/PKB and Bcl-2. CD133 expression was increased in mouse and human SCLC after chemotherapy, an observation confirmed in clinical specimens isolated longitudinally from a patient receiving chemotherapy. We discovered in CD133+ SCLC cells an increased expression of the mitogenic neuropeptide receptors for gastrin releasing peptide and arginine vasopressin. Notably, these cells exhibited increased sensitivity to the growth inhibitory and pro-apoptotic effects of a novel broad spectrum neuropeptide antagonist (related to SP-G) which has completed a Phase I clinical trial for SCLC. Our results offer evidence that this agent can preferentially target chemoresistant CD133+ cells with CSC character in SCLC, emphasizing its potential utility for improving therapy in this setting.
Introduction

Lung cancer is the most common fatal malignancy in the developed world. Small cell lung cancer (SCLC) accounts for 10-20% of all lung cancers (1-3) and is highly aggressive, killing around 20,000 people/year in the US. SCLC is a neuroendocrine cancer that secretes and responds to a wide variety of mitogenic peptide growth factors, typically gastrin releasing peptide (GRP) and arginine vasopressin (AVP) (4, 5). Patients with SCLC normally become symptomatic only once widespread metastasis has occurred therefore are precluded from surgical intervention. Once diagnosed, chemotherapy with cis-platinum and etoposide are standard therapy but although patients initially respond well to treatment the tumors invariably relapse within months with resistant tumors resulting in mortality at 2 years of greater than 95%.

The CSC theory proposes that tumor growth can be driven by a population of self-renewing cells which are able to differentiate and give rise to the heterogenous cell types that constitute the tumor (6, 7). Putative cancer stem cell-like cells have been isolated from various malignancies including brain, breast and non-small cell lung cancers (8-10). CD133 (prominin-1), a five-transmembrane glycoprotein, initially described as a marker specific for human hematopoietic progenitor cells has been described as a marker of cancer-initiating cells in different tumor types (11-15). In non-small cell lung cancer (NSCLC) CD133+ cells are more tumorigenic than CD133− cells and express genes involved in stemness, adhesion, motility, and drug efflux (6). Moreover NSCLC cells resistant to cisplatin show increased expression of CD133 (10, 16).

Expression of neuropeptides has been associated with neoplastic transformation (17) and there is evidence that lung CSC originate from neuroendocrine cells in the lung.
which show an increased expression and response to neuropeptides (18-21). We have shown that the development of chemoresistance to conventional chemotherapy in SCLC cells is accompanied by an increase in expression of neuropeptide receptors and subsequently increased sensitivity to broad-spectrum neuropeptide antagonists (22-24). Suggesting that targeting neuropeptide receptors on SCLC cells may provide a new strategy to target chemoresistant disease.

In this study we show that CD133+ SCLC cells exhibit CSC properties, form tumors in immunocompromised mice and are resistant to etoposide. In addition, these cells demonstrate increased neuropeptide receptor expression and increased sensitivity to growth inhibition by a novel neuropeptide antagonist. Moreover we show that in human biopsy specimens there is an increase in CD133 and GRP-R expression that correlates with chemoresistance. This suggests that neuropeptide antagonists may selectively target chemoresistant CSCs and provide a novel therapy for resistant SCLC.
**Methods:**

**Materials**

All cell lines were mycoplasma free. SCLC cells; NCI-H69 and NCI-H345 (ECACC, Health Protection Agency, Porton Down, UK) and GLC14 and GLC16 cells (22, 25) grew as free floating aggregates and were positive for NCAM by western blot. The GLC14 and GLC16 cells were previously profiled by short tandem repeat profiling to confirm their origin from a single patient (25, 26). GRP-R and V$_{1A}$-R expressing CHO-K1 cells (23) were confirmed receptor positive by western blot. RPMI-1640, polyclonal rabbit anti-human AVP-R (V$_{1A}$), was purchased from Chemicon International, rabbit anti-human GRP-R (Thermo Scientific), CD133/1-APC, CD133/2-PE, CD133/1, mouse IgG1-APC and mouse IgG1-PE (Miltenyi Biotec). Oct3/4 (c-2) (Santa Cruz Biotechnology), mouse monoclonal anti-Bcl-2 (Millipore), phospho-Akt (ser473) (Cell Signaling). Alexa-conjugated secondary antibodies were from Invitrogen.

**Cell culture**

SCLC cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum (FCS) 50 U ml$^{-1}$ penicillin, 50 µg ml$^{-1}$ streptomycin and 5 µg ml$^{-1}$ L-glutamine. For experimental purposes SCLC cells were cultured in SITA medium consisting of RPMI 1640 medium supplemented with 30 nM selenium, 5 µg ml$^{-1}$ insulin, 10 µg ml$^{-1}$ transferrin and 0.25% (w/v) bovine serum albumin (BSA). CHO-K1 cells expressing the human GRP and V$_{1A}$ receptors were cultured as we described previously (23).
Flow cytometry

Viable cells (10^6 cells/ml) were incubated with monoclonal anti-CD133/1 (AC133)-APC or CD133/2 (AC141)-PE (1:100 dilution) and incubated for 10 min at 4°C and washed twice with PBS. Flow cytometry was performed using a FACSCalibur (BD Biosciences). Dead cells were eliminated using 1 μg/ml PI staining (Sigma-Aldrich). Data was processed using FlowJo software.

CD133 selection

Cells were stained with CD133-1-APC as described above. For cell sorting the top 7% most brightly stained cells or the bottom 7% most dimly stained cells were selected and isolated using a FACSaria (BD Bioscience). Dead cells were eliminated using PI. The purity of sorted cells was evaluated by staining the sorted cells with CD133-2 which detects a second epitope of CD133 antigen.

Chemosensitivity assay

SCLC cells were seeded at 5 x 10^4 cells per well of a 96 well plate in the presence or absence of etoposide for 72 hours. MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide) formazan production (Sigma) was used to measure viability as per manufacturer’s instructions.

Liquid growth

SCLC cells (5 x 10^4 cells per well) in the presence or absence of mediators were grown for 24 days and cell number determined using a NucleoCounter NC-100 (ChemoMetec).
Clonogenic assay
SCLC cells were suspended in RPMI media containing 0.3% agarose in the presence or absence of mediators and layered over a solid base of 0.5% agarose in 35mm plastic dishes. The cultures were incubated at 37°C for 30 days. Colonies from 10 separate fields were counted using a microscope with a 4× objective. Cloning efficiency was calculated as the percentage of original number of seeded cells forming colonies of > 20 cells.

Apoptosis assay
SCLC cells treated with etoposide or Peptide-1 for 72 hours were cytocentrifuged onto glass slides and stained with May-Grunwald-Giemsa in which apoptotic cells were assessed morphologically for the appearance of condensed nuclei. Apoptosis was also evaluated using Ethidium bromide/acridine orange staining.

Western Blotting
Cells were lysed as described previously (23) and equilibrated for protein using Pierce BCA protein assay reagent. Lysates were resolved on 12% SDS-PAGE gels and electro-blotted onto nitrocellulose membranes. Blots were probed with primary antibody followed by the appropriate horseradish peroxidase-labeled goat IgG (DAKO, UK). Bands were visualized using enhanced chemiluminescence (ECL, Amersham).

Determination of intracellular Ca\(^{2+}\) concentration
Intracellular Ca\(^{2+}\) concentration was evaluated using the fluorescent indicator Fura-2-tetraacetoxymethylester AME (FURA-2-AM 1 μM) as described (27). Ratiometric
fluorescence was monitored in a Perkin-Elmer Fluorometric Spectrophotometer with dual excitation wavelengths of 340 and 380 nm and emission wavelength of 510 nm.

**Real-time RT-PCR**

Total RNA from sorted cells was extracted using RNeasy kits (Qiagen) and reverse transcribed into cDNA using TaqMan® reverse transcription reagents (Applied Biosystems). The expression levels of human GRP-R and human V1A-R in isolated cells was quantified using TaqMan® gene expression assay kit (Applied Biosystems). The q-PCR reaction was carried out in a 7500 Fast Real-Time PCR system and data was analysed using the System detection software (7500 Fast system SDS software, Applied Biosystems).

**Xenografts**

Six week old female CD1 nu/nu mice (Harlan, UK) were maintained in 12-hour light/12-hour dark cycles with free access to food and water. All procedures were performed in accordance with Home Office guidelines [Animals (Scientific Procedures) Act 1986]. SCLC cells were injected subcutaneously and mice were randomized into control and treatment groups containing 6-8 mice. Treatment started when the xenografts reached a diameter of 3-10 mm with the first day of peptide administration designated as day 0. Peptide-1 was dissolved in sterile distilled water and administered by s.c. injection at a dose of 25 mg/kg twice weekly for a total of 5 injections. Etoposide was administered at 15 mg/kg. Controls received the same dose schedule of vehicle. Xenografts were measured twice weekly by means of calipers and tumor volume (V) was then calculated by the formula \( V = \frac{\pi}{6} \times L \times W^2 \), where L is the longest diameter and W the diameter perpendicular to L.
Human SCLC sections and immunohistochemistry

Biopsy specimens were obtained by endobronchial biopsy from patients attending Papworth Hospital Department of Thoracic Oncology and were confirmed histologically as SCLC. Paraffin-embedded sections of mouse and human tissue were processed for immunohistochemistry and stained for CD133 and GRPR followed by secondary biotinylated species-specific antibodies (Dako). Positive staining was visualized using Vectastain Elite RTU reagent and liquid diaminobenzamine (DAB). For immunofluorescence staining species-specific Alexa-488- or Alexa-594-conjugated secondary antibodies were used (Invitrogen, UK) and sections were analyzed by confocal microscopy (Olympus FV1000).

HPLC

Mouse S9 liver prep

Livers from female C57BL/6 mice were homogenized in potassium phosphate buffer (0.1 M, pH 7.4) at 4°C to give a final homogenate consisting of 25% w/v tissue. Samples were centrifuged at 9000 × g for 20 minutes at 4°C and the supernatant (S9) was stored on ice. SP-G and peptide-1 at a final concentration of 0.1 mg/ml was added and reactions were incubated at 37°C. Aliquots were added to 0.2ml eluting buffer (methanol:1M ammonium acetate (90:10 v/v). Cleared supernatants were frozen at -80°C until analysis by High Performance Liquid Chromatography (HPLC). HPLC spectra were recorded using an Agilent 1100 Series coupled to a Polymer Lab 100 ES Evaporative Scattering Detector (ELSD), with a Phenomenex Luna C18, 5μm, 10cm column, using 1mL/min of water/acetonitrile/trifluoroacetic acid 90:10:0.1, raising to 30:70:0.1 after 8 min and detection by UV at 220, 254, 260, 282 and 495 nm.
Statistical Analysis

Results are presented as means ± S.E.M. Significance of the differences between means was assessed using Student's t-test or by ANOVA for comparison between groups. When ANOVA showed a significant treatment effect, Dunnet’s post hoc test was used to compare individual means. Differences were considered statistically significant at P<0.05.
Results:

Expression of CD133 on SCLC cell lines

SCLC cell lines H345 and H69 expressed the stem cell marker CD133 (Figure 1A). Both H345 and H69 cells exhibited a broad Gaussian distribution staining pattern. A gate set relative to the isotype control revealed 18.1 ± 2.1 % of H345 cells and 23 ± 2.2 % of H69 cells showed high expression CD133. Treatment of H345 and H69 with 30 μg/ml etoposide for 72 hours enriched the percentage of CD133 expressing cells in both cell lines to 24 ± 3.4 % and to 36 ± 3.1 % respectively (Figure 1B). CD133 high (top 7%) and low (bottom 7%) were sorted based on CD133-1 fluorescence and the purity of the sorted cells was validated by flow cytometry using CD133-2 (Figure 1C,D). Additionally, CD133+ cells demonstrated high CD133 expression as measured by western blot analysis compared to unsorted and CD133- cells (Figure 1E). Sorted CD133+ cells from both H69 and H345 cells demonstrated higher expression of the stem cell marker Oct-4 (Figure 1E) indicating their stem cell-like nature. H345 and H69 CD133+ and CD133- cells were viable and there was no significant difference in viability following 72 hours culture (Figure 1F).

CD133+ cells form colonies in vitro and tumors in vivo

Proliferation of CD133+ and CD133- SCLC cells was examined in cells cultured in serum free SIT supplemented media. CD133+ cells were significantly more proliferative compared to their negative counterpart (Figure 2A). Colony formation in semi solid media is an in vitro model of tumorigenicity. CD133- cells failed to form colonies (Figure 2B) whilst CD133+ cells displayed increased colony formation compared to unsorted cells (cloning efficiency 5.41 and 7.20 % in CD133+ compared
to 1.91 and 2.95% in unsorted H345 and H69 cells respectively, n=4). We then examined growth of CD133 expressing cells in vivo. $1 \times 10^4$, $1 \times 10^5$ and $5 \times 10^5$ CD133+, CD133- and unsorted H345 cells were injected into the flanks of nu/nu mice and tumor growth was measured over 55 days. Consistent with data obtained from colony assay, CD133- cells failed to grow tumors in nu/nu mice whereas CD133+ cells readily formed tumors (Figure 2C). $1 \times 10^5$ and $5 \times 10^5$ CD133+ cells formed larger tumors than equivalent numbers of unsorted H345 cells (Figure 2C) indicating their tumorigenic potential.

**CD133+ cells are resistant to etoposide**

Both H345 and H69 SCLC cell lines have been cultured from patients that had originally undergone a period of chemotherapy and are therefore relatively resistant to etoposide in culture. Etoposide sensitivity was assessed by MTT assay following 72 hours treatment (Figure 3A). Unsorted cells showed IC$_{50}$ values for etoposide of 11.5 and 14.1 μg/ml for H345 and H69 cells respectively. In both cell lines the CD133+ population were significantly more resistant to etoposide compared to the CD133- cells (IC$_{50}$ = 200 vs 3.5 μg/ml for CD133+ vs CD133- H345 cells and 111 vs 10 μg/ml in H69 cells respectively (Figure 3A)). In contrast, CD133- cells were significantly more sensitive to etoposide-induced cytotoxicity compared to both CD133+ cells and unsorted cells (P<0.05). In line with the cell viability assay, CD133+ cells demonstrated reduced etoposide-induced apoptosis compared to CD133- cells (Figure 3B and Supplemental Figure S1). Resistance to apoptosis in the positive population was associated with increased phosphorylation of Akt and increased expression of the anti-apoptotic protein Bcl-2 (Figure 3C).
CD133+ cells retain CD133 expression in SIT supplemented media but readily differentiate in serum containing media

An essential characteristic of stem cells and CSCs is their ability to self-renew and differentiate. To examine if CD133+ SCLC cells retain such characteristics, we analysed the CD133 expression on sorted H345 cells after 21 days culture in media containing 10% FCS or serum-free SIT supplemented media. CD133+ sorted cells cultured in SITA medium maintained CD133 expression whereas CD133+ cells cultured in FCS containing medium differentiated and generated a mixed cell population of CD133+ and CD133- cells (Figure 3D). To confirm that CD133+ cells differentiate in the presence of serum, sorted cells cultured in SITA or FCS containing media for 21 days were analysed for their colony forming ability. Figure 3E shows that whilst culturing in SITA media maintained the increased colony forming efficiency of CD133+ cells, CD133+ cells subsequently cultured in FCS containing media had lost this high colony forming ability and showed equal colony formation to unsorted cells. This would suggest that CD133+ sorted cells lose their increased clonogenicity in FCS due to differentiation into CD133- cells.

CD133+ cells express increased neuropeptide receptors

Our previous work has shown that chemoresistant cells show increased sensitivity to neuropeptides GRP and AVP and substance P analogues (22, 28). We aimed to establish whether the chemoresistant CD133+ cells expressed increased GRP and V1A receptors. GRP-receptor expression was measured in CD133+, CD133- and unsorted H345 cells (Figure 4A). GRP-R transcript expression was significantly higher in CD133+ H345 cells compared to CD133- H345 cells (P<0.01) as determined by transcript expression and western blot. CD133- cells did not form colonies in semi
solid media but CD133+ cells formed significantly more colonies compared to unsorted cells both basally and in response to GRP further supporting higher levels of GRP-R expression in the CD133+ cells compared to their negative counterpart (Figure 4B). V$_{1A}$ receptor expression was examined in CD133 sorted H69 cells. Similar to data obtained in H345 cells, CD133+ H69 cells showed increased expression of V$_{1A}$ receptor transcriptionally and translationally and formed more colonies in response to vasopressin compared to CD133- H69 cells (Figure 4C,D).

The GLC14 and GLC16 cell lines are SCLC cells that have been established from a single patient during the progression of chemoresistance (25). The GLC16 line has subsequently been shown to be more chemoresistant than the GLC14 cells (26). We have shown previously that the GLC16 line expresses more neuropeptide receptors, which correlates with an increased response to SP-G (22). We therefore sought to examine the expression of CD133 on the GLC14 (chemosensitive) and GLC16 (chemoresistant) cells and show that the GLC16 cells express more CD133, GRPR and activated AKT in comparison to the GLC14 counterpart (Figure 4E).

**CD133 and GRP-R expression is increased in human SCLC lung biopsy samples following chemotherapy**

We then examined human bronchial endoscopy samples from patients with confirmed SCLC. We analysed 11 pre-chemotherapy diagnosis samples and 2 samples from patients following treatment with etoposide and platinum based chemotherapy including one set of paired samples from the same patient (Figure 5A,B). CD133 and GRP-R was expressed in tumor cells from all patients but the extent of CD133 and GRP-R expression was markedly increased in the post-chemotherapy samples (Figure
5). Immunofluorescence staining revealed that both the percentage of positive cells and the intensity of staining was increased post-chemotherapy (Figure 5C).

**CD133+ cells are more sensitive to a novel substance P analogue**

The substance P analogue SP-G which underwent a successful phase 1 clinical trial was shown to have a short half life and poor bioavailability (29, 30) and therefore did not provide a viable clinical candidate (31). We synthesised and characterised a panel of modified analogues based on the structure of SP-G and SP-A (32) (Supplemental Table 1) and tested their efficacy on SCLC cell viability. One analogue, (Peptide-1) with a D-Leu at position 10 was 3 fold more potent than SP-G in H345 and H69 SCLC lines *in vitro* (Figure 6A and Supplemental Table 1) and showed increased inhibition of cell growth (Figure 6B). Peptide-1 also inhibited Ca\(^{2+}\) mobilization in response to GRP and AVP in receptor expressing CHO-K1 cells with potencies of 0.3 and 1.1 uM for GRP-R and V\(_{1A}\)R respectively (Figure 6C) and inhibited colony formation in response to AVP and GRP in H69 and H345 cells respectively (Figure 6D). Peptide-1 was more effective in inducing apoptosis in both H345 and H69 cells compared to SP-G (Figure 6E). The stability of Peptide-1 was examined in an *in vitro* mouse liver microsome preparation. As described previously SP-G was metabolised rapidly by mouse liver (29, 30) with t1/2 = 9.32 ± 1.37 min. and was completely metabolised following 30 min. Peptide-1 in contrast had a t1/2 = 41.5 ± 2.5 min (n=4) and was not completely metabolised until after 100 mins exposure to mouse liver microsomes (Figure 6F). This suggests that Peptide-1 is approximately 4 times more stable than SPG *in vitro*. 
The novel Peptide-1 inhibits SCLC tumor growth in vivo

Peptide-1 was tested in a H345 SCLC xenograft model in vivo. The peptide was administered daily by s.c. injection at 25mg/kg for a total of 5 injections once tumors were established and compared to and in combination with etoposide (15mg/kg) (Figure 7A). Administration of Peptide-1 alone produced a significant reduction in tumor volume for the duration of the study and was as least as efficacious as etoposide alone. A complete tumor response was observed in 3 mice receiving Peptide-1. Tumor tissue was excised and CD133 immunofluorescence staining showed clusters of CD133 expressing cells in the tumors of control mice. The number of CD133 expressing cells was markedly increased by approximately 3 fold in tumour from mice treated with etoposide (Figure 7B,C). The increased CD133 staining in the etoposide group was associated with cells that co-expressed GRP-R. Confocal microscopic analysis of CD133 (green) and GRP-R (red) showed colocalisation of these markers in human xenografts sections (Supplemental Figure S1). In contrast, tumors from mice treated with Peptide-1 showed only very few CD133 positive cells (Figure 7B,C). We next examined the effect of Peptide-1 in CD133 sorted cells in vitro. In contrast to etoposide, CD133+ cells were 3 fold more sensitive to Peptide-1 in vitro (Figure 7D) compared to unsorted cells whilst CD133- cells were > 4 fold less sensitive. These results show that chemoresistant CD133 expressing SCLC cells are more sensitive to substance P analogues and suggest these analogues may have potential as anti-cancer agents with potential in resistant disease.
Discussion:

In this study we used CD133 to identify a population of etoposide-resistant human SCLC cells \textit{in vitro}. We show that the CD133+ sub-population was resistant to etoposide and had augmented proliferative and clonogenic capacity \textit{in vitro} and significantly increased tumor initiation potential \textit{in vivo}. These cells exhibited some stem cell characteristics including Oct-4 expression, the ability to maintain their stem cell-like characteristics \textit{in vitro} and to differentiate into a mixed population of CD133+ and CD133- cells. We also show that expression of CD133 increases in mouse and patient samples following chemotherapy indicating new therapeutic strategies are urgently required for the treatment of SCLC patients.

Several studies have implicated CD133+ cells as the cause of cancer chemoresistance (6, 10, 33, 34) however its role in colorectal cancer and brain tumors is controversial (35). In lung cancer, CD133 expression has been associated with poor prognosis in NSCLC patients and predicts shorter progression-free survival of patients treated with platinum-based regimens (6). Although no study has directly evaluated the relation of CD133 expression with SCLC patient prognosis, \textit{in vitro} studies performed on SCLC cells from primary tumors and cell lines have illustrated the importance of CD133 in selecting cells with an aggressive, chemoresistant and tumorigenic phenotype (10, 33, 36).

Based on the CSC theory, not all the cells within a neoplasm are clonogenic and tumorigenic and only a rare fraction of cells with a stem cell-like phenotype propagate tumor development, metastasis and treatment resistance (37). However the generality of this hypothesis can be questioned as the frequency of CSCs appears to be highly variable between cancers of the same type (38). Eramo et al., (10) showed that 0.33-
22% of lung cancer cells were CSCs. In our studies we did not identify a rare discrete subset but rather showed a Gaussian distribution pattern. Similar diffuse CD133 expression pattern has been observed in various other cancers (39-41). Additionally, CD133 expression has been shown to be up-regulated by the basic helix-loop-helix transcription factor achaete-scute complex homologue 1 (ASCL1) which identifies a relatively abundant rather than a small discrete population of cells with tumor initiating capacity in SCLC (42), which is in agreement with the present study. Our higher than expected CD133 expression could be a function of the cells having derived chemoresistance \textit{in vivo} as both H345 and H69 cells are derived from post-chemotherapy patients. Additionally, chemotherapy can enrich the CSC population (39, 43, 44). In breast cancer patients after primary therapy an increase in the proportion of CSCs was observed (45). However, the fundamental concept underlying the CSC hypothesis is not associated with the absolute CSC frequency but is based on the functional heterogeneity within tumors where a sub-population of cells have tumor initiating capacity whereas the rest of the tumor cells are devoid of it. The SCLC cell lines used in this study have acquired anchorage independence and are propagated as non-adherent aggregates in liquid culture and therefore show some characteristics of stem-like cells. However our extensive characterization of these cells have shown that not all SCLC cells are able to form colonies in semi solid agar (5, 46, 47). Confirming heterogeneity of cells in the cell line even after extensive passaging \textit{in vitro}.

Although CSCs are often considered to be slowly proliferating cells based on the assumption that they are derived from tissue stem cells, their expansion can be controlled by factors in the microenvironment or “niche”. We show that CD133+
SCLC cells exhibited significantly greater proliferation in serum-free medium compared to the CD133- cells, which is in agreement with other studies on hepatocellular carcinoma (39). Contributing factors promoting CSC growth may be attributed to over-expression of growth factors by the CSCs. Levina et al., (33) demonstrated higher expression of various growth factors in CSCs than non-CSCs in lung cancer cell lines. SCLC cell lines produce and secrete various extracellular matrix (ECM) proteins and neuropeptides (48-50) which can protect cells from chemotherapy (48, 51, 52), and could contribute to a permissive environment for CSC expansion. We have shown previously that the PI3-K/Akt pathway is constitutively active in SCLC cells (49) and provides a mechanism whereby SCLC cells exhibit chemoresistance (48), however other studies have suggested that Akt is inducible in SCLC (53). The increased activation of Akt in CD133+ cells could explain their increased resistance to etoposide. In SCLC cells and other cell types CD133 expression is localized to plasma membrane protrusions and its function may be associated with plasma membrane remodeling and may effect cell migration (54) and communication with ECM proteins that can influence resistance to chemotherapy.

The current strategies to target CSCs have focussed primarily on pathways that regulate normal stem cell growth and differentiation such as the Hedgehog (Hh), Notch, and Wnt signaling pathways, and there is emerging evidence that targeting these pathways may show some benefit (55). However these pathways have not been extensively studied in SCLC. A previous study has shown that CD133 may be a marker of CSCs in SCLC (56) and that increased CD133 expression is associated with chemoresistance and worse clinical outcome in SCLC and NSCLC (10, 57). As we have previously shown that chemoresistant SCLC cells are more sensitive to
neuropeptides, we examined the hypothesis that CD133+ SCLC cells are also sensitive and showed increased expression of GRP and V1A receptors and increased GRP and AVP induced signaling and growth. Our new analogue Peptide-1 inhibited SCLC cell growth \textit{in vitro} and \textit{in vivo} and was more efficacious in CD133+ cells, extending our previous work and suggesting that the chemoresistant CD133+ CSC-like cells show increased neuropeptide responsiveness. We also show even in our small patient set that in human samples post-chemotherapy there is increased expression of CD133 and GRP-R, suggesting that the percentage of CD133 positive cells increases with chemoresistance. This was further corroborated in xenograft studies which showed clearly defined CD133 negative and positive tumor cell populations, with the fraction of CD133+ cells increasing 3 fold in etoposide treated mice which co-expressed GRP-R. In another approach we demonstrated that the chemoresistant GLC16 cell line expressed higher CD133 and GRPR than it’s paired chemosensitive GLC14 line. Further prospective studies in pre- and post-chemotherapy SCLC samples are required to explore this interesting finding in more detail.

Our finding that GRP-R receptor and Peptide-1 sensitivity increases with resistance to etoposide suggests that strategies directed at neuropeptide receptors may selectively target chemoresistant cells. Our new analogue, which has higher affinity and improved stability, may therefore have additional benefit as an adjunct to chemotherapy or as a second line treatment of resistant disease. We believe that this property makes them very exciting and novel potential therapies for chemoresistant SCLC.
Acknowledgments:

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References


Figure legends

Figure 1 SCLC cells express CD133. A) H69 and H345 cells were stained with anti-CD133-1-APC or isotype control and examined by flow cytometry. B) Culturing SCLC cells with 3 or 30 μg/ml etoposide for 72h increased % CD133 expression (*P<0.05, n=4). C) Gating strategy for sorting live CD133+ and CD133- cells using anti-CD133-1-APC. D) Purity check on sorted CD133+ and CD133- cells using anti-CD133-2-PE confirming CD133 expression status post sorting. E) Representative western blots of lysates from unsorted and CD133+ and CD133- H69 and H345 cells were probed for CD133, oct-4 and β-actin. F) CD133+ and CD133- sorted H69 and H345 cells were cultured for 72 hours and viability assessed by Propidium iodide staining (n=3).

Figure 2 CD133+ cells are more proliferative than CD133- cells. A) CD133+ H345 (left) and H69 (right) cells proliferated more rapidly in culture than CD133- cells (***P<0.01, *P<0.05, n=4). B) CD133- cells failed to form colonies and CD133+ cells formed significantly more colonies compared to unsorted cells (***P<0.01, *P<0.05, n=4). B1) Representative pictures of CD133+, unsorted and CD133- colonies after 14 days culture in 0.3% agarose. C) CD133+ cells form tumors in nu/nu mice. Female nu/nu mice received CD133 sorted H345 cells and tumor size measured over 55 days.

Figure 3 CD133+ cells are resistant to etoposide. A) CD133+, CD133- and unsorted H345 and H69 cells were incubated with etoposide for 72 hours and viability was assessed by MTT assay (n=4). B) Quantification of apoptosis of CD133+ and CD133- H345 cells treated for 72 hours with etoposide (**P<0.01, *P<0.05, n=4). D) Representative western blots of CD133+, CD133- and unsorted H345 cells probed for
anti-CD133, Akt, p-Akt, Bcl-2 and β-actin. C) CD133 sorted H345 cells were plated in the presence of 10% FCS or in serum free SITA medium for 21 days and CD133 expression assessed by flow cytometry. D) CD133 sorted H345 cells incubated for 21 days in either SITA medium or 10% FCS medium were assayed for colony forming ability in 0.3% agarose for a further 21 days. CD133+ cells formed significantly more colonies than unsorted cells when cultured in SITA but not in FCS media (**P<0.05, n=4).

Figure 4 CD133+ cells express increased neuropeptide receptor expression. A) CD133+, CD133- and unsorted H345 and H69 cells were analysed for expression of GRP and V1A receptor C) by qPCR (***P<0.005, **P<0.01, *P<0.05, n=4). Representative western blots confirm changes in receptor expression at the protein level. CHO-K1 cells transfected with human GRP (G6A) and V1A (V3B) receptors were used as positive controls. CD133+ and unsorted cells were grown in 0.3% agarose in the presence of either B) GRP (H345) or C) AVP (H69). Co-incubation with neuropeptide increased colony formation in CD133+ cells (**P<0.01, *P<0.05, n=4). E) Representative western blots of GLC14 and GLC16 cell lysates probed for GRP-R, pAKT and β-actin. FACS analysis of CD133 expression shows increased CD133 expression in GLC16 cells (n=3).

Figure 5 Immunohistochemical staining of SCLC sections from one patient prior to (A1 and A2) and following (B1 and B2) standard etoposide and platinum based chemotherapy regimen. Serial sections were immunostained for CD133 and GRP-R. Increased staining intensity for both CD133 and GRP-R was observed in post-chemotherapy samples. Immunofluorescence staining for CD133 in pre- (C1) and
post- (C2) chemotherapy samples. The percentage of CD133+ cells and the intensity of CD133 expression is significantly increased in post-chemotherapy samples.

Figure 6 A novel substance P analogue Peptide-1 inhibits growth of SCLC cells. SCLC cells were incubated with SPG or Peptide-1 and viability A) was assessed after 72 hours and proliferation B) over 14 days. C) Peptide-1 inhibited GRP- and AVP-induced calcium mobilization in transfected CHO-K1 cells expressing GRP (G6A) and V1A (V3B) receptors. Results are representative of 4 experiments. D) Peptide-1 inhibited basal and neuropeptide stimulated colony formation in H345 (top) and H69 (bottom) cells. E) Peptide-1 induced apoptosis in H345 and H69 cells and was more effective than equivalent concentration of SP-G (*P<0.05, n=4). F) SP-G and Peptide-1 were incubated with mouse liver microsomes for up to 140 min and the presence of the non-metabolised parent compound analysed by HPLC. Results are representative of 3 experiments.

Figure 7 Peptide-1 inhibits growth of SCLC in vivo and inhibits growth of CD133+ cells in vitro. A) Peptide-1 was administered at 25mg/kg s.c. twice weekly for 5 injections either alone or in combination with 15mg/kg etoposide after H345 xenografts were established in mice (n=6 mice per group). B) Representative sections from xenografts from control, etoposide and Peptide 1 treated mice immunostained for CD133. C) Quantification of CD133+ cells per high power field (**P<0.01, *P<0.05 compared to control n=4). D) CD133+ and CD133- and unsorted H345 cells were incubated with Peptide-1 for 72 hours and viability assessed by MTT assay. CD133+ cells were more sensitive to Peptide-1 than unsorted and CD133- cells (n=4).
Figure 1

(A) Flow cytometry analysis of CD133 expression in H345 and H69 cell lines. 

(B) Bar graph showing the percentage of CD133+ cells in different conditions.

(C) Scatter plot illustrating the distribution of cells in different quadrants, with overlays indicating single and live cell populations.

(D) Histogram showing the distribution of CD133 expression in sorted and unsorted samples.

(E) Western blot analysis comparing CD133, Oct-4, and β-actin expression levels in H345 and H69 cell lines.

(F) Graph depicting cell viability (% of control) for CD133- and CD133+ cells, along with unsorted controls.
Figure 2

A

H345

Cell number $\times 10^4$/ml

0 10 20 30

Days

H69

CD133+  CD133-

0 100 200 300

0 10 20 30

H345

Cloning efficiency (%)

0 2 4 6

CD133-  CD133+  Parent

H69

CD133-  CD133+  Parent

B

CD133-  Unsorted  CD133+

B1

C

Tumour volume (mm$^3$)

0 100 200 300 400 500

0 10 20 30 40 50 60

Days

5x10$^5$ CD133+

1x10$^5$ CD133+

1x10$^4$ CD133+

5x10$^5$ unsorted

5x10$^5$ CD133-
Figure 3

A

Cell viability (% of control)

Etoposide Concentration (log g/ml)

H69

- unsorted
- CD133+
- CD133-

B

Apoptosis (% of live)

Etoposide Concentration (µg/ml)

H345

- CD133-
- CD133+

H69

- CD133-
- CD133+

C

CD133-
CD133+
- unsorted

pAkt
Akt
β-actin
Bcl-2
β-actin

D

Day 0

CD133-1

CD133-2

21 days in SITA medium
21 days in 10% FCS medium

E

Cloning Efficiency (%)

H345

- CD133-
- CD133+
- unsorted

H69

- CD133-
- CD133+
- unsorted
Figure 4

A

Unsorted

CD133+

CD133-

Fold Increase

H345

**

G6A P + -

GRP-R

β-actin

CD133- CD133+ Parent

B

Unsorted

CD133+

Cloning Efficiency (%)

H345

**

G6A P + -

GRP-R

β-actin

Control 1 nM 10 nM 100 nM

GRF concentration (nM)

C

Unsorted

CD133+

Fold Increase

H69

**

V3B P + -

V1A-R

β-actin

CD133- CD133+ Parent

D

Unsorted

CD133+

Cloning Efficiency (%)

H69

**

V3B P + -

V1A-R

β-actin

Control 1 nM 10 nM 100 nM

AVP concentration (nM)

E

GLC 14

GLC 16

GRP-R

pAK

β-Actin

GLC16 Control

GLC14 Control

GLC16

GLC14

CD133-1
Figure 5

Pre-chemotherapy  Post-chemotherapy

CD133

GRP-R

DAPI  CD133
Figure 7

A

![Graph showing tumor volume over time](image)

- etoposide + peptide 1
- etoposide
- peptide 1
- control

B

![Images of control, etoposide, and peptide-1 treated samples](image)

CD133 DAPI

C

![Bar graph showing number of CD133+ cells](image)

Unsorted

D

![Graph showing cell viability over peptide-1 concentration](image)

Unsorted

CD133+

CD133-
CD133+ cancer stem-like cells in small cell lung cancer are highly tumorigenic and chemoresistant but sensitive to a novel neuropeptide antagonist

Sana Sarvi, Alison C Mackinnon, Nicolaos Avlonitis, et al.