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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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 subpopulation of cancer stem-like cells (CSC) that exhibit multiple drug resistance. In this study, we characterized 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 proapoptotic 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. Cancer Res; 74(5); 1554–65. ©2014 AACR.

Introduction
Lung cancer is the most common fatal malignancy in the developed world. Small cell lung cancer (SCLC) accounts for 10% to 20% of all lung cancers (1–3) and is highly aggressive, killing around 20,000 people per year in the United States. 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; refs. 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 is a 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 more than 95%.

The cancer stem-like cell (CSC) theory proposes that tumor growth can be driven by a population of self-renewing cells that are able to differentiate and give rise to the heterogeneous 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 (NSCLC; refs. 8–10). CD133 (prominin-1), a five-transmembrane glycoprotein, initially described as a marker specific for human hematopoietic progenitor cells has been designated as a marker of CSCs in different tumor types (11–15). In 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 originates 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, peptide-1. 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.

Materials and Methods

Materials

All cell lines were mycoplasma free. SCLC cells: NCI-H69 and NCI-H345 (European Collection of Animal Cell Cultures, Health Protection Agency, Porton Down, United Kingdom) and GLC14 and GLC16 cells (22, 25) grew as free floating aggregates and were positive for neuronal adhesion molecule by Western blot analysis. 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 V1A-R-expressing CHO-K1 cells (23) were confirmed receptor positive by Western blot analysis. RPMI-1640, polyclonal rabbit anti-human AVP-R (V1A), 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) were from Santa Cruz Biotechnology, mouse monoclonal anti–Bel-2 was from Millipore, and phospho-Akt (ser473) was from Cell Signaling Technology. 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 penicillin, 50 μg/mL streptomycin, and 5 μg/mL L-glutamine. For experimental purposes, SCLC cells were cultured in RPMI-1640 medium supplemented with 30 nmol/L selenium, 5 μg/mL insulin, 10 μg/mL transferrin, and 0.25% (w/v) bovine serum albumin. CHO-K1 cells expressing the human GRP and V1A receptors were cultured as described previously (23).

Flow cytometry

Viable cells (10⁶ cells/mL) were incubated with monoclonal anti–CD133/1 (AC133)-APC or CD133/2 (AC141)-PE (1:100 dilution) for 10 minutes 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 propidium iodide staining (Sigma-Aldrich). Data were 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 Biosciences). Dead cells were eliminated using propidium iodide. The purity of sorted cells was evaluated by staining the sorted cells with CD133-2, which detects a second epitope of CD133 antigen.

Chemoresistance assay

SCLC cells were seeded at 5 × 10⁴ cells per well of a 96-well plate in the presence or absence of etoposide or peptide-1 for 72 hours. MTT formazan production (Sigma) was used to measure viability as per the manufacturer’s instructions.

Liquid growth

SCLC cells (5 × 10⁴ cells/well) in the presence or absence of mediators were grown for 24 days and cell number was determined using a NucleoCounter NC-100 (ChemoMetec).

Clonogenic assay

SCLC cells were suspended in RPMI media or SITA media containing 0.3% agarose in the presence or absence of mediators and layered over a solid base of 0.5% agarose in 35 mm 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 electroblotted onto nitrocellulose membranes. Blots were probed with primary antibody followed by the appropriate hors eradish peroxidase-labeled goat immunoglobulin G (IgG; Dako). Bands were visualized using enhanced chemiluminescence (Amersham).

Determination of intracellular Ca²⁺ concentration

Intracellular Ca²⁺ concentration was evaluated using the fluorescent indicator Fura-2-tetraacetoxymethyleneester AME (FURA-2-AM 1 μmol/L) as described (27). Ratiosmetric 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 were quantified using TaqMan gene expression assay kit (Applied Biosystems). The quantitative PCR reaction was carried out in a 7500 Fast Real-Time PCR system and data were analyzed using the System detection software (7500 Fast system SDS software, Applied Biosystems).
Xenografts
Six-week-old female CD1 nu/nu mice (Harlan) 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 to 8 mice. Treatment was started when the xenografts reached a diameter of 3 to 10 mm with the first day of drug administration designated as day 0. Peptide-1 was dissolved in sterile distilled water and administered by subcutaneous injection at a dose of 25 mg/kg twice weekly for a total of five 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 = π/6 × L × W², 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 (Cambridge, United Kingdom) and were confirmed histologically as SCLC. Paraffin-embedded sections of mouse and human tissue were processed for immunohistochemistry and stained for CD133 and GRP-R followed by secondary biotinylated species-specific antibodies (Dako). Positive staining was visualized using Vectastain Elite RTU reagent and liquid 3,3′-diaminobenzidine. For immunofluorescence staining, species-specific Alexa-488- or Alexa-594-conjugated secondary antibodies were used (Invitrogen) 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 mol/L, pH 7.4) at 4°C to give a final homogenate consisting of 25% w/v tissue. Samples were centrifuged at 9,000 g for 20 minutes at 4°C and the supernatant (S9) was stored on ice. SP-G and the supernatant (S9) was stored on ice. SP-G and peptide-1 were homogenized at potassium phosphate buffer (0.1 mol/L, pH 7.4) at 4°C and the supernatant (S9) was stored on ice. SP-G and the supernatant (S9) was stored on ice. SP-G and peptide-1 was dissolved in sterile distilled water and administered by subcutaneous injection at a dose of 25 mg/kg twice weekly for a total of five 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 = π/6 × L × W², where L is the longest diameter and W the diameter perpendicular to L.

Expression of CD133 on SCLC cell lines
SCLC cell lines H345 and H69 expressed the stem cell marker CD133 (Fig. 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 ± 2.9% of H69 cells were positive for CD133 expression. 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 (Fig. 1B). CD133 high (top 7%) and low (bottom 7%) were sorted on the basis of CD133-1 fluorescence and the purity of the sorted cells was validated by flow cytometry using CD133-2 (Fig. 1C and D). In addition, CD133+ cells demonstrated high CD133 expression as measured by Western blot analysis compared with unsorted and CD133− cells (Fig. 1E). Sorted CD133+ cells from both H69 and H345 cells demonstrated higher expression of the stem cell marker Oct-4 (Fig. 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 (Fig. 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 SITA medium. CD133+ cells were significantly more proliferative compared with their negative counterpart (Fig. 2A). Colony formation in semi solid media is an in vitro model of tumorigenicity. CD133− cells failed to form colonies (Fig. 2B), whereas CD133+ cells displayed increased colony formation compared with unsorted cells (cloning efficiency 5.41% and 7.20% in CD133+ compared with 1.91% and 2.95% in unsorted H345 and H69 cells, respectively, n = 4). We then examined growth of CD133-expressing cells in vivo. A total of 1 × 10⁵, 1 × 10⁶, and 5 × 10⁵ CD133+ cells, 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 (Fig. 2C). A total of 1 × 10⁵ and 5 × 10⁵ CD133− cells formed larger tumors than equivalent numbers of unsorted H345 cells (Fig. 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 (Fig. 3A). Unsorted cells showed IC₅₀ 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 with the CD133+ cells (IC₅₀ = 200 vs. 3.5 μg/mL for CD133− vs. CD133+ H345 cells and 111 vs. 10 μg/mL in CD133− vs. CD133+ cells used to compare individual means. Differences were considered statistically significant at P < 0.05.
H69 and H345 cells, respectively; Fig. 3A). In contrast, CD133− cells were significantly more sensitive to etoposide-induced cytotoxicity compared with both CD133+ cells and unsorted cells (P < 0.05).

In line with the cell viability assay, CD133+ cells demonstrated reduced etoposide-induced apoptosis compared with CD133− cells (Fig. 3B and Supplementary Fig. 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 (Fig. 3C).

CD133+ SCLC cells are sensitive to neuropeptide antagonist

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 72 hours increased the percentage of CD133+ cells (*, 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 blot analyses of lysates from unsorted, CD133+, and CD133− H69 and H345 cells were probed for CD133, Oct-4, and β-actin. F, CD133+, CD133−, and unsorted H69 and H345 cells were cultured for 72 hours and viability assessed by propidium iodide staining (n = 3).

CD133+ cells retain CD133 expression in SITA 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 whether CD133+ SCLC cells retain such characteristics, we analyzed the CD133 expression on sorted H345 cells after 21 days culture in media containing 10% FCS or serum-free SITA 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 (Fig. 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 analyzed for their colony forming ability. Figure 3E shows that although 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-/C0, and unsorted H345 cells (Fig. 4A).

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**Figure 2.** CD133+ cells are more proliferative, clonogenic, and tumorigenic 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 with 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 various numbers of CD133+, CD133-, and unsorted H345 cells and tumor size was measured over 55 days.
CD133\(^+\) H345 cells compared with CD133\(^-\) H345 cells \((P<0.01)\) as determined by transcript expression and Western blot analysis. CD133\(^+\) cells did not form colonies in semi solid media but CD133\(^-\) cells formed significantly more colonies compared with unsorted cells both basally and in response to GRP further supporting higher levels of GRP-R expression in the CD133\(^+\) cells compared with their negative counterpart (Fig. 4B). \(V_{1A}\) receptor expression was examined in 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 with unsorted H69 cells (Fig. 4C and 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, GRP-R, and activated AKT in comparison to the GLC14 counterpart (Fig. 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 analyzed 11 prechemotherapy diagnosed samples and two samples from patients...
following treatment with etoposide- and platinum-based chemotherapy, including one set of paired samples from the same patient (Fig. 5A and 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 postchemotherapy samples (Fig. 5A and B). Immunofluorescence staining revealed that both the percentage of positive cells and the intensity of staining were increased postchemotherapy (Fig. 5C).

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

The substance P analogue SP-G, which underwent a successful phase I 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 synthesized and characterized a panel of modified analogues based on the structure of SP-G and SP-A (Supplementary Table S1; ref. 32) 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 their efficacy on H345 and H69 SCLC lines (Fig. 6A and Supplementary Table S1) and showed increased inhibition of cell growth compared with SP-G and SP-A (Supplementary Table S1; ref. 32) and tested in vitro (Fig. 5A and B). Immunoexpression was markedly increased in the postchemotherapy tumor cells from all patients but the extent of CD133 and GRP-R expression shows increased neuropeptide expression in CD133+ cells (*, *P < 0.01; **P < 0.005; n = 4). Representative Western blot analyses confirm changes in receptor expression at the protein level. CHO-K1 cells transfected with human GRP (G6A) and V1α (V3B) receptors were used as positive controls. B and D, CD133+ and unsorted cells were grown in 0.3% agarose in the presence of either GRP (H345; B) or AVP (H69; D). Coincubation with neuropeptide increased colony formation in CD133+ cells (*, *P < 0.01; **P < 0.005; n = 4)., E, representative Western blot analyses of GLC14 and GLC16 cell lysates probed for GRP-R, pAKT, and β-actin. F, FACS analysis of CD133 expression shows increased CD133 expression in GLC16 cells (n = 3).

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 twice weekly by subcutaneous injection at 25 mg/kg for a total of five injections once tumors were mobilized in vivo. CD133+ cells express increased neuropeptide receptors. A and C, CD133+, CD133−, and unsorted H345 and H69 cells were analyzed for expression of GRP (A) and V1α receptor (C) by quantitative PCR (*, *P < 0.01; **P < 0.005; n = 4), respectively. Representative Western blot analyses confirm changes in receptor expression at the protein level. CHO-K1 cells transfected with human GRP (G6A) and V1α (V3B) receptors were used as positive controls. B and D, CD133+ and unsorted cells were grown in 0.3% agarose in the presence of either GRP (H345; B) or AVP (H69; D). Coincubation with neuropeptide increased colony formation in CD133+ cells (*, *P < 0.01; **P < 0.005; n = 4)., E, representative Western blot analyses of GLC14 and GLC16 cell lysates probed for GRP-R, pAKT, and β-actin. F, FACS analysis of CD133 expression shows increased CD133 expression in GLC16 cells (n = 3).
CD133+ SCLC cells are sensitive to neuropeptide antagonist

were established and compared with and in combination with etoposide (15 mg/kg; Fig. 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 three mice receiving peptide-1. Tumor tissue was excised and CD133 immunostained for CD133 and GRP-R. Increased staining intensity for both CD133 and GRP-R was observed in postchemotherapy 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 postchemotherapy samples.

Figure 5. Immunohistochemical staining of SCLC sections from one patient before (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 postchemotherapy 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 postchemotherapy samples.

may have potential as anticancer agents with greater efficacy in resistant disease.

Discussion

In this study, we used CD133 to identify a population of etoposide-resistant human SCLC cells in vitro. We show that the CD133+ subpopulation was resistant to etoposide and had augmented proliferative and clonogenic capacity in vitro and significantly increased tumor initiation potential in vivo. These cells exhibited some stem cell characteristics, including Oct-4 expression, the ability to maintain their stem cell–like characteristics 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 that new therapeutic strategies are urgently required for the treatment of patients with SCLC.

Several studies have implicated CD133+ cells as the cause of cancer chemoresistance (6, 10, 33, 34); however, its role in colorectal cancer and breast tumors is controversial (35). In lung cancer, CD133 expression has been associated with poor prognosis in patients with NSCLC 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, 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).

On the basis of 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 seems to be highly variable between cancers of the same type (38). Eramo and colleagues (10) showed that 0.33% to 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). In addition, CD133 expression has been shown to be upregulated by the basic helix–loop–helix transcription factor achaete–scute complex homologue 1, 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 in vivo as both H345 and H69 cells are derived from postchemotherapy patients. In addition, chemotherapy can enrich the CSC population (39, 43, 44). In patients with breast cancer 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 subpopulation of cells has 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...
nonadherent aggregates in liquid culture and therefore show some characteristics of stem-like cells. However, our extensive characterization of these cells has 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 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 with the CD133− cells, which is in agreement with other studies on hepatocellular carcinoma (39). Contributing factors promoting CSC growth may be attributed to overexpression of growth factors by the CSCs. Levina and colleagues (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 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 PI3K/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 extracellular matrix 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 V\(_{1A}\) receptors and increased GRP and AVP induced signaling and growth. Our new analogue peptide-1 inhibited SCLC cell growth in vitro and 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 postchemotherapy, 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 that showed clearly defined CD133-negative and -positive tumor cell populations, with the fraction of CD133\(^+\) cells increasing 3-fold in etoposide-treated mice that coexpressed GRP-R.

Our finding that GRP-R and V\(_{1A}\) receptors 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Sarvi, A.C. Mackinnon, M. Bradley, C.D. Gregory, T. Sethi
Development of methodology: S. Sarvi, A.C. Mackinnon, N. Avlonitis, W. Wang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Sarvi, R.C. Rintoul, D.M. Rassl

Figure 7. Peptide-1 inhibits growth of SCLC in vivo and inhibits growth of CD133\(^+\) cells in vitro. A, peptide-1 was administered twice weekly by subcutaneous injection at 25 mg/kg for a total of five injections once tumors were established and compared with and in combination with etoposide at 15 mg/kg (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 (\(\times 100\); \(P < 0.01\); \(P < 0.05\) compared with 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 (\(n = 4\)).
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis) S. Sarvi, A.C. Mackinnon, W. Wang, S.J. Forbes, C.D. Gregory, T. Sethi
Writing, review, and/or revision of the manuscript S. Sarvi, A.C. Mackinnon, W. Wang, S.J. Forbes, C.D. Gregory, T. Sethi
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CD133^+ SCLC cells are sensitive to neuropeptide antagonist


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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