The FACT inhibitor CBL0137 Synergizes with Cisplatin in Small-Cell Lung Cancer by Increasing NOTCH1 Expression and Targeting Tumor-Initiating Cells

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Abstract

Traditional treatments of small-cell lung cancer (SCLC) with cisplatin, a standard-of-care therapy, spare the tumor-initiating cells (TICs) that mediate drug resistance. Here we report a novel therapeutic strategy that preferentially targets TICs in SCLC, in which cisplatin is combined with CBL0137, an inhibitor of the histone chaperone facilitates chromatin transcription (FACT), which is highly expressed in TICs. Combination of cisplatin and CBL0137 killed patient-derived and murine SCLC cell lines synergistically. In response to CBL0137 alone, TICs were more sensitive than non-TICs, in part, because CBL0137 increased expression of the tumor suppressor NOTCH1 by abrogating the binding of negative regulator SP3 to the NOTCH1 promoter, and in part because treatment decreased the expression of stem cell transcription factors. The combination of cisplatin and CBL0137 greatly reduced the growth of a patient-derived xenograft in mice and also the growth of a syngeneic mouse SCLC tumor. Thus, CBL0137 can be a highly effective drug against SCLC, especially in combination with cisplatin.

Significance: These findings reveal a novel therapeutic regimen for SCLC, combining cisplatin with an inhibitor that preferentially targets tumor-initiating cells. Cancer Res; 78(9); 2396–406. ©2018 AACR.

Introduction

Lung cancer is the leading cause of cancer-related deaths in the world, with more than 1.3 million fatalities annually (1). Small-cell lung cancer (SCLC), which accounts for about 20% of all lung cancer, is an aggressive neuroendocrine tumor, characterized by rapid expansion and metastasis (2). Genomic characterization of SCLC tumors has not yet identified effective targets for therapy. Thus, standard chemotherapy remains the backbone of SCLC treatment and has changed little over the past three decades (3). The commonly used chemotherapeutic agents, including cisplatin, are highly cytotoxic and kill the majority of tumor cells initially, but the tumors recur rapidly. The cytotoxic activity of cisplatin is mediated by the formation of DNA-damaging adducts that activate several different signaling pathways, leading to apoptosis or cell-cycle arrest (3). However, intrinsic or acquired resistance to cisplatin remains a major limitation to curative therapies. Several mechanisms are believed to be responsible for resistance, including enhanced DNA damage repair (4), resulting in reduced apoptosis (5). For any potential new therapy to succeed, it is important to understand the molecular mechanisms that facilitate cell killing.

Tumor-initiating cells (TIC), important contributors to disease recurrence and metastasis, have been identified within most solid tumors and are associated with increased resistance to therapies, including cisplatin (6, 7). Therapies that kill non-TICs, but not TICs, may temporarily reduce the volume of a tumor, but relapse occurs because therapy-resistant TICs escape treatment and result in a drug-resistant recurrent tumor (7). TICs express specific markers, including CD133 and CD44, at much higher levels than the bulk tumor cell population (8). These markers are useful for the isolation and functional characterization of SCLC TICs and non-TICs as separate populations. Similarly to normal stem cell populations, TICs self-renew, differentiate, and express many of the same core transcription factors (9). SCLCs contain a much higher percentage of TICs than non-TICs, >65%–75%, compared with <15%–20% for non-small cell lung cancers (NSCLC; ref. 10). Improved clinical responses in SCLC may be achieved, therefore, by improved targeting of TICs, which are relatively insensitive to chemotherapy and lead to the growth of resistant tumors.

The experimental drug CBL0137 (11) is currently undergoing multicenter phase I clinical trials in metastatic or unresectable advanced solid neoplasms or refractory lymphomas (NCT01905228). CBL0137 (or the related drug quinacrine) has been shown to have potent anticancer activity in NSCLC (12), pancreatic cancer (13), breast cancer (14), and neuroblastoma (15). CBL0137 targets facilitates chromatin transcription (FACT), a histone chaperone that is expressed at high levels in tumors. Inhibition of FACT is toxic for most cancer cells.
because it is needed for the NFκB-induced expression of many genes, and activated NFκB is required for the survival of virtually all tumors (11). FACT is essential for the survival of glioblastoma (GBM) TICs (16), and also plays a critical role in cisplatin resistance by facilitating the repair of DNA damage (17). Interestingly, CBL0137 exhibits strong synergy with cisplatin in neuroblastoma by blocking the FACT-mediated repair of DNA damage (15). Therefore, targeting DNA repair is a potentially important strategy to enhance the effectiveness of cisplatin in SCLC.

The NOTCH signaling pathway regulates the self-renewal and survival of TICs (18). NOTCH1 is a transmembrane receptor that is activated upon ligand binding through a series of proteolytic cleavages. Once cleaved, the NOTCH1 intracellular domain translocates to the nucleus, where it binds to DNA and activates the transcription of target genes, including HEY1 and HEY2 (19), whose increased expression in turn downregulates the expression of the transcription factor achaete-scute homolog-1 (ASCL1; ref. 20), which plays an important role in the proliferation and survival of SCLC cells (21). NOTCH1 can act as either a tumor suppressor or an oncogene. The tumorigenic or tumor-suppressive activities of NOTCH in different tumor types reflect its different roles in promoting or repressing the undifferentiated status of stem cells in the corresponding tissues (18). The oncogenic role of NOTCH has been identified in many cancers, including NSCLC (22), T-ALL (23), and GBM (24). In contrast, NOTCH1 signaling is suppressed in neuroendocrine tumor cells, including SCLC (25, 26), indicating that inducing its expression is an attractive strategy for treating these tumors.

The SP/KLF family of transcription factors consists of proteins with three highly conserved DNA-binding zinc finger domains, which recognize GC/CACCC boxes present in many GC-rich promoters (27). SP3 belongs to this family, which also includes SP1, 2, and 4, all of which bind to GC-rich NOTCH1 promoters (28). In human keratinocytes, KLF4 binds to the NOTCH1 promoter and, together with SP3, functions as a negative regulator of transcription, affecting recruitment of the Pol II preinitiation complex (28). Furthermore, knockdown of KLF4 and SP3 led to upregulation of NOTCH1 expression in HeLa cervical carcinoma and skin squamous carcinoma cells (SCC13) (28).

We have investigated a novel therapeutic strategy for SCLC by combining CBL0137 with cisplatin in patient-derived SCLC cells and xenografts. We tested the impact of CBL0137 on SCLC TICs in comparison with non-TICs, and the potential role of FACT in maintaining the stem cell phenotype of TICs. We also investigated the role of CBL0137 in increasing NOTCH1 expression, activating a core inhibitory signaling pathway in TICs. On the basis of previous findings and our current study, CBL0137 is a very potent anticancer drug. It inhibits FACT and NFκB activation in several different cancers (11), preferentially kills TICs, and targets NOTCH1 activation in SCLCs. CBL0137 synergizes with cisplatin in SCLCs, greatly increasing the sensitivity to this traditional chemotherapeutic agent.

Materials and Methods

Cell lines and reagents

The SCLC cell lines NCI-H82 (H82), NCI-H526 (H526), and NCI-H446 (H446) were obtained from ATCC, three years before being used in this study. The Rb/p53–mutant mouse SCLC KP1 cell line was a generous gift from Dr. Julien Sage (Stanford University, Stanford, CA), received a month before being used. The cells were maintained in culture for no longer than 2–3 months, and were routinely assayed for mycoplasma. The cells were grown in RPMI1640 medium supplemented with 5% (v/v) heat-inactivated FBS. For experimental purposes, the cells were cultured in SITA medium, consisting of RPMI1640 medium supplemented with 30 nmol/L selenium, 5 μg/mL insulin, 10 μg/mL transferrin, and 0.25% (w/v) BSA, EGF, and FGF (29). All cultures were incubated in 5% (v/v) CO₂ at 37°C. CBL0137 (lot # 10-106-88-30) was provided by Incuron, LLC. EGF and basic FGF were from PeproTech. Selenium, insulin, transferrin, and BSA were purchased from Sigma Chemicals. Antibodies against SOX2 (1:1,000) and OCT4 (1:500) were from Cell Signaling Technology. SSRP1 antibody (1:2,000) was from BioLegend, and β-actin antibody was from Sigma Aldrich. For immunofluorescence assays, antibody against CD133, anti-CD133/1 (AC133) conjugated with phycocerythrin (PE), and mouse IgG1-PE were from Milenyi Biotec; and anti-CD44 conjugated with brilliant blue 515 (BB) was from BD Biosciences. The CyQUANT Direct Cell Proliferation Assay Kit was from Thermo Fisher Scientific. shRNAs to SP3 and scrambled shRNAs were obtained from Sigma Chemicals.

The H82 and H526 cells were authenticated. DNA extraction, short repeat profiling, and comparison with known cell line profiles from ATCC were performed by Genetica DNA Laboratories. The H446 cells from ATCC were not further authenticated.

Isolation and culture of TICs

Flow cytometry was performed using a FACSemia II Cell Sorter (BD Biosciences) to isolate TICs from H82, H526, or H446 cells. To obtain CD133<sup>high</sup> and CD133<sup>low</sup> cells, individual cells were labeled with PE-conjugated mAb against CD133. To isolate populations of CD44<sup>high</sup> and CD44<sup>low</sup> cells, the cells were labeled with BB-conjugated CD44 antibody. Dead cells were eliminated by DAPI staining (1 μg/mL, added immediately prior to sorting). The CD133 or CD44<sup>high</sup> cells were cultured in SITA medium (29). CD133 or CD44<sup>low</sup> cells were cultured in RPMI1640 with 5%–10% FBS. CD133 or CD44<sup>high</sup> and CD133 or CD44<sup>low</sup> cells in cell proliferation assays (CyQuant) were maintained in the SITA medium.

Cell survival assay

Cell survival was determined using the CyQUANT Fluorescent Assay (Thermo Fisher Scientific), according to the manufacturer's instructions. Briefly, the reagent was added directly to the culture medium in clear bottom black-wall 96-well plates. After a 2-hour incubation at 37°C, plates were centrifuged at 200 × g for 5 minutes, and fluorescence was read with a plate reader at excitation 480 nm and emission 535 nm. The combination index was assessed using CompuSyn software (ComboSyn, Inc; ref. 30).

Limiting dilution assay and sphere formation

For tumosphere formation assays, TICs were FACS-sorted and plated at different dilutions in ultralow adherent 96-well plates, in supplemented SITA medium. Tumospheres were counted after 2–3 weeks under a phase contrast microscope. Wells with a tumorsphere were counted as positive and the...
Quantitative real-time PCR
Quantitative real-time PCR was performed as described previously (32). DNA was synthesized from total RNA, using a random hexamer and SuperScript III (Invitrogen). The expression levels of human SOX2, NANOG, OCT4, NOTCH1, HEY1, HES1, and ASCL1 mRNAs or control 18S rRNA were examined by using the EvaGreen qPCR master mix (Bullseye) in a LightCycler 480 (Roche). Gene-specific primers were: SOX2, forward CACACCTGCCCCTCTCCTAC, and reverse TCCATCTGTCTTCTA-CTCCCT; NANOC, forward GAATCCTCAACCTCCACGC, and reverse GCCGTCACACCATGCTATTC; OCT4, forward TCTCCCATGCTACAACTCTGAC, and reverse CCTTTGGTTCCCAATAGAAGC; HES1, forward AGGCTGGAGAGGCGGCTAAG, and reverse GACCAGGCGAACGCTCTCC; HEY1, forward CTG; and reverse 5'-GCACTAGTGAGGCTCAGAGT-3'.

Electrophoretic mobility shift assays
Whole or nuclear extracts were prepared with the Nuclear Extract Kit (Active Motif). The NOTCH1 probe containing 28 nucleotides (5'-CGGCCCCGCCCCGCCCGGCCCGCCCGCCCGCCCGCGC-3') and (5'-GCCCCGGCCCGCCCGCCCGCCCGCCCGCCCGCCCGCCCGC-3') was labeled with the Biotin 3' End DNA Labeling Kit (Thermo Fisher Scientific). The DNA binding reaction and native polyacrylamide gel electrophoresis were performed following the instructions for Gelshift Chemiluminescent EMSA (Active Motif). The protein composition of complexes treated with CBL0137 (or untreated) was determined by performing a supershift assay, using antibodies to SP3 (D20, Santa Cruz Biotechnology). In different experiments 2 or 6 μg of SP3 antibody was used. The competition EMSA was done with a 100 fold molar excess of unlabeled probe.

Immunoblotting
Whole cell extracts were prepared by incubating cell pellets in lysis buffer containing 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP-40, and a mixture of protease and phosphatase inhibitors (Roche). After incubation for ice on 2 minutes, cell debris was removed by centrifugation. Chromatin fractions were extracted using a subcellular protein fractionation kit (Thermo Fisher Scientific). Cell extracts containing equal quantities of proteins, determined by the Bradford method, were separated by SDS/PAGE (10% gel). After transfer, the membranes were incubated with primary antibody for 2 hours, followed by incubation with secondary antibody for 1 hour at room temperature, and developed by using enhanced chemiluminescence solution (Perkin-Elmer). Chromatin immunoprecipitation assays
These assays were performed with the Agarose ChIP Kit (Thermo Fisher Scientific), according to the manufacturer’s instructions. In brief, 2 x 10^5 cells were fixed with 1% formaldehyde for 10 minutes at room temperature in supplemented SIFA medium, followed by quenching with 125 mmol/L glycine for 5 minutes. The cells were lysed and the chromatin was fragmented by partial digestion with Micrococcal Nuclease. DNA/protein complexes were precipitated by overnight incubation with 4 μg anti-SP3 antibody (D-20, Santa Cruz Biotechnology), or 4 μg normal rabbit anti-IgG antibody (Santa Cruz Biotechnology), and then incubated with Protein A/G agarose beads for 2 hours. After reversal of protein-DNA cross-links, the DNA was purified and the abundance of the NOTCH1 promoter was analyzed by qPCR using NOTCH1-specific primers, forward 5'-AAGCAGAAGTGTCTCCAGCG-3' and reverse 5'-GCACATAGCTGAGCTAGAGT-3' as before (33). The PCRs for the NOTCH1 promoter sequence were performed using 2X Phusion Master Mix (New England Biolabs).

Statistical analysis
Results from SCLC cell lines are represented by means ± SD. Data were analyzed using Student t test or by two-tailed ANOVA,
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Inhibiting DNA repair is likely to enhance the effectiveness of cisplatin, and we hypothesized that combining cisplatin with CBL0137, to inhibit FACT, would be a logical approach. We evaluated the synergy between cisplatin and CBL0137 in the established patient-derived SCLC cell lines H82, H526, and H446, and also in the murine SCLC cell line KP1, combining the two drugs at constant molar ratios of 1:1, using values above and below the IC_{50} for each drug. By CyQUANT Direct assay, which measures proliferation as well as cytotoxicity (36), we show that the cells are more sensitive to the combination than to treatment with either drug alone (Fig. 1A–D, top). Synergism was determined by the Chou–Talalay method, which measures the median–drug effect and quantifies the combination indices of two drugs based on the growth inhibition curves of each drug alone, or of both in combination (30). The combination of cisplatin and CBL0137 was synergistic in all the cell lines, when combined at 1:1 ratios, as indicated by combination indexes substantially below 1.0 (Fig. 1A–D, bottom).

The in vivo effect of drug combination was then assessed in the H82 SCLC xenograft, murine SCLC syngeneic, and SCLC PDX models. In the H82 xenograft model, CBL0137 in combination with cisplatin significantly inhibited H82 tumor growth (P < 0.05), compared with CBL0137 alone, cisplatin alone, or vehicle control (Fig. 1E; Supplementary Fig. S1). Strikingly, there was no significant tumor growth until day 30 in mice treated with the combination of cisplatin and CBL0137, and the growth rate was much slower than with control or single-agent treatment. Mice treated with vehicle or single agents survived for 34–40 days, whereas mice receiving the combination survived for 51 days (Fig. 1E). In another experiment (Supplementary Fig. S1), tumor growth was monitored for 32 days of treatment. In immunocompetent mice, CBL0137 in combination with cisplatin substantially inhibited tumor growth, compared with CBL0137 alone (P < 0.05), cisplatin alone (P < 0.05), or vehicle control (P < 0.05; Fig. 1F). In 35 days, the tumors in vehicle or CBL0137 or cisplatin-treated mice reached a maximum size of approximately 1,000–2000 mm³, whereas the tumors did not grow further in mice treated with the drug combination, and the tumors were found to have regressed (Fig. 1F). Importantly, in the PDX study as well, CBL0137 in combination with cisplatin substantially inhibited tumor growth, compared with CBL0137 alone, cisplatin alone, or vehicle control (Fig. 1E; Supplementary Fig. S1).

Using GraphPad Prism software. Differences between tumor volumes are represented by means ± SE, and compared pairwise using Student t test. P values of <0.05 are considered statistically significant. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant.

Results

CBL0137 and cisplatin inhibit SCLCs synergistically in vitro and in vivo

CBL0137 Sensitizes SCLC to Cisplatin in vitro and in vivo. A-D, Top, the patient-derived SCLC cell lines, human H82, H526, and H446, and murine SCLC KP1 cells were plated at 1 × 10⁵ cells/well in 96-well plates in triplicate. After 24 hours, the cells were treated with the indicated concentrations of CBL0137 alone, cisplatin alone, or CBL0137 plus cisplatin at a 1:1 ratio, for 6 hours. Cell viability was evaluated by CyQUANT Direct assay after 72 hours of drug treatment and normalized to controls (n = 3). A-D, The degree of drug synergy of the combination of cisplatin and CBL0137 was determined by calculating combination indices (CI), based on the dose–response data, using ComboSyn software, where CI < 1 indicates synergy (bottom). Each experiment in A–C was carried out three times, and D was carried out twice independently. E–G, In vivo studies. E, H82 SCLC cells, mixed with Matrigel (1:1), were inoculated subcutaneously into the flanks of NSG mice. F, Murine SCLC KP1 cells mixed with Matrigel (1:1) were inoculated subcutaneously into the flanks of B6.129S mice. G, SCLC PDX tumor fragments (2 mm) were inoculated subcutaneously into the flanks of NSG mice. Once the tumors reached approximately 20 mm³, the mice were randomized to treatment with vehicle control, CBL0137 (CBL) alone (60 mg/kg i.v., weekly), cisplatin (Cis) alone (5 mg/kg i.p., weekly), or CBL0137 plus cisplatin (CBL+Cis). Tumor diameters were measured 3 times a week for 51 days for H82, 35 days for KP1, and 50 days for PDX. The results are represented as means ± SE. *, P < 0.05 versus the other three treatment groups.
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sion levels of cells. Previously, we showed that CBL0137 decreases the expres-
ted in Fig. 2C, although CBL0137 reduced both CD133 high or CD44 high and CD133 low or CD44 low cell populations in a dose-dependent manner, it was more potent against the CD133 high or CD44 high cells, emphasizing the preferential targeting of TICs by this drug.

Self-renewal is an important characteristic of tumor-initiating cells, defined by their ability to form tumors from single cells in vitro (39). We assessed whether treatment of SCLC TICs with CBL0137 affected their ability to form tumors in an extreme limiting dilution assay, which permits quantification of TIC frequencies (31). We exposed TICs to DMSO or CBL0137 for 24 hours, washed the drug out, and then plated the cells at densities of 5, 10, 20, or 50 cells per well in 96-well plates. Tumorsphere formation was evaluated after 14–21 days. Self-renewal of the TICs was markedly decreased by a single pretreatment with CBL0137 (Fig. 2D). These findings together suggest that CBL0137 exerts a potent inhibitory effect on TICs derived from SCLC cell lines, by targeting FACT and stem cell transcription factors that are required for TIC survival.

CBL0137 targets the SCLC tumor suppressor NOTCH1 in tumor-initiating cells

Overexpression of NOTCH1 in SCLC cells decreased cell proliferation and increased apoptosis (26, 40). Whole-genome sequencing of SCLC samples revealed inactivating mutations of NOTCH family genes in about 25% of the cases, suggesting a tumor-suppressive role of NOTCH (26). In this study, we observed that the expression of NOTCH1 mRNA was dramatically lower in SCLC TICs compared with non-TICs derived from H82 and H526 cells (Fig. 3A and B). Importantly, CBL0137 treatment significantly increased NOTCH1 mRNA expression in the TICs, in both cell lines (Fig. 3A and B). Next, we determined the effect of CBL0137 on the kinetics of NOTCH1 gene expression in the TICs, observing that treatment increased NOTCH1 mRNA expression after only 4 hours (Fig. 3C). Furthermore, treatment with CBL0137 significantly increased expression of the NOTCH1 target mRNAs HEY1 and HES1 in the TICs (Fig. 3D–G). Although CBL0137 also moderately increased the levels of mRNAs encoding HES1 and HEY1 in the non-TICs, the increases in the TICs were more dramatic (Fig. 3D–G).

NOTCH1 activation targets ASCL1, which is highly expressed in SCLC cells and regulates tumor-initiating capacity in these cells (26, 41). We observed a higher level of ASCL1 mRNA expression in TICs compared with non-TICs, and treatment with CBL0137 significantly reduced ASCL1 mRNA levels in these cells (Fig. 3H and I). We conclude that CBL0137 increases NOTCH1 expression and activates NOTCH1 signaling.

SP3 negatively regulates the NOTCH1 pathway in SCLC TICs

The above findings prompted us to investigate the mechanism by which CBL0137 activates NOTCH1 in SCLC TICs. We postulated that CBL0137 treatment might downregulate a negative regulator of NOTCH1, which in turn would increase NOTCH1 expression. It has been reported previously that the transcriptional repressors KLF4 and SP3 can modulate NOTCH1 expression by binding to its promoter (28). On the basis of this information, we determined the expression level of SP3 in TICs and non-TICs, finding it to be higher in the non-TICs (Fig. 4A). By performing chromatin immunoprecipitation assays, we show increased binding of SP3 to the endogenous NOTCH1 promoter in TICs, compared with non-TICs derived from H82 SCLC cells, and that this binding was eliminated in 2 hours by CBL0137, determined by PCR (Fig. 4B and C, top) as well as by qPCR (Fig. 4B and C, bottom). We further confirmed the effect of CBL0137...
on the binding of SP3 to the NOTCH1 promoter by electro- 
phoretic mobility shift assays (EMSA). The cells were treated 
with CBL0137 for different times, and EMSAs were performed 
with nuclear (Fig. 4D) or whole-cell lysates (Fig. 4E). In both 
experiments, CBL0137 impaired the binding of SP3 to the 
NOTCH1 promoter, although the levels of SP3 remained 
Figure 2.
Exposure to CBL0137 kills TICs preferentially and attenuates self-renewal. A, Previously sorted CD133high or CD133low H82 or H526 cells, or CD44high or 
CD44low H446 cells, were assayed to confirm CD133 or CD44 expression. The sorted cells were stained with anti-CD133-1-PE, or anti-CD44-BB or isotype 
controls and examined by flow cytometry. The expression levels of CD133high and CD133low or CD44high and CD44low were compared by overlaying 
the expression levels of each populations by using FlowJo software version 10. B, TICs isolated from SCLC cells can differentiate. CD44high or CD133high H446, H526, 
or H82 cells were plated in medium with 5% FBS. CD44low or CD133low H446, H526, or H82 cells were plated either in serum-free SITA medium 
or medium plus 5% FBS for 21 days and the levels of SOX2 were assessed by the Western method. C, SCLC TICs are more sensitive to CBL0137 than non-TICs. 
CD133high or CD44high or CD44high low cells were seeded at 3,000 cells/well in 96-well plates and allowed to attach for 24 hours. The cell viability after 72 hours 
of CBL0137 treatment was determined by CyQuantDirect assay and normalized to controls (n = 4). The experiments were repeated thrice and each 
measurement was performed in triplicate. Data are expressed as means ± SD. D, Acute exposure to CBL0137 attenuates TIC self-renewal. In vitro 
extreme limiting dilution assays for TICs from H82 and H526 cells were performed by plating the cells at 10, 20, 30, 40, or 50 cells per well, 12 wells for each, 
in ultralow adherent 96-well plates. Each group of cells was pretreated with vehicle (DMSO) or 1 μmol/L CBL0137 for 24 hours before plating. Fourteen 
to 21 days later, the tumor spheres in each well were counted. TIC frequencies were determined based on the numbers of wells with no colony, and the 
estimated frequencies for each condition are indicated. P values indicate the significance of differences in TIC frequencies between groups, by the χ2 test.
unchanged (Fig. 4F). Next, we explored whether CBL0137 treatment could prevent the binding of SP3 to the NOTCH1 promoter in vitro, using whole-cell lysates from H82 and H526 TICs for EMSAs with a NOTCH1 probe. In control lysates, SP3 bound to the NOTCH1 probe [Fig. 5A (lane 2) and B (lane 1)]. However, when the lysates were treated in vitro with CBL0137 at different concentrations, SP3 did not bind to the probe. When the cell lysates were mixed with the probe first, and then treated with CBL0137 [Fig. 5A (lanes 4–6) and B (lanes 3–5)], SP3 binding was decreased dramatically, even at 200 nmol/L, the
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Discussion

Resistance to chemotherapy is a major obstacle to successful treatment of SCLC. There are currently no targeted approaches to treat this disease that are similar to those used successfully against NSCLC, and there have been no significant advances in the last 30 years (43). The standard-of-care platinum-based drugs have the ability to kill the bulk tumor, but fail to eliminate the TICs, resulting in tumor recurrence (7, 44). The most immediate therapeutic improvements against this cancer will depend on our ability to prevent or delay the emergence of chemoresistance. CBL0137 sensitizes with the DNA-damaging drugs cisplatin and etoposide in neuroblastoma (15). In addition, CBL0137 increases the sensitivity to cisplatin in neuroblastoma because FACT, the target of CBL0137, is required for DNA repair (15). Therefore, targeting DNA repair is a promising stratagem to enhance cisplatin effectiveness, and provides a strong rationale for combining cisplatin with CBL0137, to inhibit FACT and achieve synergistic lethality.

The combination of CBL0137 with cisplatin is determined to be synergistic at their respective IC50 ratios (30) in patient-derived SCLC cell lines as well as in a murine SCLC cell line. We demonstrate extensively that these therapeutic strategies are also effective in vivo, in experiments with SCLC xenographs, and a PDX model in immunocompromised mice, and syngeneic SCLC tumors in immunocompetent mice. Importantly, the PDX specimen we used is derived from the tumor of a patient who relapsed after initial response to the combination of cisplatin and etoposide, the standard-of-care therapy. Our data reveal that CBL0137 helps to overcome resistance to cisplatin. CBL0137 is currently in the final stages of multicenter phase I clinical trials in advanced or metastatic solid tumors and lymphomas (NCT01905228), and it has not yet exhibited dose-limiting toxicity. Therefore, using CBL0137 in combination with cisplatin in SCLC is a novel therapeutic strategy for this cancer that can be employed soon.

By using the cell surface markers CD133 or CD44, TICs have been identified in a variety of human cancers, including lung cancers (7, 29). TICs show elevated expression levels of genes encoding transcription factors that are associated with stemness, including SOX2, OCT4, and NANOG (16). We demonstrate that...
CD133<sup>high</sup> or CD44<sup>high</sup> SCLC cells have increased levels of SOX2, NANOG, and OCT4, compared with CD133<sup>low</sup> or CD44<sup>low</sup> cells, consistent with the tumor-initiating characteristics of these cells. As in previous reports (16, 29), we also show that culturing CD133<sup>high</sup> or CD44<sup>high</sup> SCLC cells in serum-containing medium leads to their differentiation, accompanied by the loss of stem cell markers.

Eradication of TICs, a major challenge for cancer therapy, can be achieved by using inhibitors that target TIC-specific pathways. We show that the FACT inhibitor CBL0137 is very potent as a single agent toward SCLC cells, and preferentially targets TICs, consistent with our previous findings in GBM (16).

FACT is required for the expression of stem cell transcription factors that are vital for TICs to self-renew (16). Treatment with CBL0137 in SCLC TICs inhibits the function of FACT by depleting soluble SSRP1 and trapping it on chromatin, decreases the expression of self-renewal genes, and dramatically reduces the self-renewal potential of the TICs. Our findings indicate that CBL0137, by targeting FACT, may have greater efficacy against tumors high in TIC content, as revealed by SOM2 expression. TICs are critical determinants of drug resistance and relapse in SCLC (45), and therefore, further studies are warranted to correlate stem cell marker expression in this disease with chemotherapeutic-responses and survival.

We discovered an additional potential therapeutic activity of CBL0137, as an activator of NOTCH1 expression in SCLC. This drug preferentially increases the expression of NOTCH1 mRNA in SCLC TICs compared with non-TICs, and also increases the expression of the NOTCH1 targets HEY1 and HES1, while decreasing the expression ASCL1, which is inhibited by NOTCH signaling (40). However, a moderate increase in the HEY1 and HES1 in the non-TICs suggests that NOTCH-independent signaling pathways might be involved in the non-TICs. The NOTCH1 signaling pathway is silenced in many neuroendocrine malignancies, including SCLC (40). Activation of NOTCH1 signaling inhibited the growth of SCLC cells (25), and reduced the number of tumors and extended the survival of mice in a preclinical SCLC mouse model (26). We show that the expression of NOTCH1, HEY1, and HES1 mRNAs is very low in SCLC TICs, compared with non-TICs, suggesting that the tumor-suppressive role of NOTCH1 is not manifest in SCLC TICs. Our finding that treatment of SCLC TICs with CBL0137 increases NOTCH1 expression reveals a novel potential therapeutic role of...
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Figure 6. Model figure showing the function of CBL0137. CBL0137 intercalates into DNA and changes chromatin conformation. FACT is recruited to the changed conformation and is immobilized there, inhibiting its ability to function, including in its role in DNA repair. NOTCH1 activation is increased because, when treated with CBL0137, the binding of SP3 to the NOTCH1 promoter is reversed. The increase in NOTCH1 transcription decreases the viability of SCLC TICs. CBL0137, in combination with the DNA-damaging agent cisplatin, has the potential to kill both SCLC non-TICs and TICs.

CBL0137 in SCLC. We conclude that CBL0137 preferentially impairs SCLC TICs not only by targeting FACT, but also by activating NOTCH1. Recently, it has been reported that the loss of even one allele of Ascl1 dramatically decreases mouse SCLC tumor growth (46), indicating the desirability to focus on therapeutic targeting of the ASCL1 pathway in this disease. Our results showing decreased ASCL1 expression in SCLC TICs upon CBL0137 treatment indicates the utility of further work, to reveal the therapeutic indications of this drug in targeting ASCL1. Another very recent study shows that, in SCLC, NOTCH signaling can be both tumor suppressive and protumorigenic (47). However, our findings together reveal that CBL0137 preferentially kills SCLC TICs by activating the tumor-suppressive role of NOTCH1 by increasing the expression of NOTCH1 and NOTCH1 target genes, and by decreasing ASCL1 expression, in SCLC TICs.

CBL0137 treatment impairs both the endogenous and in vitro binding of SP3 to the NOTCH1 promoter, revealing the mechanism of CBL0137-induced NOTCH1 activation in SCLC TICs. SP3 can act as an activator (48) or a repressor of transcription (49). It is upregulated in cancer cells, for example, cervical carcinomas and keratinocyte-derived squamous cell carcinomas, where NOTCH1 expression is downmodulated (28). We observe that acute exposure to CBL0137 not only prevents the binding of SP3 to the NOTCH1 promoter, but also reverses the binding in SCLC TICs. Downregulation of SP3 increased NOTCH1 expression in the TICs, confirming the role of SP3 as a negative regulator of NOTCH1 (28).

Genomic profiling of SCLC is still in its infancy, delaying the development of molecularly targeted therapies. Our approach to use combination therapy with CBL0137 and cisplatin is an important means to circumvent the development of resistance to standard therapy (Fig. 6). Another promising approach is the use of CBL0137 as a TIC-targeting therapy to prevent the emergence of tumor recurrence by eradicating TICs (Fig. 6). Our novel finding of the role of CBL0137 in activating NOTCH1 also has therapeutic implications, opening an important new avenue to further explore in the clinical use of this drug.

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

Authors’ Contributions
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