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

Sarmishtha De1, Daniel J. Lindner2, Claire J. Coleman1, Gary Wildey3, Afshin Dowlati3, and George R. Stark1

Abstract

Traditional treatments of small-cell lung cancer (SCLC) with cisplatin, a standard-of-care therapy, spare the tumor-initiating cells (TIC) 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 high 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 HES1 and HEY1 (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 cancer (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 Miltenyi 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 FACSaria 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> cell 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-well 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 by CompuSyn software (CompuSyn, Inc; ref. 30).

Limiting dilution assay and sphere formation
For tumorsphere formation assays, TICs were FACS-sorted and plated at different dilutions in ultralow adherent 96-well plates, in supplemented SITA medium. Tumorspheres 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 CACACATGCCCTCCTCAG, and reverse TCTCATGTGTTCCCAAT; NOTCH1, forward GAAATACCTCCAGCCTCCAG, and reverse GCCGACACCATCGCTATTC; OCT4, forward TCTCCCATGCTACCAACTCAG, and reverse CCGTTGTCAGTCCATCCTGTC; NOTCH1, forward CAAATGGATGCCGAGTTGG, and reverse CACACATGCCCTCCTCAG; and HES1, forward AGGCTGGAGAGGCGGCTAAG, and reverse AGCTATCAATCTGTCAATCCTGTC. The NOTCH1 probe containing 28 nucleotides (5'-GGCCCCGCCCCGCCGGGGGGCCCCCGGCGC-3') and (5'-GGCCCCGCCCCGCCGGGGGGCCCCCGGCGC-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.

Lentiviral transduction

Lentiviral transduction was performed as described earlier (32) using lentiviral plasmids encoding shRNAs against SP3 (TRCN 0000280421, # 1; TRCN 0000280370, # 2) or scrambled shRNA control.

In vivo studies

All animal experiments were approved by the Cleveland Clinic Foundation Institutional Animal Care and Use Committee and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Six-week-old NOD scid gamma (NSG) mice, obtained from Biological Research Unit, Cleveland Clinic, and B6.129S mice from Jackson Laboratories, were maintained in 12-hour light/12-hour dark cycles with free access to food and water. B6.129S mice were used as hosts in the immunocompetent syngeneic model with KP1 cells (34). For the patient-derived xenograft (PDX) study, we received a generous gift of PDX tumor (MSK-LX95), derived from a SCLC patient who previously received chemotherapy and relapsed (35), from Dr. Charles M. Rudin (Memorial Sloan Kettering Cancer Center, New York, NY). The PDX tumor fragments were passaged and maintained in NSG mice.

The mice were inoculated subcutaneously in the flanks with H82 cells (5 x 10^5), or murine KP1 cells (5 x 10^5), in medium containing 50% growth factor reduced, phenol red-free Matrigel (Corning). For the PDX experiment, 2-mm diameter fragments were inoculated subcutaneously in the flanks using a trocar. Tumors were measured by caliper 3 times weekly for the duration of the experiment. When tumors reached 3—4 mm diameter (~20 mm³), the mice were divided into a control group that received both vehicles, and treatment groups that received either CBL0137 alone, cisplatin alone, or a combination of CBL0137 and cisplatin (n = 8 per group). Treatments were started on day 14 for H82 xenografts, KP1 syngeneic tumors, and for the PDX study, with vehicles alone; or CBL0137 (60 mg/kg, i.v.), or cisplatin (5 mg/kg, i.p.), or CBL0137 in combination with cisplatin. In the combination group, the mice received CBL0137 4 hours before treatment with cisplatin. Treatments were given once per week, either for 3 weeks, or until the tumors reached approximately 1,500—2,000 mm³, at which time mice were euthanized. Tumor volumes (V) were calculated using the volume for a prolate spheroid: 
\[ V = \frac{4}{3} \pi a^2 b \]
where a = minor radius, b = major radius.

Statistical analysis

Results from SCLC cell lines are represented by means ± SD. Data were analyzed using Student t test or by two-tailed ANOVA,
CBL0137 Sensitizes SCLC to Cisplatin and Kills TiCs

Results

CBL0137 and cisplatin inhibit SCLCs synergistically in vitro and in vivo

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 mm^3, 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, and vehicle controls (P < 0.05 versus the other three treatment groups).

Figure 1.

CBL0137 exhibits synthetic lethality in combination with cisplatin in SCLC in vitro and in vivo. A–D, Top, the patient-derived SCLC cell lines, human H82, H526, and murine SCLC KP1 cells were plated at 1 × 10^4 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 mm^3, 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.
alone (P < 0.05), cisplatin alone (P < 0.05), or vehicle control (P < 0.05; Fig. 1G). There was no significant difference between control and single-agent treatment at the time of euthanasia (d50; P > 0.2; Fig. 1G). These results together indicate that CBL0137 in combination with cisplatin dramatically decreased SCLC tumor growth in SCLC cell lines as well as in xenograft and syngeneic tumors, revealing a novel potential combination therapy for this cancer.

CBL0137 preferentially kills TICs isolated from SCLCs and attenuates self-renewal

TICs help to account for tumor recurrence after chemotherapy (37). Previously we showed that FACT is essential for the survival of GBM TICs, and that CBL0137 preferentially targets them (16). Because CBL0137 increases the sensitivity to cisplatin in SCLC, we thought that it might also target SCLC TICs. To test this idea, we assessed the effect of CBL0137 in TICs and non-TICs as separate populations (7, 29). We isolated tumor-initiating populations by sorting the cells for CD133 high or CD44 high (top 8%–10%). Differentiation is another important characteristic of TICs (16, 29). We found that the expression levels of OCT4, SOX2, KLF4, and SP3 increased in SCLC TICs compared with non-TICs derived from H82 SCLC cells (Fig. 3A and B). Next, we determined the effect of CBL0137 on the kinetics of Tumorsphere formation after 14 days and syngeneic tumors, revealing a novel potential combination therapy for this cancer.

CBL0137 preferentially kills TICs isolated from SCLCs and attenuates self-renewal

TICs help to account for tumor recurrence after chemotherapy (37). Previously we showed that FACT is essential for the survival of GBM TICs, and that CBL0137 preferentially targets them (16). Because CBL0137 increases the sensitivity to cisplatin in SCLC, we thought that it might also target SCLC TICs. To test this idea, we assessed the effect of CBL0137 in TICs and non-TICs as separate populations (7, 29). We isolated tumor-initiating populations by sorting the cells for CD133 high or CD44 high (top 8%–10%). Differentiation is another important characteristic of TICs (16, 29). We found that the expression levels of OCT4, SOX2, KLF4, and SP3 increased in SCLC TICs compared with non-TICs derived from H82 SCLC cells (Fig. 3A and B). Next, we determined the effect of CBL0137 on the kinetics of Tumorsphere formation after 14 days and syngeneic tumors, revealing a novel potential combination therapy for this cancer.
on the binding of SP3 to the NOTCH1 promoter by electrophoretic 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...
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...
were repeated twice, and the values represent means derived from H82 were treated with CBL0137 at 1 antibody against SP3 (4 NSCLC, and there have been no significant advances in the last 30 years (43). The standard-of-care platinum-based drugs have the capacity 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 synergizes 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 synergetic 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 xenografts, 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. 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

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 synergizes 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 synergetic 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 xenografts, 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

Figure 4.

CBL0137 inhibits endogenous SP3 binding to the NOTCH1 promoter in SCLC TICs. A, SP3 protein level was determined in CD133high or CD133low cells derived from H82 by Western blot analysis. B, Chromatin immunoprecipitation was performed in CD133high or CD133low cells derived from H82 using an antibody against SP3 (4 µg) or rabbit IgG (4 µg), followed by PCR (top) and qPCR analyses (bottom) of the promoter of the NOTCH1 gene. C, CD133high cells derived from H82 were treated with CBL0137 at 1 µmol/L for 2 hours, and a chromatin immunoprecipitation assay was performed as above. The experiments were repeated three times, and the values represent means ± SD. D and E, EMSAs using a NOTCH1 probe. H82 CD133high cells were treated with CBL0137 (5 µmol/L) for different times, and EMSAs were performed with nuclear (D) or total cell extracts (E). F, SP3 protein levels were determined by immunoblotting in H82 CD133high cells after treatment with CBL0137. All values represent means ± SD. All experiments were repeated three times.

lowest concentration of CBL0137 [Fig. 5A (lane 4), and B (lane 5)], and was completely abolished at higher concentrations [1 or 5 µmol/L; Fig. 5A (lanes 5 and 6) and B (lanes 3 and 4)]. Controls with unlabeled competitor DNA [Fig. 5A (lane 1) and B (lane 6)] and with antibodies to SP3 [Fig. 5A (lane 3) and B (lane 2)] show that the indicated band is indeed SP3. In another experiment (Fig. 5C and D), the NOTCH1 probe was incubated with CBL0137 first, and then the whole-cell lysates of H82 or H526 TICs were added to the NOTCH1-CBL0137 mixtures. Results similar to those in Fig. 5A and B were obtained, where SP3 binding was decreased or abolished with CBL0137 treatment (Fig. 5C and D, lanes 4–6), compared with the control (Fig. 5C and D, lane 3). We conclude that CBL0137 treatment prevents the in vitro binding of SP3 to the NOTCH1 promoter in TICs and reverses binding when added to an SP3–probe complex. These results indicate that CBL0137 treatment abrogates the binding of a negative regulator to the NOTCH1 promoter, facilitating NOTCH1 transcription, which in turn facilitates SCLC cell death (26, 40, 42). To test whether downregulating SP3 could increase NOTCH1 expression, we transduced H82 SCLC cell death (26, 40, 42). To test whether downregulating SP3 could increase NOTCH1 expression, we transduced H82 TICs with two different shRNAs to SP3 or scrambled shRNA and determined the expression levels of NOTCH1 mRNA. Depletion of SP3 significantly increased the level of NOTCH1 in these cells, indicating that SP3 is a negative regulator of NOTCH1 expression (Fig. 5E and F).

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 synergizes 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 synergetic 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 xenografts, 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\textsuperscript{high} or CD44\textsuperscript{high} SCLC cells have increased levels of SOX2, NANOG, and OCT4, compared with CD133\textsuperscript{low} or CD44\textsuperscript{low} cells, consistent with the tumor-initiating characteristics of these cells. As in previous reports (16, 29), we also show that culturing CD133\textsuperscript{high} or CD44\textsuperscript{high} 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 SOX2 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 of 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 with increased levels of ASCL1 mRNA, 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
CBL0137 Sensitizes SCLC to Cisplatin and Kills TiCs

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 ASCL1 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
Conception and design: S. De, A. Dowlati, G.R. Stark
Development of methodology: S. De, D.J. Lindner, A. Dowlati
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. De, D.J. Lindner, C. Coleman, G. Wildey, A. Dowlati
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. De, D.J. Lindner, A. Dowlati, G.R. Stark
Writing, review, and/or revision of the manuscript: S. De, D.J. Lindner, C. Coleman, G. Wildey, A. Dowlati, G.R. Stark
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. De, C. Coleman
Study supervision: S. De, G.R. Stark

Acknowledgments
This research was supported by a VeloSano Pilot grant from Cleveland Clinic (to G.R. Stark). We are extremely thankful to Drs. Andrei Gudkov and Andrei A. Purmal for providing CBL0137. We thank the Cleveland Clinic Flow Cytometry Core for excellent technical support. We would also like to thank Ms. Yvonne Parker for technical support and Dr. Katerina V. Garova for helpful comments.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 30, 2017; revised December 5, 2017; accepted February 9, 2018; published first February 13, 2018.

References
2406 Cancer Res; 78(9) May 1, 2018 Cancer Research


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

Sarmishtha De, Daniel J. Lindner, Claire J. Coleman, et al.

Cancer Res 2018;78:2396-2406. Published OnlineFirst February 13, 2018.