Targeting Transcriptional Addictions in Small Cell Lung Cancer with a Covalent CDK7 Inhibitor

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SUMMARY

Small cell lung cancer (SCLC) is an aggressive disease with high mortality, and the identification of effective pharmacological strategies to target SCLC biology represents an urgent need. Using a high-throughput cellular screen of a diverse chemical library, we observe that SCLC is sensitive to transcription-targeting drugs, in particular to THZ1, a recently identified covalent inhibitor of cyclin-dependent kinase 7. We find that expression of super-enhancer-associated transcription factor genes, including MYC family proto-oncogenes and neuroendocrine lineage-specific factors, is highly vulnerability to THZ1 treatment. We propose that downregulation of these transcription factors contributes, in part, to SCLC sensitivity to transcriptional inhibitors and that THZ1 represents a prototype drug for tailored SCLC therapy.

INTRODUCTION

Lung cancer accounts for nearly 30% of all cancer-related deaths. In 10% to 15% of lung cancer cases, patients are diagnosed with small cell lung cancer (SCLC), the most malignant subtype of lung cancer, characterized by aggressive growth, early onset of metastasis, and rapid development of chemorefractory disease. The 5 year survival rate for SCLC patients is less than 5%, a poor prognosis that has not changed in four decades, because of the lack of advances in SCLC therapeutics (Sato et al., 2007; William and Glisson, 2011). In contrast to non-small cell lung cancer (NSCLC), for which therapeutics designed to target known oncogenic drivers such as EGFR and ALK have been extremely effective, the poor understanding of SCLC disease etiology has precluded the identification of therapeutic targets and effective treatments (William and Glisson, 2011). Recent efforts to collect and sequence SCLC tissues have revealed that these tumors display a strikingly high rate of protein-changing mutations (Pfeifer et al.,...
SCLC cells (as well as PNECs) exhibit high, sustained expression of many neuroendocrine genes, in particular transcription factors that regulate neuroendocrine development and differentiation in various tissues (Pedersen et al., 2003; Reynolds et al., 2000; Travis, 2009). Achaete-scute homolog 1 (ASCL1) is a master regulator of neuroendocrine differentiation in lung development (Borges et al., 1997; Ito et al., 2000) and has been shown to regulate tumor-initiating capacity and survival pathways in SCLC (Jiang et al., 2009; Osada et al., 2005), hence underscoring the interplay between neuroendocrine signaling and SCLC pathogenesis. Furthermore, the lineage-specific transcription factor NEUROD1 has been reported to govern survival pathways in SCLC cells (Osborne et al., 2013). Further, SCLC cells exhibit various chromosomal and focal amplifications leading to increased target gene expression and possible gain of function. Fifty to eighty percent of SCLC tumors exhibit mutually exclusive increased target gene expression and possible gain of function, various chromosomal and focal amplifications leading to increased target gene expression and possible gain of function.

2012; Rudin et al., 2012); however, paradoxically, no targetable mutations have been identified to guide therapeutic decisions for SCLC, and efficient treatment paradigms are urgently needed.

SCLC is defined by the near ubiquitous inactivation of both PS3 and RB (Peifer et al., 2012; Rudin et al., 2012; Sato et al., 2007); however, recent reports indicate that the cell of origin is equally important for the development of SCLC disease. Conditional inactivation of ps3 and Rb in the adult mouse lung, using a genetically engineered mouse (GEM) model, is sufficient to develop murine SCLC (mSCLC) resembling human disease (Meuwissen et al., 2003). Importantly, though, SCLC is firmly established only if PS3 and RB are inactivated in the small population of pulmonary neuroendocrine cells (PNECs) (Sutherland et al., 2011). In contrast, PS3 and RB loss confined to the abundant nonneuroendocrine cell population in the murine lungs causes low penetrance of SCLC and a significant increase in disease latency (Park et al., 2011; Sutherland et al., 2011). Thus, the PNEC is the major cell of origin of SCLC, suggesting that neuroendocrine pathways collaborate with PS3 and RB loss to initiate and drive SCLC tumorigenesis.

SCLC cells (as well as PNECs) exhibit high, sustained expression of many neuroendocrine genes, in particular transcription factors that regulate neuroendocrine development and differentiation in various tissues (Pedersen et al., 2003; Reynolds et al., 2000; Travis, 2009). Achaete-scute homolog 1 (ASCL1) is a master regulator of neuroendocrine differentiation in lung development (Borges et al., 1997; Ito et al., 2000) and has been shown to regulate tumor-initiating capacity and survival pathways in SCLC (Jiang et al., 2009; Osada et al., 2005), hence underscoring the interplay between neuroendocrine signaling and SCLC pathogenesis. Furthermore, the lineage-specific transcription factor NEUROD1 has been reported to govern survival pathways in SCLC cells (Osborne et al., 2013). Further, SCLC cells exhibit various chromosomal and focal amplifications leading to increased target gene expression and possible gain of function. Fifty to eighty percent of SCLC tumors exhibit mutually exclusive amplification of the proto-oncogene MYC, MYCN, or MYCL (Brennan et al., 1991; Huijbers et al., 2014; Johnson et al., 1987; Kim et al., 2006; Peifer et al., 2012; Rudin et al., 2012; Voortman et al., 2010). MYC is misregulated in the majority of human cancers, leading to uncontrolled proliferation possible through augmentation of existing gene expression programs (Lin et al., 2012). In contrast to MYC, MYCN and MYCL misregulation occurs only in high-risk cancers of neuroendocrine origin, such as SCLC (Huijbers et al., 2014; Johnson et al., 1987; McFadden et al., 2014), neuroblastoma (MYCN) (Huang and Weiss, 2013), and medulloblastoma (MYCN, MYCL) (Northcott et al., 2012). Moreover, many SCLC tumors have focal amplifications or increased expression of SOX-family genes (Voortman et al., 2010), including SOX2, which is critical for lung development and self-renewal. SOX2 amplification and increased expression levels in tumors correlate with accelerated disease stage, and silencing of SOX2 inhibits growth of SCLC cells (Rudin et al., 2012).

Thus, misregulated and amplified lineage-specific and proto-oncogenic transcription factors appear to govern SCLC initiation and disease evolution, and downregulation of such factors could form the basis for SCLC targeted therapy.

Using an unbiased small-molecule screen approach, we indeed observed that SCLC is highly sensitive to transcription-targeting drugs and in particular to a covalent inhibitor of cyclin-dependent kinase 7 (CDK7), THZ1, which can drastically reduce RNAPII-mediated gene transcription (Kwiatkowski et al., 2014). Here, we sought to investigate the therapeutic potential of THZ1 in SCLC preclinical models and further use THZ1 as a chemical tool to dissect the malignant transcriptional programs driving SCLC state.

RESULTS

High-Throughput Small-Molecule Drug Screen Identifies THZ1 as a Highly Potent Inhibitor of SCLC Viability

To identify small molecules that suppress SCLC cell growth, we performed an unbiased high-throughput screen in SCLC cell lines using a library of greater than 1,000 annotated small-molecule inhibitors composed of both experimental compounds and early or advanced stage clinical candidates. To minimize artifacts that may arise from long-term culture of SCLC cell lines, we used low-passage (passage 5–10), primary mSCLC cell lines, established from mSCLC tumors isolated from the GEM model of SCLC consisting of the Rb<sup>L/L</sup>·p53<sup>L/L</sup> (RP) allelic genotype (Meuwissen et al., 2003) (Figure 1A). To account for tumor heterogeneity, a total of three mSCLC cell lines (termed mSCLC1, mSCLC2, and mSCLC3), each from a different tumor-bearing RP mouse, were used to establish cell lines for the screen (Figure 1A). Importantly, all established mSCLC cell lines displayed morphology that is characteristic of SCLC by growing as suspension cells in floating aggregates and spheres (Figure 1A). Furthermore, the established mSCLC cells expressed classical SCLC-specific (neuroendocrine) markers (Figure S1A available online). Using a cell viability assay, compounds were initially screened at a single dose on the mSCLC cells. Forty-five compounds elicited greater than 50% reduction in growth and were triaged for further inspection (Figure 1B). Fifteen compounds resulted in a mean antiproliferation half maximal inhibitory concentration (IC<sub>50</sub>) across three SCLC cell lines of less than 300 nM (Figure 1C).

The top-ranked inhibitors fell broadly into three major putative categories of inhibitors of (1) transcription (THZ1, flavopiridol, CGP60474, JQ1, NVP-AUY922, 17-DMAG, and BI2536), (2) cell cycle function (BI2536, CGP60474, AZD7762, ON-01910, PF477736, XMD16-144, and THZ1), and (3) the mTOR-Pi3K pathway (GSK2126458, PIK75, and Torin 2) (Figures 1C and 1D).

SCLC cell lines demonstrate particular sensitivity to transcriptional inhibition with the top 3 scoring small-molecule inhibitors from the screen (NVP-AUY922, BI2536, and THZ1) targeting proteins involved in transcriptional regulation. NVP-AUY922 targets HSP90, which has recently been shown to be involved in regulating RNAPII pausing and transcriptional activation (Sawarkar et al., 2012). BI-2536, known as a Polo-like 1 (PLK1) kinase inhibitor, has recently been shown to cross-react with bromodomain (BRD) 4, a general coactivator of gene expression (Ember et al., 2014). The BRD inhibitor JQ1 (Filipakopoulos et al., 2010) also ranked within the top 15 inhibitors (Figure 1C). Disappointingly, however, despite displaying efficacy in tumor cell lines, clinical trials with both HSP90 inhibitors and PLK1 inhibitors have in general fared poorly. As a result, we focused much of our attention on THZ1, a recently identified covalent CDK7 inhibitor with additional cross-reactivity against CDK12/13. THZ1 has recently
been demonstrated to decrease T cell acute lymphoblastic leukemia viability, in part, through downregulation of key oncogenic transcription factors (Kwiatkowski et al., 2014). We speculated that the high ranking of THZ1 in the drug screen could indicate that SCLC exhibits similar dependencies for the expression of oncogenic transcription factors.

We compared the observed effects of THZ1 in mSCLC cells with that of mutant Kras-driven murine NSCLC (mNSCLC) cell lines (Liu et al., 2013). THZ1 displayed an IC50 value of 75 to 100 nM against mSCLC cells, while mNSCLC cells had a higher IC50 of about 750 nM (Figure 1E). These data suggest that lung cancer types addicted to mutated kinases are relatively less vulnerable to transcription-targeting drugs than SCLC. When testing several commercially available pan-CDK inhibitors targeting one or more of the higher order transcriptional CDKs such as CDK7, CDK8, and CDK9, we found that apart from flavopiridol and dinaciclib, all compounds had an IC50 exceeding 500 or 1,000 nM (Figures S1B–S1D). Dinaciclib and flavopiridol showed potency at a nanomolar scale similar to that of THZ1 but did not exhibit SCLC specificity, as Kras-driven NSCLC cell

![Figure 1. High-Throughput Drug Screen in Primary mSCLC Cell Lines Identifies THZ1 as a Highly Potent Inhibitor of SCLC Cell Viability](image-url)
Figure 2. THZ1 Treatment of RP Mice Causes Significant Tumor Response and Increased Survival

(A) Schematic overview of treatment trial with vehicle (control), THZ1, or Cis-Eto compared with vehicle (control) in RP mice with confirmed (by MRI) SCLC disease. THZ1 is dosed at 10 mg/kg twice daily, and Cis-Eto is dosed as follows: cisplatin 5 mg/kg once per week, etoposide 10 mg/kg three times per week. Cis-Eto is given 1 to 2 weeks on (pending weight and toxicity) and thereafter 2 to 3 weeks off, followed by 1 to 2 weeks retreatment (pending weight, toxicity, and tumor burden according to MRI). After treatment start, MRI was performed biweekly in all treatment cohorts.

(B) Tumor volume change % (normalized to pre-treatment volume) over 2 weeks and 4 weeks. The results show significant differences in tumor volume change between the treatment groups.

(C) Graph showing the percentage of cohort with different responses: Progressive disease (PD), Stable disease (SD), Partial response (PR), and Toxicity. THZ1 and Cis-Eto groups have a higher percentage of PR compared to vehicle.

(D) Kaplan-Meier survival analysis showing the percentage of cohort surviving from confirmed MRI disease. THZ1 and Cis-Eto groups have a higher percentage of survival compared to vehicle.

(F) Images showing pre-treatment and 2 weeks post-treatment of heart and tumor sections for vehicle, THZ1, and Cis-Eto groups.

(G) Images showing TUNEL staining of liver and lung tumors for vehicle and THZ1 groups.

(H) Graph showing the mean IC50 (nM) of THZ1 for primary and metastatic tumors. THZ1 has a lower IC50 for both primary and metastatic tumors compared to vehicle.

(I) Scatter plot showing the mean IC50 (nM) of etoposide (µM Cisplatin) for chemotheraputically naive and chemotheraputically treated tumors. THZ1 has a lower IC50 for chemotheraputically naive tumors compared to chemotheraputically treated tumors.

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lines were equally sensitive to compounds (Figures S1B and S1C). As previously observed (Kwiatkowski et al., 2014), THZ1 facilitated irreversible (covalent) binding to CDK7 (Figure S1E), and a noncovalent version of THZ1 (THZ1-R) was significantly less potent (Figure S1F). Hence, THZ1 potently and selectively inhibits SCLC cell viability, and its induced cellular effects result, in part, from its irreversible covalent inhibition of CDK7. Both flavopiridol and THZ1 have been shown to cross-react with CDK12/13 at higher concentrations (Böskens et al., 2014; Kwiatkowski et al., 2014); therefore, this shared pharmacology may also affect the transcriptional and phenotypic response of SCLC to these inhibitors.

Mice with Aggressive Autochthonous SCLC Disease Show Significant Tumor Response following THZ1 Treatment

To establish the translational significance of the observed potency of THZ1 in mSCLC cells, we tested THZ1 in vivo in the RP GEM model. This autochthonous mouse model offers a pre-clinical platform with remarkable resemblance to the human SCLC (hSCLC) disease (Meuwissen et al., 2003). The concomitant loss of p53 and Rb function in the adult murine lung tissue allows for a stochastic and heterogeneous disease course with aggressively growing primary lung tumor and metastasis. Disease course in RP mice was followed by MRI of the thorax region, and upon detectable lung tumor burden, mice were started on treatment with either THZ1 or vehicle. We additionally treated a cohort of mice with cisplatin and etoposide (Cis-Eto) to allow comparison with the standard-of-care chemotherapeutic regimen used in the clinic for SCLC patients. MRI was performed every 2 weeks after treatment began in the three different treatment cohorts (Figures 2A–2F). All vehicle-treated mice showed progressive disease (PD; a more than 20% increase in tumor volume compared with baseline), with mean tumor volume doubling after a 2-week period (Figures 2B and 2F). THZ1-treated mice had significant tumor response compared with control at the 2-week time point (Figures 2B, 2C, 2F, and S2A). Three of nine THZ1-treated mice showed partial response (PR; a more than 30% tumor volume reduction compared with baseline), and two of those had near complete response, with more than 90% reduction in tumor burden. Four THZ1-treated mice had stable disease (SD; neither a reduction nor an increase in tumor volume to classify as PR or PD), while two THZ1-treated mice had PD. Further, the majority of THZ1-treated mice with SD or PR at the 2-week time point had similar disease responses at the 4-week time point (Figures 2B, 2C, 2F, and S2A). In contrast, most vehicle-treated mice succumbed to their tumor burden before the 4-week time point (four of six mice), emphasizing the aggressive disease course in the SCLC GEM model (Figures 2B and 2E). Overall, THZ1-treated mice had longer survival compared with control-treated mice (p = 0.0011) (Figure 2E). Furthermore, we observed that response rates and survival obtained from single-agent THZ1 treatment were comparable with tumor response rates upon standard-of-care chemotherapy (Figures 2B–2F). However, THZ1 treatment did not result in treatment-related toxicity in RP mice in contrast to Cis-Eto treatment (Figure 2D). The lack of observed toxicity in THZ1-treated mice suggests that THZ1 treatment allows a broader therapeutic window in cancer versus normal cells compared with chemotherapy.

To investigate whether single-agent activity of THZ1 or Cis-Eto could be augmented by a combined treatment regimen, we treated an additional cohort of RP mice with THZ1 combined with Cis-Eto. Our results indicate that mice treated with THZ1 and Cis-Eto do not experience superior tumor response at the 4-week time point compared with mice receiving THZ1 or Cis-Eto alone (Figures S2B–S2E). Of note, we did not observe increased toxicity in mice treated with THZ1 and Cis-Eto compared with Cis-Eto treatment alone (Figure S2C).

THZ1 treatment resulted in decreased RNA Pol II CTD phosphorylation in SCLC tumor tissue compared with vehicle-treated mice (Figure S2F), consistent with THZ1 binding and inhibition of CDK7 in tumor tissue. Tissue from both lung tumors (n = 3) and liver metastases (n = 1) isolated from THZ1-treated mice had significantly more apoptotic cells than vehicle-treated tumor tissue as measured by TUNEL staining (Figures 2G and S2G). Additionally, mSCLC cells established from SCLC lung tumors and from liver metastases showed equal sensitivity to THZ1, indicating that both primary and metastatic SCLC lesions are responsive to THZ1 treatment (Figure 2H). Importantly, we found that mSCLC cells established from tumors from chemotreated (resistant) SCLC RP mice had similar sensitivity to THZ1 compared with mSCLC cell lines established from untreated
(chemonaive) RP mice (Figure 2I). Thus, these data suggest that both chemonaive and chemorefractory SCLC disease is sensitive to THZ1 treatment.

**Cellular and Tumor Models of hSCLC with Distinct Genotypes and Phenotypes Are Highly Sensitive to THZ1**

To further confirm THZ1 as a drug candidate for patient disease, we investigated THZ1 potency in a panel of genotypically-distinct hSCLC cell lines. hSCLC cell lines are known to harbor a complex genome with a high mutation rate, frequent copy number gains and losses, and translocations (Peifer et al., 2012; Pleasance et al., 2010; Rudin et al., 2012). The major identified alterations in the hSCLC cell lines, apart from the mutations in *PS3* and *RB*, include amplification of *MYC, MYCL*, or *MYCN* (Johnson et al., 1987). We used a panel of hSCLC cell lines harboring amplifications in each of the MYC genes. Additionally, we investigated SCLC tumor cells from chemonaive and chemorefractory treated patients as well as those isolated from primary lung sites and from metastatic lesions (Figure 3A). All hSCLC cell lines exhibited high sensitivity to THZ1, with IC50 values in the range of 5 to 20 nM (Figure 3A [all], Figure 3B, top [representative]). The NCI-H69, GLC16, and NCI-H82 cell lines established from chemorefractory patients were less sensitive to Cis-Eto treatment than the chemonaive cell line NCI-H209 (Figure 3B, bottom). THZ1 treatment of tumor xenografts established from NCI-H69 and GLC16 cells resulted in significant tumor growth reduction compared with control-treated tumors, indicating that chemorefractory disease is sensitive to THZ1 treatment (Figure 3C). As in GEM mice, no overt toxicity was observed from THZ1 treatment (Figure 3C, insets). In contrast to hSCLC cells, immortalized tracheobronchial epithelial (hTBE) cells showed very little sensitivity to THZ1 but similar sensitivity to Cis-Eto (Figure 3B), reinforcing previous observations that THZ1 treatment confers a superior therapeutic window between cancer and noncancer cells compared with chemotherapy.

Substantial induction of apoptosis was observed in hSCLC cells as early as 6 hr after exposure to THZ1 (Figure 3D), and inhibition of proliferation occurred at 24 and 48 hr after exposure (Figure 3E). The appearance of apoptotic markers appeared concomitant with the decrease of RNAPII CTD phosphorylation (Figures 3D and 3F). In line with these results, we observed that the classical sphere-forming morphology of SCLC cells was dramatically disrupted after only 12 hr of exposure to THZ1 in 3D culture (Figure S3A). Similar differential sensitivity was observed between hSCLC and hNSCLC cells (Figures S3B–S3E), as previously noted in the murine cellular lung cancer models (Figure 1E). As observed in the mSCLC cell lines, most pan-CDK inhibitors tested showed either less potency in hSCLC cells or less SCLC specificity (Figures S3C–S3E). Furthermore, we observed significantly lower potency of THZ1-R compared with THZ1, again confirming that the covalent mechanism of action of THZ1 is critical for drug potency (Figure S3F–S3G).

**THZ1 Confers Preferential Repression of Transcription-Regulating Genes in SCLC Cells**

Intrigued by the significant therapeutic response in various preclinical models of SCLC disease, we aimed to understand the mechanisms governing SCLC sensitivity to THZ1. CDK7 is a component of the general transcription factor IIH, which activates RNAPII-mediated transcription. Consistent with this notion, high-dose treatment with THZ1 in Jurkat cells was previously shown to cause pronounced global downregulation of gene expression; however, low-dose THZ1 produced gene-selective effects on the Jurkat interconnected transcriptional core regulatory circuitry (Kwiatkowski et al., 2014). To investigate THZ1-induced transcriptional effects in SCLC, we performed gene expression profiling in selected hSCLC cell lines (NCI-H69, GLC16, and NCI-H82), following treatment with 25 and 100 nM THZ1, which are concentrations at which limited and substantial inhibition of RNAPII phosphorylation is achieved, respectively (Figure 3F). Across SCLC cell lines, treatment with THZ1 led to a reduction in steady-state mRNA levels with 0.2% to 5% and 13% to 27% of mRNAs showing greater than 2-fold reduction at 25 and 100 nM, respectively (Figures 4A and 4B; Tables S1 and S2). Gene ontology (GO) analysis revealed a significant enrichment of genes involved in transcription among the top 5% of downregulated genes after 100 nM THZ1 treatment (Figure 4C). In particular the major part of THZ1-sensitive transcripts is associated with DNA-dependent transcription, indicating transcription factor function (Figure 4C). Moreover, gene set enrichment analysis of transcription factor binding sites showed that genes containing binding motifs for transcription factors such as E2F, NRF1, and CREB were preferentially affected by THZ1 treatment (Figure S4A). In particular, we observed that short-lived transcripts of the E2F family were heavily downregulated and that E2F-regulated gene sets were significantly enriched in THZ1-downregulated transcripts (Figures S4B and S4C). Interestingly, E2F transcription factor binding sites are significantly enriched in the SCLC cells compared with the NSCLC cells (Figures S4D and S4E), suggesting that E2F transcription factors have a more prominent role in SCLC biology.

**Proto-Oncogenic and Lineage-Specific Transcription Factors Are Associated with Super-Enhancers in SCLC**

Little is known about the transcription factors and cofactors that govern the pathogenesis of SCLC. Genes encoding important regulators of cell state, including key oncogenic transcription factors, were recently shown to be preferentially associated with unusually large transcriptional regulatory domains known as super-enhancers (Hnisz et al., 2013; Lovén et al., 2013). Furthermore, previous work has demonstrated that expression of super-enhancer-associated genes can be especially vulnerable to transcriptional inhibitors (Chapuy et al., 2013; Hnisz et al., 2013; Kwiatkowski et al., 2014; Lovén et al., 2013; Whyte et al., 2013). Therefore, the identification of super-enhancers in SCLC may enumerate candidate SCLC oncogenes and focus our search for THZ1-responsive transcripts whose downregulation lead to loss of SCLC cell state.

To identify super-enhancers in SCLC cells, we performed chromatin immunoprecipitation sequencing (ChIP-seq) against the lysine 27 acetylated form of histone H3 (H3K27Ac) in untreated hSCLC cells. H3K27Ac defines active enhancers and has been used previously to determine the positions and sizes of super-enhancers (Hnisz et al., 2013). Using this approach, we identified 130 super-enhancer-associated genes in NCI-H69, 103 in GLC16, and 70 in NCI-H82 (Figures 5A–5D; Table S3).

We found that many super-enhancer-associated genes encode gene products involved in regulation of RNAPII-mediated...
transcription (Figures 5A and 5B). This indicates that many transcription factors are important to SCLC state and might represent candidate oncogenes. The super-enhancer landscape recapitulated the embryonic and neural signature common to SCLC cells. Many super-enhancers were found to be associated with genes encoding lineage-specific transcription factors that are central regulators of embryogenesis and neural development (Figures 5A and 5B). Consistent with previous investigations observing differential gene expression profiles between the so-called classical and variant neuroendocrine SCLC cells, THZ1 was tested in a panel of SCLC cell lines and xenografts. THZ1 is a potent inhibitor of cell viability and tumor growth in a panel of genotypically and phenotypically distinct SCLC cell lines and xenografts. The IC50 values for THZ1 in the panel of SCLC cell lines are presented as mean with confidence interval (C.I.). Phenotype profile information on cell lines comprises the following: origin—organ of origin of tissue specimen from which cell line was established; chemotherapy—whether patients had received chemotherapy before cell line establishment (yes or no). Genetic profile information on cell lines includes P53 and RB mutations (both leading to loss of function) and MYC gene family amplification (C-MYC, MYCN, or MYCL). Asterisk indicates that cell lines have previously been classified as “variant” neuroendocrine SCLC cell line on the basis of the lack of expression of “classical” neuroendocrine genes such as ASCL1, NCAM, and SCG2.

Figure 3. THZ1 Is a Potent Inhibitor of Cell Viability and Tumor Growth in a Panel of Genotypically and Phenotypically Distinct SCLC Cell Lines and Xenografts

(A) Summary of THZ1 IC50 values in the full panel of SCLC cell lines with genotype and phenotype status. IC50 data are presented as mean with confidence interval (CI). Phenotype profile information on cell lines comprises the following: origin—organ of origin of tissue specimen from which cell line was established; chemotherapy—whether patients had received chemotherapy before cell line establishment (yes or no). Genetic profile information on cell lines includes P53 and RB mutations (both leading to loss of function) and MYC gene family amplification (C-MYC, MYCN, or MYCL). Asterisk indicates that cell lines have previously been classified as “variant” neuroendocrine SCLC cell line on the basis of the lack of expression of “classical” neuroendocrine genes such as ASCL1, NCAM, and SCG2.

(B) Representative hSCLC cell lines (from A) and the hTBE cell line were exposed to increasing doses of THZ1 (top) or Cis-Eto (bottom), and IC50 values were determined by cell viability assay. Each data point is shown as mean ± SEM. Mean IC50 values to Cis-Eto treatment are reported next to cell line name (bottom).

(C) Tumor volume index of hSCLC xenografts grown on nude mice (NCI-H69, left; GLC16, right) as normalized to pretreatment volume (day 0). Mice were treated with vehicle or THZ1. THZ1 was dosed at 10 mg/kg twice daily and vehicle twice daily. Insets show weights from mice on treatment.

(D) Apoptosis assay measuring caspase-3/7 activity in the SCLC cell line NCI-H69 after THZ1 treatment for indicated time points.

(E) Proliferation assay measuring BRDU incorporation in SCLC cell line NCI-H69 after THZ1 treatment for indicated time points.

(F) Western blot detecting RNAPII, CTD phosphorylation (SER-2, SER-5, and SER-7), and CDK7 along with the apoptotic markers caspase-3 and PARP in total protein lysates from NCI-H69 cell lines exposed to indicated doses of THZ1. ACTIN serves as a loading control. See also Figure S3.
variant SCLC cell lines (Johnson et al., 1987; Pedersen et al., 2003), we found that the “classical” GLC16 and NCI-H69 cell lines had significant overlap in identified super-enhancer genes, while the “variant” NCI-H82 cell line had a super-enhancer profile distinct from that of the classical cell lines (Figures 5B and 5C).

In both NCI-H69 and GLC16 cell lines, we identified super-enhancers associated with SOX2 and NFIB (Figures 5A and 5D), consistent with their proposed role as proto-oncogenic transcription factors in SCLC (Dooley et al., 2011; Rudin et al., 2012). Similarly, the neuroendocrine master transcription factor gene ASCL1 (Borges et al., 1997; Ito et al., 2000) was associated with one of the largest typical enhancers (TEs) (Figures 5A and 5D; Table S2). Moreover, we identified many super-enhancer-associated transcription factor genes in GLC16 and NCI-H69 whose functions were not previously described in the context of SCLC biology. Among them was Insulinoma-associated 1 (INSM1) (Figures 5A and 5D; Table S3), whose expression represents a highly predictive marker of SCLC disease (Figure S5) (Christensen et al., 2010; Lan and Breslin, 2009; Pedersen et al., 2003). In the NCI-H82 cell line, we identified a super-enhancer at the OTX2 gene, which encodes a transcription factor involved in neural differentiation and development. Similar to INSM1, the functional role of OTX2 in SCLC biology is unknown.

Super-enhancers were found to be associated with genes that had focal amplification. The C-MYC amplified cell lines GLC16 and NCI-H82 and the MYCN amplified cell line NCI-H69 (Figure 3A) had super-enhancers associated with C-MYC and MYCN gene loci, respectively (Figures 5A and 5D). In the NCI-H69 cell lines, the MYCN super-enhancer ranked second largest, and in NCI-H82, the two largest super-enhancers were both found in the C-MYC gene loci (Table S3). Interestingly, we also
Figure 5. Super-Enhancers in hSCLC Cell Lines Are Associated with Proto-Oncogenic and Lineage-Specific Transcription Factor Genes

(A) Distribution of H3K27Ac signal at enhancers. Enhancer regions are plotted in increasing order on the basis of their input-normalized H3K27Ac signal (length x density) in NCI-H69, GLC16, and NCI-H82 cell lines.

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found evidence for focal amplification of OTX2 in the NCI-H82 cell line (Figure 5D).

**Super-Enhancer-Associated Genes Are Disproportionately Vulnerable to THZ1 Treatment**

Next we aimed to investigate whether THZ1 conferred preferential downregulation of super-enhancer-associated genes in SCLC (Figures 6A–6C). Enrichment analysis of THZ1-sensitive transcripts (Figure 4; Table S2) and super-enhancer-associated genes (Table S3) showed that THZ1-targeted transcripts are enriched in the gene sets with associated super-enhancers (false discovery rate < 0.05) (Figure 6A; Table S4). Moreover, mean transcript abundance associated with super-enhancers was disproportionately reduced upon THZ1 exposure compared with that of TEs (Figure 6B). This disproportionate reduction was observed even at the low dose (25 nM) of THZ1, which produces limited repression of RNAPII-mediated transcription (Figure 3F). In addition, genes associated with the top-ranked (largest) TE (TOPTE) were more sensitive to THZ1 treatment than genes associated with a general TE (Figure 6B), emphasizing that enhancer size is positively correlated with THZ1 vulnerability.

In order to focus our search for SCLC oncogenic transcription factors, we identified a leading-edge gene set from our enrichment analysis (Figure 6A), consisting of super-enhancer-associated core enrichment genes that were downregulated by THZ1 (Figure 6C). Because oncogenic transcription factors are often highly expressed, we further filtered our leading-edge gene set (Figure 6C) to contain the top 5% actively expressed genes (Figure 6D). Confirmative of this scheme, the MYC-family of proto-oncogenes placed among the leading-edge gene set and was among the top 5% most actively expressed genes in the analyzed hSCLC cells (Figure 6D). Using this approach, we further identified SOX2, SOX4, INSM1 (NCI-H69, GLC16), and OTX2 (NCI-H82). ASCL1 (NCI-H69, GLC16) and NEUROD1 (NCI-H82) both associated with a TOPTTE but were both highly reduced in THZ1-treated cells and ranked among the top 5% actively expressed genes (Figures 6C and 6D). The downregulation of the transcription factor genes was confirmed on the RNA level by quantitative PCR (Figure 6E), and selected transcripts further confirmed on the protein level (Figure 6F). The reduced transcript level of SCLC-specific transcription factors observed in vitro was furthermore confirmed in vivo in THZ1-treated RP mice (Figure S6).

**DISCUSSION**

Here, we addressed a high therapeutic need by identifying THZ1 (Kwiatkowski et al., 2014) as a promising drug candidate for the treatment of SCLC. Although recent sequencing efforts revealed that SCLC tumors contain a high rate of protein-altering mutations, the mapping of the mutational landscape did not guide in therapeutic decisions for this fatal disease (Peifer et al., 2012; Rudin et al., 2012). Using an unbiased small-molecule screen approach, we observed that SCLC cells were highly vulnerable to perturbation of the transcriptional state as several transcriptional inhibitors were potent inhibitors of SCLC cell viability. The high potency of THZ1 in SCLC cells was further confirmed in vivo in the RP GEM mice model, which represents a highly translational preclinical platform for the investigation of SCLC therapeutics (Meuwissen et al., 2003). The RP GEM model mimics the stochastic and heterogeneous pathogenesis in human disease, and mSCLC tumors have been shown to accumulate additional alterations, as observed in human disease, such as amplification of MYCL (McFadden et al., 2014; Peifer et al., 2012). We observed significant tumor responses in SCLC GEM mice treated with THZ1 compared with control-treated mice and further noted that standard-of-care chemotherapy caused similar response rates in SCLC GEM mice. However, although chemotherapy was associated with toxicity, THZ1 treatment did not cause any overt toxicity in treated mice. Furthermore, our treatment data in both murine and human preclinical models of SCLC indicate that both metastatic and chemoresistant SCLC disease are sensitive to THZ1 treatment.

The encouraging preclinical results led us to investigate the transcriptional addictions of SCLC. Because of the preponderance of misregulated transcription factors in SCLC (Dooley et al., 2011; Jiang et al., 2009; Johnson et al., 1987; Osborne et al., 2013; Pedersen et al., 2003; Rudin et al., 2012; Voortman et al., 2010), we aimed to identify a candidate list of transcription factor genes associated with super-enhancers (Chapuy et al., 2013; Hnisz et al., 2013; Kwiatkowski et al., 2014; Lovén et al., 2013; Whyte et al., 2013) and whose high-level expression was particularly vulnerable to THZ1 treatment. We hypothesized that such a list would represent candidate oncogenic transcription factors in SCLC disease.

Confirmative of this scheme, we identified that the focally amplified C-MYC (in GLC16 and NCI-H82 cells) and MYCN (in NCI-H69 cells) proto-oncogenes (Brennan et al., 1991; Voortman et al., 2010) were associated with large super-enhancers and were highly vulnerable to THZ1. We did not observe differential sensitivity to THZ1 between SCLC cells with genomic amplification of MYCN, C-MYC, or MYCL and cell lines without amplification, therefore suggesting that MYC amplification is not a biomarker per se for THZ1-sensitive SCLC. Because focal amplification of MYC gene members is associated with later stage disease and, possibly, chemoresistant disease (Brennan et al., 1991; Johnson et al., 1987), these data further support that THZ1 treatment will be effective in progressive SCLC disease stages.

In contrast to MYC amplifications, the transcriptional-amplified neuroendocrine gene program is an early and possible contributing event of SCLC disease, in which PNECs are believed to be the cell of origin (Park et al., 2011; Sutherland et al., 1987).
Targeting Transcriptional Addictions in SCLC

A

GLC16 Super-enhancers

NCI-H69 Super-enhancers

NCI-H82 Super-enhancers

THZ1 versus DMSO

Log2-fold change in gene expression

25 nM

100 nM

B

C

THZ1:

SE:

TOP TE:

NCI-H69

GLC16

NCI-H82

D

LEADING-EDGE GENE SET (PANEL C) ■ TOP 5 %

NCI-H69

INSM1

SOX2

MYCN

TCF4

ASCL1

NCI-H82

GLC16

INSM1

SOX2

SOX4

MYC

ISL1

OTX2

NCI-H82

GLC16

INSM1

SOX2

SOX4

MYC

ISL1

OTX2

NEUROD1

E

Relative expression (normalized to INSC)

F

THZ1 (nM) - 25 100 - 25 100 - 25 100

INSM1

ASCL1

MYCN

ACTIN

NCI-H69

NCI-H82

GLC16

THZ1 (nM) - 25 100 - 25 100

INSM1

ASCL1

C-MYC

ACTIN

NCI-H69

NCI-H82

GLC16
et al., 2011). The PNECs are abundant in the developing lung, where they play a role in lung branching morphogenesis and oxygen sensing. The purpose and function of the small population (<0.5%) of PNECs maintained in the adult lung epithelium is not known (Reynolds et al., 2000; Travis, 2009). Although the neuroendocrine signature of SCLC has been a central diagnostic tool, only a few functional studies have been performed to explore if and how the malignant neuroendocrine factors contribute to SCLC disease (Ball, 2004; Lan and Breslin, 2009). We identified a TOPTE associated with ASCL1, in support of its established role as a master transcription factor in lung neuroendocrine differentiation (Borges et al., 1997) and as a proposed candidate oncogene in SCLC (Jiang et al., 2009). Our results greatly corroborate these findings as we observe that ASCL1 is among the top 2% most downregulated genes in THZ1-treated cells. We further identified several candidate oncogenic transcription factors in SCLC. One example is the neuroendocrine transcription factor INSM1, which is representative of lineage-defining traits of SCLC. INSM1 phenocopies ASCL1 function in murine neural development, but it is not known whether the same functional link exists in SCLC biology (Ball, 2004; Lan and Breslin, 2009). Future work should investigate the role of INSM1 and other transcription factors in the malignant transcriptional gene programs that drive and maintain SCLC disease.

Our chemical screen revealed that 7 of 15 compounds identified as potent inhibitors of SCLC cell viability are transcriptional inhibitors. Hence, further pharmacological studies of small molecules targeting the general transcription apparatus should be performed to advance the field of SCLC-tailored therapeutics and to uncover the transcriptional addictions of SCLC.

EXPERIMENTAL PROCEDURES

Generation of GEM Model of SCLC and mSCLC Cell Lines
Mice were bred to contain conditional p53 floxed (L) allele (Jonkers et al., 2001) and Rb floxed (L) allele (Meuwissen et al., 2003) to a final genotype of RP. All experimental mice were maintained on a mixed genetic background (C57Bl/6, Balb-c, and C129). From 6 weeks of age, mice were induced with adenovirus-Cre recombinase (Ad-Cre) by intratracheal intubation (DuPage et al., 2009) to allow cre-lox-mediated recombination of floxed p53 and Rb alleles. Mice were aged 8 to 12 months to allow SCLC disease development. The Animal Care and Use Committee of the Dana-Farber Cancer Institute approved all in vivo experiments performed in this study. mSCLC cell lines were established from RP tumors upon mechanical dissection of tissue. Cells were established and maintained in HITES medium (Carney et al., 1981) with 10% fetal bovine serum and cultured at 37°C in a humidified chamber with 5% CO2. Extracted DNA from cell lines was evaluated for p53 and Rb recombination as previously described (Jonkers et al., 2001; Meuwissen et al., 2003). Cell stocks were frozen at very low passage (p = 5) for use in screen experiments.

High-Throughput Small-Molecule Screen
Using a semiautomated platform, we tested a >1,000 small-molecule annotated library in three mSCLC cell lines (termed mSCLC1, mSCLC2, and mSCLC3). Cells were seeded in a 384-well format and treated with a concentration of 800 nM of individual compounds before evaluating cell viability after 120 hr (two doubling times) using CellTiter-Glo Luminesscent assay (Promega). Further evaluation of compounds that allowed more than 50% inhibition of cell viability was performed in a five-point 2-fold dilution series of each compound before evaluating cell viability after 120 hr using CellTiter-Glo (experimental repeat 1) or MTS-based CKK-8 assay (Dojindo) (experimental repeat 2). See Supplemental Experimental Procedures.

GEM Dosing Studies and MRI
Mice were imaged using a 7 T Biospec Bruker BioSpin with MRI protocols optimized for image resolution of pulmonary parenchyma and vessels in healthy mice. Upon determination of tumor growth, mice were randomized into treatment groups receiving THZ (Kwiatkowski et al., 2014), Cis-Eto, or vehicle. See Supplemental Experimental Procedures.

RNA Extraction and Synthetic RNA Spike-In
In short, total RNA from hSCLC cells was prepared using ERCC RNA Spike-In Mix (Ambion) as previously described (Lovén et al., 2012). See Supplemental Experimental Procedures.

Microarray Sample Preparation and Analysis
Gene expression analysis on spiked-in RNA samples was performed using Human PrimeView array (Affymetrix) as previously described (Kwiatkowski et al., 2014). See Supplemental Experimental Procedures.

Chromatin Immunoprecipitation, Sequencing, and Analysis
hSCLC cells were crosslinked and incubated with magnetic bead bound-antibody for H3K27Ac (Abcam) before sonication to achieve DNA fragment of 200 to 250 bp. Crosslinks of precipitated protein-DNA complexes were hereafter reversed, and sequencing was performed using an Illumina HiSeq 2000. See Supplemental Experimental Procedures.

Additional Procedures
A full description of methods for cellular viability, proliferation, and apoptosis; 3D matrix in vitro assay; western blotting; Bio-THZ1 pull-down, cDNA synthesis, and quantitative PCR; immunohistochemistry; xenograft dosing studies; gene set enrichment analysis; and functional gene ontology (GO) analysis is provided in Supplemental Experimental Procedures.

ACCESSION NUMBER
The Gene Expression Omnibus accession number for the microarray and ChIP-seq data reported in this paper is GSE862614.
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SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.ccell.2014.10.019.

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