Targeting histone deacetylase SIRT1 selectively eradicates EGFR TKI-resistant cancer stem cells via regulation of mitochondrial oxidative phosphorylation in lung adenocarcinoma

Abstract

Lung adenocarcinoma (LAD) is a human malignancy successfully treated with the tyrosine kinase inhibitor (TKI) gefitinib; however, the enrichment of therapy resistant cancer stem cells (CSCs) in such patients is assumed to be a source of treatment failure. Evaluation of LAD cell populations treated with the TKI inhibitor gefitinib identified unique aspects of a subpopulation of tumor cells exhibiting stemlike properties and mitochondrion-specific metabolic features along with their reliance on sirtuin 1 (SIRT1) for survival advantage. This addiction to bioenergetic metabolism in LAD treated with EGFR-targeted therapy suggests that mitochondrial targeting should be synthetically lethal using established cytotoxic therapies. Accordingly, loss of the phenotype present in resistant CSC clones either by targeting the energy metabolism with tigecycline, a mitochondrial DNA translation inhibitor, or tenovin6 (TV6), a SIRT1 inhibitor, inhibited their dependency on mitochondrial oxidative phosphorylation (mtOXPHOS) and sensitized them for a more pronounced and longlasting TKI therapeutic effect. The results specifically demonstrated that combined therapy with TV6 and gefitinib resulted in tumor regression in xenograft mouse models, whereas administration of a single agent showed no such efficacy. Importantly, combined treatment with TV6 also decreased the effective dose of gefitinib necessary for treatment response. Clinical analysis demonstrated that highprofile SIRT1 and mtOXPHOS proteins were associated with recurrence and poor prognosis in LAD patients. These observations support the CSC hypothesis for cancer relapse and advocate use of mitochondria-targeting inhibitors as part of combinatorial therapy in a variety of clinical settings, as well as for reducing frontline TKI dosage in LAD patients.

Introduction

Targeting oncogenedriven signaling pathways is a clinically validated approach for treating several devastating diseases. Gefitinib is a selective inhibitor of epidermal growth factor receptor (EGFR) mutation and constitutes a paradigm shift in lung adenocarcinoma (LAD) therapy [1].
Unfortunately, acquired resistance and early relapses, which unavoidably occur, represent a major limitation to clinical responses [2]. To explain this phenomenon, the cancer stem cell (CSC) hypothesis suggests that tumors contain a small number of tumorforming and self-renewing stem cells that are less responsive to gefitinib and other tyrosine kinase inhibitors (TKIs) and constitute a critical target population for acquisition of drug resistance [3–5]. Therefore, strategies to prevent the reemergence of CSCs have been devised to augment the impact of anticancer therapy.

The comprehensive evidence for bioenergetic metabolism reprogramming of tumor cells has received increased attention during the previous decade [6]. Studies demonstrated mitochondriaspecific oxidative features as a specific vulnerability of cells exhibiting therapy selective quiescence (TSQ), which when exploited, should be amendable to established cytoxic interventions and provide the basis for developing more effective therapies to combat lethal disease [7,8]. Minimally, this would apply to lymphomas undergoing TSQ in response to cytotoxic therapy [7], minimal residual leukemia resistant to TKIs [9], slowcycling melanoma cells selected for a JARID1Bhigh phenotype by cytotoxic agents [10], BRAF-mutant melanomas escaping therapy with BRAF inhibitors [11], and a subsystem of dormant pancreatic cancer cells surviving KRAS oncogene ablation [12]. These examples strongly suggest that reliance on mitochondrial metabolism might represent a measure of drugable susceptibility by multidrugresistant cancers. Comprehensive evidence for a mitochondrial role in LAD cells exhibiting TSQ and the genes responsible for this process is inconclusive [13,14].

The nicotinamide adenine dinucleotide (NAD)dependent histone deacetylase sirtuin 1 (SIRT1) is a nuclear protein expressed in almost all cell types and that plays a major role in stemness maintenance and TKI resistance, as well as being involved in modulating gene expression, aging, development, and many metabolic and stressresponse pathways [15–17].

The aim of this study was to elucidate the mechanism(s) involved in cellular perturbations accompanying acquired gefitinib resistance in order to provide new therapeutics to target TSQ cells and achieve therapy free remission in LAD patients. Additionally, we evaluated new possibilities of the synergism of tenovin6 (TV6) [18–20], a smallmolecule compound that inhibits SIRT1, with reduced doses of conventional cytotoxic therapies currently ineffective as monotherapies for future application in clinical settings to treat LAD.

Materials & Methods

Study participants

From 2007 to 2014, a multicenter research of lung adenocarcinoma drew subjects primarily from the Taihang Mountain region of Henan provinces, including the First Affiliated Hospital of Henan University of Science and Technology (HUST; Luoyang, Henan, China) and Anyang Tumor Hospital (ATH; Anyang, Henan, China) and Anyang Tumor Hospital (ATH; Anyang, Henan, China). Tumor specimens containing the EGFR exon 19 deletion or L858R sensitive mutation were collected, for isolation and identification of ALDH1+ ALDCs from postrelapsed LAD patients under gefitinib treatment. The aim of this study was to elucidate the mechanism(s) involved in cellular perturbations accompanying acquired gefitinib resistance in order to provide new therapeutics to target TSQ cells and achieve therapy free remission in LAD patients. Additionally, we evaluated new possibilities of the synergism of tenovin6 (TV6) [18–20], a smallmolecule compound that inhibits SIRT1, with reduced doses of conventional cytotoxic therapies currently ineffective as monotherapies for future application in clinical settings to treat LAD.

High-throughput RNA sequencing and data analysis

The constructed libraries were sequenced using an Illumina HiSeq 2500 system (Illumina). Lowquality reads, short reads, rRNAs, and reads containing primer/adaptor contamination were removed. The remaining highquality reads were mapped to a reference genome (mm10) with two mismatches using TopHat (v.2.0.9; https://ccb.jhu.edu/software/tophat/index.shtml), and results were expressed as fragments per kilobase of transcript per million mapped reads (FPKM). The genes in the libraries were produced using the Cufflinks tool (v.2.1.1; http://cole-trapnell-lab.github.io/cufflinks/) according to the reference annotation set (mm10). Fold changes for the genes in different samples were estimated based on their FPKM values, and the significance threshold was determined according to the false discovery rate (FDR). In this study, a differentially expressed gene was defined as a gene whose expression changed more than twofold among the different samples with an FDR 0.05.

Gene set enrichment analysis (GSEA)

Ranked lists of differentially expressed genes (fold change 1.5; t test p 0.05) were assessed through the GSEA desktop application using the GSEAPreranked tool (http://software.broadinstitute.org/cancer/software/genepattern/modules/docs/GSEAPreranked/1). Curated gene sets (C2) from the Molecular Signatures Database (>50; http://software.broadinstitute.org/geo/musigdb/index.jsp) were interrogated, and after filtering out genes not in the expression dataset, gene sets of <15 genes or >3000 genes were excluded from analysis. GSEA was run using 1000 gene set permutations to generate FDRs. Default settings were used for normalizing the enrichment scores (NES).

In vivo studies

We used a patientderived xenograft (PDX) model, in which 1 to 2mm fragments of tumors from postrelapsed LAD patients under gefitinib treatment were orthotopically implanted into the mammary fat pad of NOD/SCID interleukin2 receptor chaindeficient (NSG) mice. After implantation, the mice were randomly divided into four different groups (n = 10/group) and treated 14 days later for a period of 3 weeks by intraperitoneal (i.p.) injection with gefitinib (100 mg/kg, daily), TV6 (50 mg/kg, daily), both drugs combined, and vehicle only twice daily. The animals dosed according to the appropriate scheme were monitored daily for up to 3 months, and the objective response rate and survival were recorded. An additional cohort of mice (n = 3/group) was included to conduct mechanistic studies. In this cohort, the mice were sacrificed on day 40 postdrug administration, and residual tumors were surgically removed before terminal escape [tumor with partial response (PR); TV6 therapy] or complete remission [tumor with complete response (CR); combined therapy]. Resected tumors were processed for Aldefluor assay and flow cytometric analysis. Flow cytometry results related to the aldehyde dehydrogenase 1 (ALDH1)− fraction were determined, and a representative mouse from each treatment group in this separate cohort is shown. For studies on tumor rechallenge, 1 to 2mm diameter pieces of tumor taken from residual tumors surgically removed on day 25 postdrug administration were implanted into another cohort of NSG mice (n = 5/group), and the engraftments were recorded. CR was defined as complete regression of the tumor without any recurrence. Primary and secondary challenge tumor growth was followed for up to 90 and 28 days, respectively. Tumor growth was monitored by caliper measurements, and tumor volume (TV) was calculated as follows: TV (mm3) = /6 × length × width2.
Treatment with gefitinib, TV6, and their combination was conducted twice daily due to toxicity to TV6 and deviation from design of the animal study. Mice suffering from progressive disease or those used for subsequent analysis were euthanized when TV was >2500 mm³. The health status and weight of the mice was monitored daily, and mice were humanely euthanized when moribund, as defined by weight loss exceeding 10% to 15%, lethargy, ruffled fur, and/or partial or hindlimb paralysis. At the end of the measurement, mice were sacrificed, venous blood was drawn from the orbit of the mice for confirmation of drug tolerability, and serum levels of liver, muscle, or cardiac enzymes were determined.

Sixto 8-week-old male nude mice were implanted subcutaneously with a total of 5 × 10⁶ PC9 green fluorescent protein (GFP)⁺ cells, administered luciferin i.p., and imaged by noninvasive bioluminescence imaging using an IVIS Lumina system (Caliper Life Sciences, Waltham, MA, USA), all of which developed tumors within 14 days (size: 55 mm³). For each of which developed tumors within 14 days (size: 55 mm³). For each experiment, mice were randomly distributed into equal groups (n = 10 mice/group) that were either untreated or treated with i.p. injections twice daily with 25 mg/kg/day or 100 mg/kg/day gefitinib, 50 mg/kg/day TV6, or TV6-based combined therapy involving low or highdose gefitinib for 21 consecutive days. The animals dosed appropriately were monitored daily for 3 months, and the objective response rate and survival were recorded. Animals were sacrificed because of progressive disease if TV was >2500 mm³ before the next measurement. CR was defined as a complete regression of the tumor without any recurrence.

Statistical analysis

Results are presented as the mean ± standard error of the mean (SEM) or standard deviation (SD). Predefined pairwise comparisons of treated group(s) with the control group for the indicated time points were conducted. Kaplan–Meier survival analysis and multivariate Cox regression were performed using the R survival package (https://cran.r-project.org/web/packages/survival/index.html) and replotted using Graphpad Prism software (v.6.00; GraphPad Software, La Jolla, CA, USA). Log ratios, unpaired t test p values, and color-coded heat maps were obtained using MATRIX. For comparisons involving progressively resistant cell lines, analyses were performed using the R package by fitting a linear regression model to gene expression data against the logtransformed IC₅₀ values as measures of drug response. Weibull parametric survival analysis was used to test the intersection effect of each combination of drugs in groups to determine synergy, additivity, less than additivity, or antagonism of survival. Unless indicated, the mean ± SD is reported. A p < 0.05 was considered significant.

Results

Residual LAD that resist oncogene ablation exhibits CSC-like features and relies on SIRT1 for survival

To establish in vitro models of LAD exhibiting EGFR TKI resistance, we applied highly selective SIRT1 inhibitors TV6 and Selisistat (EX 527), findings that PC9 (Figure 1G) and HCC827 (Figure 1H) T20 cells showed higher sensitivity to these inhibitors as compared with parental cells. We then tested the pharmacologic sensitivity of ALDH1bri⁺ cells derived from relapsed LAD patients to SIRT1 inhibitors, and found that the IC₅₀ values for this cell population after incubation were in a lower micromolar range than their ALDH1low counterparts (Figure 1I).

We then evaluated SIRT1 expression levels in ALDH1bri⁺ cells from eight relapsed LAD patients, resistant to gefitinib, and sorted by ALDH activity. ALDH1bri⁺ cells displayed enhanced SIRT7 expression as compared with that in ALDH1low counterparts (Supplementary Figure S2B). We then queried the entire progressively resistant PC9 (Supplementary Figure S2C) and HCC827 (Supplementary Figure S2D) series and found a consistent increase in SIRT1 expression along with increasing resistance to TKI therapy.

To evaluate whether the hypersensitivity of TKI-resistant cells was specific to SIRT family inhibitors or represented a general epigenetic susceptibility, we tested other compounds inhibiting JmjC lysine demethylase specific to SIRT family inhibitors or represented a general epigenetic susceptibility. We did not observe significant differences in IC₅₀ values for any of these drugs between parental and resistant variants (Supplementary Figure S3A). These results uncovered a specific, targetable vulnerability to SIRT inhibition that can be exploited to treat LADs exhibiting resistance to TKI therapy.
LAD treated with EGFR inhibitors are addicted to mitochondrial oxidative metabolism

GSEA of the rankordered genes associated with drug sensitivity revealed prominent expression of genes governing hypoxia, autophagy, lysosome activity, and insulin secretion, as well as strong reliance on mitochondrial respiration for cellular energetics (Supplementary Table S2). Considering that malignant transformation involves cellular metabolic changes, which could in turn render the transformed cells susceptible to specific assaults in a selective manner, we searched for such vulnerabilities in LAD.

Accordingly, in resistant PC9 and HCC827 T20 cells, the basal and maximal respiratory capacities of their CSC fractions were higher than those in their parental counterparts, suggesting activated mitochondrial (mt)OXPHOS (Figure 2A). Consistent with this result, mitochondrial membrane potentials (mtMPs) were also upregulated in PC9 and HCC827 T20 cells (Figure 2B). We then measured the levels of reactive oxygen species (ROS), which can be elevated through the activation of the

Figure 1. Residual LAD resistant to EGFR ablation exhibit CSC features and rely on SIRT1 for survival. A and B, Dose-response curves for PC9 (A) and HCC827 (B) cells after long-term treatment with gefitinib. P, parental cell line; T[n], resistant variant generated after n cycles of gefitinib. Values on the X-axis indicate gefitinib concentration (M). Each data point represents the mean SD of three replicates. C and D, Relative percentage of the ALDH1\textsuperscript{bri} subpopulation of PC9 and HCC827 parental and T20 cells. E and F, Sphere-formation assay showing that PC9 and HCC827 T20 cells acquired an increased ability to form spheres in suspension culture relative to parental cells. G–I, PC9 (G), and HCC827 (H) T20 cells showed hypersensitivity to TV-6, as well as EX 527, as compared with parental cells. The result was the same as that for ALDH1\textsuperscript{bri} cells (I) derived from relapsed LAD patients under gefitinib treatment. Each data point represents the mean SD of three replicates per drug dose.
electron transport chain in the mitochondria. Hydrogen peroxide (H$_2$O$_2$) and mitochondrial superoxide levels were elevated in PC9 and HCC827 T20 cells as compared with parental cells, which was in line with hyper-active mtOXPHOS (Figure 2C and D). Moreover, ALDH1$^{bri}$ cells derived from residual LAD tumors following TKI therapy also exhibited elevated mtOXPHOS capacity, mtMPs, ROS levels, and mitochondrial superoxide levels as compared with their ALDH1$^{low}$ counterparts (Figure 2A–D). PC9 and HCC827 T20 cells were then sorted for high
versus low mtMPs by the intensity of MitoTracker Red CMX Ros and plated as spheres, revealing that sphere formation was markedly higher in cells with high mtMPs as compared with low mtMPs (Figure 2E).

To test the survival dependency of resistant clones on mtOXPHOS, we employed the mitochondriadirected inhibitor tigecycline [22], which suppress mitochondrial DNA (mtDNA) translation, and oligomycin A [10], an ATP synthase inhibitor (Supplementary Figure S4A). PC9 (Supplementary Figure S4B) and HCC827 (Supplementary Figure S4C) T20 cells were severalfold hypersensitized to mitochondriadirected inhibitors as compared with their corresponding parental cells. Moreover, targeting mitochondria with oligomycin A attenuated sphere formation (Supplementary Figure S4D) and the ALDH1<sup>hi</sup>-subpopulation (Figure 2F and Supplementary Figure S4E) by resistant CSC clones. Similarly, tigecycline reduced sphere formation (Supplementary Figure S4F) and the ALDH1<sup>hi</sup>-fraction (Supplementary Figure S4G). Even treatment with extremely low doses of mitochondriadirected inhibitors blocked the enrichment of ALDH1<sup>hi</sup>-cells. These results suggested that resistant clones exhibiting CSC features relied upon mtOXPHOS for survival. We then determined whether inhibition of mitochondrial respiration could counteract the intrinsic drug resistance mediated by CSC clones. At lowdoses, singleagent oligomycin A or tigecycline administration did not induce substantial cell death, as measured by flow cytometry in PC9 T20 cells; however, when combined with gefitinib, the overall number of dead cells significantly increased. Indeed, in the presence of gefitinib, the lower dose of mitochondriadirected inhibitors was equally effective as the higher dose (Figure 2G and Supplementary Figure S4H). These findings suggested that resistant CSCs were susceptible to the inhibition of the mitochondrial respiratory chain, and that oxidative metabolism could represent a prime target for overcoming TKI resistance in LAD.

The role of SIRT1 in the mitochondrial metabolism phenotype of TKI-resistant CSCs in LAD

To identify potential factors that regulate mtOXPHOSmediated metabolic changes that occur in response to TKI exposure, we evaluated the effects of lentiviral vectormediated shorthairpin (sh)RNA knockdown of SIRT1 in PC9 and HCC827 T20 cells, which was confirmed by immunoblot after use of two independent shRNAs for each gene (Supplementary Figures S5A and B). Knockdown of SIRT1 reduced mitochondrial respiratory capacities in PC9 and HCC827 T20 cells (Figure 3A). Additionally, mtDNA content was reduced upon SIRT1 ablation in PC9 and HCC827 T20 cells (Figure 3B). Moreover, transfection of SIRT1

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Inhibition of SIRT1 inhibits the mtOXPHOS dependency of CSCs. A, OCRs were determined by a Seahorse XFe96 extracellular flux analyzer. B, mtDNA levels were measured by real-time quantitative PCR. C, ROS levels were determined by ROS-Glo. D, LAD cell lines were stained with MitoTracker Red CMX Ros and then analyzed by flow cytometry. E, Relative percentage of the ALDH<sup>hi</sup> subpopulation in PC9 T20 cells under TV-6 treatment at the indicated concentrations. F, PC9 T20 cells were seeded for sphere assays (40×) and were treated with DMSO or tigecycline DOX. The inhibitory effect of tigecycline on sphere formation was abolished in PC9 T20 cells expressing DOX-inducible SIRT1 as compared with uninduced cells. Scale bar = 500 m. G, Flow cytometric determination of the frequency of the Annexin V<sup>+</sup> cell fraction in PC9 T20 cells treated with gefitinib (Gef) and/or TV-6 at the indicated concentrations. Annexin V was used as a marker of cell death. A representative result from three independent flow cytometry experiments is shown.
shRNA into PC9 and HCC827 T20 cells reduced ROS (Figure 3C) and mtMPs (Figure 3D) levels, and reductions in sphere formation (Supplementary Figure S5C) and the ALDH1bri+ fraction (Supplementary Figure S5D) were also observed in PC9 and HCC827 T20 cells following SIRT1 ablation.

We then determined whether SIRT inhibitors could produce similar antitumor effects as observed with genetic strategies. To this end, we used the SIRT1 inhibitors TV6 and EX 527. Similar to SIRT1 ablation, TV6 reduced sphere formation (Supplementary Figure S5E) and the ALDH1bri+ fraction (Figure 3E and Supplementary Figure S5F) of PC9 and HCC827 T20 cells and was associated with decreased oxygen consumption rate (OCR) (Supplementary Figure S5G), and mtMP (Supplementary Figure S5H). Similarly, EX 527 decreased sphere formation (Supplementary Figure S5I) and the ALDH1bri+ fraction (Supplementary Figure S5J) of PC9 and HCC827 T20 cells and inhibited mitochondrial respiratory capacity as assessed by OCR (Supplementary Figure S6A) and mtMP (Supplementary Figure S6B).

Additionally, we found that the inhibitory effect of tigecycline on sphere formation was rescued in PC9 T20 cells expressing doxycycline (DOX)inducible SIRT1 as compared with uninduced control cells (Figure 3F). Furthermore, the inhibitory effect of oligomycin A on stemness maintenance was also abolished in PC9 T20 cells stably overexpressing SIRT1 as compared with control cells, suggesting that the tumorinitiating capacity potentiated by SIRT1 relies on mtOXPHOS (Supplementary Figure S6C). These results implied that SIRT1 potentiated mtOXPHOS and subsequent CSC enrichment.

Consequently, we then determined whether eliminating SIRT1 expression could affect the responsiveness of LAD cells to anticancer drugs. Indeed, highdose TV6 modestly increased apoptosis in PC9 T20 cells resistant to TKI treatment, with this accompanied by a significant sensitization to gefitinib therapy. At a lowerdose, singleagent TV6 did not induce substantial cell death in PC9 T20 cells; however, the sensitization to anticancer approaches with lowdose TV6 in resistant cells was comparable to that achieved by highdose treatment (Figure 3G). Additionally, we confirmed SIRT1 inhibition as a combinatorial strategy to sensitize gefitinibinduced cell death using EX 527 (Supplementary Figure S6D).

Collectively, these findings indicated that SIRT1 inhibition enhanced the targeting of resistant CSCs in combination with molecular-targeted drugs in LAD.

**TV-6 selectively targets CSCs and acts together with gefitinib to block tumor growth and prolong remission**

We tested the in vivo effect of TV6, gefitinib, and the combination of both on primary human PDxIs initiated from a sample from a TKIrefractory LAD patient and engrafted in NSG mice. We first evaluated the pharmacokinetics of TV6 in mice (Supplementary Figure S7A), subsequently choosing a treatment schedule of twice daily i.p. injections. Following engraftment, mice (n = 10 mice/group) were treated after 14 days with a 3week course of gefitinib (100 mg/kg/day), TV6 (50 mg/kg/day), both drugs combined and vehicle only (Supplementary Figure S7B). We observed a synergistic response from combined TV6 and gefitinib treatment of tumors arising after 14 days. After 15 days of treatment, the drug combination virtually eliminated tumors, whereas injection of gefitinib alone had little effect on tumor remission. Moreover, mice treated with TV6 alone showed reduced tumor growth relative to that observed in untreated or gefitinibtreated mice, and after day 20, we observed only modest tumor regression. Furthermore, combined treatment resulted in further reductions in TV after an additional 20 days. In mice treated with TV6 alone, tumor growth was suppressed until day 40, after which tumor growth resumed and/or showed signs of relapse at rates near those observed in untreated control mice, whereas the major

TV-6 reduces the gefitinib dose necessary to prolong remission and decrease lung colonization

Because cytotoxic therapy is toxic and causes unwanted and often serious side effects in cancer patients [23], a major challenge is to lower the doses of TKIs without decreasing their effectiveness. We reasoned that the combinatorial effect of TV6 would permit lowering of the gefitinib dose. To test this hypothesis, we transplanted nude mice subcutaneously with PC9 cells carrying a luciferase reporter and evaluated the effects of a 3week course of scheduled treatments starting 14 days later when tumors were already palpable (n = 10 mice/group). We administered gefitinib (100 mg/kg/day), TV6 (50 mg/kg/day), both drugs combined, as well as TV6based combinatorial therapy involving a fourfold reduced concentration of gefitinib (25 mg/kg/day instead of 100 mg/kg/day) (Supplementary Figure S8A). As monotherapies, treatment of PC9 xenografts with highdose gefitinib suppressed tumor growth but did not prevent relapse. However, the reduced dose of gefitinib alone was less effective, as tumor regression was not observed. Interestingly, combined treatment with TV6 and the reduced dose of gefitinib resulted in complete tumor regression and no detectable relapse for at least 65 days. Indeed, in the presence of TV6, the lower dose of gefitinib was equally as effective as the higher dose in prolonging remission (Figure 5A–C).

We then determined the effect of different dosing schedules on the lung engraftment of PC9 cells intravenously injected into the tail of nude mice (Supplementary Figure S8B). For imaging of lung metastatic activity and distribution, mice were viewed dorsally and ventrally by bioluminescence imaging weekly, as well as by examination of the lungs at necropsy.
A single dose of TV6 was more effective at inhibiting tumor colonization than gefitinib alone at a dose of 25 mg/kg. Moreover, 100 mg/kg gefitinib as a single agent potently lowered lung bioluminescence signals, whereas lowdose gefitinib mice were moribund and presented enlarged lung engraftment of GFP+ cells, which did not occur in mice receiving highdose therapy. Indeed, decreased engraftment upon treatment was sharply accelerated by combined treatment with TV6 and gefitinib, with a slightly more pronounced effect observed with the higher gefitinib dose than the lower dose. However, the absence of relapse due to TV6 therapy was comparable at both doses of gefitinib for the period of the experiment. A double dose of gefitinib plus TV6 substantially reduced longterm lung engraftment, and the number of colonies remained at almost undetectable levels, even after up to 53 days (Figure 5D and E; Supplementary Figure S8C). These preclinical observations suggested the possibility of using SIRT1 inhibitors to lower the gefitinib dose in LAD patients.

Similar to the results obtained from bioluminescence lungcolonization experiments, mice treated with highdose gefitinib monotherapy or combined TV6 and gefitinib showed a dramatic decrease in the number of nodules and incidence of lung metastasis as compared with mice treated with other schedules (Figure 5F). Furthermore, decreased frequency of metastasis was confirmed by a significant reduction in histologically determined organ infiltration of tumor cells into the lungs of these mice relative to that observed in those bearing more nodules. The infiltration of tumor cells between different dosages was more pronounced at 21 days after drug withdrawal when lowdose gefitinib treated mice were moribund and displayed massive lung infiltration of tumor cells, which did not occur in those receiving highdose therapy and/or combined treatment (Figure 5G).

These results indicated that adding mitochondrial inhibitors to firstline TKI treatment significantly diminished the number of cells with high repopulation and invasion potential, and that even relatively lowdose TKI treatment could yield a sustained antitumor effect.

Inhibition of metabolic activity prevents the development of drug resistance

Given the hypersensitivity of TKIresistant cells to metabolic inhibitors and the metabolic reprogramming present in resistant cells, we determined whether metabolic inhibitors and TKI therapy could synergistically pre-
vent the emergence of acquired drug resistance. We examined the ability of a subset of putative anticancer compounds to prevent the emergence of tolerant colonies by cotreating cultures continuously with gefitinib and these compounds. Among the tested compounds, three different SIRT1 inhibitors as well as mitochondrial metabolism inhibitors virtually eliminated the emergence of drug-tolerant colonies from PC9 cells during anti-tumor treatment, whereas other tested agents did not in the presence of gefitinib (Figure 6A and B). This result presented a new therapeutic opportunity for not only targeting resistant LADs but also to possibly prevent the emergence of resistant subpopulations and achieve greater response from sensitive LADs treated with first-line TKIs. Upon pretreatment of naive PC9 cells with tigecycline for 5 days, followed by immediate removal of the inhibitor before isolating individual cells by transfer to sixwell plates, we were unable to detect any TKI-tolerant clones (Figure 6C). However, pretreatment of naive cells and removal of the inhibitor 72 h prior to isolating individual cells allowed detection of TKI-tolerant clones in a proportion similar to that observed in control naive cells (Figure 6D), suggesting that drug-tolerant cells were continuously replenishing in the absence of agents eliminating them. This suggested that preexisting TKI-tolerant cells in navel LAD populations are highly metabolism dependent.

Clinical significance of the expression of SIRT1 and mtOXPHOS components in LAD patients

We used an independent series of tumors derived from LAD patients sensitive to first-generation TKIs or exhibiting relapse characteristics under gefitinib treatment to determine levels of SIRT1, ALDH1, and widely accepted stem cell markers of lung cancer and a subset of mtOXPHOS components by immunohistochemistry (IHC) (Figure 7A). These mtOXPHOS proteins include, cytochrome C oxidase IV, a component of mitochondrial complex IV and a frequently used marker for mitochondrial content, PGC1α, a transcriptional coactivators that promotes energy metabolism and ROS detoxification, and mitochondrial ribosomal protein S5, which localizes to the mitochondria and promotes complex I function and NAD+ generation to enhance mitochondrial respiration. Our results identified markedly higher levels of SIRT1, ALDH1, and mtOXPHOS components in sections from postrelapse tumor tissues as compared with TKInave samples.

We then analyzed the clinical relevance of SIRT1 and CSC marker expression in human LAD samples. Based on the expression levels, samples were divided into two groups (SIRT1/ALDH1high and SIRT1/ALDH1low). SIRT1 and ALDH1 expression was significantly associated
with multiple aggressive clinicopathological characteristics, such as large tumor size, high recurrence and metastasis rates, and cancer embolus (Figure 7B). Moreover, Kaplan–Meier survival analysis indicated that compared with the SIRT1/ALDH1 low profile, LAD patients with the SIRT1/ALDH1 high profile had a much worse prognosis (Figure 7C).

These data suggested that the SIRT1/mtOXPHOS protein axis plays a critical role in predicting resistance to TKI and poor prognosis in LAD patients.

**Discussion**

Targeting oncogene-driven signaling pathways represents a clinically validated approach to treating several devastating diseases; however, despite such treatments resulting in significant tumor shrinkage, the frequency of relapse remains. Insights into the limitations of targeted kinase therapy were obtained from understanding that LAD comprises differentiated cells, as well as a higher fraction of spherogenic and tumorigenic undifferentiated cells that survive shutdown of oncogenic signaling and are capable of propagating the disease [1,3]. Using multiple treatment cycles, we developed a series of progressively resistant cell lines representing different oncogenotypes, followed by molecular characterization of the entire series and identification of novel pathways involving an increased dependency on mitochondrial biogenesis and oxidative phosphorylation. Previous studies also revealed a metabolic vulnerability to tumor recurrence following neoadjuvant chemotherapy via molecular profiling of residual disease [24,25].

Tumor cells reprogram a variety of central metabolic and bioenergetic pathways to maintain exacerbated growth and survival rates [6]. Several recent reports explored mitochondrial respiration specificities as targetable susceptibilities of cytotoxic drug-selected CSCs, which when exploited, should be synthetically lethal when combined with established therapies [8]. For example, residual breast cancers after conventional chemotherapy display mtOXPHOS, as well as tumorigenerating features [26]. Mammospheres obtained from hormone-dependent, estrogenreceptorpositive breast cancer exhibit upregulation of key mitochondrial enzymes involved in oxidation and ketone metabolism [27], suggesting that CSC metabolism

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**Figure 6.** Preventing the Establishment of Drug-Tolerant Clones. A, PC9 cells were either untreated or treated alone with the indicated pharmacological agents for 7 days (top rows) or with Gefitinib (Gef) alone or the combination of the indicated agent with Gefitinib for 40 days (bottom rows). Fresh media with drugs were provided every 3 days. Following treatment, plates were fixed and stained. All experiments were performed in triplicate and representative plates are shown. B, Individual colonies were counted and the quantified results were graphed. In some cases the colonies were too numerous to count (indicated as > 500 colonies). Error bars represent standard deviations from the mean of three independent experiments. C, PC9 cells were either treated with Gefitinib (Gef) for 30 days or pretreated for 5 days with Tigecycline and then immediately treated with Gefitinib for 30 days, plates were fixed and stained. D, PC9 cells were either treated with Gefitinib for 30 days, in addition, pretreated for 5 days with Tigecycline, then removed the inhibitor 72 hours prior to Gefitinib treatment for 30 days. Plates were fixed and stained. The experiment was performed in triplicate and representative plates are shown.
relied on mitochondrial respiration. Vazquez et al. [28] report reduced glycolysis and increased OXPHOS in certain melanomas, revealing metabolic plasticity rather than stable Warburg pathophysiology. Furthermore, Haq [11] reported that increased OXPHOS is required for melanomas to survive BRAF inhibition, suggesting the potential benefit of investigating therapeutic combinations of BRAF inhibitors with metabolism-related inhibitors. Other studies [9,29,30] showed that leukemic primitive cells rely more heavily on OXPHOS to supply their energetic demands as compared with bulk tumor cells, thereby suggesting a link between mitochondrial pathophysiology and therapy resistant clones and representing an attractive topic for future investigation. Furthermore, using singlecell RNA sequencing coupled with a highly sensitive BCR-ABL detection method, Giustacchini et al. [31] showed that primitive BCR-ABL+ cells from chronic myelogenous leukemia (CML) patients presented an overexpression of OXPHOS and fatty acid oxidation-related genes as compared with BCR/ABL counterparts. This suggests that the oxidative phenotype characterized in the present study is specific to leukemia stem cells in patients, further highlighting the clinical relevance of our findings. Our findings identified an adaptive metabolic program that limits the therapeutic efficacy of EGFR inhibitors. Intrinsic resistance conferred by metabolic specificities could represent an additional protective mechanism that helps cells survive the initial contact with various cytotoxic agents and provides cells with added survival time to establish longer-lasting secondary resistance mechanisms (Figure 7D).

A previous study demonstrated that SIRT1 deacetylates the mitochondrial master regulator PGC1α to enhance mtDNA replication and biogenesis [32]. Interestingly, PGC1α inhibition did not directly inhibit CML hematopoiesis, suggesting that additional SIRT1-related mechanisms besides regulation of mitochondrial respiration are also involved in promoting CML stem cell growth. Although the role of SIRTs in oncogenesis varies based on tissue type, recent evidence revealed a critical function for SIRT1 in regulating p53 deacetylation in CML-specific escape from imatinib therapy [20]. To gain further insights into SIRT1, they extended their findings and proposed a mechanism involving a positive feedback
loop between SIRT1 and cMYC in regulating acute myeloid leukemia (AML) stem cells harboring a FLT3 internal tandem duplication mutation [19]. In the future, it will be of interest to explore interactions between SIRT1 regulation of metabolism and its regulation of p53 and cMYC and those effects on CSCs in LAD. Although changes in chromatin in our study were reminiscent of an established model of drug tolerant lung cancer cells, the underlying mechanisms appeared distinct, as we did not observe enrichment of KDM5A in LAD cells that survived acute, short-term exposure to an EGFR inhibitor [33]. It is possible that additional chromatin-modifying enzymes contribute to TKI tolerance in various experimental contexts. A plausible explanation is the involvement of unknown oncogenic signaling pathways activated by EGFR mutants that arise from the adaptation of cells from prolonged exposure to TKIs. The discrepancy further highlights the importance of understanding under which cellular context and protein complexes these enzymes act and suggests that differences in genetic backgrounds, culture conditions, specific oncogenes present, and the use of knockout constructs versus the study of knockout cells must be taken into account.

Interestingly, it was recently reported that a subpopulation of pancreatic cancer cells that had survived Kras ablation were also mainly dependent on OXPHOS [12]. Similarly, treatment of BRAF mutated melanomas with BRAF inhibitors renders them addicted to OXPHOS [10,11]. This observation together with the results of the present study indicate that in oncogene-expressing tumors, there exists a small subpopulation endowed with tumorigenic potential, self-renewal capabilities, intrinsic resistance to targeted therapies, high hyperpolarized mitochondria, and increased consumption of oxygen. By contrast, another study found that human CSCs derived from a large panel of PDX models carrying activating KRAS mutations identical to those found in their bulk counterparts bear distinct metabolic phenotypes with limited plasticity and controlled by MYC [34]. Strikingly, CSCs from a second PDX model carrying wildtype KRAS showed a similar metabolic phenotype, further corroborating that the lack of plasticity is independent of KRAS mutational status. Moreover, KRAS-ablated cells require reactivation of mutant KRAS for proliferation and tumor relapse, suggesting that the observed OXPHOS phenotype is restricted to oncogene-ablated dormant cells (as described in the present study), as opposed to the highly tumorigenic CSCs studied by others. The metabolic phenotype of CSCs appears not to be universal but rather varies according to context. Elucidating the molecular basis for this specificity with regard to differential oncogenic reprogramming of cellular metabolism will be the next critical step in understanding tumor heterogeneity and complexity.

The CSC hypothesis is based on the differential tumorforming properties and responses to well-defined therapy [35]. A prediction of this model, heretofore untested, is that drugs that selectively inhibit CSCs should function synergistically with well-defined drugs to prolong the therapeutic response and make them more susceptible to a second drug. Additionally, it might have a potential use in preventing the development of drug tolerance, as opposed to treating resistance that has already occurred. Based on our findings, the addiction to OXPHOS treated with EGFR-targeted therapy suggests that mitochondrial inhibitors should be evaluated in combination with EGFR pathway inhibitors in vivo. Upon drug withdrawal, we observed regression of transplanted tumors following singleagent treatment within 2 to 3 weeks, followed by relapse after 5 to 6 weeks; however, combinatorial therapy involving TV6 prevented relapse for at least 2 months and indeed might have even represented a cure for these xenograftgenerated tumors. Another major problem that cancer patients face is the high toxicity of conventional and molecularly targeted drugs that manifest as anemia, appetite changes, fatigue, hair loss, nausea, vomiting, and fertility changes [23]. By contrast, lower doses of these well-defined therapeutics are ineffective at suppressing tumor burden. Therefore, identification of agents that can be combined with lower doses of existing therapeutics is of high clinical relevance. Although TV6 is not a candidate for drug development targeting tumor regression, our observations support further investigation of SIRT1 inhibition as an approach for targeting LAD CSCs in combination with TKI treatment. TV6 exhibited comparable effects in preventing relapse and prolonging the therapeutic response when combined with a fourfold reduced dose of gefitinib, which was ineffective as a monotherapy. Therefore, TV6 exhibited broad anticancer effects of potential utility in a wide variety of clinical contexts for both cancer treatment and lowering the toxicity associated with frontline TKI therapy. Recent studies focusing on tolerance to SIRT1 inhibitors showed that TV6 appeared more tolerable to normal hematopoietic progenitor cells according to multiple endpoints, and that applying it as a treatment in combination with the BCRABL TKI imatinib promoted eradication of CML stem cells in mouse models [19,20]. Within the limited scope of the present study, we observed no changes in body or spleen weight or signs of toxicity during treatment, and total bone marrow cellularity was unaffected in all experimental arms, thereby confirming excellent tolerability. These observations, although preliminary, indicate promising avenues for further investigation toward using a combination of TV6 and gefitinib to eliminate residual CSCs, as well as determine the safety and tolerability in patients with LAD who are in cytoreductive remission with evidence of residual EGFR+ cells. Furthermore, our model identified clinically relevant mechanisms of drug resistance; therefore, the mouse model could be a surrogate for acquired TKI drug resistance, as it captures cells that persist following a single cycle of TKI therapy and could, therefore, mimic a patient that clears the vast majority of the bulk population but experiences regrowth of disease within months of starting therapy. Additionally, the model could offer insight into mechanisms of relapse. The high OXPHOS status not only correlated with the probability of achieving remission with induction TKI therapy in an independent dataset of patients with LAD but also correlated with overall survival, thereby supporting the clinical relevance of our findings. Moreover, this model is compatible with recent findings by Farge et al. [29], which demonstrated an in vivo approach to identifying primary AML cells that persist in the bone marrow after chemotherapy. In that study, mice were engrafted with tumor cells and then treated with cytarabine at an appropriate dose and schedule to reduce the level of tumor burden. On the time interval of maximal depletion of tumor cells, the residual disease that persisted in the bone marrow was isolated and studied.

Some potential limitations should be considered in our findings. First, the in vitro and in vivo studies of CSC response to TKIs and SIRT1 inhibitors were confined to a relatively small number of patient samples. Consequently, these data are not robust, and the results must, therefore, be interpreted with due caution. Additionally, LAD CSC populations vary from patient to patient, which can directly affect the response or resistance of these cells to single and combined treatments. Another consideration is the off-target effects of the SIRT1 inhibitor. All SIRT inhibitors, including TV6, suppress more than SIRT1 [36]; therefore, results from use of SIRT inhibitors should be interpreted with multiple enzymes in mind. This raises the question as to whether functional redundancy exists within SIRT families based on sequence homologies and architectural similarities. Although difficult to reconcile, discrepancies might at least be due to the fact that no study has demonstrated selective inhibition of the SIRT1 deacetylase, despite recent studies of several mechanism-based inhibitors targeting SIRT histone deacetylases. Although we cannot exclude the possibility that TV6 also suppresses other SIRTs, these results could also be faithfully reproduced using an independent SIRT1 shRNA approach and tumor cells to distinguish the phenocopying effects of the chemical inhibitor. This could support SIRT1 as at least one target mediating TV6 effects. Even with these considerations, the clinical implication of this work provides a supportive rationale to blocking the mTORPHOS pathway in EGFRmutant LAD patients and specifically in those who
developed resistance under anti-EGFR therapy. Further work will reveal whether there are practical opportunities for synthetic lethality by pharmacology limiting the adaptive ability of transformed cells to upregulate OXPHOS when facing stress, such as oncogenic kinase inhibition.

Authors contributions

Conception and design: Xiang Yuan, Jiangtao Sun;
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Acquisition of data (acquired and managed patients, provided facilities, etc.): Jiangtao Sun, Guifang Li, Kaifang Song;
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Jinyu Kong, Yiwen Li, Guifang Li, Huaxu Li, Kaifang Song;
Writing, review, and/or revision of the manuscript: Jiangtao Sun, Xiang Yuan, Daxing Zhu, Xiaojun Tang;
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Xiang Yuan, Guifang Li, Yiwen Liu, Jinyu Kong, Mingyang Ma;
Study supervision: Xiang Yuan.

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Appendix A. Supplementary data

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