

# A four-gene signature identifies TKI-induced persister cells and uncovers a ZLN005-induced pyroptotic vulnerability via the GSDME pathway in EGFR-mutant lung cancer

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**Abstract.** Drug-tolerant persister cells represent a major barrier to durable responses in cases of epidermal growth factor receptor (EGFR)-mutant lung adenocarcinoma treated with tyrosine kinase inhibitors. To define the molecular features and actionable vulnerabilities of this cell state, public transcriptomic

datasets from tumour cell-based models treated with EGFR inhibitors or other targeted agents within the receptor tyrosine kinase (RTK)/RAS/MAPK pathway were integrated with machine-learning-based feature selection, survival analysis and Cancer Dependency Map-based pharmacogenomic screening. The present study also performed experimental validation of identified genes and drugs in PC-9 and HCC827 cell models. A four-gene persister-associated gene signature comprising B-cell translocation gene 1 protein, inhibitor of growth protein 4, proline-rich nuclear receptor coactivator 1 and calcium-binding and coiled-coil domain-containing protein 1 was identified, which defined a targeted therapy-induced persister state and associated with poor patient survival. Functional validation using reverse transcription-quantitative PCR, doxycycline-inducible short hairpin RNA knockdown and  $\beta$ -galactosidase staining assays demonstrated rapid induction of these genes during osimertinib treatment and showed that depletion of each gene markedly impaired drug-tolerant persister cell formation. Pharmacogenomic analysis identified ZLN005, a peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  agonist, as a candidate metabolic modulator that synergized with osimertinib to reduce persister cell viability. Mechanistically, ZLN005 enhanced mitochondrial oxidative metabolism and reactive oxygen species accumulation, leading to caspase-3 activation and gasdermin E-associated pyroptotic cell death. Collectively, these findings defined the transcriptional and metabolic features of targeted therapy-induced persister cells and revealed a metabolic vulnerability to ZLN005 in EGFR-mutant lung adenocarcinoma.

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**Abbreviations:** DTP, drug-tolerant persister; EGFR, epidermal growth factor receptor; RTK, receptor tyrosine kinase; TKI, tyrosine kinase inhibitor; NSCLC, non-small cell lung cancer; LASSO, least absolute shrinkage and selection operator; SVM-RFE, support vector machine-recursive feature elimination; ROC, receiver operating characteristic; AUC, area under the curve; OS, overall survival; PPS, post-progression survival; FP, first progression; HR, hazard ratio; ROS, reactive oxygen species; PBST, PBS containing 0.1% Tween-20; PI, propidium iodide; GSEA, gene set enrichment analysis; FDR, false discovery rate; TNMplot, tumour-normal-metastatic plot

**Key words:** drug-tolerant persister cells, EGFR, lung adenocarcinoma, TKIs, metabolism, machine learning, pyroptosis, gasdermin E, osimertinib, peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  agonist ZLN005

## Introduction

Globally, lung cancer remains the principal cause of cancer-related mortality; non-small cell lung cancer (NSCLC)

in particular has been shown to account for ~85% of lung cancer cases (1-4). In patients with epidermal growth factor receptor (EGFR)-mutant NSCLC, treatment with tyrosine kinase inhibitors (TKIs) has been shown to markedly improve clinical outcomes. However, despite notable initial treatment responses, the majority of patients eventually develop disease progression driven by acquired and adaptive resistance to TKI treatment (5-7).

In addition to genetically acquired resistance mechanisms, a small subset of tumour cells has been shown to survive initial EGFR-TKI exposure through reversible, non-genetic adaptation mechanisms (8-10). These TKI-tolerant cells, which are known as drug-tolerant persister (DTP) cells, do not initially exhibit stable resistance mutations; however, these cells can serve as a reservoir from which stable resistance eventually emerges (11-13). As such, DTP cells have become increasingly recognized as key contributors to minimal residual disease and the subsequent relapse of NSCLC (14,15).

Previous studies have shown that DTP cells undergo notable epigenetic and metabolic remodelling, such as chromatin reprogramming, increases in reliance on oxidative phosphorylation and the activation of stress-adaptation pathways (16-21). However, to the best of our knowledge, a well-defined transcriptional signature of the DTP state in EGFR-mutant lung adenocarcinoma (LUAD) has yet to be elucidated, limiting efforts to identify robust biomarkers and therapeutically actionable vulnerabilities for this disease.

Since the DTP cell state is likely governed by coordinated programs rather than single-gene programs, integrative machine-learning approaches may be particularly useful for identifying robust transcriptional signatures of EGFR-mutant NSCLC persistence (22-25). To identify conserved features of the persister state, the present study integrated publicly available transcriptomic datasets and subsequently performed experimental validation of these features in EGFR-mutant LUAD models. To the best of our knowledge, such strategies have not yet been systematically applied to define DTP-associated transcriptional programs in the context of EGFR-mutant NSCLC.

Beyond defining the regulatory basis of the DTP cell state, it is important to identify the vulnerabilities that can be exploited to eliminate DTP cells (14). Increasing evidence suggests that persister cells undergo mitochondrial rewiring and experience elevated oxidative stress, raising the possibility that metabolic overactivation may push these cells beyond their adaptive capacity. As the mode of therapy-induced cell death may influence therapeutic outcomes, the present study also investigated whether such metabolic perturbation could promote gasdermin E (GSDME)-associated pyroptotic elimination rather than apoptosis alone (26-30), a possibility that, to the best of our knowledge, has not been examined in EGFR-mutant TKI-induced persister cells.

The present study sought to define the transcriptional signature associated with the DTP cell state in EGFR-mutant LUAD and to determine whether this adaptive state exposes therapeutically actionable metabolic vulnerabilities. The present study used machine learning-based transcriptomic analysis together with experimental validation to identify

a four-gene transcriptional signature linked to the DTP phenotype. The present study further investigated whether pharmacological modulation mitochondrial metabolism using the peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) agonist ZLN005 could enhance therapeutic vulnerability of DTP cells to osimertinib. Together, these findings established a transcriptional metabolic framework for the persister state and suggested a potential strategy for eliminating DTP cells in EGFR-mutant LUAD.

## Materials and methods

*Public gene expression omnibus (GEO) dataset selection and transcriptomic analysis.* Publicly available RNA-sequencing (RNA-seq) datasets were downloaded from the GEO database. The GEO accession numbers and basic characteristics of these datasets are summarized in Table I (31-34). Datasets were included according to the following criteria; i) Use of tumour cell-based experimental models; ii) treatment with EGFR inhibitors or other targeted agents within the receptor tyrosine kinase (RTK)/RAS/MAPK pathway; iii) a clearly defined DTP state; iv) the presence of an appropriate control group; and v) availability of well-annotated transcriptomic data suitable for downstream analysis. Datasets that did not meet these criteria were excluded. For each included dataset, raw count matrices were retrieved directly from the GEO repository. Quality control, normalization and downstream analyses were performed in R (version 4.3.0; Posit Software, PBC) using standard bioinformatics workflows. Differential gene expression analysis between drug-treated and control samples was conducted separately for each dataset using the 'voom' transformation in the 'limma' package (version 3.58.1; Bioconductor) to model mean-variance relationships. Raw P-values were adjusted using the Benjamini-Hochberg false discovery rate (FDR) method. Genes with an absolute  $\log_2(\text{fold-change}) \geq 1$  and an adjusted P-value  $< 0.05$  were considered significantly differentially expressed. Results from individual datasets were then cross-compared to identify genes that were consistently upregulated or downregulated across independent experiments. Visualisation and statistical analyses were performed using the 'ggplot2' (version 3.5.2; CRAN) and 'pheatmap' packages (version 1.0.12; CRAN) in R.

As the present study was based on the retrospective analysis of publicly available datasets rather than prospectively enrolled subjects, a formal *a priori* sample size calculation was not applicable. Instead, all eligible samples meeting the predefined inclusion criteria were included to ensure the efficient use of available data, enhance the statistical robustness of the analysis and minimize selection bias. The integrated discovery cohort used for feature selection and receiver operating characteristic (ROC) analysis comprised 56 control samples and 60 treated samples.

For the temporal expression analysis shown in Fig. 1G, publicly available time-course transcriptomic data from GSE193258 were further analysed. The dataset included EGFR-mutant or TKI-responsive LUAD cell lines, including H1975, HCC827, HCC2935 and PC-9, with samples annotated according to the original metadata as untreated control, acute TKI exposure, established DTP state and short- or long-term

Table I. Summary of publicly available transcriptomic datasets analysed to identify the DTP gene signature in EGFR-mutant lung adenocarcinoma.

A, GSE193258, <https://ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE193258>

| Cell line (disease)           | Replicates | Treatment (dose)     | Drug type            | Duration |
|-------------------------------|------------|----------------------|----------------------|----------|
| H1975 (lung adenocarcinoma)   |            |                      |                      | 21 days  |
| Control                       | 3          | N/A                  |                      |          |
| DTP                           | 3          | Osimertinib (500 nM) | EGFR T790M inhibitor |          |
| HCC2935 (lung adenocarcinoma) |            |                      |                      | 21 days  |
| Control                       | 3          | N/A                  |                      |          |
| DTP                           | 3          | Osimertinib (500 nM) | EGFR T790M inhibitor |          |
| HCC827 (lung adenocarcinoma)  |            |                      |                      | 21 days  |
| Control                       | 3          | N/A                  |                      |          |
| DTP                           | 3          | Osimertinib (500 nM) | EGFR T790M inhibitor |          |
| PC-9 (lung adenocarcinoma)    |            |                      |                      | 21 days  |
| Control                       | 3          | N/A                  |                      |          |
| DTP                           | 3          | Osimertinib (500 nM) | EGFR T790M inhibitor |          |

B, GSE155341, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE155341>

| Cell line (disease)                 | Replicates | Treatment (dose)        | Drug type      | Duration |
|-------------------------------------|------------|-------------------------|----------------|----------|
| BT474 (invasive breast carcinoma)   |            |                         |                | 14 days  |
| Control                             | 2          | N/A                     |                |          |
| DTP                                 | 2          | Lapatinib (2.5 $\mu$ M) | EGFR inhibitor |          |
| EFM192A (invasive breast carcinoma) |            |                         |                | 14 days  |
| Control                             | 2          | N/A                     |                |          |
| DTP                                 | 2          | Lapatinib (2.5 $\mu$ M) | EGFR inhibitor |          |
| HCC1419 (invasive breast carcinoma) |            |                         |                | 14 days  |
| Control                             | 2          | N/A                     |                |          |
| DTP                                 | 2          | Lapatinib (2.5 $\mu$ M) | EGFR inhibitor |          |
| SKBR3 (invasive breast carcinoma)   |            |                         |                | 14 days  |
| Control                             | 2          | N/A                     |                |          |
| DTP                                 | 2          | Lapatinib (2.5 $\mu$ M) | EGFR inhibitor |          |

C, GSE153183, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE153183>

| Cell line (disease)        | Replicates | Treatment (dose)        | Drug type            | Duration |
|----------------------------|------------|-------------------------|----------------------|----------|
| PC-9 (lung adenocarcinoma) |            |                         |                      | 9 days   |
| Control                    | 2          | N/A                     |                      |          |
| DTP                        | 2          | Osimertinib (2 $\mu$ M) | EGFR T790M inhibitor |          |

D, GSE114647, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114647>

| Cell line (disease)           | Replicates | Treatment (dose)   | Drug type      | Duration  |
|-------------------------------|------------|--------------------|----------------|-----------|
| HCC827 (lung adenocarcinoma)  |            |                    |                | 2-3 weeks |
| Control                       | 3          | N/A                |                |           |
| DTP                           | 3          | Gefitinib (300 nM) | EGFR inhibitor |           |
| HCC4006 (lung adenocarcinoma) |            |                    |                | 2-3 weeks |
| Control                       | 3          | N/A                |                |           |
| DTP                           | 3          | Gefitinib (300 nM) | EGFR inhibitor |           |

Table I. Continued.

D, GSE114647, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114647>

| Cell line (disease)          | Replicates | Treatment (dose)   | Drug type      | Duration  |
|------------------------------|------------|--------------------|----------------|-----------|
| H1975 (lung adenocarcinoma)  |            |                    |                | 2-3 weeks |
| Control                      | 3          | N/A                |                |           |
| DTP                          | 3          | WZ4002 (300 nM)    | EGFR inhibitor |           |
| PC-9 (lung adenocarcinoma)   |            |                    |                | 2-3 weeks |
| Control                      | 2          | N/A                |                |           |
| DTP                          | 4          | Gefitinib (300 nM) | EGFR inhibitor |           |
| MGH119 (lung adenocarcinoma) |            |                    |                | 2-3 weeks |
| Control                      | 2          | N/A                |                |           |
| DTP                          | 4          | Gefitinib (300 nM) | EGFR inhibitor |           |

E, GSE89127, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE89127>

| Cell line (disease)                  | Replicates | Treatment (dose)        | Drug type           | Duration |
|--------------------------------------|------------|-------------------------|---------------------|----------|
| A375 (melanoma)                      |            |                         |                     | 7 days   |
| Control                              | 3          | N/A                     |                     |          |
| DTP                                  | 3          | Vemurafenib (1 $\mu$ M) | RAF inhibitor       |          |
| A549 (lung adenocarcinoma)           |            |                         |                     | 7 days   |
| Control                              | 3          | N/A                     |                     |          |
| DTP                                  | 3          | GSK1120212 (200 nM)     | MEK inhibitor       |          |
| H3122 (lung adenocarcinoma)          |            |                         |                     | 7 days   |
| Control                              | 3          | N/A                     |                     |          |
| DTP                                  | 3          | Crizotinib (250 nM)     | ALK tyrosine kinase |          |
| N87 (tubular stomach adenocarcinoma) |            |                         |                     | 7 days   |
| Control                              | 3          | N/A                     |                     |          |
| DTP                                  | 3          | Lapatinib (100 nM)      | EGFR inhibitor      |          |
| PC-9 (lung adenocarcinoma)           |            |                         |                     | 7 days   |
| Control                              | 3          | N/A                     |                     |          |
| DTP                                  | 3          | Erlotinib (1 $\mu$ M)   | EGFR inhibitor      |          |
| RT112 (bladder urothelial carcinoma) |            |                         |                     | 7 days   |
| Control                              | 3          | N/A                     |                     |          |
| DTP                                  | 3          | BGJ398 (1 $\mu$ M)      | FGFR inhibitor      |          |

F, GSE100751, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE100751>

| Cell line (disease)        | Replicates | Treatment (dose)      | Drug type      | Duration |
|----------------------------|------------|-----------------------|----------------|----------|
| PC-9 (lung adenocarcinoma) |            |                       |                | 8 days   |
| Control                    | 3          | N/A                   |                |          |
| DTP                        | 3          | Erlotinib (1 $\mu$ M) | EGFR inhibitor |          |

Public RNA-sequencing datasets of tyrosine kinase inhibitor-treated lung adenocarcinoma cell lines were curated for integrative analysis. The table summarizes experimental model, treatment agent, concentration, exposure duration and exposure duration for each dataset. EGFR, epidermal growth factor receptor; DTP, drug-tolerant persister; N/A, not applicable; ALK, anaplastic lymphoma kinase; T790M, threonine-to-methionine substitution at amino acid position 790; FGFR, fibroblast growth factor receptor.

drug-washout phases. Expression values were normalized to the corresponding control group within each dataset before comparison.

Additional public datasets used for cross-context validation of the four-gene signature were also included, including GSE162045, GSE103021 and GSE229070. These datasets



contained kinase inhibitor-treated cell lines, including PC-9, SK-MEL-28, NCI-H358, H23 and H358 cells, and were used to examine whether the four-gene expression pattern was observed across different RTK/RAS/MAPK pathway inhibition contexts.

*Cancer Dependency Map (DepMap)-based transcriptomic and pharmacogenomic analysis.* DepMap-based analyses were performed using the publicly available DepMap Portal (Broad Institute <https://depmap.org/portal/>) and the DepMap Public 25Q4 release, which integrates cancer cell-line molecular profiling data, including gene-expression datasets, and pharmacological response data from the PRISM Repurposing secondary screen. As these analyses were performed directly through the DepMap Portal, no individual external dataset accession numbers were applicable.

Single-sample gene set enrichment analysis (ssGSEA) scores were obtained using the pathway activity analysis module available in the DepMap Portal based on DepMap gene-expression profiles. These scores were used to evaluate pathway activity associated with the four-gene signature. Cell lines were stratified into high- and low-signature-expression groups according to the combined expression score of *BTG1*, *ING4*, *PNRC1* and *CALCOCO1*. Pathway activity differences between high- and low-signature-expression groups were evaluated to identify biological programs associated with the DTP-associated transcriptional state.

For DepMap analyses, LUAD and breast cancer cell lines were selected based on their biological relevance to the present study and the availability of well-annotated models. Breast cancer cell lines were included because the integrated transcriptomic discovery datasets contained lapatinib-treated breast cancer persister models, thereby allowing evaluation of whether the identified four-gene signature and associated pharmacological vulnerabilities were reproducible across multiple targeted-therapy-associated tumour contexts. Cell lines were ranked according to the expression of the four-gene signature, which comprised B-cell translocation gene 1 protein (*BTG1*), inhibitor of growth protein 4 (*ING4*), proline-rich nuclear receptor coactivator 1 (*PNRC1*) and calcium-binding and coiled-coil domain-containing protein 1 (*CALCOCO1*). The top 25 and bottom 25 cell lines in each cancer type were defined as the high- and low-signature groups, respectively, to enable robust contrast of drug-response data between cell lines of variable gene-signature expression while maintaining sufficient sample size for downstream analyses. Drug sensitivity was assessed using area under the curve (AUC) values from the Profiling Relative Inhibition Simultaneously in Mixtures (PRISM) repurposing secondary screen dataset available through the DepMap Portal. Cell lines with ambiguous lineage annotation or duplicated or incomplete records were excluded. Group comparisons were conducted using the DepMap portal (<https://depmap.org>) via the 'Custom Analysis' module using the 'Two class comparison' setting. For each compound, the analysis returned an effect size, P-value and q-value drug-response profiles between the high- and low-signature-expression cell-line groups. The q-value was used to control for multiple testing, and compounds with  $q \leq 0.05$  were considered statistically significant. Because lower AUC values correspond with increased drug sensitivity, a negative effect

size was interpreted as an indicator of enhanced sensitivity. Therefore, compounds showing significantly lower AUC values in the high-signature-expression cell lines compared with low-signature-expression cell lines were considered selectively effective and prioritized for experimental validation.

*Gene set enrichment analysis (GSEA).* Differentially expressed genes were ranked by  $\log_2$ (fold-change) (treatment vs. control) and analysed using the 'clusterProfiler' package (version 4.8.3; Bioconductor) in R (version 4.3.0; Posit Software, PBC). Multiple curated gene set collections from the Molecular Signatures Database (MSigDB; <https://www.gsea-msigdb.org/gsea/msigdb>) (35) were used, including C2; CP; Kyoto Encyclopedia of Genes and Genomes MEDICUS (release 7.4), C3; TFT (release 2023.1. Homo sapiens (Hs)), C5; Gene Ontology (release 7.4) and C6; Oncogenic Signatures (release 2023.1.Hs). Gene enrichment was computed using the 'GSEA' function with default parameters, and statistical significance was determined based on normalised enrichment scores and adjusted P-values (Benjamini-Hochberg FDR<0.25). Visualisation of the top enriched pathways was performed using the 'gseaplot2' function in the enrichplot R/Bioconductor package (version 1.28.4; Bioconductor), showing the top five positively and negatively enriched pathways.

*Machine learning analysis.* To identify robust genetic features distinguishing DTP cells from control samples, three complementary machine-learning algorithms were implemented in R (version 4.5.0; Posit Software, PBC); i) Least absolute shrinkage and selection operator (LASSO); ii) random forest; and iii) support vector machine-recursive feature elimination (SVM-RFE). Differentially expressed genes from GEO-derived datasets were transposed into a sample-by-gene matrix, and class labels (control vs. treatment) were extracted from sample names. LASSO regression was performed using the 'glmnet' package (family, 'binomial';  $\alpha$ , 1; version 4.1; CRAN) with 10-fold cross-validation, and genes exhibiting non-zero coefficients at the optimal penalty ('lambda.min') were retained. Random forest classification was conducted with the 'randomForest' package (version 4.7; CRAN) using 500 trees, followed by refinement to the tree number corresponding to the minimum out-of-bag error. Variable importance was ranked by mean decrease in Gini index, and genes with importance scores >2 were defined as random forest-selected features. SVM-RFE was implemented using the 'e1071' package (version 1.7; CRAN) and a recursive feature elimination wrapper script (k, 10; 'halve.above', 50) to iteratively remove the least informative genes until the minimal cross-validation error was achieved. The intersection of the three feature lists obtained from LASSO, random forest and SVM-RFE analyses was defined as the consensus machine-learning signature. Model performance and feature stability were evaluated by cross-validation error curves and variable importance plots generated in 'ggplot2' (version 4.0; CRAN), and reproducibility was ensured by setting random seeds ['set.seed(123)' and 'set.seed(12345)'].

*Prognostic analysis.* The prognostic significance of the four-gene signature, comprising *BTG1*, *ING4*, *PNRC1* and *CALCOCO1*, was evaluated using the Kaplan-Meier plotter

online database (<https://kmplot.com>) (36). Clinical and transcriptomic data for LUAD were obtained from the integrated GEO, European Genome-phenome Archive (EGA) and The Cancer Genome Atlas (TCGA) datasets within the Kaplan-Meier Plotter platform. Patients were included if expression data for all four genes were available and were stratified into high- and low-expression groups according to the combined expression score of the four-gene signature, as implemented in the platform.

For the primary survival analysis, the lung cancer mRNA dataset was filtered for LUAD/adenocarcinoma cases, and survival endpoints included overall survival (OS), post-progression survival (PPS) and first progression (FP). The ‘auto select best cutoff’ option was used to determine the optimal threshold. This option systematically tested all possible cut-offs between the lower and upper quartiles of expression and applied Benjamini-Hochberg FDR correction to the data to reduce multiple-testing bias. The cut-off value yielding the lowest FDR was selected for survival analysis; if multiple cut-off values showed equal significance, the one with the highest hazard ratio (HR) was chosen. Kaplan-Meier survival curves were generated for overall survival (OS), post-progression survival (PPS) and first progression (FP), and HRs with 95% confidence intervals (CIs) and log-rank P-values were obtained from the platform. The selected cut-off values and the corresponding grouping parameters for each analysis are provided in Table II.

In addition, subgroup survival analyses were performed using the same Kaplan-Meier plotter platform to further validate the prognostic relevance of the four-gene signature in clinically relevant subsets of patients with LUAD, including: i) Male patients; ii) female patients; iii) smokers after exclusion of never-smokers; and iv) patients with stage I, stage II or stage III disease. OS was compared between the high- and low-expression groups according to the grouping settings provided by the platform, and HRs with log-rank P-values were obtained directly from the Kaplan-Meier plot output. The dataset module, endpoint, subgroup filters, selected cut-off values and grouping parameters used for the primary and subgroup analyses are summarized in Table II.

**Comparative expression analysis.** Comparative expression analysis between tumour and normal tissues was conducted using the tumour-normal-metastatic plot (TNMplot, V2) web platform (<https://tnmplot.com/analysis/>) (37). TNMplot is a web-based transcriptomic analysis platform that integrates public tumour and normal tissue datasets, including TCGA, Genotype-Tissue Expression (GTEx) and GEO dataset. For the present analysis, the ‘RNA-seq (TCGA + GTEx)’ analysis mode was selected to evaluate the expression patterns of the four-gene signature in LUAD. No individual datasets were manually selected or downloaded, and no search terms were used to identify datasets. Instead, the analysis was performed by selecting the LUAD tumour-normal comparison within the predefined RNA-seq (TCGA + GTEx) module of the TNMplot V2 platform and entering *BTG1*, *ING4*, *PNRC1* and *CALCOCO1*. According to the TNMplot platform description and reference, RNA-seq data within the platform are processed using a DESeq2-based normalization pipeline for cross-cohort comparison; however, no raw data were downloaded and no

local R, DESeq2 or other statistical software was used in the present study for this analysis. Statistical differences in gene expression between tumour and normal samples were assessed using the non-parametric Mann-Whitney U-test, as implemented by the TNMplot platform, and  $P < 0.05$  was considered statistically significant. Combined signature expression was visualised as a boxplot, whereas the median expression levels of individual genes were illustrated using radar plots comparing normal and tumour tissues.

**TCGA-based clinicopathological and survival analyses.** Clinical, transcriptomic and survival data for TCGA-LUAD were obtained from the UCSC Xena Browser TCGA-LUAD cohort, including the TCGA-LUAD.star\_tpm.tsv.gz, TCGA-LUAD.clinical.tsv.gz and TCGA-LUAD.survival.tsv.gz files (<https://xenabrowser.net/datapages/>). Cases with available gene expression data, OS data and clinicopathological annotations were included. Only primary tumour samples, defined by TCGA sample type code ‘01’, were retained for expression-based analyses. Ensembl gene identifiers were converted to gene symbols using the AnnotationDbi and org.Hs.eg.db Bioconductor packages. A weighted four-gene signature based on *BTG1*, *ING4*, *PNRC1* and *CALCOCO1* was calculated by multiplying the z-score-standardized expression of each gene by its corresponding coefficient from single-gene Cox proportional hazards regression and summing the resulting weighted components across genes. The resulting composite score was then standardized to express the final signature score on a per-standard-deviation scale for downstream Cox regression, subgroup analysis and visualization. This second standardization was applied to the composite weighted score and did not alter the relative ranking of patients. Patients were classified into high- and low-score groups using the cohort-specific median standardized weighted score unless otherwise specified.

OS was assessed by Kaplan-Meier analysis using the log-rank test, and HRs with 95% CIs were estimated using Cox proportional hazards models. Univariate Cox analysis was used to evaluate the weighted score as continuous variable and to assess the prognostic effect of each individual signature gene. Multivariable Cox regression was subsequently performed with adjustments for age and disease-stage groupings. Stage groups were categorized as early [American Joint Committee on Cancer (AJCC) stage I-II] or advanced (AJCC stage III-IV) (<https://training.seer.cancer.gov>) and age subgroups were defined by the cohort-specific median age at diagnosis; the younger age subgroup was defined by patients below the median age, whereas the older subgroup included patients whose age was equal or greater than the median cohort age.

Correlations between the weighted score and continuous clinical variables were evaluated using Spearman's correlation analysis, whereas group comparisons were performed using the Wilcoxon rank-sum test for two-group comparisons or the Kruskal-Wallis test for comparisons involving more than two groups. The Kruskal-Wallis test was used as a global test, and no post hoc pairwise comparisons were performed because no pairwise group differences were interpreted. Subgroup and interaction analyses were conducted according to age group and stage group, and the proportional hazards assumption was

Table II. Kaplan-Meier plotter parameters and selected cut-off values used for survival analyses of the four-gene signature.

| Analysis        | KM<br>plotter module | Dataset type | Endpoint | Disease/histology filter | Subgroup filter                     | Cut-off option          | Selected cut-off<br>value (range) | Follow-up<br>restriction |
|-----------------|----------------------|--------------|----------|--------------------------|-------------------------------------|-------------------------|-----------------------------------|--------------------------|
| Primary OS      | Lung cancer          | mRNA         | OS       | LUAD/adenocarcinoma      | None                                | Auto select best cutoff | 694                               | 180 months               |
| Primary PPS     | Lung cancer          | mRNA         | PPS      | LUAD/adenocarcinoma      | None                                | Auto select best cutoff | 758.4                             | 180 months               |
| Primary FP      | Lung cancer          | mRNA         | FP       | LUAD/adenocarcinoma      | None                                | Auto select best cutoff | 997.2                             | 180 months               |
| Male subgroup   | Lung cancer          | mRNA         | OS       | LUAD/adenocarcinoma      | Male                                | Auto select best cutoff | 1469.5                            | 180 months               |
| Female subgroup | Lung cancer          | mRNA         | OS       | LUAD/adenocarcinoma      | Female                              | Auto select best cutoff | 1481.5                            | 180 months               |
| Smoker subgroup | Lung cancer          | mRNA         | OS       | LUAD/adenocarcinoma      | Smokers; never-<br>smokers excluded | Auto select best cutoff | 1469.5                            | 240 months               |
| Stage I         | Lung cancer          | mRNA         | OS       | LUAD/adenocarcinoma      | Stage I                             | Auto select best cutoff | 1550.5                            | 240 months               |
| Stage II        | Lung cancer          | mRNA         | OS       | LUAD/adenocarcinoma      | Stage II                            | Auto select best cutoff | 2208                              | 240 months               |
| Stage III       | Lung cancer          | mRNA         | OS       | LUAD/adenocarcinoma      | Stage III                           | Auto select best cutoff | 2430.25                           | 240 months               |

The table summarizes the Kaplan-Meier plotter settings used for the primary and subgroup survival analyses of the four-gene signature. All analyses were performed using the lung cancer mRNA module with the LUAD/adenocarcinoma histology filter. Patients were stratified into high- and low-expression groups using the 'auto select best cutoff' option implemented in the Kaplan-Meier plotter platform. The selected cut-off values represent the platform-derived thresholds for the combined expression score of BTG1, ING4, PNRC1 and CALCO1. Follow-up restriction indicates the maximum follow-up time included in each analysis. For the smoker subgroup analysis, never-smokers were excluded according to the subgroup filter available in the platform. OS, overall survival; PPS, post-progression survival; FP, first progression; LUAD, lung adenocarcinoma; mRNA, messenger RNA.

assessed using time-varying coefficient analysis. Unless otherwise specified,  $P < 0.05$  was considered statistically significant. All TCGA-based analyses were performed in R (version 4.6.0; R Foundation for Statistical Computing) using the following packages; data.table (1.18.4), dplyr (1.2.1), tidyr (1.3.2), stringr (1.6.0), ggplot2 (4.0.3), survival (3.8.6), survminer (0.5.2), broom (1.0.13), forcats (1.0.1), AnnotationDbi (1.74.0) and org.Hs.eg.db (3.23.1).

**Reagents.** DMEM (cat. no. 12100046) and RPMI-1640 medium (cat. no. 31800022) were purchased from Thermo Fisher Scientific, Inc. Foetal bovine serum was obtained from PAN-Biotech GmbH (cat. no. P30-3302), and penicillin-streptomycin solution (100X) was obtained from Bio-Channel/Nanjing Shenghang Biotechnology Co., Ltd. (cat. no. BC-CE-007). Trypsin was purchased from Thermo Fisher Scientific, Inc. (cat. no. 27250018). Propidium iodide was obtained from Beijing Solarbio Science & Technology Co., Ltd. (cat. no. P8080), Calcein acetoxymethyl ester (Calcein-AM) was obtained from Shanghai Macklin Biochemical Co., Ltd. (cat. no. C794029), and DCFH-DA was obtained from MedChemExpress (cat. no. HY-D0940). Z-DEVD-FMK was purchased from MedChemExpress (cat. no. HY-12466). Securinine (cat. no. S861357), 6-diazo-5-oxo-L-norleucine (cat. no. D915139; CAS no. 157-03-9) and clofazimine (cat. no. C830484) were obtained from Shanghai Macklin Biochemical Co., Ltd. ZLN005 (cat. no. Z170591), propranolol (cat. no. P612948) and triamcinolone acetonide (cat. no. T101294) were obtained from Shanghai Aladdin Biochemical Technology Co., Ltd. Adapalene (cat. no. BD17446), tandutinib (cat. no. BD152374), nitisinone (cat. no. BD61457) and phenazone (cat. no. BD01397972) were obtained from Shanghai Bide Pharmatech Co., Ltd. Piretanide was obtained from TargetMol Chemicals Inc. (cat. no. T19788).

**Cell culture.** The human PC-9 (cat. no. SCSP-5085) and HCC827 (cat. no. TCHu153) cell lines, which contain deletions in *EGFR* exon 19 (E746-A750del and E746-A750del, respectively), were obtained from the Cell Bank of the Chinese Academy of Sciences (<http://www.cellbank.org.cn>). Cells were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% foetal bovine serum (PAN-Biotech GmbH), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin at 37°C in a humidified incubator with 5% CO<sub>2</sub>. All cell lines were authenticated by short tandem repeat profiling and routinely tested negative for mycoplasma contamination. Cells were used within 20 passages after thawing to ensure genomic stability and reproducibility.

**Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.** Parental PC-9 cells or PC-9 Tet-on-sh*CALCOCO1* cells were cultured and lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.4) and 1% SDS. Lysates were placed on an ice-water mixture and sonicated at 20% power using cycles of 2 sec on and 4 sec off for a total sonication time of 2 min to ensure complete protein solubilization. Cell debris was removed by centrifugation at 17,000  $\times$  g for 15 min at room temperature. Protein concentrations were determined using a Bicinchoninic Acid protein kit (BCA, cat. no. WB6501, NCM Biotech) and the resulting supernatants

(~2 mg/ml total protein) were collected for analysis. Equal amounts of protein (50  $\mu$ g per line) were mixed with 6X SDS sample buffer, boiled at 95°C for 10 min and separated by SDS-PAGE on gels of 10% acrylamide concentration. Proteins were then transferred onto PVDF membranes using a wet transfer system operated at 100 V for 1 h at 4°C. Membranes were blocked with 5% bovine serum albumin (BSA, cat. no. abs9157; Absin Bioscience Inc.) in PBS containing 0.1% Tween-20 (PBST) for 1 h at room temperature to prevent non-specific binding. Blots were subsequently incubated overnight at 4°C with primary antibodies diluted in PBST with 5% BSA. After washing three times with PBST for 10 min each, membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Excess antibodies were removed by three additional PBST washes for 10 min each. Protein bands were visualised using the SuperSignal West Pico PLUS Chemiluminescent Substrate (cat. no. 34578; Thermo Fisher Scientific, Inc.). GAPDH served as the internal loading control to ensure equal protein loading.

Antibodies and dilutions used in the present study were as follows; GAPDH (1:100,000; cat. no. AC033; ABclonal Biotech Co., Ltd.), goat anti-rabbit HRP (1:5,000; cat. no. 31460; Thermo Fisher Scientific, Inc.), goat anti-mouse HRP (1:5,000; cat. no. AS003; ABclonal Biotech Co., Ltd.), poly(ADP-ribose) polymerase (PARP; 1:1,000; cat. no. 9532S; Cell Signaling Technology, Inc., recognizing full-length PARP1 and cleaved PARP1), gasdermin D (GSDMD; 1:1,000; cat. no. 39754S; Cell Signaling Technology, Inc.), GSDME (1:1,000; cat. no. F1701; Selleck Chemicals, recognizing full-length GSDME and the N-terminal cleavage fragment), caspase-3 (1:1,000; cat. no. 9662S; Cell Signaling Technology, Inc., recognizing full-length and cleaved caspase-3) and caspase-8 (1:1,000; cat. no. HA722181; HUABIO, recognizing full-length and cleaved caspase-8).

**Phase-contrast microscopy.** Cells were treated with osimertinib, the indicated candidate compounds or their combinations as described for the corresponding cell viability experiments. After treatment, cell morphology was examined using an inverted phase-contrast microscope (Olympus CKX53; Olympus Corporation), and representative images were captured to assess morphological changes, including cell detachment, rounding and membrane rupture.

**Propidium iodide (PI) and calcein acetoxymethyl ester (calcein-AM) staining.** PC-9 cells were seeded in 6-well plates at an initial density of 20-30% confluency 1 day before drug treatment. Cells were then treated for 48 or 72 h with osimertinib (0.5  $\mu$ M; cat. no. HY-15772; MedChemExpress), ZLN005 (5, 10 or 20  $\mu$ M; cat. no. Z170591; Shanghai Aladdin Biochemical Technology Co., Ltd.), propranolol (10  $\mu$ M; cat. no. P612948; Shanghai Aladdin Biochemical Technology Co., Ltd.), tandutinib (5  $\mu$ M; cat. no. BD152374; Shanghai Bide Pharmatech Co., Ltd.), nitisinone (10  $\mu$ M; cat. no. BD61457; Shanghai Bide Pharmatech Co., Ltd.) and phenazone (10  $\mu$ M; cat. no. BD01397972; Shanghai Bide Pharmatech Co., Ltd.). Following treatment, cells were maintained at 37°C in a humidified incubator with 5% CO<sub>2</sub> for the indicated time periods. At each designated time point, both the culture supernatant and adherent cells were collected to ensure the inclusion

of floating dead cells. The cell suspension was centrifuged at 450 x g for 5 min at room temperature, and the resulting pellet was gently resuspended in PBS containing PI (4  $\mu$ g/ml) and calcein-AM (1  $\mu$ g/ml). The suspension was then returned to the corresponding well and incubated at 37°C for 30 min in the dark. After this staining step, cells were washed once with PBS to remove excess dye and immediately visualized using a fluorescence microscope. Calcein-AM selectively labelled viable cells with green fluorescent signals, whereas PI was used to stain membrane-compromised or dead cells with red fluorescence.

**Flow cytometry for PI staining of cells.** For quantitative assessment of cell death, PC-9 cells were treated with vehicle control (0.1% (v/v) DMSO), osimertinib (0.5  $\mu$ M; cat. no. HY-15772; MedChemExpress), or osimertinib in combination with ZLN005 (5  $\mu$ M; cat. no. Z170591; Shanghai Aladdin Biochemical Technology Co., Ltd.), propranolol (10  $\mu$ M; cat. no. P612948; Shanghai Aladdin Biochemical Technology Co., Ltd.), tandutinib (5  $\mu$ M; cat. no. BD152374; Shanghai Bide Pharmatech Co., Ltd.), nitisinone (10  $\mu$ M; cat. no. BD61457; Shanghai Bide Pharmatech Co., Ltd.) or phenazone (10  $\mu$ M; cat. no. BD01397972; Shanghai Bide Pharmatech Co., Ltd.) for 72 h at 37°C in a humidified incubator with 5% CO<sub>2</sub>. After treatment, both adherent and floating cells were collected, washed twice with PBS and resuspended in 500  $\mu$ l PBS containing 2  $\mu$ g/ml PI. Cells were incubated at room temperature for 30 min in the dark to allow for dye uptake. Fluorescence intensity was measured using a flow cytometer (BD FACSCanto II; BD Biosciences), with PI-positive cells representing membrane-compromised or pyroptotic populations. Data acquisition and analysis were performed using FlowJo software (version 10.8.1; BD Biosciences).

**Flow cytometry for reactive oxygen species (ROS).** PC-9 cells were seeded in 6-well plates at ~25% confluency and cultured for 24 h at 37°C in a humidified incubator with 5% CO<sub>2</sub>. The cells were then treated with DMSO (vehicle control), 0.5  $\mu$ M osimertinib, 10  $\mu$ M ZLN005 or a combination of 0.5  $\mu$ M osimertinib and 10  $\mu$ M ZLN005 for 5 h at 37°C in a humidified incubator with 5% CO<sub>2</sub>. After treatment, cells were harvested by trypsinization 1 min at 37°C in a humidified incubator with 5% CO<sub>2</sub> and the collected cells incubated with 2  $\mu$ M 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), a cell-permeable probe used to detect intracellular ROS, at room temperature for 30 min in the dark. The cell suspension was subsequently filtered through a 300-mesh nylon membrane to remove aggregates prior to analysis. Intracellular ROS levels were determined using a flow cytometer (BD FACSCanto™ II; BD Biosciences), and data were processed using FlowJo software (version 10.8.1; BD Biosciences).

**Lactate dehydrogenase (LDH) cytotoxicity assay.** Cell death was evaluated using an LDH Cytotoxicity Detection Kit (cat. no. C0017; Beyotime Biotechnology) according to the manufacturer's instructions. Briefly, cells were seeded in 12-well plates and pre-incubated with or without the caspase-3 inhibitor Z-DEVD-FMK (50  $\mu$ M; cat. no. HY-12466; MedChemExpress) for 1 h, followed by potential co-treatment with ZLN005 (10  $\mu$ M; cat. no. Z170591; Shanghai Aladdin

Biochemical Technology Co., Ltd.) and osimertinib (0.5  $\mu$ M; cat. no. HY-15772; MedChemExpress) for 48 h at 37°C in a humidified incubator with 5% CO<sub>2</sub>. At the end of the treatment period, the culture plates were centrifuged at 400 x g for 5 min at 4°C to pellet cell debris. The supernatant was then collected, and 120  $\mu$ l from each sample was transferred into a fresh 96-well plate. After incubation with the LDH reaction working solution for 30 min at room temperature in the dark, the absorbance was measured at 490 nm using a microplate reader (Cytation 3 Cell Imaging Multi-Mode Reader; BioTek Instruments; Agilent Technologies, Inc.). The percentage of LDH release in each treatment group was calculated as a measure of cell death rate using the following formula; LDH release (%)=[optical density (OD)<sub>490</sub> of sample-medium blank]/(OD<sub>490</sub> of maximum enzyme activity-medium blank) x100.

**Tetracycline (Tet)-on inducible short hairpin RNA (shRNA) knockdown.** 293T cells (obtained from the National Collection of Authenticated Cell Cultures) were used for lentiviral packaging. Cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified incubator with 5% CO<sub>2</sub>. A second-generation lentiviral packaging system was used. 293T cells were co-transfected with Tet-inducible shRNA or sh-negative control (NC) plasmids (Tet-pLKO-puro; cat. no. 21915; Addgene, Inc.), the packaging plasmid psPAX2 (cat. no. 12260; Addgene, Inc.), and the envelope plasmid VSV-G (cat. no. 14888; Addgene, Inc.) using polyethyleneimine (PEI; cat. no. 24765-1; Polysciences, Inc.) according to the manufacturer's protocol. For each 6-cm dish, a total of 5  $\mu$ g plasmid DNA was used at a shRNA plasmid; psPAX2;pMD2.G ratio of 5:3:1. Transfected cells were maintained at 37°C in a 5% CO<sub>2</sub> incubator, and viral supernatants were collected 48 h after transfection. The supernatants were clarified by centrifugation at 500 x g for 10 min at 4°C and filtered through a 0.45- $\mu$ m membrane filter before use.

PC-9 cells were transduced with lentiviral particles encoding Tet-inducible shRNA constructs targeting human *ING4*, *BTG1*, *PNRC1* or *CALCOCO1*. The shRNA oligonucleotide sequences were as follows; *CALCOCO1*, 5'-CAGGAG AACCATCACTTAAAT-3'; *PNRC1*, 5'-CATACTTGAGAG GTATATTAT-3'; *BTG1*, 5'-GGAGCTGCTGGCAGAACATTA-3'; *ING4*, 5'-GCTAGGTGTGATCAACACTTT-3'; and control, 5'-CAACAAGATGAAGAGCACCAA-3'. Following lentiviral transduction at a multiplicity of infection (MOI) of 5 in the presence of 8  $\mu$ g/ml polybrene (cat. no. HY-112735; MedChemExpress) at 37°C for 24 h. Following transduction, cells were allowed to recover in fresh medium for 24 h before the addition of puromycin. Stably transduced PC-9 cells were selected with puromycin (1  $\mu$ g/ml) for 7 days. To induce shRNA expression, cells were treated with doxycycline (1  $\mu$ g/ml) for 48 h at 37°C in a humidified incubator with 5% CO<sub>2</sub>, whereas cells cultured in the absence of doxycycline served as the uninduced control. Doxycycline was added for 48 h to induce shRNA expression before all functional assays. The total time from the start of lentiviral transduction to these assays was approximately 10-12 days, which includes the transduction, recovery, puromycin selection and doxycycline induction periods.



**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was isolated and purified using a custom guanidinium isothiocyanate-silica column extraction method developed in-house. Briefly, cells (PC-9 or HCC827) were lysed in guanidinium isothiocyanate buffer (cat. no. G110926; Shanghai Aladdin Biochemical Technology Co., Ltd.; final concentration, 4M), followed by RNA adsorption onto a silicon-based spin column at room temperature for 1 min, sequential washing to remove contaminants and elution in RNase-free water. The purity and concentration of RNA were determined spectrophotometrically (NanoDrop 2000, Thermo Fisher Scientific, Inc.) [absorbance ( $A_{260}/A_{280}$  and  $A_{260}/A_{230}$  ratios >2.0]. Complementary DNA (cDNA) was synthesised from 1  $\mu$ g total RNA using the HiScript<sup>®</sup> II 1st Strand cDNA Synthesis Kit with gDNA Wiper (cat. no. R212; Vazyme Biotech Co., Ltd.) to eliminate genomic DNA contamination. Reverse transcription was performed according to the manufacturer's recommended protocol. qPCR was performed using the ChamQ Blue Universal SYBR qPCR Master Mix (cat. no. Q312; Vazyme Biotech Co., Ltd.) on a StepOnePlus<sup>™</sup> Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows; initial denaturation at 95°C for 30 sec; 40 cycles of 95°C for 10 sec and 60°C for 30 sec; followed by a dissociation stage (95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec) for melting curve analysis. The relative expression levels of target genes were calculated using the  $2^{-\Delta\Delta C_q}$  method (38), with  $\beta$ -actin serving as the internal normalization control. Each reaction was performed in triplicate, and data were obtained from three independent biological replicates. Results are presented as mean  $\pm$  SD. Primer specificity was experimentally verified via melting curve analysis, which demonstrated a single amplification peak for each primer under the assay conditions. Primer sequences used for qPCR were as follows;  $\beta$ -actin, forward 5'-CACCATTGGCAATGAGCGGTTC-3', reverse 5'-AGG TCTTTGCGGATGTCCACGT-3'; BTG1, forward 5'-CAT CTCCAAGTTTCTCCGCACC-3', reverse 5'-GCGAATACA ACGGTAACCCGATC-3'; CALCOCO1, forward 5'-TGCTGA CGAAGGAAGTGGAGCT-3', reverse 5'-TCCTGTTGTGCC ACTTGGAGCT-3'; ING4, forward 5'-CAGGAAGCCTAT GGCAAGTGCA-3', reverse 5'-TGAGATCAGCCTCAAAAC GGGC-3'; and PNRC1, forward 5'-GCCAAACCCCTCA GGAAAG-3', reverse 5'-GTGTATACCATGAACAAGCTG GC-3'.

**Senescence-associated  $\beta$ -galactosidase ( $\beta$ -gal) staining.** To evaluate the DTP-like state, senescence-associated  $\beta$ -gal staining was performed using a Cellular Senescence  $\beta$ -Galactosidase Staining Kit (cat. no. C0602; Beyotime Biotechnology) according to the manufacturer's instructions. PC-9 cells with stable knockdown of *ING4*, *BTG1*, *PNRC1* or *CALCOCO1* were seeded in 6-well plates and treated with DMSO or 1 or 2  $\mu$ M osimertinib for 72 h at 37°C in a humidified incubator with 5% CO<sub>2</sub>. The culture medium was then aspirated, and cells were washed once with PBS. Cells were subsequently fixed with 1 ml fixation solution (provided with the kit; no further dilution required) per well at room temperature for 15 min, followed by three washes with PBS for 3 min each. After complete removal of the washing buffer, 1 ml freshly prepared staining working solution was added to each

well, and cells were incubated overnight at 37°C in a CO<sub>2</sub>-free environment. Following staining, the working solution was aspirated and cells were rinsed with 2 ml PBS. Representative images were then acquired via light microscopy (Olympus CKX53, Olympus Corporation).

**Drug synergy assay.** A total of 10,000 PC-9 cells were seeded per well in a 96-well plate, arranged in six rows and 10 columns. PBS was added to the outermost wells to reduce edge effects. After 20–24 h, cells were treated with osimertinib and co-treated with DMSO, ZLN005, propranolol, tandutinib, nitisinone or phenazone at 37°C in a humidified incubator with 5% CO<sub>2</sub>. Osimertinib was serially diluted from the highest concentration (a top concentration of 30  $\mu$ M in combination with ZLN005, propranolol, tandutinib or nitisinone, or a top concentration of 10  $\mu$ M in combination with phenazone) to 0  $\mu$ M from right to left using a 1:3 dilution ratio, whereas the aforementioned drugs used for co-treatment were serially diluted from 30 to 0  $\mu$ M from bottom to top using a 1:5 dilution ratio. DMSO was used as the vehicle control, and the final concentration of DMSO in the culture medium was maintained at 0.1% (v/v) in all experimental conditions. After 48 h of treatment, MTT assays were performed at a single timepoint and the resulting purple formazan crystals were dissolved in DMSO and OD values at 595 nm were measured using a microplate reader (Cytation 3 Cell Imaging Multi-Mode Reader; BioTek Instruments; Agilent Technologies, Inc.). Cell viability values were normalized to vehicle control wells, and synergy scores were calculated using the Bliss independence model implemented in SynergyFinder (<https://synergyfinder.org/>) (39). Drug concentrations used in mechanistic and validation experiments were selected based on preliminary dose-response and synergy-optimization analyses, aiming to preserve robust combination activity while minimizing excessive single-agent cytotoxicity.

**Statistical analysis.** Data are presented as the mean  $\pm$  SD of at least three independent biological replicates. For comparisons among multiple groups involving one independent variable, one-way ANOVA followed by Tukey's multiple-comparisons test was used. For experiments involving two independent variables, two-way ANOVA was performed, followed by Tukey's multiple-comparisons test.  $P < 0.05$  was considered to indicate a statistically significant difference. All statistical analyses were performed using GraphPad Prism software (version 10.4.1; Dotmatics).

## Results

**Machine-learning identification of a core gene signature associated with the DTP state.** To delineate the molecular basis of the DTP phenotype, a multi-algorithm machine-learning analysis was conducted using publicly available RNA-seq datasets of TKI-treated tumour cells (Table I). Principal-component analysis of the integrated DTP vs. control log<sub>2</sub>(fold-change) matrix showed that independent DTP models were distributed according to similarities in their global transcriptional responses across multiple types of cancer and treatment contexts (Fig. 1A). Differential-expression analysis identified numerous upregulated genes in DTP cells [ $\log_2$ (fold-change) > 2;

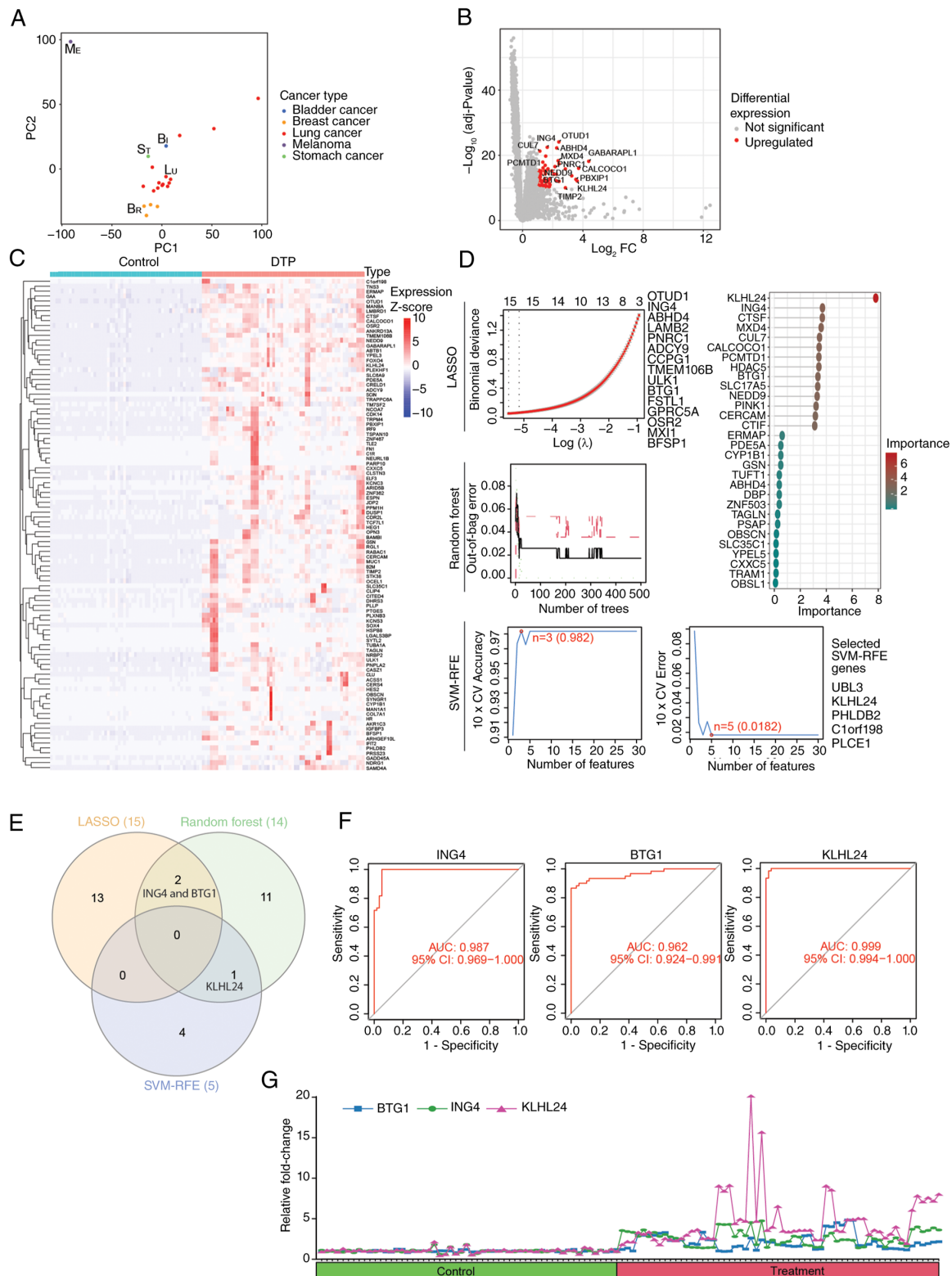


Figure 1. Machine-learning identification of a gene signature associated with the DTP state. (A) Principal-component analysis of control and TKI-treated tumour samples demonstrated distinct clustering of DTP cells, indicating a conserved transcriptional reprogramming pattern. (B) Volcano plot of differential gene expression [ $\log_2$ (fold-change) vs.  $\log_{10}$ (adj.P-value)] showing significantly upregulated genes (*BTG1*, *ING4*, *CALCOCO1* and *PNRC1*) in DTP cells compared with control cells. (C) Heatmap of the expression of candidate genes across datasets revealed consistent upregulation in DTP samples. (D) Feature-selection curves from LASSO, random forest and SVM-RFE analyses showing variable-importance scores and cross-validation error. (E) Venn diagram illustrating overlap among the three algorithms, identifying *BTG1*, *ING4* and *KLHL24* as core genes common to all approaches. (F) Receiver-operating characteristic curves evaluating the predictive performance of key genes identified in the integrated model (AUC, 0.96-0.99) for identifying DTP cells. (G) Cross-dataset comparative expression analysis showing consistent upregulation of the core genes in TKI-treated/DTP samples relative to the corresponding DMSO/control samples after within-dataset normalization. DTP, drug-tolerant persister; PC1, principal component 1; PC2, principal component 2; LogFC, logarithmic fold change; LASSO, least absolute shrinkage and selection operator; SVM-RFE, support vector machine-recursive feature elimination; AUC, area under the curve; TKI, tyrosine kinase inhibitor; adj.P-value, adjusted P-value; *BTG1*, B-cell translocation gene 1; *ING4*, inhibitor of growth family member 4; *CALCOCO1*, calcium-binding and coiled-coil domain-containing protein 1; *PNRC1*, proline-rich nuclear receptor coactivator 1; *KLHL24*, Kelch-like protein 24.

$P < 1 \times 10^{-10}$ ]; several genes emerged as consistently elevated across datasets (Fig. 1B). Heatmap visualization demonstrated the consistent upregulation of these genes across independent cohorts (Fig. 1C), defining a reproducible transcriptional signature of the TKI-induced persister state.

To assess the reproducibility of these transcriptomic features across datasets, the present study performed independent integrated pathway enrichment analyses. Individual dataset-level enrichment analyses revealed conserved patterns of metabolic quiescence, oxidative-stress adaptation and transcriptional reprogramming (Fig. S1A-C), whereas a combined meta-analysis of all DTP datasets identified a core set of oxidative-phosphorylation and stress-response pathways shared across DTP models (Fig. S2A-D). These results validated the robustness of the dataset integration strategy used in the present study and provided a mechanistic framework for subsequent machine-learning-based feature selection.

Subsequently, three complementary machine-learning algorithms, LASSO, random forest and SVM-RFE, were applied to identify the most discriminative genes associated with the DTP state (Fig. 1D). Each method produced a ranked gene list, and the intersection of the three models yielded a consensus subset comprising *BTG1*, *ING4* and Kelch-like protein 24 (*KLHL24*) (Fig. 1E). These genes showed the highest feature-importance scores and minimal cross-validation errors, indicating a strong predictive power for distinguishing DTP cells from control cells. ROC curve analysis supported the notable diagnostic accuracy of these genes, with AUC values ranging from 0.96 to 0.99 (Fig. 1F).

Lastly, comparative expression analysis showed that these genes were markedly and consistently upregulated under DTP vs. control conditions across multiple datasets, reinforcing the reproducibility and potential relevance of these findings (Fig. 1G). Taken together, these results identified a set of robust candidate genes associated with the TKI-induced persister phenotype, providing a computational foundation for defining a refined gene signature in subsequent analyses.

*Identified DTP gene signature is persistently upregulated during the formation and maintenance of DTP cells.* To determine the temporal dynamics of the candidate genes identified by the machine-learning pipeline in the present study, publicly available time-course transcriptomic data from GSE193258 were analysed throughout the course of DTP cell formation and recovery. This dataset included four EGFR mutant or TKI-responsive LUAD cell lines (H1975, HCC827, HCC2935 and PC-9), with samples annotated according to the following sequential phases; Untreated control, acute TKI exposure, established DTP state and short and long drug-washout periods.

Heatmap visualisation revealed that the genes identified by the three machine-learning algorithms (LASSO, random forest and SVM-RFE) were rapidly and co-ordinately upregulated upon acute TKI treatment; this high expression was maintained during the DTP stage and remained partially elevated even after drug removal (Fig. 2A). This pattern was reproducible across all four cell lines, indicating that these genes participated in a conserved stress-adaptive transcriptional program rather than a transient response.

Quantitative expression profiling further supported the persistent induction of *BTG1*, *ING4*, *CALCOCO1*, *PNRC1* and several other top-ranked genes in DTP cell formation across cell lines (Fig. 2B). Notably, the expression of these genes was induced during acute TKI exposure and/or the established DTP state, and declined gradually upon long-term drug withdrawal, suggesting their involvement in the formation and maintenance of the persister phenotype. Collectively, these results demonstrated that the transcriptional signature identified by the multi-algorithm approach in the present study was not only predictive of DTP identity but also dynamically engaged in the establishment and maintenance of TKI tolerance.

*A four-transcription-factor signature defines the core transcriptional program of DTP cells.* To refine the DTP-associated gene set and identify key regulatory components, the present study integrated results from multiple computational analyses. Differential gene-expression profiling of DTP and control samples revealed a distinct DTP-associated transcriptional landscape, with a subset of genes showing notable and consistent upregulation across datasets (Fig. 3A). By intersecting three analytical approaches, including machine-learning analyses (LASSO, random forest and SVM-RFE), individual-group differential analyses and  $\log_2$ (fold-change) normalization, the present study obtained a consensus set of 10 genes (Fig. 3B). For functional annotation and subsequent signature refinement, these 10 consensus genes were analysed together with *BTG1*, which was retained as a machine-learning-prioritized gene supported by the preceding expression analyses, resulting in 11 prioritized genes shown in Fig. 3C. Functional annotation clustered these genes into three biological categories; i) Protein stability and post-translational modification, including *KLHL24*, OTU domain-containing protein 1 and cathepsin F; ii) signalling regulation, such as neural precursor cell expressed developmentally downregulated protein 9 and follistatin-related protein 1; and iii) transcriptional control, for example *BTG1*, *ING4*, *PNRC1* and *CALCOCO1* (Fig. 3C).

Among these genes, four transcription factors, *BTG1*, *ING4*, *PNRC1* and *CALCOCO1*, emerged as central and reproducible regulators of the DTP state, as these genes were consistently enriched across multiple datasets and exhibited the highest discriminative power for the DTP phenotype. ROC curve analysis supported the strong predictive performance of this four-gene signature, with AUC values ranging from 0.96 to 0.99 (Fig. 3D).

Subsequently, the present study validated the expression of these four genes in DTP cells using multiple independent datasets from TKI-treated cell lines. In LUAD models (HCC2935, HCC827, H1975 and PC-9 cells) exposed to osimertinib, the expression of *BTG1*, *ING4*, *PNRC1* and *CALCOCO1* was rapidly induced during acute drug exposure and remained notably elevated during the DTP and short-washout phases (Fig. 3E). Consistent upregulation of these genes was also observed in other cancer types treated with different TKIs, including gefitinib in PC-9 cells and dabrafenib in the SKMEL28 melanoma cell line (Fig. 3F). This expression pattern was further examined in NCI-H358 cells treated with the KRAS G12C inhibitor ARS-1620 or the MEK inhibitor trametinib, both of which target the RTK/RAS/MAPK signalling axis (Fig. 3G). In an additional dataset involving prolonged treatment of H23 and H358



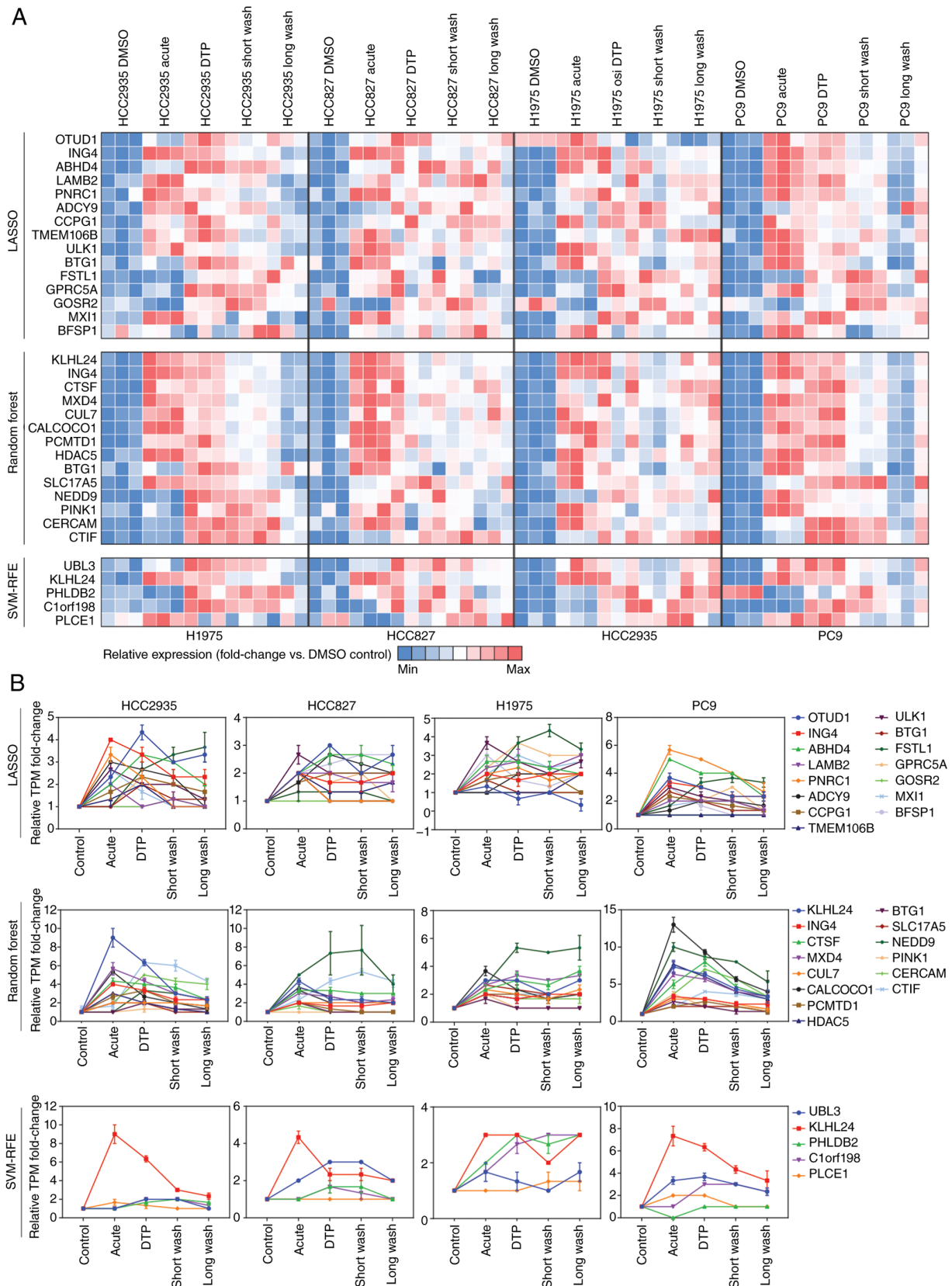
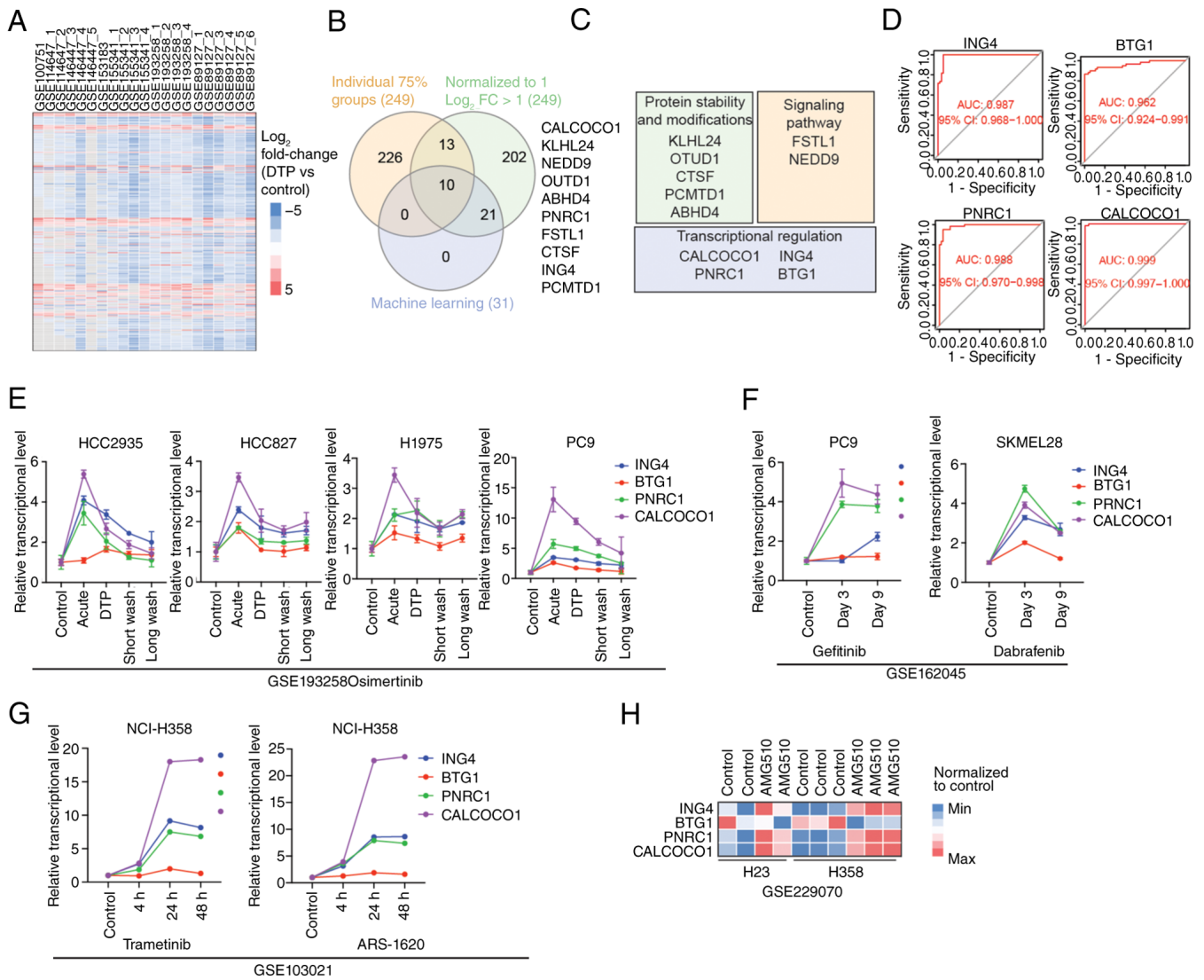


Figure 2. Identified DTP gene signature is persistently upregulated during the development and maintenance of DTP cells. (A) Heatmaps showing the expression dynamics of candidate genes identified by LASSO, random forest and SVM-RFE analyses across four lung adenocarcinoma cell lines (H1975, HCC827, HCC2935 and PC-9). Samples were collected at five stages of DTP cell formation and recovery; Control (DMSO), acute TKI treatment, established DTP state and short- and long-washout phases. Red indicates higher gene expression whereas blue indicates lower expression (scaled TPM). (B) Line-plot representation of gene-expression trajectories for top-ranked genes from each machine-learning algorithm. Genes such as *BTG1*, *ING4*, *KLHL24* and *CALCOCO1* exhibited rapid upregulation upon TKI exposure and a gradual decrease in expression throughout the DTP and washout stages. Data represent mean  $\pm$  SD from three biological replicates. DTP, drug-tolerant persister; LASSO, least absolute shrinkage and selection operator; SVM-RFE, support vector machine-recursive feature elimination; BTG1, B-cell translocation gene 1; ING4, inhibitor of growth family member 4; CALCOCO1, calcium-binding and coiled-coil domain-containing protein 1; KLHL24, Kelch-like protein 24; TKI, tyrosine kinase inhibitor; TPM, transcripts per million. SD, standard deviation.

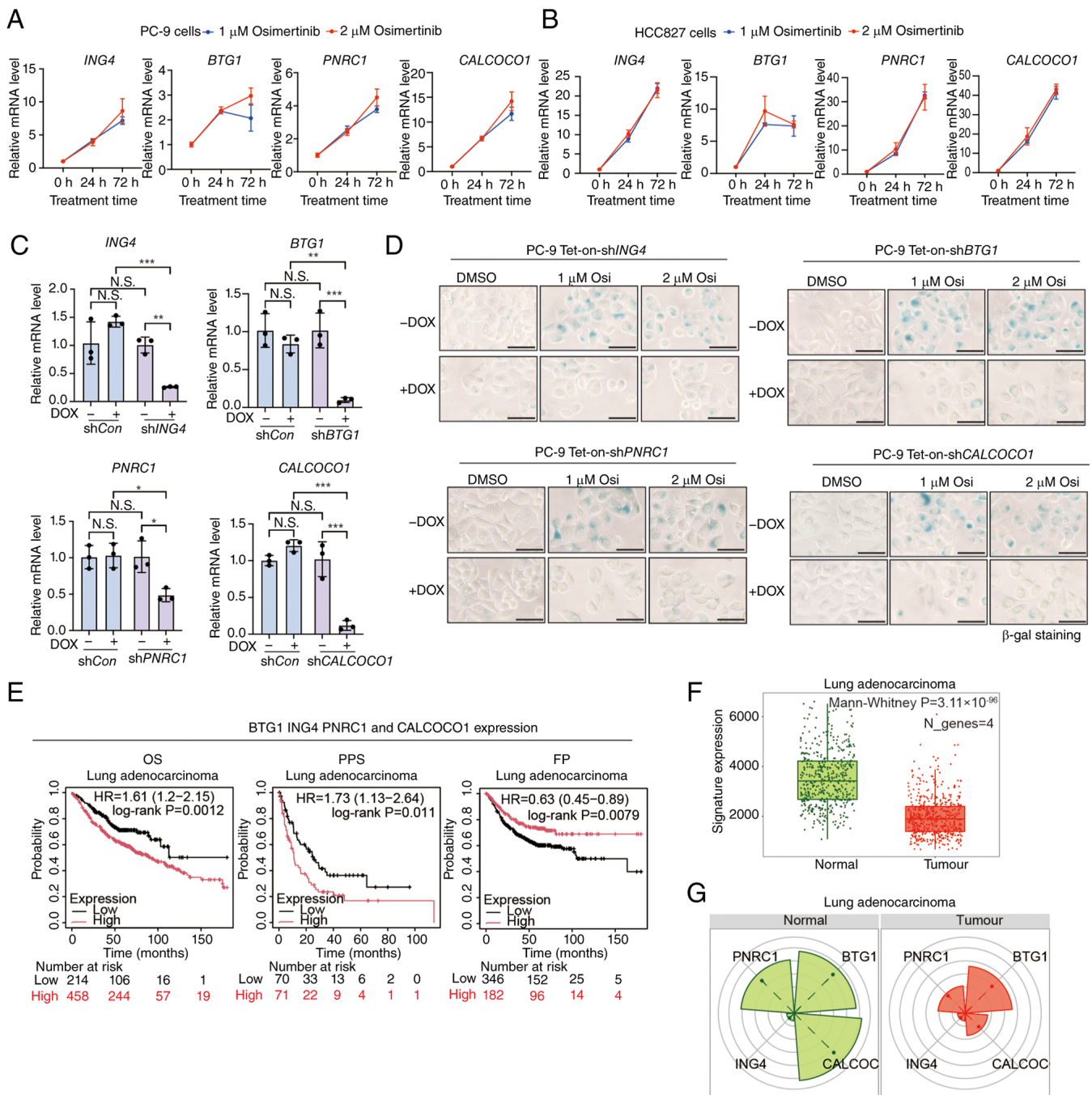


**Figure 3.** A four-transcription-factor signature defines the core transcriptional program of DTP cells. (A) Heatmap showing differential gene expression ( $\log_2$  fold-change) between DTP and control samples across datasets, highlighting persistently upregulated genes. (B) Venn diagram integrating three analytical strategies (machine-learning analysis, individual-group analysis and normalised fold-change filtering) to identify a 10-gene consensus set. (C) Functional classification of the 11 prioritized genes, including the 10-gene consensus set and BTG1, into three modules; i) Protein stability and post-translational modification; ii) signalling pathways; and iii) transcriptional regulation. (D) Receiver-operating characteristic curves demonstrating high predictive accuracy for BTG1, ING4, PNRC1 and CALCOCO1 for the DTP state. (E) Validation of differential DTP-signature gene expression at different stages of drug exposure in four lung adenocarcinoma cell lines (HCC2935, HCC827, H1975 and PC-9) treated with osimertinib, showing notable upregulation of signature genes during DTP formation and washout phases. (F and G) Independent datasets showing similar trends of transcriptional induction of the four-gene signature in cells treated with gefitinib, dabrafenib, trametinib or ARS-1620, indicating a conserved TKI-induced response. (H) Heatmap validation of signature gene expression across additional TKI-treated cell lines (H23 and H358). Data represent mean  $\pm$  SD of biological replicates. DTP, drug-tolerant persister; SD, standard deviation. ING4, inhibitor of growth family member 4; CALCOCO1, calcium-binding and coiled-coil domain-containing protein 1; PNRC1, proline-rich nuclear receptor coactivator 1.

cells with the KRAS G12C mutation inhibitor AMG510, ING4, PNRC1 and CALCOCO1 showed a similar induction pattern, whereas BTG1 was reduced, indicating context-dependent regulation of individual genes within the four-gene signature (Fig. 3H). Collectively, these results indicated that BTG1, ING4, PNRC1 and CALCOCO1 were conserved transcriptional signatures that defined the TKI-induced persister state, functioning as coordinated regulators that sustained cellular survival under targeted therapy.

**Functional validation of the four-gene signature in mediating the osimertinib-induced DTP state.** To validate the expression of the four transcription factors (BTG1, ING4, PNRC1

and CALCOCO1) identified as the core DTP signature, the present study examined their transcriptional response to the third-generation EGFR-TKI osimertinib in two EGFR-mutant LUAD cell lines (PC-9 and HCC827). RT-qPCR analysis revealed that all four genes were rapidly and robustly upregulated following osimertinib treatment in a time-dependent manner (Fig. 4A and B). The expression levels of these genes increased markedly within 24 h and remained elevated at 72 h under both 1 and 2  $\mu$ M osimertinib concentrations, and demonstrated a dose-independent but sustained activation pattern. These results were consistent with the computational prediction that these genes acted as early responders and transcriptional regulators in the DTP program.



**Figure 4.** Four-gene signature is upregulated in osimertinib-treated lung adenocarcinoma cells and associates with poor prognosis. (A) RT-qPCR analysis of *ING4*, *BTG1*, *PNRC1* and *CALCOCO1* expression in PC-9 cells treated with 1 or 2  $\mu$ M osimertinib for 24 and 72 h. (B) RT-qPCR validation in HCC827 cells treated with 1 or 2  $\mu$ M osimertinib for 24 and 72 h, showing consistent upregulation of all four genes. (C) RT-qPCR analysis of *ING4*, *BTG1*, *PNRC1* and *CALCOCO1* mRNA levels following doxycycline-inducible Tet-on knockdown in PC-9 cells. Inducible non-targeting shCon cells with and without doxycycline treatment were included as controls. Doxycycline-induced expression of the corresponding short hairpin RNA sequences resulted in efficient target-gene knockdown, whereas doxycycline treatment alone did not significantly alter gene expression in shCon cells. Statistical significance was determined by two-way ANOVA followed by Tukey's multiple-comparisons test. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . N.S., Not significant. (D) Senescence-associated  $\beta$ -gal staining of PC-9 cells after knockdown of DTP-signature genes followed by osimertinib treatment for 72 h.  $\beta$ -gal-positive cells were characterised as DTP cells. Scale bar, 50  $\mu$ m. (E) Kaplan-Meier survival analysis of patients with lung adenocarcinoma in the TCGA cohort stratified by high vs. low expression of the four-gene signature. High expression was associated with significantly shorter OS, reduced PPS and shorter FP intervals. (F) Boxplot comparison showing lower basal expression of the four-gene signature in tumour vs. normal lung tissue. (G) Radar plots illustrating differential expression profiles of *BTG1*, *ING4*, *PNRC1* and *CALCOCO1* between normal and tumour samples. Data represent mean  $\pm$  SD from  $\geq 3$  biological replicates.  $\beta$ -gal, beta-galactosidase; OS, overall survival; PPS, post-progression survival; FP, first progression; SD, standard deviation; *ING4*, inhibitor of growth family member 4; *CALCOCO1*, calcium-binding and coiled-coil domain-containing protein 1; *PNRC1*, proline-rich nuclear receptor coactivator 1; *BTG1*, B-cell translocation gene 1.

To directly test whether these genes were functionally required for the establishment of the DTP state, the present study knocked down these signature genes in PC-9 cells via a

doxycycline-induced Tet-on shRNA system targeting each of the four genes. RT-qPCR confirmed the efficient knockdown of each target gene (Fig. 4C). Subsequently, the present study

assessed the results of staining with  $\beta$ -gal, a widely used surrogate marker for DTP cells, in shRNA-treated cells after 72-h osimertinib treatments. As shown in Fig. 4D, knockdown of any of the four genes markedly reduced the proportion of  $\beta$ -gal-positive cells compared with control cells, indicating that all four genes were necessary for osimertinib-induced persister cell formation. Collectively, these results supported the causal role of the *BTG1/ING4/PNRC1/CALCOCO1* gene signature in mediating the DTP phenotype.

*Clinical relevance and robustness of the four-gene signature across independent cohorts.* To assess the clinical relevance of this four-gene signature, the present study analysed survival data using the Kaplan-Meier plotter TCGA-LUAD cohort. Patients with a high combined expression of *BTG1*, *ING4*, *PNRC1* and *CALCOCO1* exhibited significantly poorer OS (HR, 1.61;  $P=0.0012$ ) and PPS (HR, 1.73;  $P=0.0110$ ) than those with low expression (Fig. 4E). Notably, the high-expression group also showed a shorter FP interval (HR, 0.63;  $P=0.0079$ ) than the low-expression group, indicating earlier disease relapse and supporting the adverse prognostic role of the four-gene signature in LUAD. In parallel, comparative expression analysis showed that the expression of the overall four-gene signature was significantly lower in tumour tissues than in normal lung tissues ( $P=3.11 \times 10^{-96}$ ) (Fig. 4F), suggesting that the marked induction of this signature may be context-dependent and preferentially triggered under therapeutic stress. Radar-plot analysis further showed that basal expression of the four-gene signature was lower in LUAD tumour tissues than in normal lung tissues (Fig. 4G). Together with the TKI-treated cell-line analyses described above, these findings suggest that this signature is not constitutively activated in untreated tumour tissue, but may be induced under therapeutic stress during DTP formation.

To further strengthen the clinical applicability of the DTP-associated gene signature, the present study analysed the TCGA-LUAD cohort using a weighted four-gene score. In this independent cohort, patients with a high signature score demonstrated significantly poorer OS (log-rank  $P=0.030$ ; HR, 1.39; 95% CI, 1.03-1.86) compared with patients with a low signature score (Fig. S3A). In the results of the Cox regression analyses, the weighted signature score remained associated with poorer survival and retained prognostic value after adjusting for age and stage group, whereas advanced stage was the strongest clinicopathological predictor (Fig. S3B and C). Notably, the weighted signature score showed no significant correlation with age and no significant difference between the early- and advanced-stage groups (Fig. S3D and E), indicating that the prognostic effect of this signature was not simply driven by these baseline variables. Subgroup and interaction analyses further supported the stability of this association, with no significant interaction with age or stage (Fig. S3F).

Additional clinicopathological analyses of TCGA-LUAD further supported the robustness of the four-gene signature. Individual genes did not consistently outperform the combined signature score (Fig. S4A), supporting the use of the integrated signature rather than any single component alone. Furthermore, the weighted signature score was not significantly associated with detailed American Joint Committee on Cancer (AJCC) stage, pathological tumour (T) category according to the

AJCC tumour-node-metastasis (TNM) staging system (Ref), sex or smoking exposure (Fig. S4B-E). Using the optimal cut-off, patients with a high weighted signature score showed significantly poorer OS than those with a low score (HR, 1.44;  $P=0.018$ ) (Fig. S4F). Stage-stratified analyses showed a similar adverse trend associated with high signature expression in both early- and advanced-stage subgroups, although statistical significance was limited in these smaller subsets (Fig. S4G and H). Analysis of pathological nodal (N) and metastatic (M) categories showed that advanced nodal involvement and metastatic disease were associated with poorer OS compared with the corresponding reference groups (Fig. S4I). The time-varying coefficient analysis did not suggest a major violation of the proportional hazards assumption (Fig. S4J).

Lastly, external validation using Kaplan-Meier plotter-based public datasets demonstrated that the adverse prognostic effect of the four-gene signature was reproducible in several clinically relevant subgroups, including male patients, smokers and patients with stage I-III disease (Fig. S5). Collectively, these results indicated that the *BTG1/ING4/PNRC1/CALCOCO1* signature was rapidly induced by osimertinib, captured a clinically relevant adaptive transcriptional state and provided prognostic information beyond conventional clinicopathological variables in LUAD.

*In silico drug screening identifies compounds selectively effective in cells with high expression of the four-gene signature.* Given that high expression of the four-gene DTP signature (*BTG1*, *ING4*, *PNRC1* and *CALCOCO1*) was associated with poor prognosis in patients with LUAD, the present study sought to identify therapeutic compounds that could selectively target this transcriptional phenotype. Using the DepMap PRISM drug-sensitivity database, the transcriptomic and pharmacological profiles of 50 LUAD and 50 breast carcinoma cell lines were ranked by the expression levels of the four-gene signature and compared.

Heatmap visualisation demonstrated distinct transcriptional clustering between the high- and low-expression groups in both cancer types, providing evidence that this signature stratified the cell lines according to their transcriptional state (Fig. 5A). Correlation analysis of gene-signature expression revealed a strong concordance between models with high and low expression of the four-gene set in the DepMap-derived datasets, with these genes forming a distinct, tightly correlated cluster that was clearly separated from the other transcripts (Fig. 5B). Notably, lung cancer cell lines exhibiting high expression of the four-gene signature displayed increased metastatic potential in the MetMap 500 dataset, particularly in lung, liver, kidney and brain tissues (Fig. 5C), suggesting that this transcriptional program may have also associated with aggressive phenotypes.

Single-sample GSEA further revealed that DNA replication-, G<sub>2</sub>/M checkpoint- and cell cycle-associated pathways showed relatively higher enrichment in four-gene low-expression models, whereas high-expression models displayed relative reduction of these proliferative programs (Fig. 5D and E). These results indicated that high-expression cells sustained a proliferative yet stress-tolerant transcriptional state, which may have rendered them particularly vulnerable to targeted therapeutic perturbations.



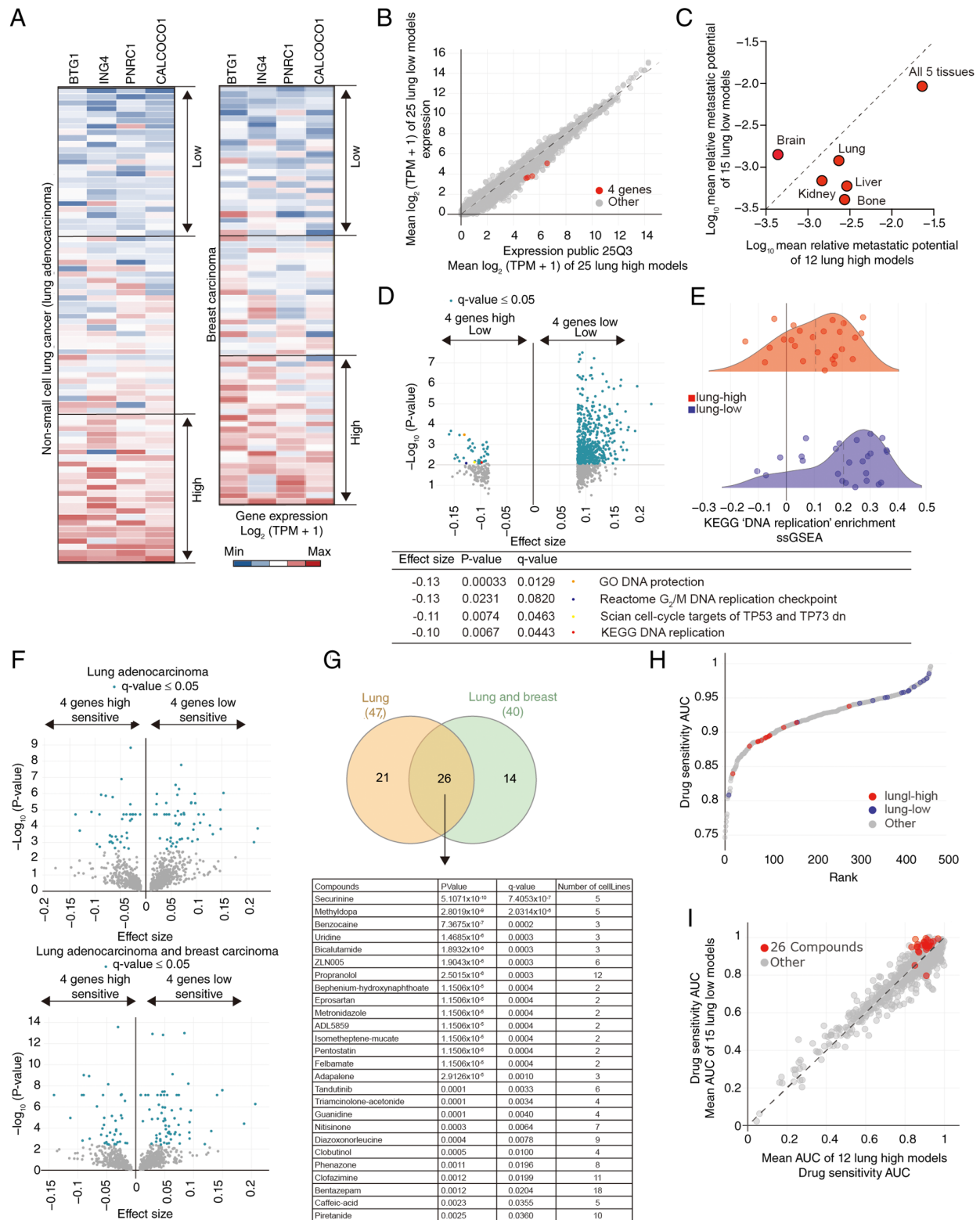


Figure 5. *In silico* drug screening identifies compounds that are selectively effective in cells with high expression of the four-gene signature. (A) Heatmaps of the expression levels of *BTG1*, *ING4*, *PNRC1* and *CALCOCO1* in high- and low-expression subgroups across non-small cell lung cancer (left) and breast carcinoma (right) cell lines from the Cancer Dependency Map database. (B) Correlation of mean gene-signature expression between the top 25 'lung-high' and 'lung-low' models from the Public 25Q3 dataset (Q3 2025 public release), showing consistent clustering of the four genes across datasets. (C) Comparison of metastatic potential among lung-high vs. lung-low cell lines across organ-specific metastasis models using data from the MetMap 500 dataset. (D and E) Differential pathway enrichment analysis (based on the Public 24Q2 dataset, that is, the public data release from Q2 2024) highlighting significant enrichment of DNA replication and G<sub>2</sub>/M checkpoint pathways in lung-high cell lines. (F) Volcano plots of drug sensitivity AUC-value differences between high and low signature-expression groups in lung (top) and lung and breast combined (bottom) datasets. (G) Venn diagram showing 26 overlapping compounds with selective sensitivity in both lung and breast cancer; table lists representative hits including securinine, metformin and ZLN005. (H) Drug-sensitivity ranking plot illustrating enhanced sensitivity of four-gene-high models to the identified 26 compounds. (I) Correlation of Profiling Relative Inhibition Simultaneously in Mixtures repurposing secondary screen results providing evidence that the 26 compounds (highlighted in red) were more effective in four-gene-high cell lines than other compounds. *ING4*, inhibitor of growth family member 4; *CALCOCO1*, calcium-binding and coiled-coil domain-containing protein 1; *PNRC1*, proline-rich nuclear receptor coactivator 1, *BTG1*, B-cell translocation gene 1; AUC, area under the curve.

Table III. Candidate compounds predicted to synergise with osimertinib in eliminating DTP cells.

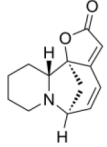
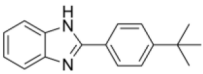
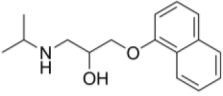
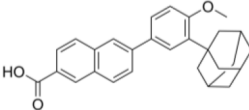
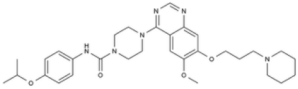
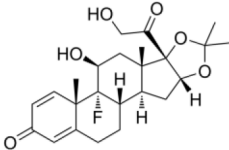
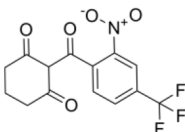
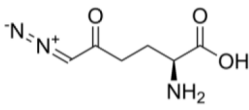
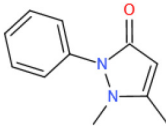
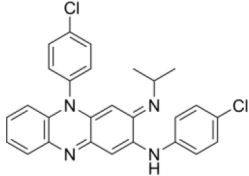
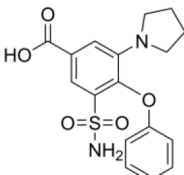
| First author/s, year                    | Drug (CAS no.)                        | Structure                                                                           | Known primary target/mechanism                                                                                                                                                                      | (Refs.) |
|-----------------------------------------|---------------------------------------|-------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------|
| Stefanowicz-Hajduk <i>et al.</i> , 2016 | Securinine (5610-40-2)                |    | GABA-A receptor antagonist                                                                                                                                                                          | (58)    |
| Zhang <i>et al.</i> , 2013              | ZLN005 (49671-76-3)                   |    | Upregulates PGC-1 $\alpha$ transcription; also reported to activate AMPK upstream; functional modulator rather than classic ligand                                                                  | (59)    |
| Duan <i>et al.</i> , 2026               | Propranolol (525-66-6)                |    | Non-selective $\beta$ -adrenergic receptor antagonist; inhibits both ADRB1 and ADRB2                                                                                                                | (60)    |
| Lee <i>et al.</i> , 2026                | Adapalene (106685-40-9)               |    | Retinoic acid receptors; preferential agonist at RAR- $\beta$ and RAR- $\gamma$ .                                                                                                                   | (61)    |
| Zhao <i>et al.</i> , 2022               | Tandutinib (387867-13-2)              |   | FLT3 tyrosine kinase inhibitor; also inhibits c-Kit) and PDGFR.                                                                                                                                     | (62)    |
| Weikum <i>et al.</i> , 2017             | Triamcinolone acetonide (76-25-5)     |  | Glucocorticoid receptor NR3C1 agonist.                                                                                                                                                              | (63)    |
| Mascarello <i>et al.</i> , 2025         | Nitisinone (104206-65-7)              |  | 4-hydroxyphenylpyruvate dioxygenase inhibitor.                                                                                                                                                      | (64)    |
| Encarnación-Rosado <i>et al.</i> , 2024 | 6-Diazo-5-oxo-L-norleucine (157-03-9) |  | Covalent inhibitor of glutamine-dependent amidotransferases; broadly blocks glutamine-utilizing enzymes, such as CTPS1/2, GMPS, PPAT, CPSII and GFAT1/2.                                            | (65)    |
| Hassan <i>et al.</i> , 2026             | Phenazone (60-80-0)                   |  | COX-1/COX-2 inhibitor; exerts analgesic/antipyretic effects.                                                                                                                                        | (66)    |
| Yano <i>et al.</i> , 2011               | Clofazimine (2030-63-9)               |  | Antimycobacterial agent that;<br>i) Binds bacterial DNA; and<br>ii) is reduced by mycobacterial NDH-2, a type II NADH dehydrogenase, causing ROS generation; also reported to affect ion transport. | (67)    |



Table III. Continued.

| First author/s, year    | Drug (CAS no.)             | Structure                                                                         | Known primary target/mechanism                                                            | (Refs.) |
|-------------------------|----------------------------|-----------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|---------|
| Löscher and Kaila, 2022 | Piretanide<br>(55837-27-9) |  | NKCC2 inhibitors. Piretanide is an orally active, relatively safe and effective diuretic. | (68)    |

List of 11 compounds prioritised from Cancer Dependency Map-based pharmacogenomic screening according to predicted sensitivity in DTP-like cell lines. Each entry shows the compound name and CAS number, chemical structure and known primary targets or mechanism of action. ADRB1, beta-1 adrenergic receptor; ADRB2, beta-2 adrenergic receptor; AMPK, AMP-activated protein kinase; c-Kit, KIT proto-oncogene receptor tyrosine kinase; COX, cyclooxygenase; CPSII, carbamoyl-phosphate synthase II; CTPS1/2, cytidine triphosphate synthase 1/2; FLT3, Fms-like tyrosine kinase 3; GFAT1/2, glutamine;fructose-6-phosphate amidotransferase 1/2; GMPS, GMP synthase; GABA-A,  $\gamma$ -aminobutyric acid type A; NDH-2, type II NADH dehydrogenase; NKCC2, sodium-potassium-chloride cotransporter 2; NR3C1, nuclear receptor subfamily 3 group C member 1; PDGFR, platelet-derived growth factor receptor; PGC-1 $\alpha$ , peroxisome proliferator-activated receptor gamma coactivator 1- $\alpha$ ; PPAT, phosphoribosyl pyrophosphate amidotransferase; RAR-beta/gamma, retinoic acid receptor beta/gamma; ROS, reactive oxygen species.

The present study subsequently performed a computational drug sensitivity comparison between the top 25 four-gene high and four-gene low cell lines in both lung and breast cancer datasets. Volcano plots highlighted a distinct subset of compounds that displayed significantly lower AUC values in cell lines with high signature expression compared with those with low signature expression, which was indicative of higher drug sensitivity in the high signature-expression groups (Fig. 5F). Cross-cancer integration of the lung and breast datasets identified 26 overlapping compounds that consistently exhibited selective toxicity toward the four gene-high models (Fig. 5G). Notably, these compounds included several metabolic and stress-modulating agents, such as securinine, a  $\gamma$ -aminobutyric acid type A (GABA-A) receptor antagonist, propranolol, a  $\beta$ -adrenergic receptor blocker, and ZLN005, a PGC-1 $\alpha$  activator.

Drug sensitivity ranking provided evidence that these 26 candidate drugs demonstrated markedly enhanced efficacy in the high-expression subgroup compared with the low-expression or unrelated models (Fig. 5H and I). Together, these analyses identified a distinct set of pharmacological agents predicted to preferentially target tumour cells characterized by elevated *BTG1*, *ING4*, *PNRC1* and *CALCOCO1* expression, providing a rational framework for combination treatment strategies against TKI-induced persister cells.

*ZLN005 synergizes with osimertinib to induce cytotoxicity in LUAD cells.* To experimentally validate the computational predictions, 11 compounds were selected from the *in silico* screen (Table III, chemical structures and drug characteristics are referenced therein) and their combinatorial effects with osimertinib in EGFR-mutant PC-9 LUAD cells were evaluated. Drug synergy was quantified using the Bliss independence model based on cell viability assays across multiple concentration gradients for each compound and osimertinib.

Among the tested candidates, five compounds exhibited positive synergy scores; ZLN005, propranolol, tandutinib, nitisinone and phenazone (mean Bliss score >0; Fig. 6A-E).

These results suggested potential positive interactions with osimertinib, although the magnitude of overall growth inhibition and the statistical support for Bliss synergy varied among compounds. ZLN005 and tandutinib produced the strongest overall combinatorial effects, achieving mean inhibition rates of 60.87 and 62.85%, with corresponding Bliss synergy scores of 1.2 ( $P=1.9\times 10^{-1}$ ) and 3.03 ( $P=7.98\times 10^{-3}$ ), respectively. Propranolol, nitisinone and phenazone also demonstrated positive mean Bliss synergy scores of 3.49 ( $P=8.62\times 10^{-3}$ ), 5.95 ( $P=1.74\times 10^{-6}$ ) and 4.08 ( $P=1.16\times 10^{-7}$ ), respectively. Although nitisinone and phenazone showed statistically supported positive Bliss scores, their overall mean inhibition rates were lower than those of ZLN005 and tandutinib. These results highlighted ZLN005 as a leading candidate for synergistic metabolic activation of osimertinib response and identified tandutinib as a potential dual-pathway sensitizer through kinase inhibition.

The aforementioned dose-response analyses provided evidence that ZLN005 alone exhibited minimal cytotoxicity at concentrations below 10  $\mu$ M; however, combined treatment with osimertinib increased overall growth inhibition. Bliss synergy matrix modelling identified a positive interaction window spanning within the tested concentration grid most evident around 3.33  $\mu$ M osimertinib combined with 6  $\mu$ M ZLN005, although the mean Bliss synergy score for ZLN005 remained modest (1.20;  $P=1.90\times 10^{-1}$ ). These findings supported ZLN005 as a candidate for further experimental validation based on its strong overall combinatorial inhibitory effect with osimertinib, rather than statistically significant global Bliss synergy.

Collectively, these results indicated that ZLN005 represented the most potent and selective synergistic partner of osimertinib, providing a strong rationale for further mechanistic and preclinical evaluation of this combination as a strategy to eradicate DTP cells in EGFR-mutant lung cancer.

*ZLN005 enhances osimertinib-induced cell death in LUAD cells.* To experimentally support the synergistic cytotoxicity

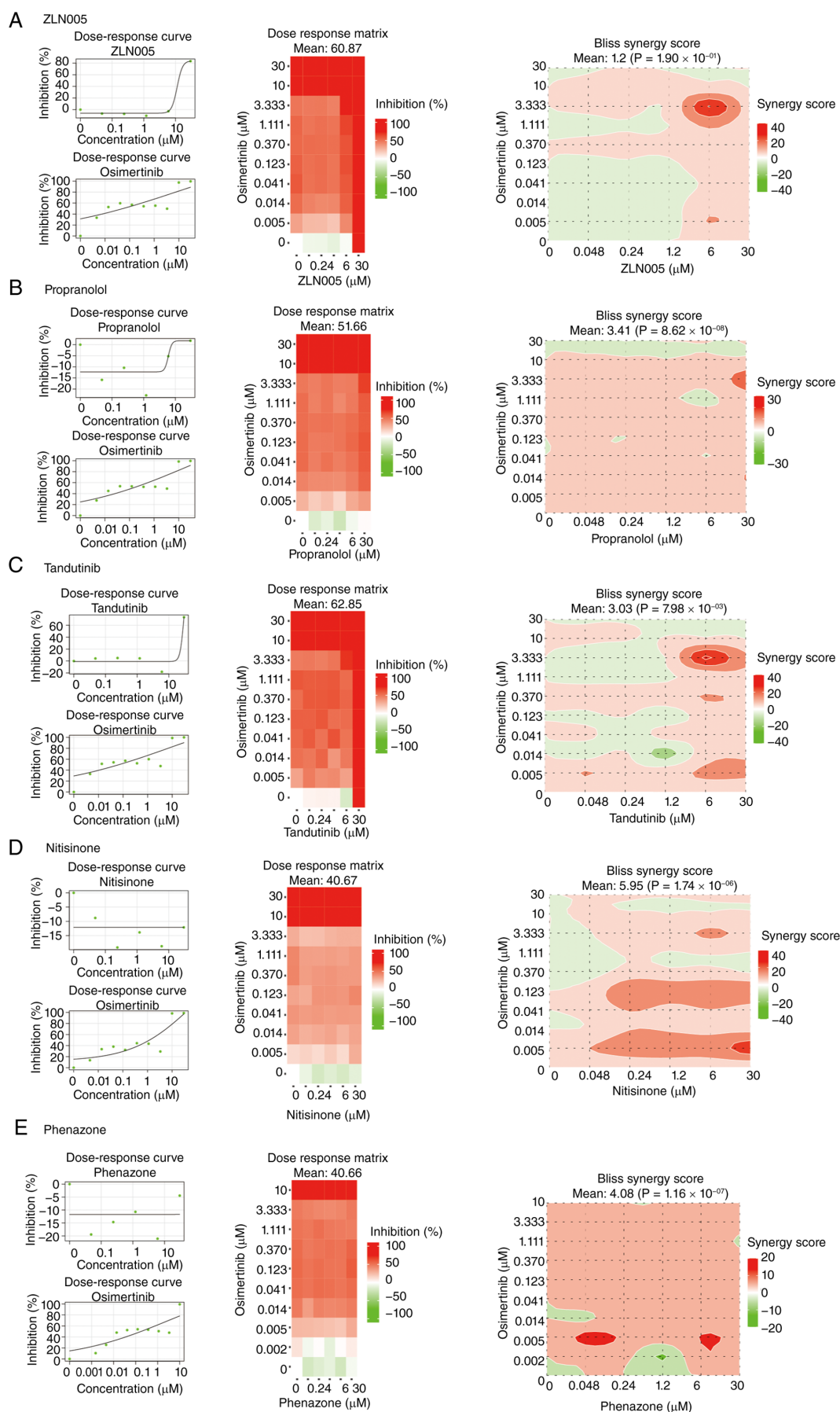


Figure 6. ZLN005 synergizes with osimertinib to induce cytotoxicity in lung adenocarcinoma cells. Synergy analysis of osimertinib with five candidate compounds in epidermal growth factor receptor-mutant PC-9 cells; (A) ZLN005, (B) propranolol, (C) tandutinib, (D) nitisinone and (E) phenazone. Left; Dose-response curves for individual compounds (0-30  $\mu\text{M}$ ; 1:5 dilution) and osimertinib (0-10/30  $\mu\text{M}$ ; 1:3 dilution). Middle; Dose-response matrices showing cell viability inhibition at different concentrations. Right; Bliss synergy heatmaps indicating synergistic interactions (red) and antagonistic effects (green).

predicted by the aforementioned computational and synergy analyses, the present study examined the cellular effects of combining osimertinib with the top five candidate compounds identified in Fig. 6. Phase-contrast microscopy revealed that treatment with osimertinib or each of the aforementioned compounds alone induced modest morphological changes, whereas their combination markedly enhanced cell death in PC-9 cells (Fig. 7A). Among all the combinations tested, ZLN005 and osimertinib showed the most pronounced cytopathic phenotype, characterized by extensive cell detachment, rounding and membrane rupture, consistent with the loss of cell viability.

To further verify these observations, dual staining was performed using calcein-AM and PI to label viable and dead cells, respectively. Fluorescence microscopy demonstrated that the combination of osimertinib and ZLN005 led to a notable increase in PI-positive (red) cells and a marked reduction in calcein-AM-positive (green) cells compared with single-agent treatment (Fig. 7B). Other tested combinations, including osimertinib with propranolol, tandutinib, nitisinone or phenazone, also increased PI-positive cell populations to varying degree. Therefore, the relative potency of the osimertinib and ZLN005 combination was further assessed by quantitative PI flow cytometry (Fig. 7C).

Quantitative flow cytometry analysis corroborated these results, showing that treatment with 0.5  $\mu$ M osimertinib alone increased PI-positive cells from 6.26% in DMSO-treated cells to 12.2%, whereas co-treatment with 5  $\mu$ M ZLN005 further elevated this proportion to 22.3% (Fig. 7C). By contrast, the other combinations exhibited minimal increases in PI-positive fractions. These data supported that ZLN005 synergistically augmented osimertinib-induced cytotoxicity, leading to enhanced cell death in EGFR-mutant LUAD cells.

*ZLN005 promotes osimertinib-induced pyroptosis through GSDME activation.* To investigate the mechanism underlying the enhanced cytotoxicity of ZLN005 in combination with osimertinib, the present study first assessed whether this drug combination induced a lytic form of cell death characteristic of pyroptosis. Calcein-AM/PI dual staining showed that treatment with either agent alone resulted in only modest levels of cell death, whereas their combination resulted in a marked, concentration-dependent increase in PI-positive cells (Fig. 8A). The proportion of PI-positive cells rose progressively with increasing ZLN005 concentrations (5–20  $\mu$ M), indicating that the drug combination promoted membrane-permeabilising cell death as reflected by loss of membrane integrity and increased PI uptake.

Phase-contrast microscopy further supported these observations; Cells co-treated with osimertinib and ZLN005 exhibited the classical morphological features of pyroptosis, including cell swelling, balloon-like membrane blebs and eventual plasma-membrane rupture (Fig. 8B). These morphological changes were consistent with gasdermin-mediated lytic death rather than apoptosis alone.

Given that ZLN005 is a PGC-1 $\alpha$  agonist known to increase mitochondrial oxidative metabolism, the present study subsequently quantified intracellular ROS levels to determine whether mitochondrial stress contributed to the enhanced cytotoxicity. DCFH-DA staining revealed a mild elevation

in ROS levels following single-agent treatments; however, the combined treatment produced a slight right-shifted fluorescence peak, indicating moderate increase (Fig. 8C). These findings suggested that ROS accumulation may contribute, at least in part, to the enhanced cytotoxicity induced by the combination of ZLN005 and osimertinib.

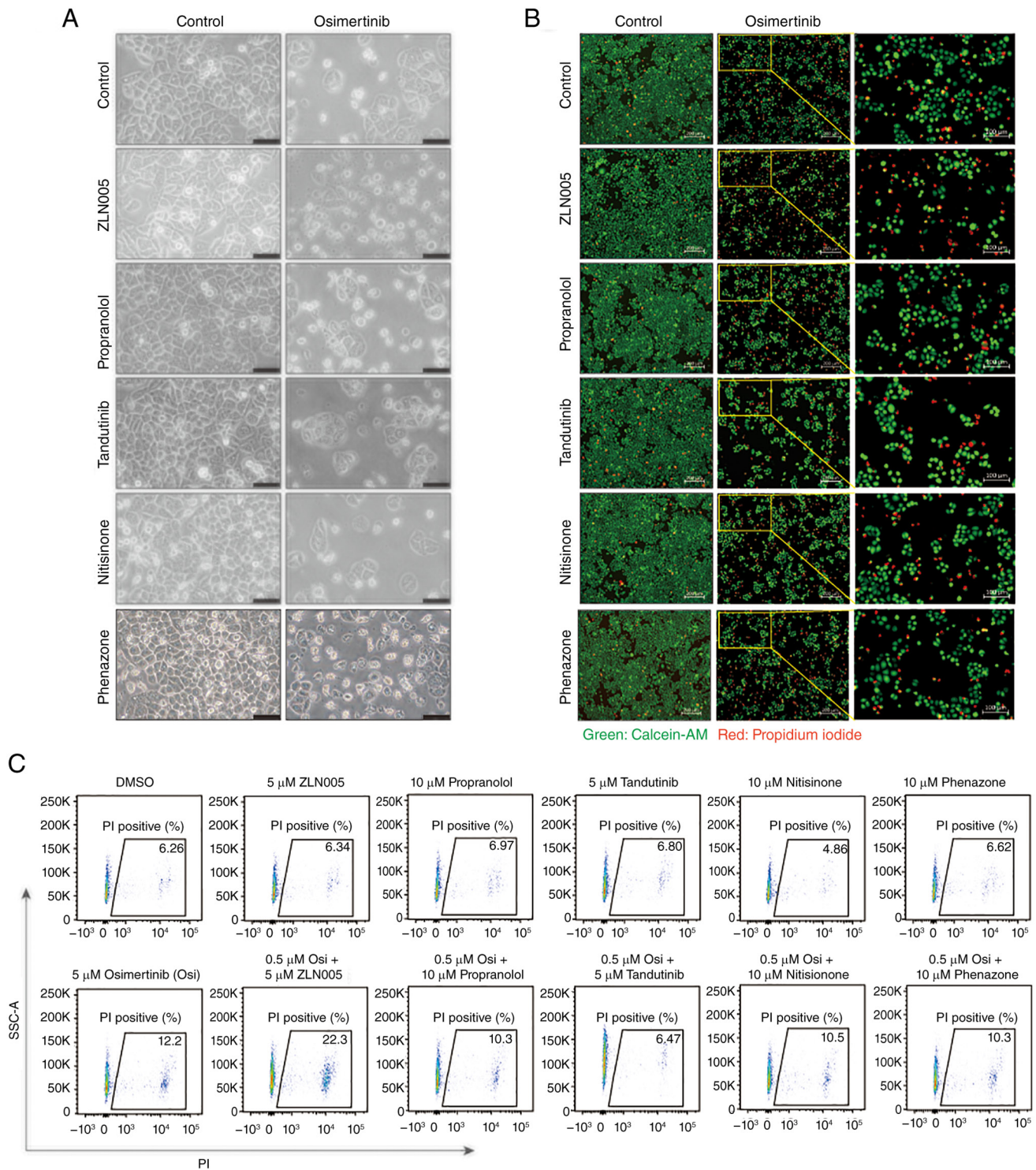
Immunoblotting was performed for canonical apoptosis and pyroptosis markers to determine the molecular basis of the observed cell death. Co-treatment markedly increased the cleavage of PARP and caspase-8 relative to single agents and was accompanied by caspase-3 processing, indicating activation of apoptotic signalling (Fig. 8D). Notably, this combination also induced robust cleavage of GSDME, generating an N-terminal pore-forming fragment that directly drives plasma-membrane rupture during pyroptosis. Although the cleaved N-terminal form of GSDMD was not detected, full-length GSDMD levels were markedly reduced following combination treatment, suggesting potential degradation or non-canonical inactivation rather than canonical inflammasome-mediated activation.

To support the causal role of caspase-3 in mediating GSDME cleavage and cell death, the present study pharmacologically inhibited caspase-3 using Z-DEVD-FMK. As shown in Fig. 8E, caspase-3 inhibition markedly suppressed the cleavage of both PARP and GSDME induced by osimertinib/ZLN005 co-treatment, indicating that caspase-3 acted upstream of GSDME activation. Consistent with these results, LDH release assays revealed that caspase-3 inhibition significantly reduced osimertinib/ZLN005-induced cell death (Fig. 8F), providing evidence that caspase-3-dependent GSDME cleavage was functionally required for the lytic phenotype.

Given that *CALCOCO1* was identified as a core component of the DTP-associated gene signature (Fig. 4), the present study tested whether its loss sensitised cells to osimertinib/ZLN005-induced death. Phase-contrast microscopy revealed that *CALCOCO1* knockdown markedly exacerbated cell death upon osimertinib/ZLN005 co-treatment, with increased cell rounding, detachment and lysis observed (Fig. 8G). This was further corroborated by calcein-AM/PI staining; *CALCOCO1*-knockdown cells exhibited markedly higher PI-positive cell fractions than control shRNA-treated cells following osimertinib/ZLN005 co-treatment (Fig. 8H). Notably, immunoblotting showed that *CALCOCO1* knockdown enhanced the cleavage of GSDME (Fig. 8I) without affecting total GSDME levels, suggesting that *CALCOCO1* acted as a negative regulator of GSDME activation in DTP cells.

Together, these findings demonstrated that ZLN005 amplified osimertinib-induced cytotoxicity by promoting ROS-driven activation of caspase-3 and the subsequent cleavage of GSDME, thereby shifting cell death toward a membrane-permeabilising lytic phenotype. This switch would be expected to enhance the elimination of DTP-like cells because GSDME-mediated pore formation causes irreversible plasma-membrane rupture, as reflected by increased PI uptake, LDH release and pyroptosis-like morphology, rather than allowing cells to remain in a drug-tolerant or non-lytic stressed state. Notably, *CALCOCO1* functioned as a key protective node in the DTP program by limiting GSDME

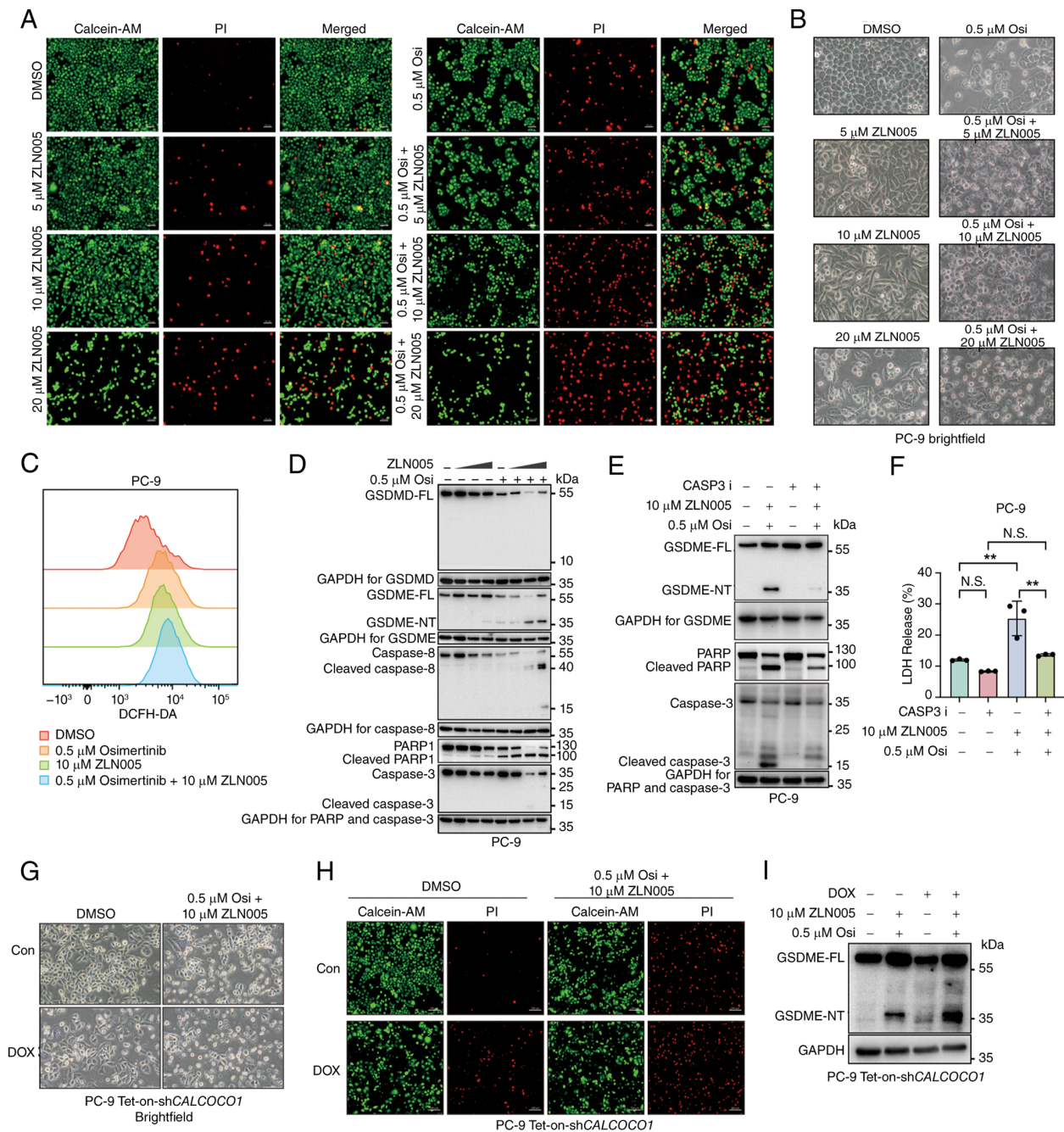




**Figure 7.** ZLN005 enhances osimertinib-induced cell death in lung adenocarcinoma cells. (A) Representative phase-contrast images of PC-9 cells treated with vehicle control, osimertinib (0.5  $\mu$ M) or osimertinib combined with 5–10  $\mu$ M of the indicated compounds (ZLN005, propranolol, tandutinib, nitisinone or phenazone) for 72 h. The combination of ZLN005 and osimertinib induced pronounced cell death and detachment compared with other treatments. Scale bar, 50  $\mu$ m. (B) Calcein-AM (green) and PI (red) staining of live and dead PC-9 cells under the aforementioned treatment conditions. The osimertinib + ZLN005 combination treatment markedly increased the proportion of PI-positive (dead) cells. (Scale bar, 200  $\mu$ m). Insets show magnified views of representative regions. (Scale bar, 100  $\mu$ m). (C) Flow cytometry analysis of PI-stained cells. Quantification of PI-positive fractions demonstrated that co-treatment with ZLN005 and osimertinib markedly increased the proportion of dead cells compared with treatment using either agent alone. Data represent mean  $\pm$  SD from three independent experiments. AM, acetoxymethyl ester; PI, propidium iodide; Osi, osimertinib; DMSO, dimethyl sulfoxide; SD, standard deviation; SSC-A, side scatter-area.

activation. Its depletion removed this brake and further sensitised persister-like cells to osimertinib/ZLN005-induced lytic death. Thus, the metabolic-stress-associated switch toward

GSDME-mediated pyroptosis provided a mechanistic explanation for the enhanced elimination of DTP cells following combined osimertinib and ZLN005 treatment.



**Figure 8.** ZLN005 promotes osimertinib-induced pyroptotic cell death through GSDME activation. (A) Calcein-AM/PI dual staining of PC-9 cells treated with increasing doses of ZLN005 (5–20  $\mu$ M), osimertinib (0.5  $\mu$ M) or their combination for 48 h. Calcein-AM (green) signals mark viable cells; PI (red) signals mark membrane-permeabilized or dead cells. Scale bar, 50  $\mu$ m. (B) Representative phase-contrast microscopy images showing morphological changes characteristic of lytic cell death, including swelling and membrane rupture, particularly under combination treatment. Scale bar, 50  $\mu$ m. (C) Flow cytometric detection of intracellular ROS using DCFH-DA. Co-treatment with osimertinib and ZLN005 showed a modest increase in fluorescence intensity compared with single-agent treatment, suggesting increased intracellular ROS levels. (D) Immunoblot analysis of apoptosis and pyroptosis markers. Osimertinib and ZLN005 co-treatment enhanced PARP and caspase-8 cleavage and robustly induced the active N-terminal fragment of GSDME. Full-length GSDMD was reduced but not cleaved. The involvement of caspase-3 in GSDME cleavage was further supported by the caspase-3 inhibitor experiments shown in (E) and (F). Target protein blots are grouped within the figure with GAPDH blots from the corresponding membrane, which served as the loading control. (E) Immunoblot analysis showing the effect of caspase-3 inhibition on GSDME and PARP cleavage. Treatment with Z-DEVD-FMK suppressed the cleavage of PARP and GSDME induced by osimertinib/ZLN005 co-treatment, indicating that caspase-3 acted upstream of GSDME activation. GAPDH bands corresponding to each membrane are grouped with their respective target protein blots as loading controls. (F) LDH release assay under caspase-3 inhibition and osimertinib/ZLN005 co-treatment. Z-DEVD-FMK reduced osimertinib/ZLN005-induced cell death. Data are presented as mean  $\pm$  SD from three independent biological replicates. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparisons test. \*\* $P$ <0.01. (G) Representative phase-contrast images of *CALCOCO1* knockdown in PC-9 cells treated with ZLN005 (10  $\mu$ M) and osimertinib (0.5  $\mu$ M) for 48 h. *CALCOCO1* knockdown enhanced cell death under combination treatment. Scale bar, 100  $\mu$ m. (H) Calcein-AM/PI staining of *CALCOCO1*-knockdown PC-9 cells treated with osimertinib/ZLN005 for 48 h. Calcein-AM (green) labelled viable cells, whereas PI (red) labelled membrane-permeabilized or dead cells. *CALCOCO1* knockdown increased the fraction of PI-positive cells under osimertinib/ZLN005 combination treatment. Scale bar, 100  $\mu$ m. (I) Immunoblot analysis of GSDME cleavage in *CALCOCO1*-knockdown cells treated with osimertinib/ZLN005 for 48 h. AM, acetoxymethyl ester; PI, propidium iodide; Osi, osimertinib; DMSO, dimethyl sulfoxide; ROS, reactive oxygen species; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; PARP, Poly(ADP-ribose) polymerase; GSDME, gasdermin E; GSDMD, Gasdermin D; NT, N-terminal fragment; FL, full-length; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CASP3i, caspase-3 inhibitor (Z-DEVD-FMK); LDH, lactate dehydrogenase; *CALCOCO1*, calcium binding and coiled-coil domain 1; ANOVA, analysis of variance; SD, standard deviation.



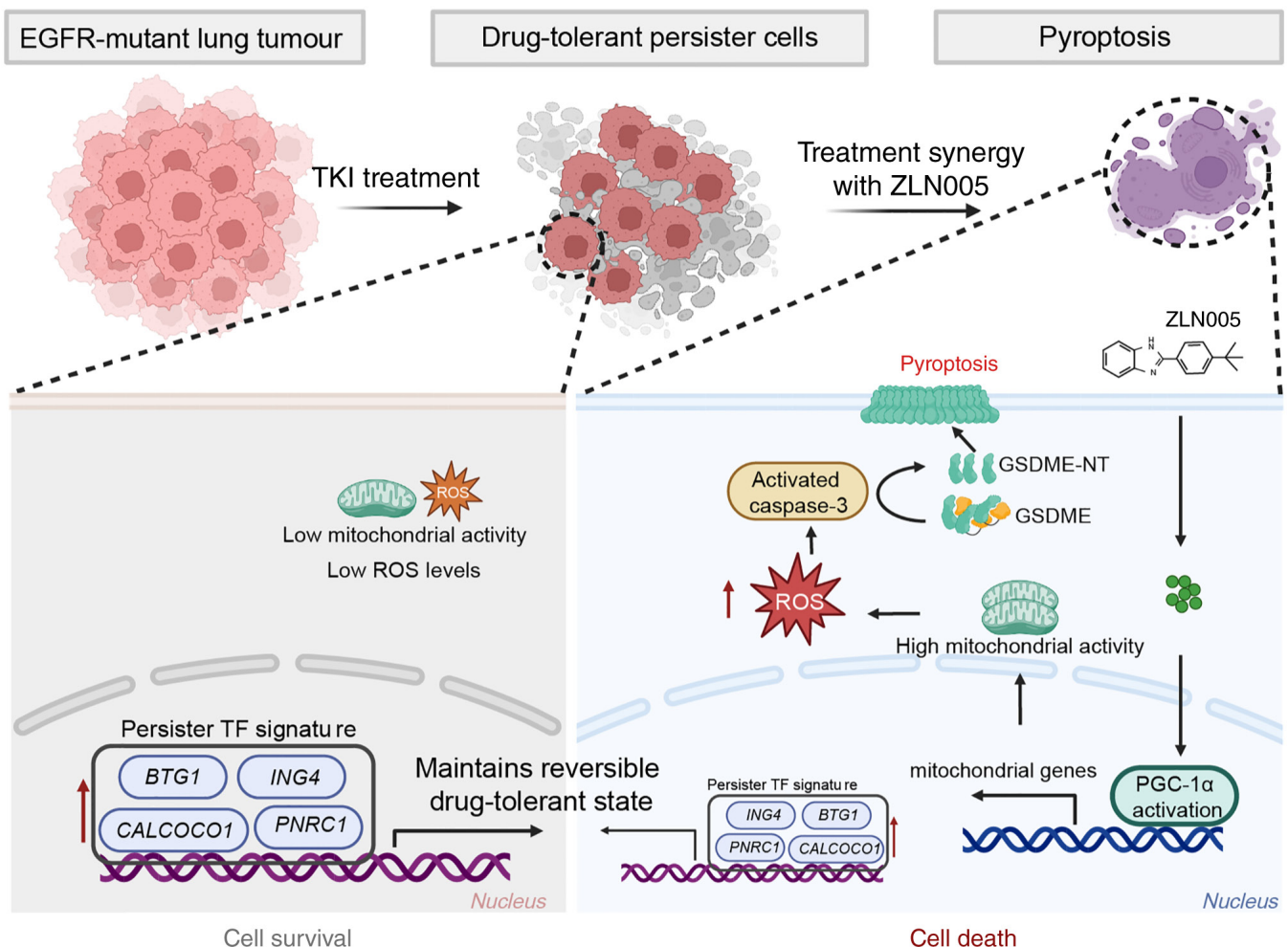


Figure 9. Mechanistic model of TKI-induced persister cells in EGFR-mutant lung cancer and their susceptibility to ZLN005-induced pyroptosis. TKI treatment drives EGFR-mutant lung tumour cells into a reversible drug-tolerant persister state characterised by reduced mitochondrial activity, low ROS levels and activation of a persister-specific transcriptional program, comprising the upregulation of *BTG1*, *ING4*, *CALCOCO1* and *PNRC1*. Treatment with the PGC-1 $\alpha$  agonist ZLN005 enhances mitochondrial activity and ROS production, synergizing with osimertinib treatment to trigger caspase-3 activation and GSDME cleavage. This converts reversible drug tolerance into irreversible pyroptotic cell death, revealing a metabolic vulnerability of persister cells that can be exploited therapeutically. EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; ROS, reactive oxygen species; GSDME, gasdermin E; NT, N terminal; *BTG1*, B-cell translocation gene 1; *ING4*, inhibitor of growth family member 4; *CALCOCO1*, calcium binding and coiled-coil domain 1; *PNRC1*, proline-rich nuclear receptor coactivator 1; PGC-1 $\alpha$ , peroxisome proliferator-activated receptor gamma coactivator 1-alpha; TF, transcription factor.

## Discussion

Targeted therapy with TKIs has transformed the therapeutic management of oncogene-driven cancers, such as EGFR-mutant LUAD; however, the emergence of DTP cells remains a notable barrier to durable clinical remission (40). These cells survive initial TKI exposure through reversible, non-genetic adaptations characterised by transcriptional reprogramming and metabolic plasticity (14,21). The present study combined machine-learning-based transcriptomic profiling with functional validation to identify a four-gene transcriptional signature, comprising *BTG1*, *ING4*, *PNRC1* and *CALCOCO1*, which was associated with the DTP state and adverse clinical outcomes. The present study further demonstrated that pharmacological activation of mitochondrial metabolism using ZLN005, a PGC-1 $\alpha$  agonist, synergistically enhanced osimertinib-induced GSDME-associated pyroptotic cell death, thereby facilitating the elimination of otherwise drug-tolerant cells (Fig. 9).

The four genes comprising the identified signature, *BTG1*, *ING4*, *PNRC1* and *CALCOCO1*, are involved in diverse cellular processes (41–44). *BTG1* and *ING4* have been implicated in chromatin remodelling, cell cycle arrest and stress-induced differentiation (45–48). *PNRC1* modulates nuclear receptor signalling (43,49) and *CALCOCO1* participates in autophagy and calcium-dependent homeostasis (50,51). In the present study, these genes were interpreted as components of a transcriptional signature associated with the DTP state rather than as a fully validated cooperative regulatory module. Their coordinated upregulation suggested that the persister phenotype may have involved a multilayered adaptive program that integrated transcriptional control, metabolic adjustment and survival signalling. However, further perturbation-based studies are required to determine whether these factors interacted to regulate DTP biology and the mechanisms behind these interactions. Collectively, these observations suggested that a subset of tumour cells may have adopted a

transcriptionally coordinated adaptive state that supported transient tolerance to TKI pressure while preserving their capacity to resume proliferation upon the cessation of treatment (21).

Clinically, high expression of the identified signature associated with reduced OS and PPS in LUAD, providing preliminary support for the potential relevance of this signature as a biomarker of residual disease and risk of relapse. More broadly, persister-associated transcriptional and metabolic states have increasingly been linked to adverse therapeutic outcomes. However, robust clinical validation of persister-cell biomarkers remains limited (52-54). Accordingly, the prognostic relevance of the four-gene signature in the present study should be interpreted as supportive, but preliminary, pending validation in independent clinical cohorts and longitudinal treatment-matched samples.

The drug-sensitivity profiling results of the present study revealed that cell lines with high expression of the four-gene signature exhibited selective susceptibility to metabolic modulators, including ZLN005, propranolol, tandutinib, nitisinone and phenazone. This observation was consistent with previous studies that have demonstrated that DTP cells frequently exhibited an increased reliance on oxidative phosphorylation and mitochondrial stress-response pathways for survival under targeted therapy pressure (16,19,21,55). In contrast to studies that have primarily described mitochondrial dependence as a feature of the persister state (56), the findings of the present study suggested that pharmacological mitochondrial overactivation may be leveraged to expose a metabolic vulnerability and promote elimination of the DTP state. Nevertheless, broader comparisons of the effects of these modulators with other mitochondrial activators and metabolic intervention strategies are limited, requiring further investigation. Among the investigated candidates, ZLN005 emerged as the strongest synergistic partner of osimertinib. Mechanistically, the data in the present study supported a model in which ZLN005-mediated activation of PGC-1 $\alpha$  enhanced mitochondrial oxidative metabolism, which may have imposed metabolic overload on DTP cells whose redox systems were already strained by TKI-induced stress. This metabolic overactivation was accompanied by increased production of ROS, a stress signal known to promote caspase-3 activation (57). Consistent with this model, the combination treatment of ZLN005 and osimertinib markedly increased intracellular ROS levels, enhanced caspase-3 cleavage and induced robust GSDME cleavage; however, GSDMD cleavage was not notably affected, indicating the preferential activation of the caspase-3/GSDME pyroptotic axis. Morphological and staining assays supported this form of lytic cell death via observations of membrane ballooning, rupture and PI uptake.

This selective engagement of GSDME-associated pyroptosis carried important mechanistic implications and may have exhibited translational relevance. Although pyroptotic cell death can proceed through different gasdermin family members depending on the cellular context (30), the data in the present study did not demonstrate detectable GSDMD activation under the conditions tested. Specifically, although full-length GSDMD levels were reduced upon combination treatment, its active N-terminal fragment was not detected,

contradicting canonical GSDMD-dependent pyroptosis. Instead, the observed cell death phenotype was consistent with a GSDME-associated process, which was supported by caspase-3 activation and robust GSDME cleavage. Therefore, the absence of detectable GSDMD-N did not exclude pyroptosis, but indicated that osimertinib/ZLN005-induced lytic death was unlikely to proceed through canonical GSDMD activation under the conditions tested. Instead, the concomitant caspase-3 processing and robust GSDME cleavage supported preferential engagement of the caspase-3/GSDME axis. Taken together, the caspase-3/GSDME axis appeared to function as the predominant execution pathway, likely through the release of the GSDME N-terminal pore-forming domain and the resulting plasma-membrane permeabilization. This mode of death may have been immunogenic and could have promoted the release of damage-associated molecular patterns, potentially influencing the tumour microenvironment and facilitating immune clearance. Thus, combining EGFR-TKIs with mitochondrial activators, such as ZLN005, may not only enhance the elimination of persister cells but also have immunomodulatory consequences, with potential implications for future integration with immunotherapies. Although GSDME appeared to be the predominant executioner in this setting, further studies on GSDMD remain relevant because full-length GSDMD was reduced after combination treatment despite the absence of detectable GSDMD-N. Proteasome inhibition, epitope mapping or ubiquitination assays may help determine whether this reduction reflects protein degradation, alternative cleavage, epitope loss or other post-translational regulation rather than canonical GSDMD activation.

A key conceptual advancement of the present study was the identification of a potential mechanistic link between mitochondrial metabolic rewiring and pyroptotic vulnerability in DTP cells. Persister cells appeared to adopt a mitochondrial state that supported oxidative phosphorylation, a metabolic configuration that may have provided survival advantages under TKI pressure while also rendering these cells sensitive to metabolic overactivation. In this context, pharmacologic activation of PGC-1 $\alpha$  by ZLN005 was proposed to further increase mitochondrial respiration and oxidative stress beyond a tolerable threshold. The present study observed that the ROS increase resulting from ZLN005 treatment coincided with caspase-3 activation and GSDME cleavage, supporting a model in which mitochondrial oxidative stress acts upstream of pyroptotic conversion. Although the data of the present study supported this mechanistic interpretation, the causality requirement of PGC-1 $\alpha$ -dependent metabolic activation and ROS accumulation for caspase-3/GSDME-mediated pyroptosis has not yet been directly established through genetic perturbation experiments, such as PPARGC1A knockdown or GSDME loss-of-function analysis. These findings suggested that the metabolic flexibility enabling DTP survival may also have exposed therapeutic vulnerabilities that can be exploited through targeted metabolic overactivation.

Despite these insights, several important limitations remain. First, the direct functional roles of *BTG1*, *ING4*, *PNRC1* and *CALCOCO1* in the coordination of drug-tolerance-associated transcriptional and metabolic programs must be defined more rigorously. Furthermore, the

functional experiments of the present study were conducted exclusively *in vitro* using cell line models, and the efficacy and mechanism of action of the osimertinib/ZLN005 combination treatment therefore remains yet to be validated *in vivo*. As such, the conclusions of the present study have been based primarily on *in vitro* and computational analyses, which cannot fully account for tumour heterogeneity, microenvironmental influences or treatment responses in more physiologically relevant settings. In particular, microenvironmental factors, including immune cell interactions, fibroblast-derived paracrine signalling, extracellular matrix remodelling and inflammatory cues, may affect the emergence, maintenance and therapeutic vulnerability of DTP cells. Therefore, these factors could also modulate the efficacy and mechanism of DTP cell response to the osimertinib/ZLN005 combination. Additionally, the restricted size of the available transcriptomic datasets and the limited number of experimental models included in a number of analyses may have affected the robustness and generalizability of the findings of the present study. Therefore, future studies using co-culture systems, organoid-based models, patient-derived xenograft models and immunocompetent mouse models are important for evaluating antitumour efficacy and validating the mechanism of action of treatments. These models are also necessary for defining how the tumour microenvironment shapes DTP cell behaviour, host immune responses and the therapeutic response to osimertinib/ZLN005 co-treatment in a more physiologically relevant context.

An additional and important limitation of the present study is that longitudinal clinical specimens, particularly paired pre- and post-EGFR-TKI treatment biopsies, were not available in the present study to validate the four-gene signature. Although the bioinformatic analyses and survival associations in the present study provided preliminary support for the potential clinical relevance of this signature, its dynamic behaviour during EGFR-TKI treatment has not yet been validated in patient-matched pre- and post-treatment samples. Such paired clinical specimens would be particularly useful for evaluating whether changes in *BTG1*, *ING4*, *PNRC1* and *CALCOCO1* expression can serve as biomarkers of adaptive drug tolerance and treatment response in clinical settings. It is also important to assess whether this EGFR-TKI treatment strategy is broadly applicable across other oncogene-driven cancers that exhibit drug-tolerant states.

In conclusion, the present study identified a four-gene transcriptional signature associated with the DTP state in EGFR-mutant LUAD and provided experimental evidence that ZLN005-mediated metabolic overactivation enhanced osimertinib-induced GSDME-associated pyroptosis *in vitro*. These findings supported the concept that metabolic vulnerabilities of DTP cells may be therapeutically exploitable. However, additional mechanistic studies, *in vivo* validations and clinical investigations are required before these findings can be translated into therapeutic strategies.

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## Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

## Authors' contributions

YS and PL designed the experiments and conceived and supervised the study. FG, YT, PZ, YY and YM supervised the studies and analysed the profiling data. WC and JY performed the biochemistry and cell biology experiments with the assistance of HZ, YY, YM, ML, WL, HY and CS. YS and PL analysed the experimental data. YS and PL confirm the authenticity of all the raw data. YS, PL and WC wrote the manuscript with input from all co-authors. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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