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Simvastatin overcomes resistance to tyrosine kinase inhibitors in patient-derived, oncogene-driven lung adenocarcinoma models

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T. Li: Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, investigation, writing–original draft, project administration, writing–review and editing.

Competing interests

T Li reports research grants from AbbVie Inc., Astellas, AstraZeneca, Genentech /LaRoche, Jounce Therapeutics, LabyRx Immuno-Oncology, Lung Cancer Mutation Consortium, Merck, OncoC4 Inc., Novartis, RasCal Therapeutics, and Tempus; and personal fee from Jessen outside the submitted work. A. Zeki is the Co-founder, former President, and current CSO/CMO of *InStatin, Inc* and *InVixa, Inc*. No disclosures were reported by the other authors.

Ethics approval and consent to participate

Animal experiments were following Institutional Animal Care and Use Committee (IACUC) approved protocol (Protocol No. 20080) at the University of California, Davis, and all authors adhered to relevant ethical regulations for animal testing and research.

Consent for publication

Not applicable.

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Abstract

There is an unmet clinical need to develop novel strategies to overcome resistance to tyrosine kinase inhibitors (TKIs) in patients with oncogene-driven lung adenocarcinoma (LUAD). The objective of this study was to determine if simvastatin could overcome TKI resistance using the *in vitro* and *in vivo* LUAD models. Human LUAD cell lines, tumor cells, and patient-derived xenografts (PDXs) from TKI-resistant LUAD were treated with simvastatin, either alone or in combination with a matched TKI. Tumor growth inhibition was measured by the MTS assay and expression of molecular targets was assessed by immunoblots. Tumors were assessed by histopathology, immunohistochemistry (IHC) stain, immunoblots and RNA sequencing. We found that simvastatin had a potent antitumor effect in tested LUAD cell lines and PDX tumors, regardless of tumor genotypes. Simvastatin and TKI combination did not have antagonistic cytotoxicity in these LUAD models. In an osimertinib-resistant LUAD PDX model, simvastatin and osimertinib combination resulted in a greater reduction in tumor volume than simvastatin alone ($P < 0.001$). Immunoblots and IHC stain also confirmed that simvastatin inhibited TKI targets. In addition to inhibiting HMG-CoA reductase, RNA sequencing and Western blots identified the proliferation, migration, and invasion-related genes (such as PI3K/Akt/mTOR, YAP/TAZ, focal adhesion, extracellular matrix receptor), proteasome-related genes, and integrin ($\alpha 3\beta 1$, $\alpha v\beta 3$) signaling pathways as the significantly downregulated targets in these PDX tumors treated with simvastatin and a TKI. The addition of simvastatin is a safe approach to overcome acquired resistance to TKIs in several oncogene-driven LUAD models, which deserve further investigation.

Keywords

statins; simvastatin; oncogene-driven lung adenocarcinoma; tyrosine kinase inhibitors; acquired resistance; patient-derived xenograft; HMGCR; EGFR mutation; ALK fusion; HER2 mutation

Introduction

Statins, a class of drugs that act as competitive inhibitors of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase (HMGCR), are widely prescribed for the treatment of hypercholesterolemia and cardiovascular disease [1]. In addition to their cholesterol-lowering effects, statins exhibit anti-cancer properties by inhibiting the mevalonate pathway via both cholesterol-mediated and non-cholesterol-mediated mechanisms [2]. As a repurposed drug, statins offer the advantages of safety, low cost, and the ability to treat comorbidities when compared to conventional cancer therapeutics.

Despite the potential benefits of statins in cancer therapy, conflicting data have emerged regarding their efficacy in improving chemotherapy outcomes for cancers [3, 4]. There are two major classes of statins, which include hydrophilic statins (such as pravastatin and

rosuvastatin) and lipophilic statins (such as simvastatin). Compared to hydrophilic statins, simvastatin has a greater ability to penetrate cell membranes through passive diffusion and has higher cytotoxic and pro-apoptotic activities [5–7]. Both epidemiological and international studies support that lipophilic statins have stronger anticancer effects than hydrophilic statins [8]. Thus, simvastatin is one of the statins that has been investigated as a potential anticancer drug.

Simvastatin has been shown to elicit a broad spectrum of anti-cancer properties. It induces apoptosis and autophagy, while suppressing proliferation by dephosphorylating sequential signaling cascades of PI3K/Akt/mTOR and MAPK/ERK pathways, thereby inhibiting breast cancer growth [9]. Moreover, simvastatin downregulates PTEN expression via NF-κB, further attenuating breast cancer cell growth [10]. In addition to these effects, simvastatin inhibits ferroptosis, pyroptosis, and angiogenesis, suggesting its potential as a versatile multi-targeted anti-cancer agent [11–14]. Its ability to modulate the tumor microenvironment further enhances its therapeutic potential [15, 16]. Simvastatin also exerts potent anti-cancer effects in lung cancers. Specifically, it disrupts growth and survival pathways in small cell lung cancer (SCLC) cells by inhibiting RAS signaling [17].

Lung adenocarcinoma (LUAD) is the most common histology of non-small cell lung cancer (NSCLC) and frequently harbor actionable, driver oncogene mutations. In LUAD cells, simvastatin prevents proliferation and osteolytic bone metastases by regulating the expression of CD44, P53, MMP family members, and inactivating the MAPK/ERK signaling pathway [18]. Furthermore, statins can break down communication between cancer cells and mesenchymal stromal cells (MSCs) by inhibiting the secretion of CCL3 by cancer cells and IL-6 and CCL2 by MSCs [19]. Moreover, the combination of simvastatin with erlotinib has been shown to synergistically induce cytotoxicity and overcome erlotinib resistance in K-RAS mutated LUAD via apoptosis [20]. In gefitinib-resistant LUAD patients, simvastatin may improve the efficacy of therapy [21]. Additionally, in patients with malignant pleural mesothelioma and advanced NSCLC, simvastatin enhances the anti-tumor effects of programmed death receptor-1 (PD-1) inhibitors [22]. Pereira et al. have reported that statins can temporarily modulate the epidermal growth factor receptor (EGFR) and prostate-specific membrane antigen (PSMA) on the surface of tumor cells, thereby enhancing the tumor-binding avidity of monoclonal antibodies such as panitumumab, cetuximab, and huJ591, and synergizing with their anti-tumor effects [23].

The use of statins as anti-cancer agents is still considered experimental, and additional research is necessary to understand their mechanism of action, determine the most effective delivery route, evaluate their safety, and establish their clinical efficacy. Our study herein examined whether simvastatin could overcome resistance to TKIs in oncogene-driven LUAD using *in vitro* and *in vivo* models.

Materials and Methods

Human LUAD cell lines

Human LUAD cell lines A549 (*KRAS* G12S), H3255 (*EGFR* L858R), and H1975 (*EGFR* L858R and T790M) were obtained from the American Type Culture Collection (Manassas,

VA). Primary tumor cells were isolated from the malignant pleural effusion of LUAD patients with oncogene-driven mutations, including EGFR mutation, HER2 mutation, and RET fusion. Patient biospecimens were collected under an institutional review board (IRB)-approved protocol (Protocol No. 226210) at the University of California, Davis. The cells were cultured in RPMI1640 growth medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37°C in a 5% CO2 humidified incubator.

Reagents and antibodies

The simvastatin sodium salt was obtained from Millipore Sigma (MA, USA), and osimertinib (AZD9291) was obtained from Selleck Chemicals (TX, USA). We chose to use the sodium salt which is an 'active form' of simvastatin, as opposed to simvastatin lactone which is the inactive pro-drug which would then require *in situ* hydroxylation and activation inside cells. The sodium salt is a carboxylate form of simvastatin active in whole cells and in cell-free preparations. The RNeasy mini kit was obtained from QIAGEN (Venlo, Netherlands). Antibodies specific to p27, BCL-XL, phospho-AKT, phospho-MEK, phospho-STAT3, phospho-EGFR, EGFR, and actin were purchased from Cell Signaling Technology (Danvers, MA, USA).

Culture of tumor cells and growth inhibition by the MTS assay

The H1975, A549, and 3255 cell lines, as well as the primary tumor cells, were seeded in 96-well plates at a density of 5×10^3 cells/well and incubated overnight. The cells were then treated with various concentrations (0, 0.001, 0.01, 0.1, 1, and 10 μM) of osimertinib or alectinib (as indicated) or simvastatin (0, 0.01, 0.1, 1, 10, 25, 50, 75, and 100 μM). The cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay as previously described. After 72 hours of incubation, the MTS solution was added, and the cell viability was measured by the absorbance at 490 nm using a SpectraMax M3 microplate reader (Molecular Devices, USA). The untreated cells served as a control. The results were presented as the average cell viability ± standard deviation [(ODtreat−ODblank)/(ODcontrol−ODblank) × 100%], calculated from triplicate wells. The data were presented as the mean ± standard deviation unless noted otherwise. All experiments were performed in triplicate, and statistical analysis was performed using Graph Prism software (version 8.21). A two-sided P-value of <0.05 was considered statistically significant.

Establishment of LUAD xenograft models

Studies in mice were conducted in compliance with Institutional Animal Care and Use Committee (IACUC) approved protocol (Protocol No. 20080) at the University of California, Davis, and all authors adhered to relevant ethical regulations for animal testing and research. PDX models were generated from patients with metastatic LUAD as described before [24]. Briefly, NOD-scid IL2Rgammanull (NSG) mice were obtained from JAX Lab (Sacramento, CA, USA), and 5×10^6 H3255 or H1975 cells were subcutaneously injected into the right flank. A PDX model was generated from patients with metastatic LUAD harboring oncogenic mutations (EGFR L858, ALK, or HER2) and implanted into the flank of NSG mice at 5–6 weeks of age. Figure S1 illustrates the key steps in generating a LUAD PDX model used in this study. Tumor size was monitored in vivo, and ex vivo

using imaging studies and calculated using the formula: ½ (length × width^2). Treatment was initiated at week three or when tumors reached an average size of 150–200 mm^3. Mice were randomized into control and treatment groups, with 4 mice per group. They received vehicle control, single-agent treatment (osimertinib, alectinib, or simvastatin), or TKI and simvastatin combination therapy. Osimertinib, brigatinib and afatinib was 25 mg/kg daily by oral gavage, respectively. In comparison, simvastatin was 10 mg/kg, administered four times per week by oral gavage. Tumor sizes were measured daily after treatment initiation. Data were presented as the mean ± SD and analyzed using Graph Prism software (Version 8.21). Statistical significance was determined using a two-sided P-value < 0.05.

Establishment of primary lung cancer cell lines from LUAD PDXs

Primary lung cancer cell lines from established patients' LUAD PDX tumors were generated as described before [25–27]. Briefly, fresh LUAD PDX tumors were minced into small pieces less than one mm^3 using sterile eye scissors. The minced tissue was washed extensively in RPMI 1640 medium and centrifuged at 300 g for 5 minutes. The tissue pieces were then incubated in an enzyme mixture, consisting of 400 U/ml collagenase type IV, 0.05 mg/ml collagenase type I, 0.025 mg/ml hyaluronidase (Sigma-Aldrich, St Louis, MO, USA), 0.01 mg/ml DNase I, and 0.2 U/ml soybean trypsin inhibitor (Boehringer Mannheim, Indianapolis, IN, USA) dissolved in RPMI 1640 for 2–4 hours at 37°C. The enzymatic digestion was stopped when most of the tissue pieces had become cell suspensions. The cells were washed in RPMI 1640 and centrifuged at 300 g for 5 minutes, then transferred into standard tissue culture-coated flasks (Corning Life Sciences, USA) and cultured in RPMI 1640 growth medium supplemented with 10% FBS (Gibco) at 37°C in the presence of penicillin, streptomycin, and amphotericin B (0.25 mg/ml; Invitrogen, USA). All tumor cells were cultured in a humidified incubator with 5% CO2 at 37°C and the culture medium was changed every 2–3 days.

Analysis of cellular proliferation and function by cell attachment assay and Western blotting analysis Western blot

The H1975 and LG2605 cell lines were treated with osimertinib and simvastatin under different conditions and collected by centrifugation at 14,000 rpm for 10 minutes. Total cellular proteins were extracted from the cell lysates using a lysis buffer (1% Triton X-100, 50 mM Tris-HCl pH 8.0, and 150 mM NaCl) with protease inhibitors. Protein concentration was determined using a BCA protein assay kit (Applygen). Thirty micrograms of protein were separated by electrophoresis on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were then probed with primary antibodies against pEGFR Y1068, EGFR, pAKT S473, AKT (40D4), pMEK1/2 S217/221, MEK1/2 47E6, pSTAT3 Y705, and STAT3 124H6 (Cell Signaling Technology, Danvers, MA) and integrin α3 (sc-374242), integrin β1 (sc-59829), and β-actin (sc-47778) (Santa Cruz Biotech) at a dilution of 1:400. The secondary antibodies used were anti-mouse IgG HRP-linked antibody (Cell Signaling Technology, 1:500; #7076) or anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology, 1:500; #7074). After blocking for 2 hours at room temperature with 5% nonfat dry milk in blotto solution (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20), the membranes were incubated overnight at 4 °C with primary antibodies. The membranes were washed (3 times, 5 minutes each) with TBST solution and

incubated with the secondary antibodies for 2 hours at room temperature. Protein expression was analyzed using Gel Doc™ software (XR+ Imager, Bio-Rad, USA). The expression of each protein was normalized to β-actin in each sample.

RNA sequencing analysis

Total RNA was isolated from 10 mg of tumor tissue using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. RNA-sequencing was performed at Novogene Corporation Inc. (https://en.novogene.com), which performed the quality control analysis and constructed the library using TruSeq Stranded Total RNA Sample Prep Kit (Illumina). The sequencing was performed on the NovaSeq 6000 system (NovaSeq PE150) at the Novogene UC Davis Sequencing Center. The raw sequencing data were obtained for analysis. Reads were aligned to Human hg38 using Salmon with standard settings.

Immunohistochemistry (IHC) and Immunofluorescence analysis

Tissue microarray (TMA) sections were subjected to de-waxing using xylene twice, followed by rehydration with 100% ethanol for 5 minutes and then with 95% and 80% ethanol for 5 minutes each. Subsequently, the TMA sections were rinsed with PBS. Antigen retrieval was carried out by incubation in 10 mM sodium citrate buffer with a pH of 6.0 at 95–100°C for 20 minutes. After cooling to room temperature, the TMA sections were rinsed once with PBS, followed by blocking endogenous peroxidase with 1% H_2O_2 for 5 minutes and blocking non-specific binding sites with Power Block (BioGenex) for 5 minutes at room temperature. The TMA sections were then incubated overnight with the specific antibody, followed by rinsing with PBS and incubation with a biotin-conjugated goat anti-rabbit IgG (BioGenex) as the second antibody. Subsequently, the TMA sections were incubated with streptavidin conjugated HRP (BioGenex) for 20 minutes at room temperature. HRP activity was detected using DAB as substrate (BioGenex) and nuclei were counterstained with hematoxylin (Cell Signaling). For immunofluorescence detection, cells were seeded at a density of 3×10^5 cells/well on round glass coverslips in 6-well plates. Following various treatments, the cells were fixed with 4% paraformaldehyde in PBS for 20 minutes. Subsequently, the cells were permeabilized using 0.2% Triton X-100 for 5 minutes at room temperature, followed by blocking with 1% BSA/PBS for 1 hour at room temperature. Detection of pEGFR, and IGTA3 was performed using their corresponding antibodies, and the images were acquired using a Zeiss Observer Z1 microscope (Zeiss, Germany).

Data and statistical analyses

Descriptive statistics for continuous and categorical variables were stratified by binding to each integrin subtype or marker. All data are shown as mean ± standard deviation (SD) with at least three independent measurements. Comparisons between groups were performed using one-way analysis of variance for multiple groups, while the two-sample t-test was employed for continuous variables. All analyses were conducted using SAS, university edition 2.5 9.4 M4 (SAS Institute, Cary, NC), and figures were made using GraphPrism software (Version 7.03). All statistical tests were two-sided, and a *p-value* less than 0.05 was considered statistically significant.

Availability of data and materials

The RNA sequencing data generated in this study are publicly available from the Gene Expression Omnibus (GEO) repository at GSE249807 before publication.

Results

Simvastatin had in vitro cytotoxicity in LUAD cell lines and patients' tumor cells.

The *in vitro* cytotoxicity of simvastatin was evaluated in gene mutation cell lines and primary tumor cells from LUAD patients. The cell viability was determined after 72 hours of treatment with simvastatin at doses ranging from 0.1 μM to 100 μM. The results showed significant inhibition of cell viability in multiple LUAD cell lines carrying oncogene mutations. Cell lines H1975 (EGFR T790M, L858R), H3255 (EGFR L858R), and H2228 (EML4-ALK fusion) were found to be more sensitive to simvastatin treatment, with IC_{50} values of approximately 25 μM (Figure 1A, S2A). The A549 cell line carrying a K-RAS G12S mutation showed less sensitivity to simvastatin, with 75% of tumor cells still having high viability at 25 μM simvastatin. Additionally, *in vitro* cytotoxicity of simvastatin was evaluated in primary tumor cells from three LUAD patients. Tumor cells with ALK or RET mutations were found to be more sensitive to simvastatin than those with EGFR exon 19 deletion (Figure 1B, S2B). These results demonstrate the *in vitro* cytotoxicity of simvastatin in LUAD cell lines and patient-derived tumor cells.

Simvastatin potentiated TKI functions in LUAD cell lines and overcame resistance in patients' primary tumor cells in vitro.

The antitumor effect of simvastatin was evaluated *in vitro* LUAD models. In the osimertinib-sensitive LUAD cell line H1975, simvastatin showed vigorous tumor-suppressing activity either as a single agent or in combination with osimertinib (Figure 1C). The effect of the drugs was also tested in a primary tumor cell line derived from a patient with EGFR exon 19 deletions, who showed resistance to osimertinib treatment. The results showed that while osimertinib monotherapy had a limited impact on cell viability, its combination with simvastatin led to a significant decrease in cell viability (Figure 1D). These findings suggest that simvastatin has the potential to potentiate the function of TKIs and overcome resistance in LUAD cell lines and patients' primary tumor cells *in vitro*.

Simvastatin enhanced the in vitro cytotoxicity and growth inhibition with osimertinib in patient-derived EGFR L858R mut osimertinib resistance cells

We examined the antitumor effect of simvastatin and osimertinib in a patient-derived cell line LG2605 resistant to osimertinib and harboring an EGFR L858R mutation. Our findings indicate that simvastatin has a potent synergistic *in vitro* cytotoxic effect when combined with osimertinib in these cells (Figure 2). Longer treatment durations (72 hours) (Figure 2B) resulted in greater inhibition of cell viability compared to 48 hours (Figure 2A) of treatment. The growth inhibition assay also revealed that simvastatin alone or combined with osimertinib effectively inhibited cell proliferation *in vitro* (Figure 2C). The morphology changes of LG2605 cells to these drug treatments under the light microscopy are shown in Figure S3A. Single-cell immunofluorescence analysis of lung cancer cells showed that

tumor cells remained intact in the control group and the group treated with osimertinib alone but not in the groups treated with simvastatin alone or in combination with osimertinib (Figure S3B). Furthermore, immunofluorescence analysis of surface biomarkers showed that simvastatin alone or combined with osimertinib effectively suppressed the expression of pEGFR (Figure S3C), ITGB1 (Figure S3D), and ITGA3 (Figure S3E). These results demonstrate the synergistic *in vitro* cytotoxicity and growth inhibition effect of simvastatin and osimertinib in the patient-derived, osimertinib-resistant, EGFR L858R mutant LUAD model.

Simvastatin enhanced the antitumor effect with osimertinib in different signaling molecules downstream of EGFR in H1975 cell lines and patient-derived, osimertinib-resistant EGFR L858R mutant cells.

We next examined the effects of simvastatin and osimertinib on the EGFR and downstream signaling pathways in both sensitive and resistant LUAD models. The results indicated that the combination of simvastatin and osimertinib, as well as simvastatin alone, effectively reduced EGFR expression and phosphorylation in the osimertinib-sensitive cell line H1975. This reduction also led to decreased phosphorylation levels of downstream signaling molecules, including STAT3, AKT, MEK, and BCL-XL, as well as increased p27 levels and Integrin α3 (Figure 3A). However, in the osimertinib-resistant patient-derived cell line LG2605, the treatment of simvastatin alone or in combination with osimertinib effectively decreased EGFR expression and phosphorylation, as well as the phosphorylation levels of downstream signaling molecules, and increased p27 expression levels. In contrast, treatment with osimertinib alone did not produce similar results (Figure 3B). These findings support that simvastatin may enhance the inhibition of the EGFR signaling pathway and overcome osimertinib resistance when used in combination with osimertinib.

Simvastatin overcame resistance to TKIs in vivo.

We investigated the impact of simvastatin and TKIs in various oncogene-driven LUAD PDX models. Mice were transplanted with tissue from patients harboring EGFR L858R mutation, HER2 exon 20 insertion mutation, or EML4-ALK fusion- LUAD, as well as with H1975 cell lines. Mice were divided into four groups: control (vehicle), simvastatin, TKIs (osimertinib, afatinib, and brigatinib), or a combination of simvastatin and a TKI. First, in the osimertinib-sensitive LUAD H1975 xenograft model, although simvastatin alone had less tumor growth inhibition compared to osimertinib alone, the addition of simvastatin to osimertinib led to statistically more tumor growth inhibition compared to osimertinib alone or simvastatin alone ($p<0.001$) (Figure 4A, B; Table S1). In the osimertinib-resistant EGFR L858R-mutant LUAD PDX model, simvastatin alone demonstrated substantial tumor inhibitory activity. However, the most pronounced tumor inhibitory effect was observed in the group treated with a combination of simvastatin and osimertinib, yielding the smallest xenograft tumor among all groups ($p<0.001$) (Figure 4C, D; Table S1). In the brigatinib-sensitive ALK-positive LUAD PDX model, simvastatin and brigatinib, either alone or in combination, inhibited tumor growth. The tumor was more responsive to brigatinib than simvastatin ($P<0.0001$). Furthermore, simvastatin monotherapy could be given as a treatment option as it reduced tumor volume by 95% (Figure 4E, F; Table S1). In the afatinib-resistant HER2-mutant LUAD PDX model, afatinib alone did not significantly

inhibit tumor growth. However, both simvastatin alone and in combination with afatinib exhibited comparable levels of tumor inhibition by 95% without any antagonistic effect ($p<0.001$) (Figure 4G, H; Table S1). No obvious weight loss was detected in the vehicle and drug treated mice across these groups (Figure S4). These findings suggest that the addition of simvastatin to TKIs might overcome resistance to TKIs without antagonistic effect in oncogene-driven LUAD models.

Heatmaps of key altered gene expression in an osimertinib- resistance, EGFR L858R-mutant PDX model

We further assessed the impact of simvastatin and osimertinib on gene expression by RNA sequencing in the osimertinib-resistant, EGFR L858R mutant LUAD PDX model (LG2605). Compared to the control group, there were 644 upregulated differentially expressed genes (DEGs) and 1191 downregulated DEGs in the combination treatment group (Figure S5A). Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analyses (Figure S5B and C) and Gene Ontology (GO) analyses (Figure S5D and E) revealed that in osimertinib-resistant PDX tumors, there were upregulation of the major oncogenic pathways, including receptor tyrosine kinase pathway (e.g., AKT1, PI3K, MAPK), angiogenesis (e.g. VEGFc), YAP/TAZ pathway, and other oncogenic markers (e.g., MYC, HMGB1, SET, BZW1, LSR) (Figure 5A, second lane). Conversely, treatment with simvastatin or a combination of simvastatin and osimertinib resulted in a significant reduction in the expression of these genes (Figure 5A, third and fourth lanes, respectively). Similar changes were observed in lipid metabolism pathways (Figure 5B). In addition, simvastatin was found to modulate a range of proteasome-related genes, including PSMC, PSMB, PSMD, and PSMG gene families, with most of these genes being downregulated by simvastatin and TKI combination treatment (Figure 5C). Consistent with our previous report that integrin α3β1 mediated resistance to EGFR TKIs (24), we observed ITGB1 and INTA3 were elevated in osimertinib-treated tumors, which were inhibited by simvastatin alone or in combination with osimertinib (Figure 5D). We further verified the expression of several key target expression in these pathways by immunohistochemical stains (Figure 5E). The combination treatment group had the lowest expression levels of p-EGFR, p-AKT, ITGA3, and ITGB1, followed by lower expression levels in the simvastatin treatment group compared to the control and osimertinib treatment groups. In the independent TCGA LUAD database, we confirmed that higher RNA expression of ACLY (Figure S6A), HMGCR (Figure S6B), HMGB1 (Figure S6C), FASN (Figure S6D), SET (Figure S6E), BZW1 (Figure S6F), PSMC (Figure S6G–I), PSMB (Figure S6J–L), and PSMD (Figure 6SM–O) genes was independently associated with a poor prognosis in NSCLC patients compared with those genes with lower expression ($p < 0.05$).

Discussion

Targeting EGFR and other oncogene trafficking in LUAD through modulation of key membrane-regulatory proteins, such as integrins, caveolins, annexins, and sortilin, has been proposed as a strategy for inhibiting the activity of oncogene-driven receptor tyrosine kinases [28, 29]. These oncogene receptors have a transmembrane lipophilic segment that may be susceptible to the effects of statins. However, the interaction between these

membrane proteins in regulating the function of RTKs beyond EGFR in LUAD has not been fully explored. This approach in targeting membrane trafficking may be particularly relevant for oncogene-driven RTKs in LUAD that are not driven by *gain-of-function* mutations in tyrosine kinase domains and those that drive ligand-independent EGFR or other RTK signaling, as these play a critical role in regulating the tumor microenvironment, immunity, inflammation, and tissue repair [30].

Simvastatin, a well-known cholesterol-lowering drug, has potential anti-cancer properties. Recently, several epidemiology and observation studies showed the use of statins improve the therapeutic outcomes and survivals in patients with breast cancer, gastric cancer, lung cancer, and melanoma [31–35]. Several clinical trials have indicated that simvastatin reduces lung cancer-specific mortality rates and improves survivals (Table S2). In preclinical studies, simvastatin reduces cancer cell migration, invasion, and inhibits tumor growth via multiple mechanisms in mouse models [36, 37]. Our study represents the first report of simvastatin's ability to overcome resistance in various TKI-resistant LUAD PDX models. We found that the combination of simvastatin and TKIs demonstrated the favorable tumor inhibitory effect in all tested NSCLC models, effectively overcoming resistance to EGFR, ALK and HER2 TKIs and enhancing their anticancer activity.

First, we found that simvastatin directly inhibits EGFR tyrosine kinase phosphorylation, leading to suppression of downstream signaling pathways (AKT, MAPK, STAT) and induction of apoptosis in cancer cells. Furthermore, simvastatin modulated EGFR resistance through regulation of anti-apoptotic protein expression, such as Bcl-XL and p27, and promotion of EGFR degradation through lipid metabolism pathways. Additionally, our study demonstrated simvastatin's inhibition of integrin α3β1 *in vitro* and *in vivo*. Integrins play a crucial role in cell behavior, including cell adhesion, migration, and signaling, and have been implicated in the development and progression of various cancers [38]. The integrin α3β1 heterodimer has been linked to the interaction between cancer cells and the extracellular matrix, and its activity has been associated with cancer cell migration, invasion, and tumor growth in previous studies [39–41]. By modulating the integrin α3β1 pathway, simvastatin may interfere with these processes, prevent cancer progression, and overcome TKI resistance.

Consistent with previous reports [42–44], this study found that a decrease in HMGCR expression following simvastatin treatment, suggesting a potential role for negative feedback mechanisms in regulating cholesterol synthesis. This reduction in HMGCR expression may be mediated through several interrelated mechanisms, including inhibition of protein prenylation, induction of apoptosis, and modulation of mevalonate metabolism. HMGCR degradation via the proteasome pathway has been described previously. Specifically, the proteasome-mediated degradation of HMGCR was triggered by sterol addition, leading to its ubiquitination [45, 46]. Moriyama et al indicated that HMGCR was cleaved by a cysteine protease associated with the ER membrane, a process which was accelerated by sterols or mevalonate, the end products of the pathway governed by HMGCR [46]. In addition, simvastatin has been shown to interact with the proteasome signaling pathway. The closed ring forms of these drugs influence various activities of the 20S proteasome [47]. Our results are consistent with these findings. Furthermore, we found that in the TCGA

database, high HCMGCR expression was associated with a poor prognosis in patients with NSCLC. Several studies have shown that the proteasome-related gene families modulate tumor microenvironment and play an important role in cancer prognosis [48, 49]. We also found that simvastatin alone or in combination with TKI downregulated the expression of Yes-associated protein (YAP) and its related genes. Our results are consistent with the data from several recent publications showing statins could modulate the YAP/TAZ pathway in pancreatic, gastric, prostate and breast cancers [50–54]. The precise mechanisms by which simvastatin or other statins inhibit or downregulate HMGCR during cancer treatment remains to be fully elucidated.

Based on these findings, we summarize the potential mechanisms of simvastatin in oncogene-driven LUAD in Figure 6. In addition to inhibiting the HMGCR pathway, simvastatin markedly interferes with critical oncogenic receptor tyrosine kinase (RTK) signaling cascades, attenuates the YAP/TAZ signaling pathway, and culminates in the significant downregulation of essential proliferative genes, such as MKI67, PCNA, and CCND1. Furthermore, simvastatin modulates the proteasome activity, promotes the apoptosis in cancer cells, and inhibits the integrin α3β1 function. The influence of statins on tumor cells is complex and depends on a variety of factors, including the biochemical pathways engaged in the tumor cells, the tumor type, the dosage of the administered statin, and the existing state of the tumors, etc. [3, 16, 42, 55]. Further pre-clinical and clinical investigation are warranted to better understand these complex interactions and their implications for cancer therapy.

There are several ongoing clinical trials evaluating the statins as anticancer drugs in multiple solid tumor types, including lung cancer (Table S3). Some preclinical studies have shown that simvastatin (10 to 40 mg) can effectively modulate EGFR signaling in cancer cells without causing significant toxicity to normal cells [22, 37, 56]. Conversely, other research has indicated that the impact of simvastatin on EGFR signaling may be dose-dependent, potentially necessitating higher doses for clinical application [37, 56]. According to established pharmacological recommendations, a ratio of 0.081 allows us to convert the drug dose in mice to human equivalent dosing based on the differences in body surface area [57]. In our study, the simvastatin dosage in mice (10 mg/kg) is equal to ~49 mg for a 60-kg human or is estimated at ~61 mg for a 75-kg human, which is approximately 1.5-fold higher than the dose typically used in the clinical setting for cardiovascular disease management, i.e., simvastatin (20–40 mg). However, the human dose range for simvastatin (orally once daily) is 10 to 80 mg, therefore, an effective human dose of 50–60 mg in our experiments was well within this range. Most clinicians avoid the higher dosing of 80 mg given the potential risk of causing side effects (e.g., myalgias, liver dysfunction). Simvastatin has various half-lives based on its formulation and administration route. In rats, the elimination half-life of simvastatin is approximately 4–5 hours [58], and in rat liver microsomes it is 33.51 minutes. In addition, the half-life of simvastatin varies according to whether it is the inactive lactone (i.e., simvastatin) or the hydroxyl acid active form (i.e., simvastatin acid). In rats, oral administration of simvastatin (at 20 mg/kg) yields a half-life of 7.62 hours, and for simvastatin acid it is 3.79 hours. The intravenous administration (at 2 mg/kg) of simvastatin yields a half-life of 42.45 minutes, and for simvastatin acid it is 31.55 minutes [59]. In contrast, simvastatin has an elimination half-life of 1.4 to 3 hours in

human [60]. , which changes how one interprets the dosing and metabolism of simvastatin in rodent models and the extrapolation of results to the human situation. Of note, in rodent models it is possible that there is relatively greater exposure to simvastatin as compared to human dosing simply based on half-life considerations. The disparity in half-lives between rats and humans might have further implications in clinical development of statins. To deliver higher doses of a statin in cancer therapy without increasing adverse effects, new formulations aiming to enhance solubility and bioavailability should be explored, including utilizing nanoparticles, liposomes, or polymeric micelles for targeted drug delivery [61]. While preliminary studies indicate nanoparticle-facilitated statin delivery may amplify anticancer efficacy while mitigating side effects associated with high-dose administration [62, 63], a comprehensive investigation into the benefits and constraints of these delivery techniques is warranted.In conclusion, our study has shown that although simvastatin had variable antitumor effects on individual PDX models, the combination of simvastatin and a TKI was safe without any antagonistic effect observed. Thus, the addition of simvastatin to a TKI might be a safe strategy to overcome acquired resistance to TKIs in patients with oncogene-driven LUAD. Further preclinical and clinical investigation is warranted to determine optimal dosing and delivery strategy of statins in lung cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

ACLY	ATP Citrate Lyase
ALK	anaplastic lymphoma kinase
AKT	protein kinase B
BCL-XL	B-cell lymphoma-extra large
BZW1	basic leucine zipper and W2 domains 1
CCL	chemokine (C-C motif) ligand
CCND1	Cyclin D1
CI	confidence interval
DEGs	differentially expressed genes
DMSO	dimethyl sulfoxide
EDTA	ethylenediamine tetraacetic acid

EGFR	epidermal growth factor receptorERK, Extracellular signal-regulated kinase
FASN	fatty acid synthase
FBS	fetal bovine serum
GO	gene ontology
HER2	human epidermal growth factor receptor 2
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HMGB1	high mobility group box1
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
IHC	immunohistochemistry
IRB	Institutional Review Board
ITG	integrin
KEGG	Kyoto Encyclopedia of Genes and Genomes
LUAD	lung adenocarcinoma
MAPK	mitogen-activated protein kinases
MEK	mitogen-activated protein kinase kinase
MKI67	marker of proliferation Ki-67
MMP	matrix metalloproteinases
MSCs	mesenchymal stromal cells
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
mTOR	mammalian target of rapamycin
N	number
NSCLC	non-small cell lung cancer
NSG	NOD-scid IL2Rgammanull
NGS	next generation sequencing
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PD-L1	programmed death-ligand 1
PDX	patient-derived xenograft

PI3K	phosphatidylinositol-3 kinase
PTEN	phosphatase and tensin homolog
PSMB	proteasome 20S subunit beta
PSMC	proteasome 26S subunit, ATPase
PSMD	proteasome 26S subunit, non-ATPase
PSMG	proteasome assembly chaperone
PSMA	prostate-specific membrane antigen
RET	rearranged during transfection
ROC	receiver operating characteristic
SCLC	small cell lung cancer
SD	standard deviation
SET	SET Nuclear Proto-Oncogene
STAT3	signal transducer and activator of transcription 3
NF-KB	nuclear factor-kappa B
TAZ	transcriptional coactivator with PDZ-binding motif
TCGA	The Cancer Genome Atlas
TKIs	tyrosine kinase inhibitors
TMA	tissue microarray
VEGF	vascular endothelial growth factor
YAP	yes-associated protein

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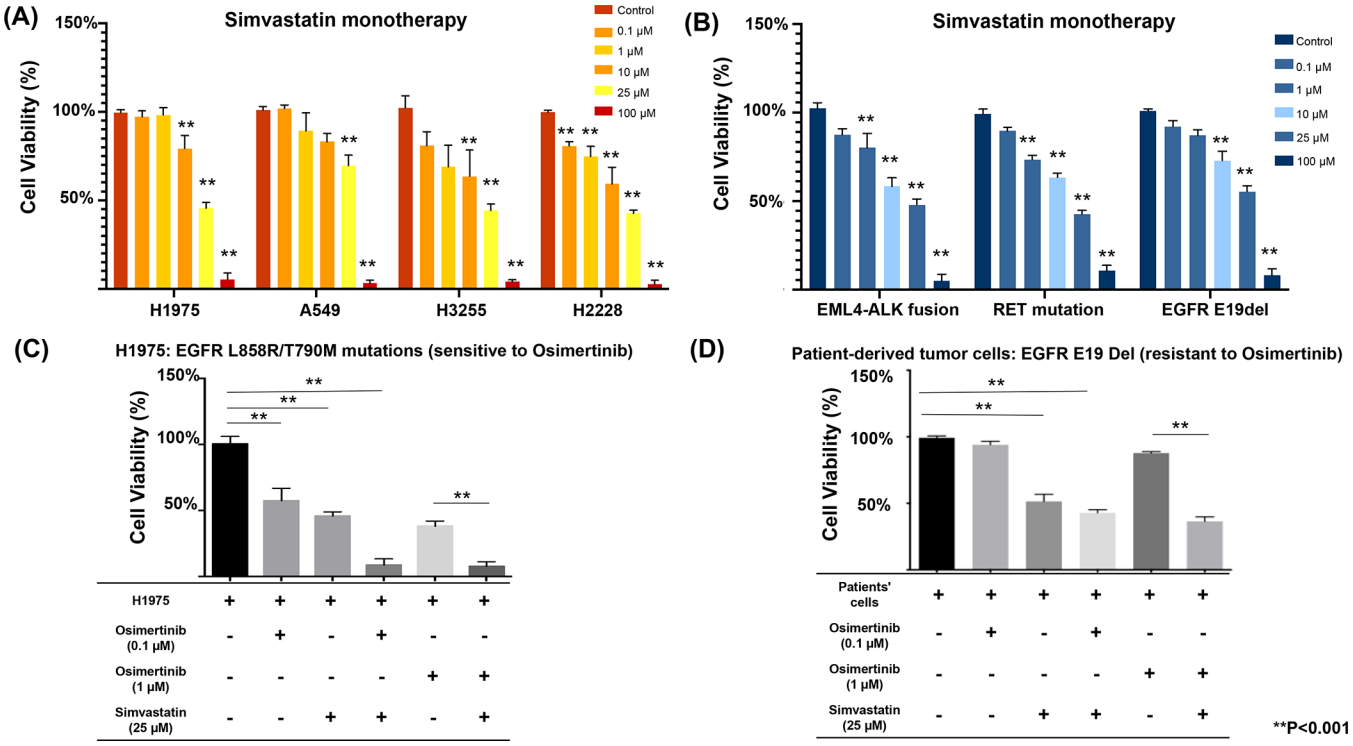

Figure 1. Simvastatin inhibited the growth of human LUAD cell lines and patient-derived LUAD cells.

(A) Growth inhibition of four LUAD cell lines, H1975, A549, H3255, and H2228. LUAD cells were treated with different doses of simvastatin (0.1 μM-100 μM) for 72 h and growth inhibitor of drugs was measured by the MTS assay using vehicle as 100% control. **(B)** Growth inhibition of LUAD cells isolated from malignant pleural effusion of patients with EGFR mutation or gene fusions of ALK, or RET. The patient-derived LUAD cells were treated with different doses of simvastatin (0.1 μM-100 μM) for 72 h. Simvastatin significantly inhibited cell growth in both human LUAD cell lines and patient-derived LUAD cells in a dose-dependent manner.

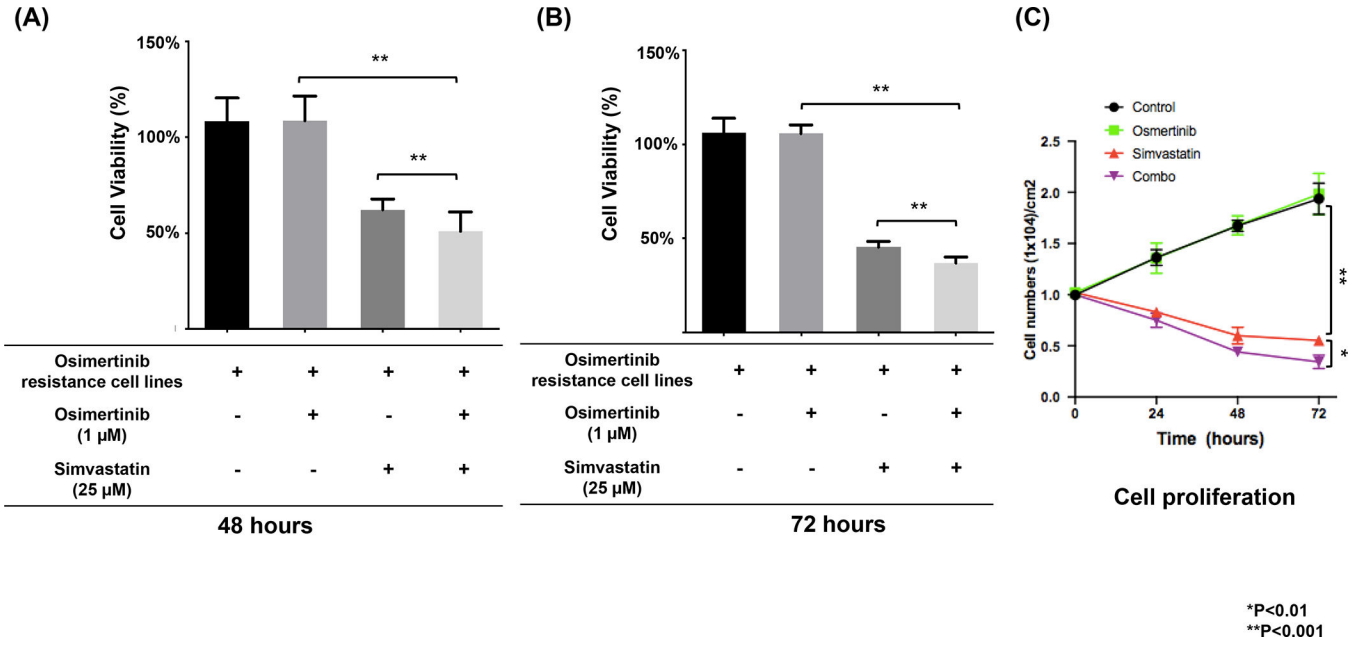

Figure 2. Simvastatin had synergistic *in vitro cytotoxicity* with osimertinib in osimertinib-resistant, patient-derived EGFR L858R mutant LUAD cells.

(A, B) Growth inhibition of Osimertinib-resistant, patient-derived EGFR L858R mutant LUAD. Osimertinib resistance LG2605 cells were treated with simvastatin (25 μM), osimertinib (1 μM), or their combination for 48 and 72 h. Simvastatin significantly inhibited cell viability in the tumor cells, demonstrating the effect of overcoming acquired resistance. **(C)** The proliferation of cells was assessed using an MTS assay, and the data were expressed as mean ± standard deviation. All data are presented as the mean of triplicate samples, and error bars indicate standard deviation (SD). Group comparisons were made using the Wilcoxon signed-rank test, with $P < 0.05$ considered statistically significant. Asterisks indicate statistically significant differences with *P <0.05 and **P<0.01.

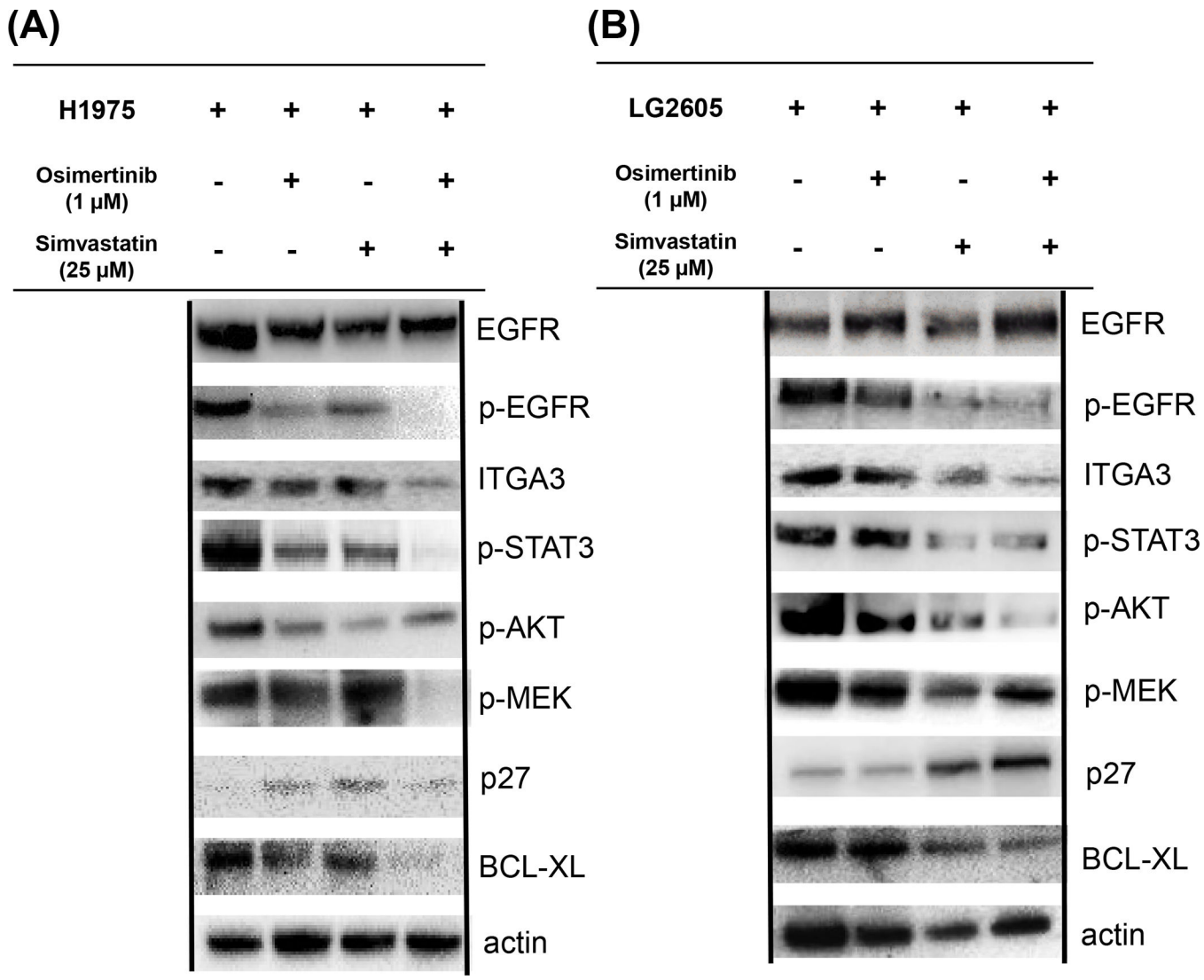

Figure 3. Simvastatin had synergistic effect with osimertinib in different signaling molecules downstream of EGFR in H1975 cell lines and Osimertinib-resistant, patient-derived EGFR L858R mutant LUAD cells.

(A) The effect of Simvastatin and Osimertinib on the expression of integrin α3, p27, BCL-XL, EGFR, and its key downstream signaling molecules in H1975 cells was analyzed after 48 h by Western blot. **(B)** The effect of simvastatin and osimertinib on the expression of the same signaling molecules was analyzed in LG2605 after 48 h by Western blot. Asterisks indicate statistically significant differences with *$p < 0.01$.

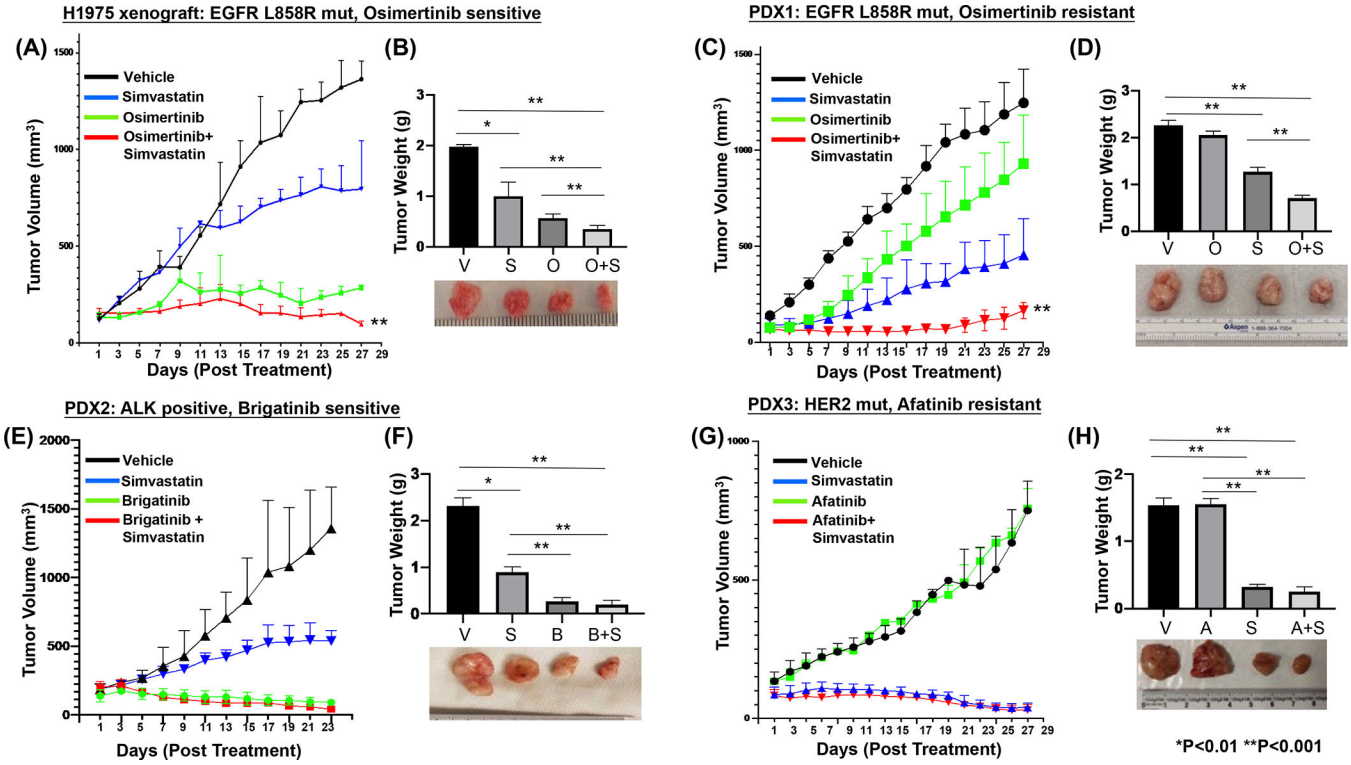

Figure 4. Lung adenocarcinoma xenografts model treated with vehicle, simvastatin, TKIs or both.

(A, C, E, G) The combination of simvastatin and TKIs (osimertinib, afatinib, and brigatinib) effectively inhibits the growth of H1975 xenograft (EGFR L858R/T790M mutant, osimertinib-sensitive LUAD), PDX1 (EGFR L858R mutant, osimertinib-resistant LUAD), PDX2 (HER2 Exon 20 insertion, afatinib-resistant LUAD), and PDX3 (EML4-ALK positive, crizotinib-resistant LUAD), respectively. PDX1 (EGFR L858R, PD-L1 IHC 95%) was established from a tumor biopsy after the patient with received osimertinib for 8 months and chemotherapy with carboplatin and pemetrexed for 5 cycles. PDX2 (HER2 A775_G776insYVMA, PD-L1 IHC negative) was established from a tumor biopsy after the patient with received afatinib for 7 months. PDX3 (EML4-ALK V5 variant, PD-L1 IHC 3%) was established from a tumor biopsy after progression on crizotinib for 13 months, ceritinib for 10 months and alectinib for 7 months. The patient had never received brigatinib. Xenografts were treated (4 times a week) with vehicle control, simvastatin, TKIs, and their combination starting on the same day after grouping for 29 consecutive days. Mice were euthanized when the tumor length reached 20 mm in any direction. The tumor dimensions were measured every 2 days. The tumor volume was calculated using the formula: 0.5 × length × width^2 (mm^3). Each measurement is mean ± SD. (B, D, F, H) At the end of the treatment, the mice were sacrificed, and the tumors were removed and weighed as indicated. The representative images of the excised tumors are shown. Asterisks indicate statistically significant differences with *p < 0.01 and **P<0.01.

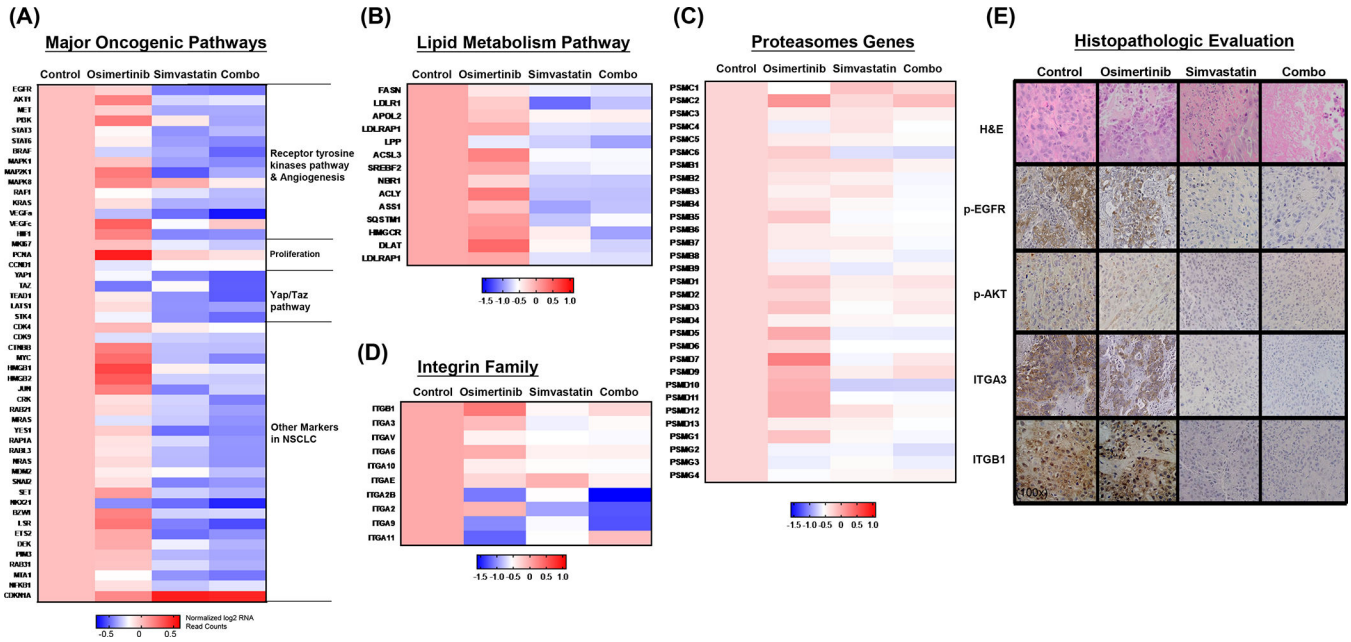

Figure 5. Heat map of key altered gene expression in osimertinib-resistant, EGFR L858R-mutant, PDX models and histopathological evaluation.

(A) RNA-seq analysis demonstrates a decrease in the expression of tumor-associated genes in the Simvastatin and combination treatment group. (**B, C, D**) Genes related to lipid metabolism, integrin family and proteasome activity are also decreasing in the simvastatin and combination treatment group. **(E)** Formalin-fixed paraffin-embedded LG2605 PDX tumor sections were stained for Hematoxylin and Eosin (H&E), pEGFR, pAKT, integrin α3, and integrin β1. More pEGFR, pAKT, integrin α3, and integrin β1 positive cells were observed in the control group, but significantly decreased in the simvastatin group and the combination group.

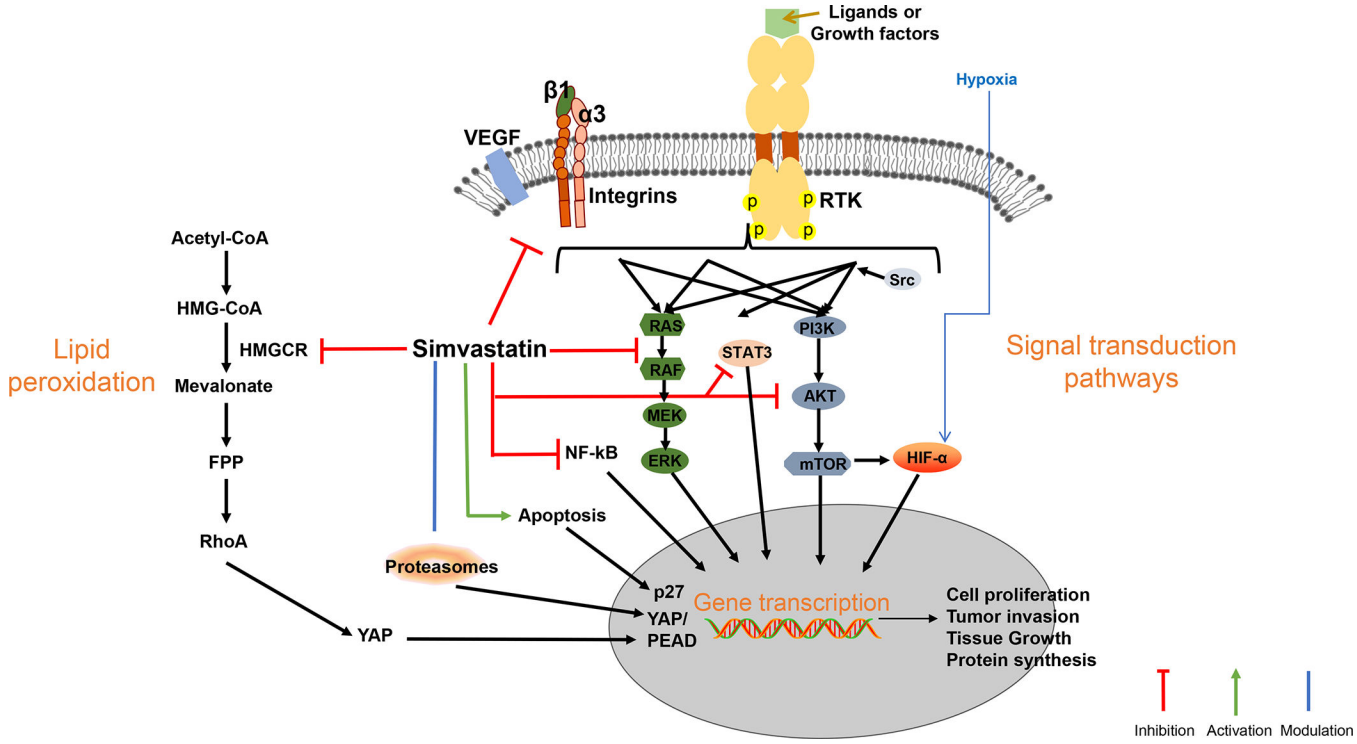

Figure 6. Schematic summary of molecular targets of simvastatin in oncogene-driven lung adenocarcinoma

Simvastatin inhibits the HMG-CoA reductase (HMGCR) pathway, modulating the essential metabolites (i.e., sterols and non-sterol intermediates) in the tumor. Concurrently, it suppresses major oncogenic receptor tyrosine kinase (RTK) signaling pathways, including PI3K, AKT, STAT3 and MAPK, mitigating the proliferative and survival cues initiated by these cascades. In addition, simvastatin has anti-angiogenic capabilities, undermining the vascularization essential for tumor growth and dissemination. It also manifests antiproliferative effects, notably by suppressing YAP/TAZ signaling, which culminates in the marked downregulation of critical proliferative genes such as MKI67, PCNA, and CCND1. This agent induces apoptosis, facilitating the programmed death of cancer cells, and modulates proteasome activity, the pivotal component in cellular processes, including the degradation of malfunctioned proteins and cell cycle regulation. Furthermore, simvastatin inhibits integrin functions, such as ITGA3, ITGB1, and ITGA6, which are vital in cancer cell adhesion, migration, and signaling, thereby exhibiting a holistic approach to curtailing tumor progression and proliferation.

Abbreviation: AKT, ATP Citrate Lyase; CCND1, Cyclin D1; ITG, integrin; HIF-a, hypoxia-inducible factor 1-alpha; FPP, farnesyl pyrophosphate; MAPK, mitogen-activated protein kinases, MKI67, marker of proliferation Ki-67; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol-3 kinase; PCNA, proliferating cell nuclear antigen; PSMB, proteasome 20S subunit beta; PSMC, proteasome 26S subunit, ATPase; PSMD, proteasome 26S subunit, non-ATPase; PSMG, proteasome assembly chaperone, STAT3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor, YAP/TAZ, yes associated protein; transcriptional coactivator with PDZ-binding motif