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Article

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Co-activation of STAT3 and YES-Associated Protein 1 (YAP1) Pathway in EGFR-Mutant NSCLC

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Abstract

Background: The efficacy of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) in EGFR-mutant non–small cell lung cancer (NSCLC) is limited by adaptive activation of cell survival signals. We hypothesized that both signal transducer and activator of transcription 3 (STAT3) and Src-YES-associated protein 1 (YAP1) signaling are dually activated during EGFR TKI treatment to limit therapeutic response.

Methods: We used MTT and clonogenic assays, immunoblotting, and quantitative polymerase chain reaction to evaluate the efficacy of EGFR TKI alone and in combination with STAT3 and Src inhibition in three EGFR-mutant NSCLC cell lines. The Chou-Talalay method was used for the quantitative determination of drug interaction. We examined tumor growth inhibition in one EGFR-mutant NSCLC xenograft model (n = 4 mice per group). STAT3 and YAP1 expression was evaluated in tumors from 119 EGFR-mutant NSCLC patients (64 in an initial cohort and 55 in a validation cohort) by quantitative polymerase chain reaction. Kaplan-Meier and Cox regression analyses were used to assess the correlation between survival and gene expression. All statistical tests were two-sided.

Results: We discovered that lung cancer cells survive initial EGFR inhibitor treatment through activation of not only STAT3 but also Src-YAP1 signaling. Cotargeting EGFR, STAT3, and Src was synergistic in two EGFR-mutant NSCLC cell lines with a combination index of 0.59 (95% confidence interval [CI] = 0.54 to 0.63) for the PC-9 and 0.59 (95% CI = 0.54 to 0.63) for the H1975 cell line. High expression of STAT3 or YAP1 predicted worse progression-free survival (hazard ratio [HR] = 3.02, 95% CI = 1.54 to 5.93, $P = .001$, and HR = 2.57, 95% CI = 1.30 to 5.09, $P = .007$, respectively) in an initial cohort of 64 EGFR-mutant NSCLC patients treated with firstline EGFR TKIs. Similar results were observed in a validation cohort.

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Conclusions: Our study uncovers a coordinated signaling network centered on both STAT3 and Src-YAP signaling that limits targeted therapy response in lung cancer and identifies an unforeseen rational upfront polytherapy strategy to minimize residual disease and enhance clinical outcomes.

Activating epidermal growth factor receptor (EGFR) kinase domain mutations are present in a meaningful number of non–small cell lung cancer (NSCLC) patients (1). Although the EGFR tyrosine kinase inhibitors (TKIs) collapse an extensive downstream signaling network in EGFR-mutant NSCLC that often elicits an initial antitumor response in patients (2,3), only approximately 5% of patients achieve more than 90% tumor reduction solely with an EGFR TKI (eg, erlotinib) and virtually all patients relapse on treatment, with a median progression-free survival of less than one year (4). Mutant EGFR inhibition in cell cultures mimics this clinical experience, with approximately 5% of cells remaining viable one week after EGFR inhibition as drug-tolerant or -resistant residual disease cells. These residual surviving cells then grow to form drug-resistant colonies that manifest as tumor relapse (acquired resistance) (3).

EGFR mutations activate phosphatidylinositol 3-kinase (PI3K)/AKT, Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3), but less so Ras/mitogen-activated protein kinase (MAPK) (5,6). EGFR inhibition produces an imbalance in EGFR signaling, promoting some signaling pathways while impairing others (6). STAT3 is activated almost immediately after erlotinib or gefitinib treatment (7,8) by tyrosine phosphorylation in part, downstream of interleukin-6 (IL-6) (9). We previously showed that EGFR inhibition induces an EGFR-TNF receptor–associated factor 2 (TRAF2) receptor interacting protein 1 (RIP1) inhibitor of nuclear factor kappa-B kinase (IKK) complex (EGFR-TRAF2-RIP1-IKK) and stimulates a nuclear factor-kappa B (NF-κB)–mediated transcriptional program that includes IL-6-STAT3 signaling upregulation (10). We also found that increased expression of the NF-κB inhibitor IκB was associated with better outcome in erlotinib-treated EGFR-mutant NSCLC patients (11). In addition to STAT3, IL-6 activates the Src family kinases (SFK; such as YES) and subsequently YES-associated protein 1 (YAP1) (12). Therefore, control of EGFR pathway activity occurs at multiple levels within the signal cascade and involves crosstalk and signal integration with other pathways such as IL-6 signaling, modifying the cellular response to EGFR TKI treatment (13).This connection between IL-6 activation and multiple downstream survival pathways including STAT3 and Src-YAP1 prompted us to explore the role of dual activation of STAT3 and Src-YAP1 in modulating the initial EGFR TKI response in lung cancer. While YAP1 activation can limit the response to RAF- and MEK-targeted therapies in BRAF- and RAS-mutant cancers (14), the role of YAP1 in limiting EGFR TKI response, particularly in concert with other key survival factors such as STAT3, has not been established.

In the current study, we hypothesized that Src-YAP1 signaling functions in conjunction with parallel STAT3 activation, potentially downstream of IL-6, to limit initial EGFR TKI response. We aimed to evaluate whether cotargeting EGFR, STAT3, and Src-YAP1 can improve responses in EGFR-mutant NSCLC models in comparison with single EGFR inhibition. Finally, in two independent cohorts of EGFR-mutant NSCLC patients treated with firstline EGFR TKIs, we explored STAT3 and YAP1 messenger RNA (mRNA) expression as predictors of progression-free survival.

Methods

Sample Collection

Pretreatment tumor specimens from advanced EGFR-mutant NSCLC patients were retrospectively collected from eight sites in Spain, France, Italy, and Colombia (Table 1). For the validation cohort, pretreatment tumor samples from advanced EGFR-mutant NSCLC patients were retrospectively collected from the Department of Oncology of Shanghai Pulmonary Hospital, affiliated with Shanghai Tongji University School of Medicine (Table 1). Clinical data were assessed in accordance with the protocol approved by the institutional review board of Germans Trias i Pujol Hospital, Badalona, Spain, and de-identified for patient confidentiality.

Chemicals and Reagents

The first-generation EGFR TKI gefitinib was purchased from Tocris Bioscience. TPCA-1 (2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide), a nonpeptidic small molecule inhibitor of the inhibitor of κB kinase-2 (IKK2) (15), and the Src homology 2-domain of STAT3 (16), was purchased from Sigma Aldrich. AZD0530 (SFK inhibitor; saracatinib) and the third-generation EGFR TKI AZD9291 were purchased from Selleck Chemicals. Drugs were prepared in dimethyl sulfoxide (DMSO) at a concentration of 10 to 100 mmol/L stock solution and stored at −20°C. Further dilutions were made in culture medium to final concentration before use. Other chemicals and reagents used are described in the Supplementary Methods (available online).

Cell Lines

Human lung adenocarcinoma PC-9 cells harboring EGFR exon 19 deletion (E746-A750) were provided by F. Hoffmann-La Roche, Ltd, Basel, Switzerland, with the authorization of Dr. Mayumi Ono (Kyushu University, Fukuoka, Japan). Human lung adenocarcinoma 11-18 cells harboring EGFR exon 21 L858R mutation were provided by Dr. Mayumi Ono, respectively. Human lung adenocarcinoma H1975 cells harboring both sensitizing L858R and resistant T790M mutations were purchased from the American Type Culture Collection (ATCC); Manassas, VA, USA. All cell lines were maintained in RPMI (Roswell Park Memorial Institute medium) 1640 supplemented with 1% penicillin/streptomycin/glutamine (Gibco) and 10% fetal bovine serum (FBS; Gibco) in a 5% CO_2 37°C cell culture incubator and routinely evaluated for mycoplasma contamination.

Cell Viability Assay

Cells were seeded on 96-well plates and treated with serial dilutions of the drugs administrated at doses typically corresponding to 1/8, 1/4, 1/2, 5/8, 3/4, 7/8, 1, 1.5, and 2 of the individual half maximal inhibitory concentration (IC50) values. Cell viability was then assessed with MTT (tetrazolium-based semiautomated colorimetric 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

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Table 1. Patient characteristics of the 64 patients of the initial cohort and the 55 patients of the validation cohort included in the present study

Clinical characteristics	Initial cohort, No. (%)	Validation cohort, No. (%)
Sex		
Male	22 (34.4)	36 (65.4)
Female	42 (65.6)	19 (34.6)
Age, y		
Median	67	58
Range	35 – 89	50 – 69
ECOG performance status		
0	15 (23.4)	2 (3.7)
1	49 (76.6)	51 (92.7)
>1	–	2 (3.6)
Smoking status		
Never smoked	42 (65.6)	49 (89.1)
Former smoker	17 (26.6)	4 (7.3)
Current smoker	5 (7.8)	2 (3.6)
Disease stage		
IIIB	12 (18.7)	7 (12.7)
IV	52 (81.3)	48 (87.3)
Brain metastasis		
No	43 (67.2)	37 (67.3)
Yes	21 (32.8)	18 (32.7)
Bone metastasis		
No	40 (62.5)	22 (40.0)
Yes	24 (37.5)	33 (60.0)
Type of EGFR mutation		
Exon 19 deletion	44 (68.8)	36 (65.5)
L858R	18 (28.1)	19 (34.5)
Other*	2 (3.1)	
Type of EGFR TKI		
Erlotinib	37 (57.8)	7 (12.7)
Gefitinib	25 (39.1)	45 (81.8)
Afatinib/icotinib	2 (3.1); afatinib	3 (5.5); icotinib
Progression-free survival		
Median (95% CI), mo	14.1 (8.8 to 16.3)	9.8 (7.1 to 11.1)
Overall survival		
Median (95% CI), mo	26.7 (17.9 to 37.1)	Not reached
Best response		
Complete response	3 (4.7)	–
Partial response	39 (60.1)	31 (56.4)
Stable disease	16 (25.8)	14 (25.5)
Progressive disease	6 (9.4)	7 (12.7)
Not evaluable	–	3 (5.4)

*Other, L861Q, G719X. CI = confidence interval; ECOG = Eastern Cooperative Oncology Group; TKI = tyrosine kinase inhibitor.

bromide) reagent. Data of combined drug effects were analyzed using the Chou and Talalay method (17) and are the average of three independent experiments. Combination index values of less than 1, 1, and more than 1 indicated synergism, additive effect, and antagonism, respectively. The mean values of the survival fractions were used to generate a set of CI values (data points) and construct the growth inhibition curves and the isobologram for a particular cell line and drug combination. The mean combination index value (mCI) for this set was reported as the summary measure of three independent experiments for each cell line. The associated 95% confidence intervals (CIs) are also reported. Further details are provided in the Supplementary Methods (available online).

Colony Formation Assay

To test the inhibitory effect of EGFR TKIs alone or in combination with other compounds, the cytotoxicity assay based on colony formation was performed, as described in the Supplementary Methods (available online).

Immunoblotting

Whole cell lysates were prepared, and equivalent amounts of protein were detected with appropriate primary antibodies. Details are provided in the Supplementary Methods (available online).

Animal Experiments

All mouse procedures were based on National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals and approved by Institutional Animal Care and Use Committee of Jiangsu Province Academy of Traditional Chinese Medicine (SYXK-2011-0017). Four- to five-week-old female nude mice were obtained from Nanjing Biomedical Research Institute of Nanjing University and maintained in a clean facility in the Jiangsu Province Academy of Traditional Chinese Medicine (Nanjing, Jiangsu, China). Details are provided in the Supplementary Methods (available online).

Real-time PCR Analysis

RNA was isolated from the tumor tissue specimens and cell lines as previously described (18). mRNA gene expression analysis was performed by TaqMan-based quantitative reverse transcription polymerase chain reaction (qRT-PCR) as described in the Supplementary Methods (available online).

The primer and probe sets for the genes analyzed were designed using Primer Express 3.0 Software (Applied Biosystems) according to their Ref Seq (http://www.ncbi.nlm.nih.gov/locuslink) (Supplementary Table 1, available online).

Statistical Analysis

Progression-free and overall survival were estimated by the Kaplan-Meier method and compared using a nonparametric log-rank test. Gene expression levels were divided into two groups according to median relative expression. A Cox proportional regression model was applied to calculate hazard ratios (HRs) and their 95% confidence intervals. Statistical analyses were performed using SAS version 9.3. Details are provided in the Supplementary Methods (available online). All statistical tests were two-sided, and a *P* value of less than .05 was considered statistically significant.

Results

Impact of STAT3 and Src-YAP1 Inhibition on the Effect of Gefitinib on EGFR Downstream Signaling Pathways

We first evaluated the effect of gefitinib and AZD9291 (a recent US Food and Drug Administration [FDA]–approved third-generation EGFR TKI, also called osimertinib, with activity against EGFR T790M), with or without TPCA-1 (a STAT3 inhibitor), on EGFR downstream signal transduction pathways.

Gefitinib suppressed EGFR, ERK1/2, and AKT phosphorylation but increased STAT3 phosphorylation on the critical tyrosine residue 705 (pSTAT3-Tyr705) in a time- and dose-dependent manner in PC-9 cells that harbor EGFR exon19 deletion (E746-A750) (Figure 1A). Gefitinib plus TPCA-1 abolished pSTAT3-Tyr705 phosphorylation (Figure 1B). PC-9 cells showed increased STAT3 and Rantes (regulated upon activation, normal T-cell expressed, and presumably secreted) mRNA levels following gefitinib treatment, an effect not observed when gefitinib was combined with TPCA-1 (Figure 1C). Rantes expression is dependent on a transcription complex of STAT3 with NF-κB (19). pSTAT3-Tyr705 was also induced by gefitinib alone in 11-18 cells that harbor EGFR exon 21 L858R mutation and was diminished with gefitinib plus TPCA-1 (Figure 1D). We then evaluated the effect of AZD9291 with or without TPCA-1 in the H1975 cell line, which harbors EGFR T790M that is resistant to first-generation EGFR TKIs (erlotinib, gefitinib). AZD9291 suppressed EGFR, ERK1/2, and AKT phosphorylation but not pSTAT3-Tyr705, while AZD9291 plus TPCA-1 abolished pSTAT3-Tyr705 (Figure 1E).

Given that IL-6 can activate STAT3 as well as other pathways such as YAP1, we hypothesized that YAP1 signaling might limit EGFR TKI response in EGFR-mutant cells. Interestingly, IL-6 can act via the kinase Src to promote YAP1 signaling in other epithelial contexts, but the role of this potential Src-YAP1 signaling has not been explored in lung cancer to our knowledge (12). Consistent with our hypothesis, we found that EGFR TKI treatment led to increased expression of the YAP1 target connective tissue growth factor (CTGF) in the treated EGFR-mutant cell population over time (Figure 2). Interestingly, we found that combined treatment with the STAT3 inhibitor TPCA-1 and gefitinib did not ablate this increase in CTGF expression (Figure 2). This observation suggested that YAP1 is engaged via another mechanism beyond solely EGFR or STAT3 signaling.

We therefore considered the alternative possibility that Src may be critical for YAP1 activation in EGFR-mutant cells as Src can phosphorylate paxillin and promote YAP1 activation in certain other epithelial cells (12). We tested the effect of the SFK inhibitor AZD0530 in this system. Gefitinib alone was unable to suppress the phosphorylation of STAT3, paxillin, or YAP1 in PC-9 cells (Figure 3A). Gefitinib plus AZD0530 blocked paxillin and YAP1 phosphorylation on tyrosines 118 and 357 but had no effect on pSTAT3-Tyr705 in PC-9 cells (Figure 3A). Gefitinib plus TPCA-1 blocked STAT3 but had no effect on paxillin and YAP1 phosphorylation. The triple combination of gefitinib, TPCA-1, and AZD0530 inhibited STAT3, paxillin, and YAP1 phosphorylation. Phosphorylation of YAP1 on serine 127, which causes cytoplasmic retention of YAP1, was not affected by any treatment (Figure 3A). Similar results were observed with the triple combination of AZD9291, TPCA-1, and AZD0530 in H1975 cells (Figure 3B). Thus, activation of Src, acting upstream of YAP1, is a previously unrecognized event in the initial biochemical adaptation to EGFR TKI treatment in EGFR-mutant NSCLC cells. These results show that the efficacy of EGFR TKIs in EGFR-mutant NSCLC cell lines might be attenuated by STAT3 and Src-YAP1 pathway co-activation and that this process can be reversed by additional STAT3 and Src inhibition.

In Vitro Effect of Gefitinib in Combination With STAT3 and Src Inhibitors

Based on our findings linking dual STAT3 and Src-YAP1 signaling to the adaptive signaling response that is induced by EGFR TKI treatment in EGFR-mutant lung cancer cells, we then assessed the growth inhibitory effects of combinations of EGFR with STAT3 and Src inhibition. As shown in Figure 3C, a 72-hour exposure to gefitinib and TPCA-1 resulted in a synergism in PC-9 cells with an mCI of 0.82 (95% CI = 0.80 to 0.84). A synergism was also observed by adding AZD0530 to gefitinib in PC-9 cells with an mCI of 0.80 (95% CI = 0.78 to 0.82) (Figure 3C). The triple combination of gefitinib, TPCA-1, and AZD0530 was highly synergistic, with an mCI of 0.59 (95% CI = 0.54 to 0.63) (Figure 3C). In addition, the triple combination of AZD9291, TPCA-1, and AZD0530 was synergistic in H1975 cells, as shown by the isobologram analysis and the representative curves in Figure 3D, where experimental data points are below the calculated additivity line, indicating synergistic effects of the combination. PC-9 cells treated with the double combinations failed to form as many colonies as cells treated with gefitinib alone, and the few cells surviving the triple combination generated even fewer colonies than cells surviving the double combinations (Figure 3E); overall, the results from the clonogenic assay were consistent with the MTT data.

Gefitinib in Combination With STAT3 and Src Inhibitors In Vivo

Given the role of co-activation of STAT3 and Src-YAP signaling in limiting the magnitude of initial EGFR TKI response and the inhibitory effect of the triple combination of EGFR TKIs with TPCA-1 and AZD0530, we explored whether this in vitro effect was translated into in vivo model systems. PC-9 cells were injected into mice and treated with gefitinib, TPCA-1, and AZD0530 alone, or double and triple combinations. Stronger tumor regression was observed with gefitinib plus TPCA-1 ($P < .001$) or AZD0530 ($P < .001$), compared with gefitinib alone. The triple combination of gefitinib, TPCA-1, and AZD0530 had greater effect than gefitinib plus TPCA-1 ($P = .01$) or gefitinib plus AZD0530 ($P < .001$) (Figure 4). In order to examine the hypothesis that there is a synergistic effect with the double and triple combinations in the PC-9 sensitive to gefitinib xenograft model, we used three different concentrations of gefitinib (2, 10, 50 mg/kg). Both the 10 mg/kg and 50 mg/kg dose groups showed tumor regression with complete disappearance after 10 days of treatment. Considering the high sensitivity of PC-9 cells to gefitinib, we selected the low dose of 2 mg/kg of gefitinib to combine with TPCA-1, AZD0530, or TPCA1 and AZD0530. This explains the increase in the tumor volume observed in all groups until the 15th day of treatment (Figure 4A). No substantial toxicity was noted with the triple therapy approach. These in vivo results support a consistent synergistic effect of EGFR, STAT3, and Src inhibition on EGFR-mutant NSCLC cells.

STAT3 and YAP1 as Biomarkers of Clinical Outcome to EGFR Inhibition in EGFR-Mutant Patients

To test whether our findings were clinically relevant, we examined the mRNA levels of STAT3 and YAP1 in baseline samples of 64 EGFR-mutant NSCLC patients treated with firstline EGFR TKI (Table 1). With a median follow-up of 26.7 months, median progression-free survival was 9.6 months (95% CI = 5.9 to 14.1) and 18.4 months (95% CI = 8.8 to 30.2) for patients with high and low STAT3 mRNA, respectively ($P < .001$; HR for disease progression = 3.02, 95% CI = 1.54 to 5.93, $P = .001$) (Figure 5A). Median progression-free survival was 9.6 months (95% CI = 7.7 to 15.2) and 23.4 months (95% CI = 13.0 to 28.1) for patients with high

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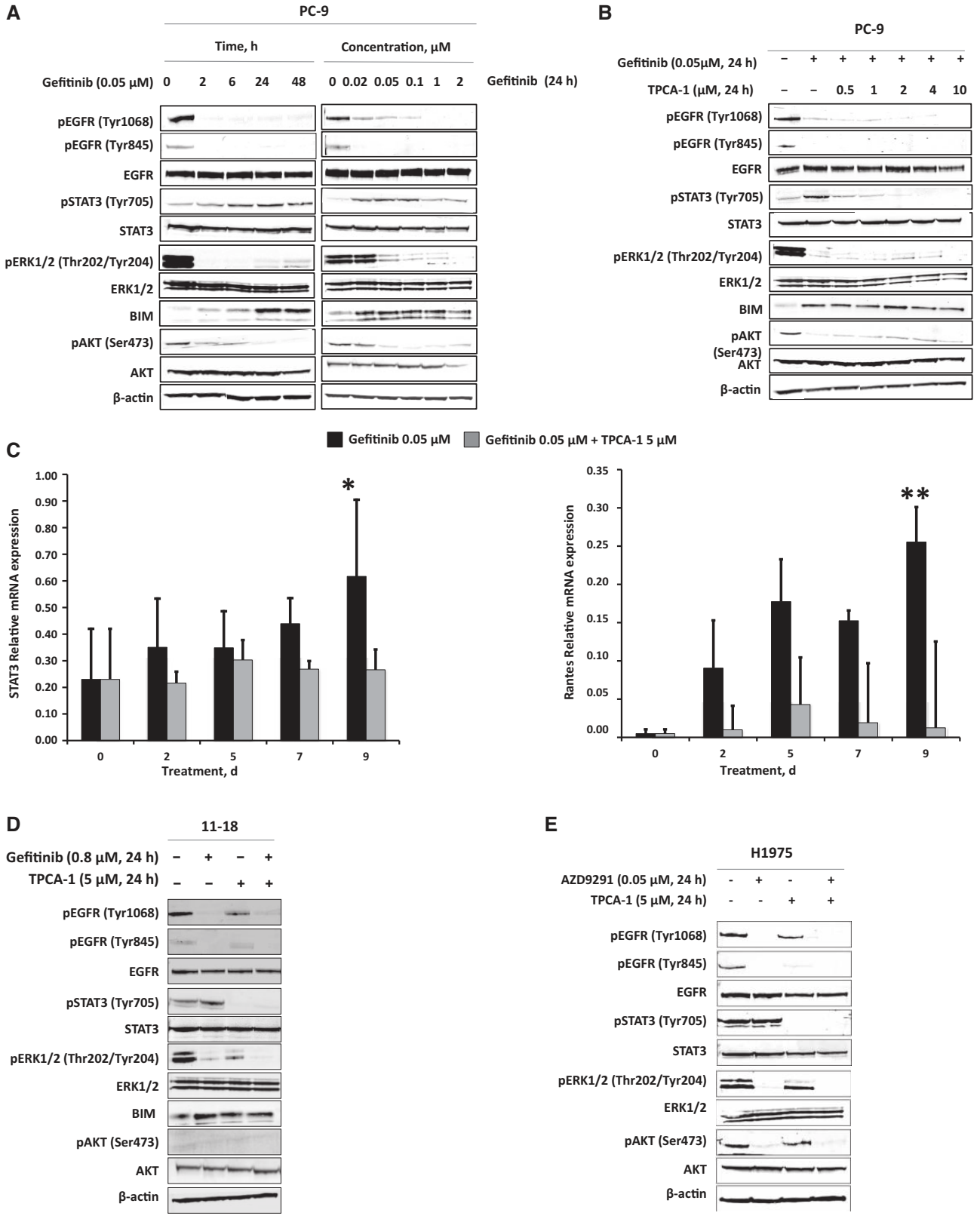

Figure 1. Effect of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) with or without TPCA-1 (signal transducer and activator of transcription 3 [STAT3] inhibitor) on EGFR-regulated signal transduction pathways. **A)** Protein lysates from the PC-9 cell line treated with gefitinib (0.05 μM) for 0 to 48 hours were collected and assessed by immunoblot analysis. PC-9 cells were treated with different doses of gefitinib for 24 hours, and protein lysates were assessed by immunoblot analysis. **B)** PC-9 cells were treated with gefitinib (0.05 μM) in the absence or presence of increasing concentrations of TPCA-1 for 24 hours. Expression of different proteins was analyzed using immunoblot analysis. **C)** STAT3 and Rantes mRNA expression were measured using quantitative reverse transcription polymerase chain reaction in PC-9 cells that were treated with 0.05 μM of gefitinib or 0.05 μM of gefitinib plus 5 μM of TPCA-1 for nine days. Data were generated from a minimum of three

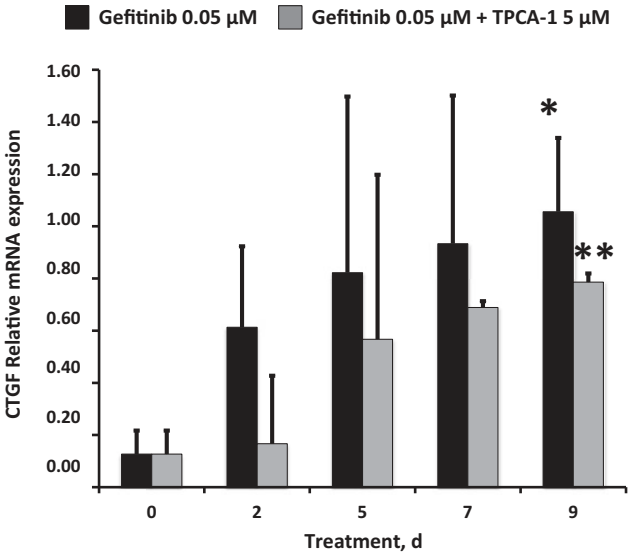

Figure 2. Effect of epidermal growth factor receptor (EGFR) and signal transducer and activator of transcription 3 (STAT3) inhibition on the YES-associated protein 1 (YAP1) signaling pathway. connective tissue growth factor mRNA expression was measured using quantitative reverse transcription-polymerase chain reaction in PC-9 cells that were treated with 0.05 μM of gefitinib or 0.05 μM of gefitinib plus 5 μM of TPCA-1 for nine days. Data were generated from a minimum of three replicates. β-actin was used to normalize gene expression. Data are presented as the means ± standard deviation; *P = .04, **P = .002 (two-sided Student's t test). CTGF = connective tissue growth factor.

and low YAP1 mRNA, respectively (P = .005; HR for disease progression = 2.57, 95% CI = 1.30 to 5.09, P = .007) (Figure 5B). Smoking status was also found to affect progression-free survival (Supplementary Table 2, available online). Using the forward selection method, the STAT3 and YAP1 mRNA levels were used for the Cox proportional hazards regression model. Only STAT3 mRNA levels remained a statistically significant predictor of progression-free survival (HR for disease progression = 2.99, 95% CI = 1.38 to 6.48, P = .005) (Supplementary Table 3, available online). Differences were observed in median overall survival according to STAT3 and YAP1 mRNA expression (Supplementary Figure 1, A and B, available online).

The time-dependent receiver operating characteristic (ROC) curve analysis (20) revealed similar performance of STAT3 and YAP1 in predicting progression-free survival (Supplementary Figure 1C, available online). Given that we noted the cooperative interplay between STAT3 and YAP1 signaling in limiting EGFR TKI response in the preclinical studies and uncovered an independent impact of STAT3 and YAP1 on clinical outcome (correlation coefficient R = 0.17, P = .31), we reasoned that the combined, rather than individual, biomarkers are better predictors of outcome. Specifically, we divided 37 patients with evaluable STAT3 and YAP1 mRNA expression into three groups that were defined according to the median expression of the two biomarkers: one high-risk group with high STAT3 and high YAP1, one low-risk group with low STAT3 and low YAP1, and one intermediate-risk group with either low STAT3 and high YAP1 or low YAP1 and high STAT3. The median progression-free survival in the low-risk, intermediate-risk, and high-risk was 25.7 months (95% CI = 8.5 to 60.9 months), 14.1 months (95% CI = 8.2 to 23.4 months), and 9.4 months (95% CI = 2.8 to 15.2 months), respectively (P = .004) (Figure 5C). Differences were observed in overall survival according to the combined biomarkers (Supplementary Figure 1D, available online).

The role of YAP1 and STAT3 was externally validated in 55 EGFR-mutant NSCLC patients treated with firstline EGFR TKI in the Department of Oncology of Shanghai Pulmonary Hospital (Table 1). With a median follow-up of 18.7 months, median progression-free survival was 6.1 (95% CI = 4.0 to 9.8) and 13.2 months (95% CI = 8.7 to 17.4) for patients with high and low STAT3 mRNA, respectively (P < .001; HR for disease progression = 3.06, 95% CI = 1.66 to 5.64, P < .001) (Figure 5D). Median progression-free survival was 6.1 months (95% CI = 5.1 to 8.2) and 12.6 months (95% CI = 10.0 to 17.4) for patients with high and low YAP1 mRNA, respectively (P = .002; HR for disease progression = 2.53, 95% CI = 1.37 to 4.66, P = .003) (Figure 5E; Supplementary Table 4, available online). The two-gene model was also reconfirmed (Figure 5F). The type of EGFR mutation was also found to predict progression-free survival in this cohort of patients. Median progression-free survival was 10.6 months (95% CI = 7.8 to 13.7) and 6.8 months (95% CI = 3.3 to 10.2) for patients with exon 19 deletion and L858R point mutation, respectively (P < .001; HR for disease progression = 0.52, 95% CI = 0.29 to 0.93, P = .03). Using the forward selection method, the STAT3 mRNA levels and the type of EGFR mutation were used for the Cox proportional hazards regression model. Both parameters remained statistically significant predictors of progression-free survival (Supplementary Table 5, available online). Median survival time was not reached for this cohort of patients. After 54 months of follow-up, 60.3% of patients (95% CI = 38.54 to 76.52) were alive. These data identify STAT3 and YAP1 levels, alone and combined, as a novel predictive biomarker of initial EGFR TKI response in lung cancer patients.

Our results showed differences in progression-free survival between patients with high and low STAT3 mRNA expression, according to the type of the EGFR mutation (P = .002) (Supplementary Table 6, available online). Among patients with the exon 19 deletion, STAT3 levels statistically significantly affected progression-free survival (P = .004). Statistically significant differences in progression-free survival were also observed between patients with high and low YAP1 mRNA expression, according to the type of the EGFR mutation (P = .02). Among patients with exon 19 deletion, YAP1 levels statistically significantly affected progression-free survival (P = .007). No statistically significant differences were noted in progression-free survival among patients with the L858R mutation at exon 21 according to STAT3 or YAP1 mRNA expression levels. Similar results were obtained in the validation cohort (Supplementary Table 6 and Supplementary Figure 2, available online).

Discussion

Our study reveals that dual STAT3 and Src-YAP1 activation limits its EGFR TKI efficacy in EGFR-mutant NSCLC. STAT3 and YAP1 mRNA levels, as well as the STAT3-YAP1 composite score, were

Figure 1. Continued
replicates. β-actin was used to normalize gene expression. Data are presented as the means ± standard deviation; *P = .04, **P = .01 (two-sided Student's t test). **D)** Extracts from the 11-18 cell line were treated with 0.8 μM gefitinib, TPCA-1 (5 μM), or gefitinib combined with TPCA-1 for 24 hours. **E)** H1975 cells were treated with AZD9291 (0.05 μM) in the absence or presence of TPCA-1 (5 μM) for 24 hours. Expression of different proteins was analyzed by immunoblot analysis. BIM = Bcl2 interacting mediator of cell death; EGFR = epidermal growth factor receptor; STAT3 = signal transducer and activator of transcription 3.

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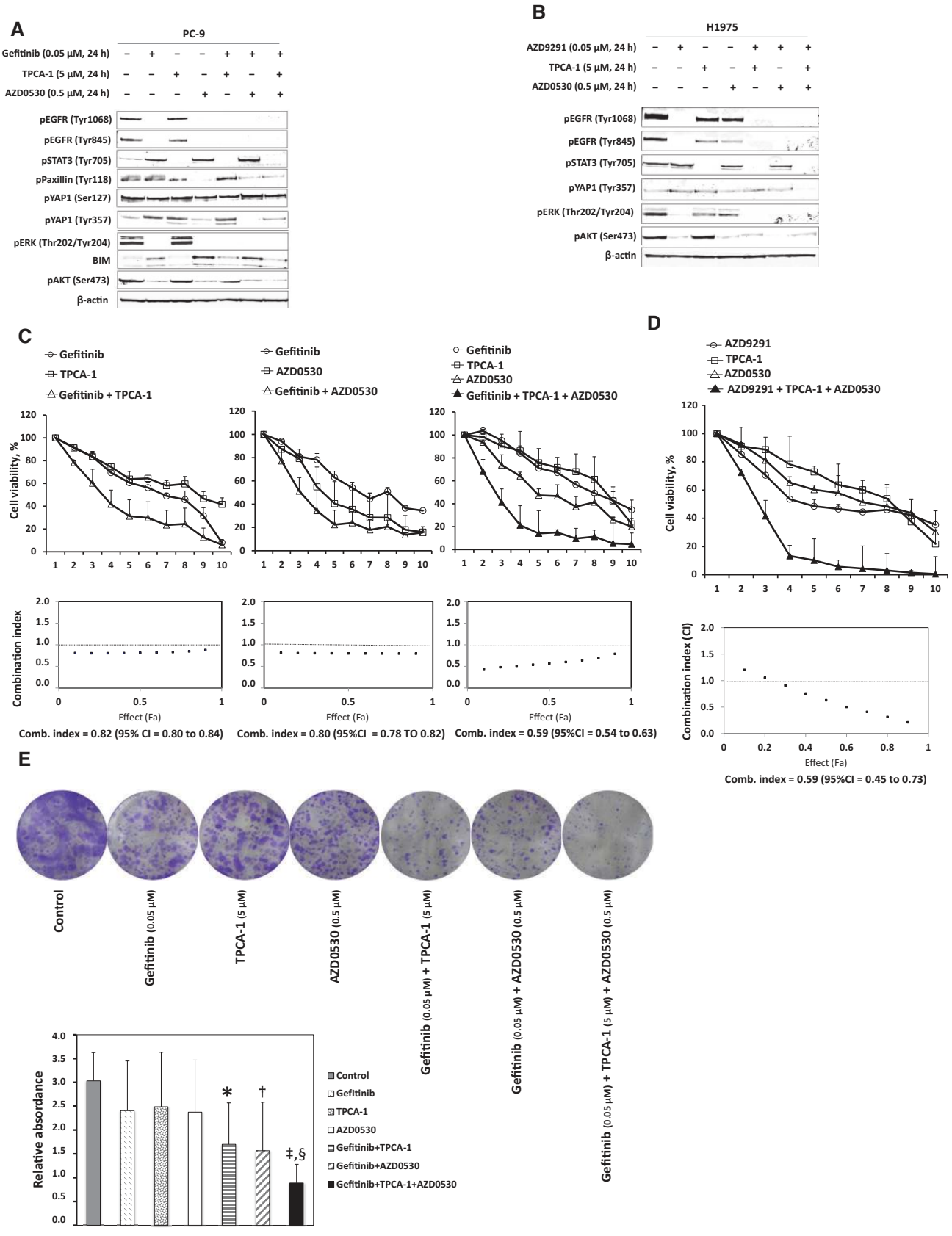

Figure 3. Effects of the triple combination of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI), TPCA-1, and AZD0530 in PC-9 and H1975 cells. Extracts from **(A)** the PC-9 cell line treated with gefitinib (0.05 μM), TPCA-1 (5 μM), or AZD0530 (0.5 μM), or double and triple combinations for 24 hours and **(B)** the H1975 cell line treated with AZD9291 (0.05 μM), TPCA-1 (5 μM), or AZD0530 (0.5 μM), or double and triple combinations for 24 hours, were analyzed for indicated antibodies as well as β-actin as a loading control to confirm equal gel loading by immunoblot analysis. Similar results were obtained in three independent experiments. **C)** PC-9 cells were treated with serial dilutions of gefitinib, TPCA-1, AZD0530 alone and with their double and triple combinations for 72 hours. The cell viability was measured by

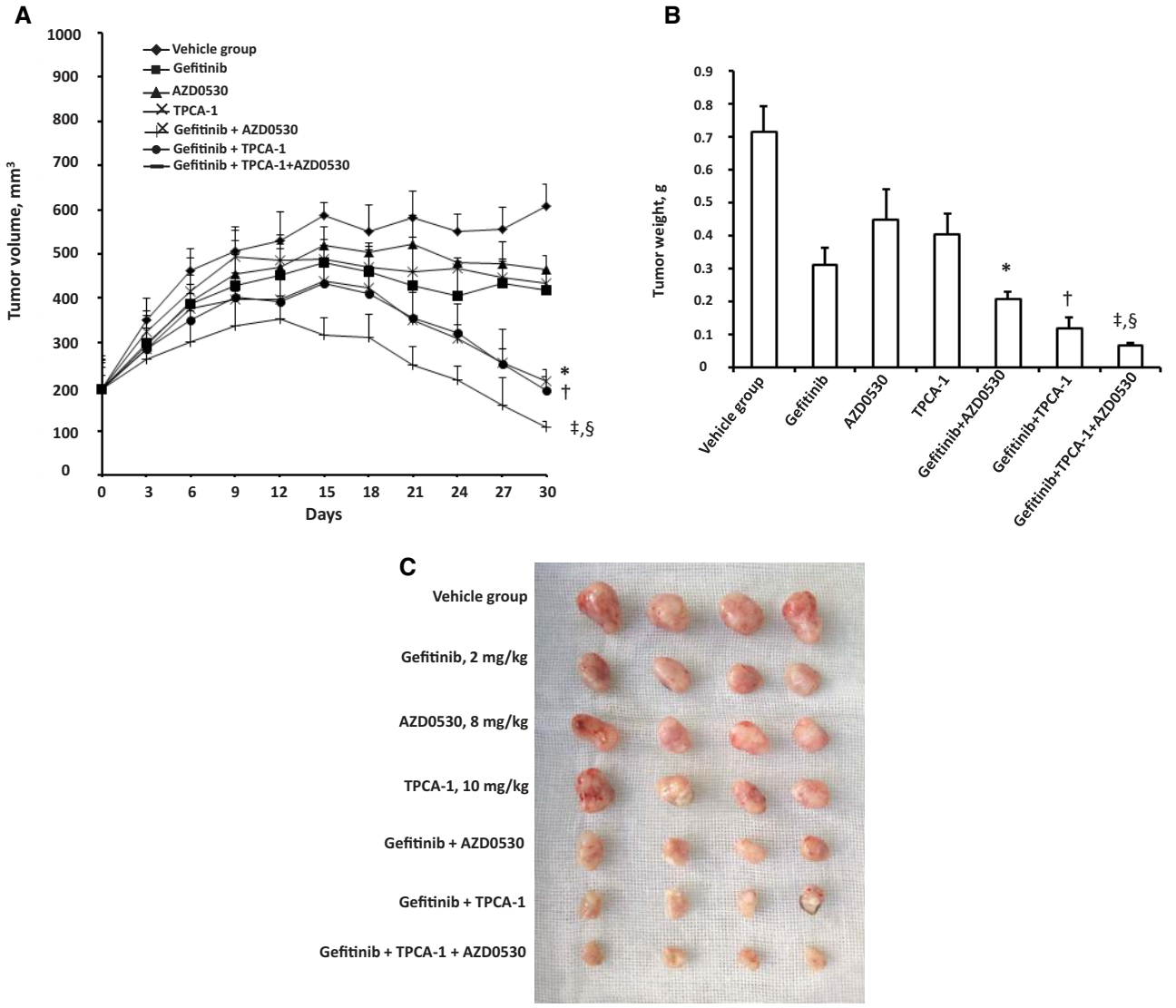

Figure 4. Effect of epidermal growth factor receptor (EGFR), signal transducer and activator of transcription 3 (STAT3), and Src co-inhibition in vivo. **A)** Mice with established PC-9 tumors were treated with vehicle control, gefitinib alone, TPCA-1 alone, AZD0530 alone, gefitinib and TPCA-1, gefitinib and AZD0530 or gefitinib, TPCA-1, and AZD0530. Each point represents the mean ± standard deviation of the tumor volume (n = 4 per group). Initially, different concentrations of gefitinib (2, 10, 50 mg/kg) were used. Both the 10 mg/kg and 50 mg/kg dose groups showed tumor regression with complete disappearance after 10 days of treatment. Considering the high sensitivity of PC-9 cells to gefitinib, a very low concentration of gefitinib (2 mg/kg) was finally used in this experiment, which explains the shape of the survival curves. Statistically significant differences on day 30 are shown for gefitinib plus AZD0530 vs gefitinib alone (*$P < .001$), gefitinib plus TPCA-1 vs gefitinib alone (†$P < .001$), and the triple combination of gefitinib, TPCA-1, and AZD0530 vs gefitinib plus AZD0530 (‡$P < .001$) and vs gefitinib plus TPCA-1 (§$P = .01$). **B)** After 30 days, the mice were killed and the tumors removed and weighed. Tumor weights were individually plotted, and comparisons between control and treatment groups were analyzed by Student's *t* test. Representative tumor pictures were taken. The reduction in tumor weight obtained with gefitinib plus TPCA-1 or gefitinib plus AZD0530 in the PC-9 xenograft model was statistically significantly different compared with gefitinib alone (†$P < .001$ and *$P = .009$, respectively). The reduction in tumor weight obtained with the triple combination of gefitinib, TPCA-1, and AZD0530 was statistically significantly different compared with gefitinib plus TPCA-1 (§$P = .03$) and gefitinib plus AZD0530 (‡$P < .001$). **C)** Representative tumors surgically removed. The two-sided Student's *t* test was used for the statistical analysis. EGFR = epidermal growth factor receptor; STAT3 = signal transducer and activator of transcription 3; YAP1 = YES-associated protein 1.

Figure 3. Continued
MTT, and the synergy between the drugs was determined using the Chou and Talalay method (Chou and Talalay plot or Fa plot). The **dotted horizontal line** at 1 indicates the line of additive effect. Effect (**Fa**) indicates the fractional inhibition for each combination index. To calculate drug concentration for each Fa point, the drugs were mixed using constant ratios corresponding to 1/8, 1/4, 1/2, 5/8, 3/4, 7/8, 1, 1.5, and 2 of the individual IC50 values for each drug in the PC-9 cell line. The results represent the means of at least three independent experiments. Data are presented as the means ± standard deviation. **D)** H1975 cells were treated with serial dilutions of AZD9291, TPCA-1, AZD0530 as a triple combination for 72 hours, a procedure similar to those described in **(C)**. **E)** PC-9 cells grown in six-well plates (1000 cells/well) for 24 hours and then left untreated or treated with gefitinib, TPCA-1, and AZD0530 alone and with their double and triple combinations. After 72 hours, media was replaced with fresh media without drugs. After seven more days, cells were washed and stained with crystal violet and then photographed. The crystal violet was extracted and assayed by spectrophotometry. The absorbance was measured at 570 nm. Gefitinib was used at 0.05 μM, TPCA-1 at 5 μM, and AZD0530 at 0.5 μM. Data are means ± standard deviation of three independent experiments. *$P = .04$, gefitinib plus TPCA-1 vs control; †$P = .003$, gefitinib plus AZD0530 vs control; ‡$P = .03$, gefitinib plus TPCA-1 plus AZD0530 vs gefitinib plus TPCA-1; §$P = .05$, gefitinib plus TPCA-1 plus AZD0530 vs gefitinib plus AZD0530 (two-sided Student's *t* test). BIM = Bcl2 interacting mediator of cell death; EGFR = epidermal growth factor receptor; STAT3 = signal transducer and activator of transcription 3; YAP1 = YES-associated protein 1.

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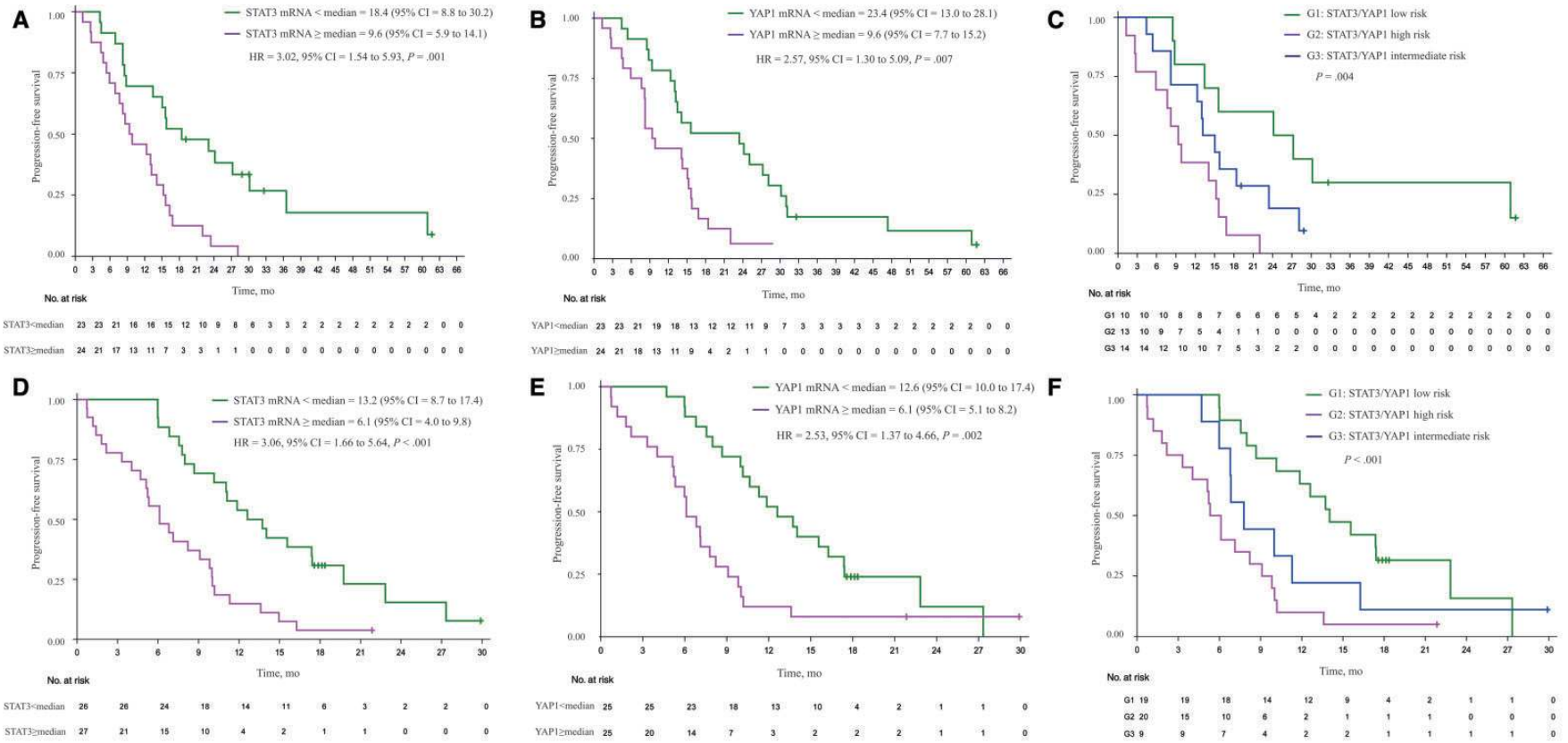

Figure 5. Signal transducer and activator of transcription 3 (STAT3) and YES-associated protein 1 (YAP1) mRNA expression as biomarkers to predict outcome to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI). **A)** Progression-free survival by STAT3 mRNA expression levels for the 64 patients with EGFR-mutant non–small cell lung cancer (NSCLC) treated with firstline EGFR TKI. STAT3 mRNA expression was evaluable in 47 out of the 64 patients. **B)** Progression-free survival by YAP1 mRNA expression levels for the 64 patients with EGFR-mutant NSCLC treated with firstline EGFR TKI. YAP1 mRNA expression was evaluable in 47 out of the 64 patients. **C)** Progression-free survival by STAT3 and YAP1 mRNA expression levels for the 64 patients with EGFR-mutant NSCLC treated with firstline EGFR TKI. Both STAT3 and YAP1 mRNA expression were evaluable in 37 out of 64 patients. STAT3 expression lower than the median combined with YAP1 expression lower than the median denotes a low-risk group. STAT3 expression higher than the median combined with YAP1 expression higher than the median denotes a high-risk group. **D)** Progression-free survival by STAT3 mRNA expression levels for the 55 patients with EGFR-mutant NSCLC treated with firstline EGFR (validation cohort). STAT3 mRNA expression was evaluable in 53 out of the 55 patients. **E)** Progression-free survival by YAP1 mRNA expression levels for the 55 patients with EGFR-mutant NSCLC treated with firstline EGFR (validation cohort). YAP1 mRNA expression was evaluable 50 out of the 55 patients. **F)** Progression-free survival by STAT3 and YAP1 mRNA expression levels for the 55 patients with EGFR-mutant NSCLC treated with firstline EGFR (validation cohort). Both STAT3 and YAP1 mRNA expression were evaluable in 48 out of 55 patients. STAT3 expression lower than the median combined with YAP1 expression lower than the median denotes a low-risk group. STAT3 expression higher than the median combined with YAP1 expression higher than the median denotes a high-risk group. Progression-free survivals were compared with a two-sided nonparametric log-rank test. CI = confidence interval; HR = hazard ratio.

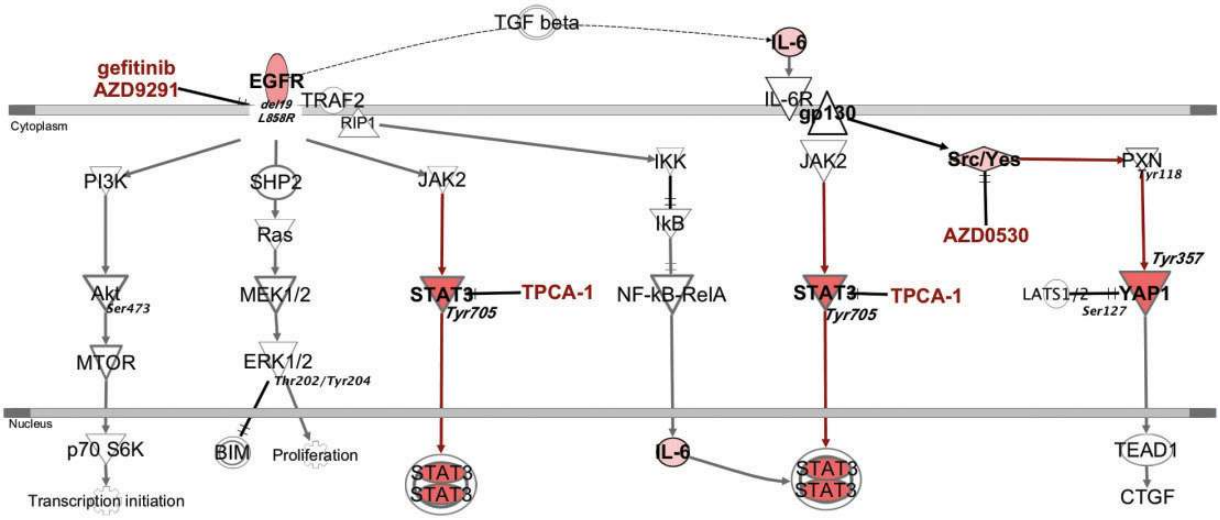

Figure 6. Epidermal growth factor receptor (EGFR), signal transducer and activator of transcription 3 (STAT3), and Src-YES-associated protein 1 (YAP1) crosstalk. EGFR-activating mutations located in the tyrosine kinase domains and mainly in the form of a base-pair deletion at exon 19 (ΔE746_A750) or a point mutation at exon 21 (L858R) enhance cell growth and invasion via tyrosine phosphorylation and lead to the activation of mitogen-activated protein kinase (MAPK), STAT3, and AKT pathways. Ras-ERK signaling promotes cell growth and decreases apoptosis-related BIM expression. SHP2 modulates signals of receptor tyrosine kinases at the level of Ras. Phosphorylation of the tyrosine residue 705 of cytoplasmic STAT3 in response to activated EGFR promotes STAT3 homodimerization, which leads to nucleus translocation and DNA binding. IL-6 signals via receptor complexes, which contain gp130, the common signal-transducing protein of the IL-6 family of cytokines and IL-6R. IL-6R is not a signal transducer, but its function is to present IL-6 to the signal-transducer gp130, resulting in phosphorylation of gp130 by JAK2 and recruitment of STAT3. In EGFR TKI–resistant cells, paracrine or autocrine stimulation of the TGF-β axis drives expression of IL-6 and activation of STAT3, unleashing the cells from their EGFR activity dependency. gp130 associates with Src and YES and triggers activation of YAP1 through phosphorylation on the tyrosine residue 357, independently of STAT3. YAP1 is normally kept inactive in the cytoplasm through phosphorylation on serine residue 127 by the Hippo effector kinase LATS. EGFR inhibition promotes immediate ubiquitination of TRAF2, which is essential for RIP1 and IKK activation, IkB phosphorylation, and degradation and NF-κB (RelA) nuclear translocation. NF-κB-induced IL-6 ensures STAT3 activation. TPCA-1 is a STAT3 inhibitor. AZD0530 (saracatinib) is a potent, orally administered small molecule that inhibits Src by blocking the ATP-binding site of Src kinases. BIM = Bcl2 interacting mediator of cell death; CTGF = connective tissue growth factor; ERK = extracellular signal-regulated kinase; gp130 = glycoprotein 130; IkB = nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; IKK = IkB kinase; IL-6 = interleukin 6; IL-6R = IL-6 receptor; JAK2 = Janus kinase 2; LATS1/2 = large tumor suppressor kinase 1 and 2; MEK = mitogen-activated protein kinase; MTOR = mechanistic target of rapamycin; P 70 S6K = ribosomal protein S6 kinase beta-1; NF-κB = nuclear factor kappa light chain enhancer of activated B cells; PI3K = phosphatidylinositide 3 kinase; P XN = paxillin; RelA = v-rel reticuloendotheliosis viral oncogene homolog A; RIP1 = receptor-interacting protein 1; Ser = serine; SHP2 = Src homology region 2–containing protein tyrosine phosphatase 2; STAT3 = signal transducer and activator of transcription 3; TEAD1 = TEA domain transcription factor 1; TGF-beta = transforming growth factor beta; Thr = threonine; TRAF2 = TNF receptor–associated factor 2; Tyr = tyrosine; YAP1 = YES-associated protein 1.

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highly predictive of progression-free survival in the original as well as in the validation cohort of EGFR-mutant NSCLC patients, a clinical biomarker discovery that warrants future prospective clinical validation.

We have shown that EGFR inhibition results in the adaptive activation of not only STAT3 but also Src-YAP1 signaling, potentially operating downstream of IL-6, to promote cell survival and limit the initial response to EGFR TKI treatment in EGFR-mutant lung cancer (Figure 6). Our data offer the first evidence, to our knowledge, that Src-YAP1 signaling limits EGFR TKI response, in conjunction with STAT3, in lung cancer. EGFR mutations activate MAPK, PI3K-AKT, and STAT3 directly or through IL-6-JAK2 (5,10,21). An IL-6-mediated crosstalk between NF-κB and STAT3 has been described in EGFR-mutant NSCLC cells (16). pSTAT3-Tyr705 is induced two hours after erlotinib or gefitinib treatment (7,8,16). Further, pSTAT3-Tyr705 is abrogated by a pan-JAK inhibitor (Pyridone 6) (21) or TPCA-1 (16) but not by EGFR TKIs or Src inhibitors (21). In our study, similar to gefitinib, the FDA-approved third-generation EGFR TKI AZD9291 (osimertinib) also failed to suppress STAT3 activation in EGFR-mutant cells. Our study sheds new light on the context in which STAT3 activation can limit EGFR TKI response, revealing a previously unappreciated interplay with other pathways, including Src-YAP1 signaling, that cooperatively buffer cells from the effects of EGFR TKI treatment (Figure 6).

We previously showed that YAP1 causes RAF and MEK inhibitor resistance in BRAF- and RAS-mutant tumors and predicts poor clinical response to RAF and MEK inhibition, but we did not previously examine the role of YAP1 in modulating EGFR TKI response in EGFR-mutant NSCLC (14). While conducting our study, it was reported that gp130 (a coreceptor for IL-6) can activate YAP1 independently of STAT3 through direct association with SFKs in colorectal cancer cells (12). An earlier report shows that cotargeting STAT3 and Src or STAT3 and EGFR induced pancreatic tumor cell growth inhibition (22). We evaluated STAT3 and Src-YAP1 signaling activity in PC-9 and H1975 cells and showed that Src-induced phosphorylation of paxillin and YAP1 at tyrosines 118 and 357, respectively (23), was inhibited with gefitinib plus AZD0530. The triple combination of gefitinib, TPCA-1, and AZD0530 fully abrogated STAT3, paxillin, and YAP1 phosphorylation. Tumors in nude mice showed near-complete suppression by the triple combination without signs of substantial toxicity, suggesting both efficacy and a wide therapeutic window for potential clinical feasibility of this polytherapy strategy.

Our study has a few limitations, such as the use of a rather small panel of in vitro and in vivo models to demonstrate the efficacy of cotargeting EGFR, STAT3, and Src-YAP1. Nevertheless, our preclinical findings accurately model EGFR-mutant NSCLC and uncover a previously unappreciated role of the coordinated activation of both STAT3 and Src-YAP1 signaling as a

compensatory response to EGFR TKI therapy. Although the assessment of STAT3 and YAP mRNA in EGFR-mutant NSCLC patients treated with EGFR TKI was retrospective, the predictive role of STAT3 and YAP mRNA was validated in an independent cohort of patients. Based on our findings, we are currently investigating the cotargeting of EGFR, STAT3, and Src-YAP1 with the aim of establishing a phase I clinical trial.

In conclusion, EGFR TKI monotherapy is inadequate for patients with EGFR-mutant NSCLC. Our data provide evidence that co-activation of a broader network of signaling events than previously appreciated limits the initial cellular response to EGFR TKI treatment by providing prosurvival signals that facilitate the survival of residual disease cells. Critically, our findings suggest that biomarker-driven clinical trials cotargeting STAT3, Src-YAP1, and mutant EGFR with existing agents are warranted to improve the initial efficacy of EGFR TKI treatment, minimize residual disease, and potentially prevent the emergence of resistance to enhance patient survival.

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Notes

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Contributors: IC, NK, TB, and RR designed the study, did the literature search, and wrote the manuscript; IC, NK, SP, JCS, XC, XL, CCS, JY, CH, CT, CL, SR, AVerg, IdA, CQ, RE, RPdlB, JLR, MAM, and PC performed the experiments; IC, NK, AD, IS, RE, PM, TB, and RR analyzed and interpreted data; NK, AFC, GLV, AVerl, JMS, MP, FdM, AP, EC, NR, CGC, JW, KJ, HD, MS, SV, CZ, and RR collected data; all authors contributed to the writing, review, and approval of the final manuscript.

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