

Original Article

Targeting Hsp90 with FS-108 circumvents gefitinib resistance in EGFR mutant non-small cell lung cancer cells

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Aim: Inhibition of heat shock protein (Hsp90) has been proven to be effective in overriding primary and acquired resistance of kinase inhibitors. In this study, we investigated the role of FS-108, a newly developed Hsp90 inhibitor, to overcome gefitinib resistance in EGFR mutant non-small cell lung cancer cells.

Methods: Cell proliferation was assessed using the SRB assay. Cell cycle distribution and apoptosis were analyzed by flow cytometry. Protein expression was examined by Western blotting. The *in vivo* effectiveness of FS-108 was determined in an NCI-H1975 subcutaneous xenograft model.

Results: FS-108 triggered obvious growth inhibition in gefitinib-resistant HCC827/GR6, NCI-H1650 and NCI-H1975 cells through inducing G₂/M phase arrest and apoptosis. FS-108 treatment resulted in a remarkable degradation of key client proteins involved in gefitinib resistance and further abrogated their downstream signaling pathways. Interestingly, FS-108 alone exerted an identical or superior effect on circumventing gefitinib resistance compared to combined kinase inhibition. Finally, the ability of FS-108 to overcome gefitinib resistance *in vivo* was validated in an NCI-H1975 xenograft model.

Conclusion: FS-108 is a powerful agent that impacts the survival of gefitinib-resistant cells *in vitro* and *in vivo* through targeting Hsp90.

Keywords: non-small cell lung cancer; gefitinib resistance; Hsp90

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Introduction

Heat shock protein 90 (Hsp90) is a highly conserved and constitutively expressed molecular chaperone that plays a crucial role in protein homeostasis by facilitating the folding and maturation of its client proteins^[1, 2]. In cancer cells, Hsp90 is ubiquitously overexpressed and interacts with diverse co-chaperones to construct fully active multi-chaperone complexes, providing the selective therapeutic rationale to recognize Hsp90 as an attractive target for cancer therapy^[3–6]. To date, dozens of Hsp90 inhibitors have been developed and are currently in phase I–III clinical trials across a broad range

of cancer types, in monotherapy or in combination with other standard therapies^[7–11].

Of note, the recent systematic analysis of Hsp90-client interactions has revealed that intrinsic thermodynamically unstable kinases represent the major population of the strongest clients of Hsp90^[12, 13]. Inhibition of Hsp90 leads to the simultaneous proteasomal degradation of these commonly deregulated onco-kinases^[14, 15]. Given its unique properties in kinome buffering and evolution, targeting Hsp90 has been proven to act as an effective strategy to thwart primary and acquired resistance of kinase inhibitors^[16, 17]. Indeed, many preclinical and limited clinical observations have shown the advantage of Hsp90 inhibition to delay and circumvent rapidly occurring drug resistance after the original kinase inhibition^[18–21]. Overall, these investigations suggested that specified populations of patients who escape from control by kinase inhibitors should be prioritized for treatment with Hsp90 inhibitors.

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Mutant EGFR is one of the most successful emerging therapeutic targets in non-small cell lung cancer (NSCLC). Inhibition of EGFR with gefitinib achieved an approximately 70% response ratio in patients harboring EGFR mutations, including in-frame deletions in exon 19 or point mutations in exon 21 (L858R)^[22–24]. However, intrinsic and acquired resistance were frequently observed, resulting in limited overall clinical benefit and promotion of disease progression^[25,26]. To date, many different mechanisms, including the T790M gatekeeper mutation, co-activation of c-Met signaling by MET amplification and up-regulation of the signals of IGF-1R and AXL, were reported to participate in resistance to gefitinib^[27–30]. Compared to the routine combination therapy that blocks the bypassed signals concomitantly, inhibition of Hsp90 offers a more feasible and effective way to overcome resistance because the kinases accounting for gefitinib resistance retain a high affinity for Hsp90 binding^[31]. Notably, multiple recent preclinical and clinical investigations have highlighted the potential of the application of Hsp90 inhibitors such as NVP-AUY922 and ganetaspib to suppress the proliferation of gefitinib-resistant cells^[32–36].

FS-108, an analog of NVP-AUY922, is a newly developed potent small-molecule inhibitor of Hsp90 (Figure 1A). Preliminary data revealed that FS-108 had effective anti-tumor activity *in vitro* and *in vivo*^[37]. In the current study, we evaluated the pharmacological ability of FS-108 to impair cell survival in gefitinib-resistant NSCLC cells. We discovered that FS-108 remarkably suppressed the viability of gefitinib-resistant HCC827/GR6, NCI-H1650 and NCI-H1975 cells through degrading essential client proteins and inducing G₂/M phase arrest and apoptosis. In addition, FS-108 significantly inhibited subcutaneous tumor growth in an NCI-H1975 xenograft model. Our data suggested that FS-108 is a powerful agent to overcome gefitinib resistance via targeting Hsp90, which might be a subject for clinical investigation in the future.

Materials and methods

Compounds and reagents

Gefitinib, SGX-523, OSI906, and AZD9291 were obtained from Selleck Chemicals (Houston, TX, USA). FS-108 was kindly provided by Dr Jing-kang SHEN (Shanghai Institute of Material Medica, Shanghai, China) and synthesized as previously described^[37]. All these compounds were dissolved to 10 mmol/L with DMSO as a stock solution and stored at -20 °C.

Antibodies against GAPDH, Cdc2, and Hsp70 were from Epitomics (Burlingame, CA, USA). Antibodies against phospho-c-Met (Y1234/1235), c-Met, phospho-EGFR (Y1068), EGFR, phospho-ErbB3 (Y1248), ErbB3, phospho-IGF1R β (T1131), IGF1R β , phospho-Cdc2 (Y15), Cdc25C, phospho-Cdc25C (S216), Akt, phospho-Akt (S473), Erk, phospho-Erk (T202/Y204), Pro-Caspase-3, cleaved Caspase-3, pro-Caspase-7, cleaved Caspase-7, and cleaved PARP were from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

The human NSCLC cell lines HCC827 and HCC827/GR6 were

a kind gift from Dr Pasi A JÄNNE (Dana-Farber Cancer Institute, Boston, MA, USA). The human NSCLC cell lines NCI-H1650 and NCI-H1975 were obtained from the American Type Culture Collection (Manassas, VA, USA). The cell lines were authenticated by STR fingerprinting and routinely maintained according to the supplier's instructions.

Cell proliferation assay

Cells were plated into 96-well plates at a density of 1500–4500 cells/well in triplicates. After incubation overnight, the cells were exposed to the indicated concentrations of compounds and incubated at 37 °C for another 72 h. Then, the cells were fixed with 10% pre-cooled trichloroacetic acid, washed with distilled water, and stained with 4 mg/mL sulforhodamine B (SRB, Sigma, St Louis, MO, USA) in 1% acetic acid. SRB in the cells was dissolved in 10 mmol/L Tris-HCl and measured at 560 nm using spectra-MAX190 (Molecular Devices, Sunnyvale, CA, USA). IC₅₀ values were calculated by concentration-response curve fitting using a SoftMax pro-based four-parameter method.

Cell cycle analysis

Cells were seeded in 12-well plates at a density of 1.5×10^5 cells/well. After 24 h, the cells were treated with DMSO or the indicated compounds for 24 h. Both adherent and floating cells were harvested and fixed in cold 70% ethanol overnight at 4 °C. Prior to a FACS analysis, the cells were centrifuged at 500 \times g for 5 min. Then, they were washed twice with cold PBS and re-suspended in PBS containing propidium iodide (100 mg/mL) and RNase A (20 mg/mL) and incubated for 30 min at 37 °C in the dark. The quantitation of cell cycle distribution was evaluated using a Becton-Dickinson FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). The data were analyzed using Flowjo (Flowjo LLC, OR, USA).

Cell apoptosis analysis

Cells were cultured in 12-well plates at a density of 1.2×10^5 cells/well. After 24 h, the cells were treated with DMSO or the indicated compounds for 72 h. Then, the cells were trypsinized and washed once with cold PBS. Aliquots of the cells were re-suspended in 100 μ L of binding buffer and stained with 5 μ L of annexin V-FITC and 5 μ L of a PI working solution (BD Biosciences) for 15 min at room temperature in the dark. Then, the cells were re-suspended in 400 μ L of binding buffer and analyzed using a Becton-Dickinson FACS Calibur flow cytometer (BD Biosciences). The data were analyzed using CELLQuest software (BD Biosciences).

Western blotting

Protein extracts were prepared by washing twice in cold PBS followed by lysis with a SDS-lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 2% SDS). Cell lysates were boiled for 10 min and cleared by centrifugation at 14000 \times g for 5 min at 4 °C. The supernatant was collected and subsequently resolved by SDS-PAGE and transferred to nitrocellulose membranes, probed with the appropriate primary antibodies and then incubated

with horseradish peroxidase-conjugated secondary antibodies. The immunoreactive proteins were detected using an ECL plus detection reagent (Pierce, Rockford, IL, USA), and images were captured with ImageQuant LAS 4000 (GE Healthcare Life Science, MA, USA).

Animal studies

Four- to six-week-old female nu/nu athymic BALB/c mice were obtained from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). All studies were conducted in compliance with the Institutional Animal Care and Use Committee Guidelines of the Shanghai Institute of Materia Medica. NCI-H1975 cells (3.5×10^6 cells/200 μ L) were suspended in RPMI-1640 medium that was subcutaneously injected into the right flank of nude mice. When the tumor volume reached 100–150 mm³, the mice were randomly assigned to control or treatment groups ($n=6$ per group). For efficacy studies, mice bearing NCI-H1975 cells were treated with the indicated doses of FS-108 or gefitinib for 21 d. Tumor volume (TV) was measured every 3 d and calculated by caliper measurements of the width (W) and length (L) of each tumor using the following formula: $V=(L \times W^2)/2$. The individual relative tumor volume (RTV) was calculated as follows: $RTV=V_t/V_0$, where V_t is the volume on each day, and V_0 represents the volume at the beginning of the treatment. The RTV is shown on the indicated days as the mean \pm SD for the groups of mice. At a designated time (d 21) following treatment, the mice were humanely euthanized, and the tumors were extracted. The tumors were snap frozen in liquid nitrogen and homogenized in 500 μ L of cold RIPA-lysis buffer (Beyotime, Haimen, China) supplemented with protease and phosphatase inhibitors (Merck, Darmstadt, Germany) and then processed for a Western blot analysis.

Statistical analysis

The data are presented as the mean \pm SD, with significance determined by Student's t -test. Differences were considered statistically significant at $P<0.05$. All statistical analyses were performed using GraphPad Prism software (California, CA, USA).

Results

FS-108 impacts the survival of gefitinib-resistant cells through degrading kinases involved in resistance

In this study, we focused our research on evaluating the effect of FS-108 in several types of gefitinib-resistant EGFR mutant non-small cell lung cancer cells. To this end, gefitinib-resistant cell lines, including HCC827/GR6 (MET amplification), NCI-H1650 (PTEN loss) and NCI-H1975 (EGFR T790M mutation), were selected for an analysis^[28, 38, 39]. The resistant cell lines were treated with the indicated concentrations of gefitinib and FS-108 for 72 h, and cell viability was determined using the SRB assay. As expected, all these cell lines were extremely resistance to gefitinib compared to sensitive HCC827 cells (Figure 1B). In contrast, FS-108 treatment significantly inhibited the proliferation of resistant HCC827/GR6, NCI-H1650

and NCI-H1975 cells to the same extent as HCC827 cells (Figure 1C).

To determine whether the decreased cell proliferation induced by FS-108 in resistant cells was the functional consequence of direct inhibition of Hsp90, we examined the expression of Hsp90 client proteins that confer gefitinib resistance in the indicated cells. Resistant cells were exposed to increased concentrations of gefitinib and FS-108 for 24 h, and the expression of various client proteins and downstream signaling pathways were investigated by Western blotting. Gefitinib clearly lost its ability to inhibit the phosphorylation of upstream EGFR, ErbB3 and c-Met and the downstream Akt and Erk signaling pathways in resistant cells (Figure 1D). Nevertheless, as expected, FS-108 treatment profoundly induced the up-regulation of the molecular co-chaperone Hsp70 and abrogated the expression of EGFR, ErbB3, c-Met and the downstream phosphorylation of Akt and Erk simultaneously (Figure 1D), suggesting the role of Hsp90 inhibition to circumvent gefitinib resistance.

Together, these results indicated that FS-108 effectively suppresses the viability of gefitinib-resistant EGFR mutant non-small cell lung cancer cells through targeting Hsp90 and degrading critical kinases involved in resistance.

FS-108 induces G₂/M phase arrest by reducing the expression of Cdc2 and Cdc25C

Next, we proceeded to gain insight into the mechanisms triggered by FS-108 in gefitinib-resistant cells. We exposed the cells to increased concentrations of gefitinib or FS-108 for 24 h and determined the cell cycle distribution by flow cytometry. FS-108 treatment induced apparent G₂/M phase arrest in resistant cell lines, whereas gefitinib had no effect on cell cycle distribution (Figure 2A).

To further explore the molecular mechanisms involved in FS-108-induced G₂/M phase arrest, the regulators of G₂/M transition were probed after FS-108 treatment. We observed that FS-108 treatment caused a universal reduction in both phosphorylation and basal expression of Cdc2 and Cdc25C in a dose-dependent manner in gefitinib-resistant cells (Figure 2B). These data suggested that FS-108 induces G₂/M phase arrest in gefitinib-resistant cells through inhibition of the expression of Cdc2 and Cdc25C.

FS-108 promotes apoptosis via a caspase-dependent pathway

Next, we asked whether FS-108 can induce apoptosis in gefitinib-resistant cells. To this end, resistant cell lines were treated with gefitinib or FS-108 at the indicated concentrations for 72 h, and apoptosis was determined using the annexin V/PI assay. Notably, gefitinib could not induce apoptosis in resistant cells compared to sensitive HCC827 cells. On the other hand, FS-108 treatment triggered apparent apoptosis in both gefitinib-sensitive and gefitinib-resistant cell lines in a dose-dependent manner (Figure 3A).

We then examined the pathways involved in FS-108-induced apoptosis. Cells were treated with the indicated concentrations of gefitinib or FS-108 for 72 h and analyzed by Western

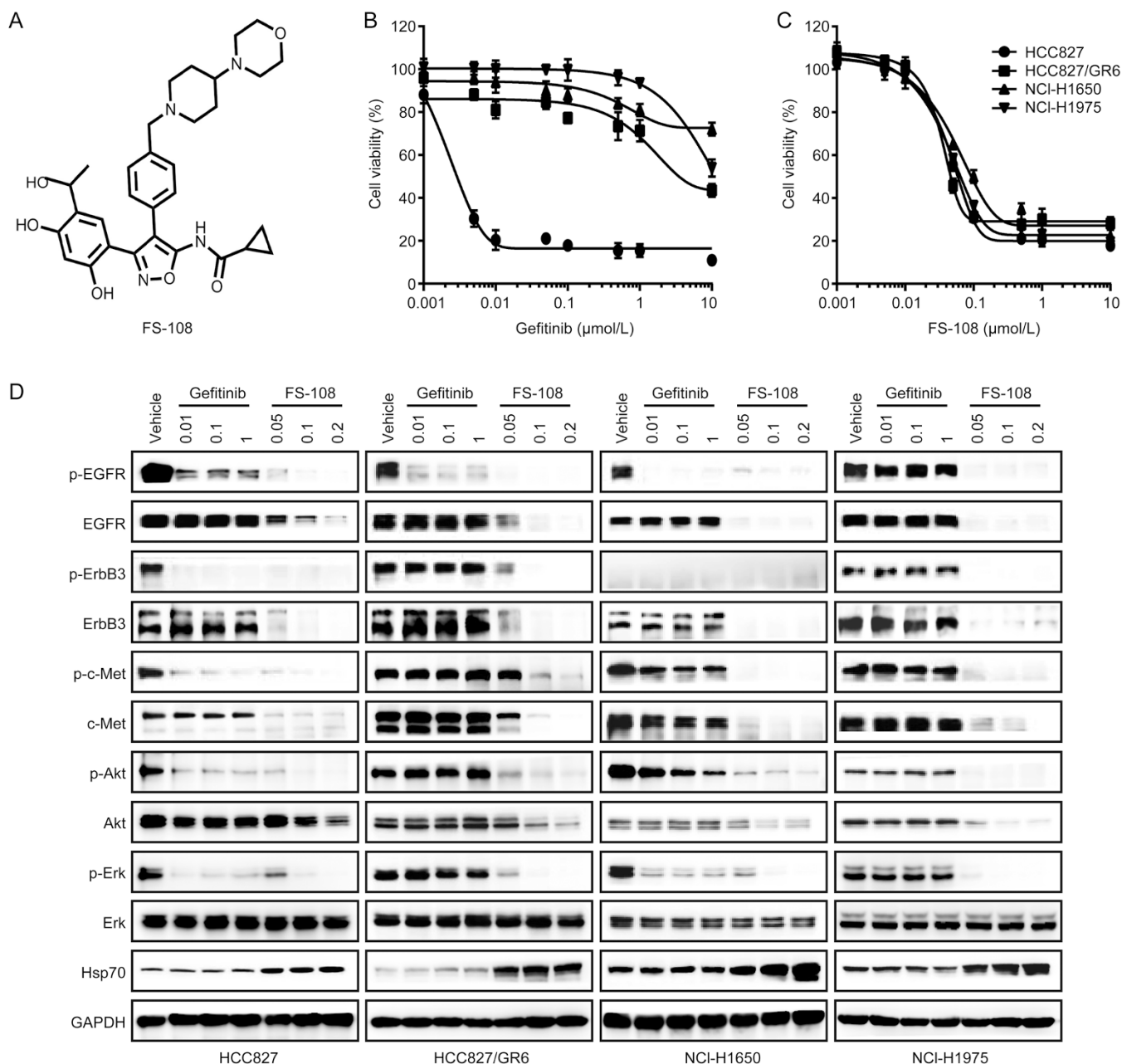


Figure 1. FS-108 impacts the viability of gefitinib-resistant cells through destabilizing kinases involved in resistance. (A) Chemical structure of FS-108. (B, C) Effects of gefitinib and FS-108 on cell proliferation. HCC827, HCC827/GR6, NCI-H1650, and NCI-H1975 cells were treated with gefitinib (B) or FS-108 (C) at the indicated concentrations for 72 h. Cell viability was assessed by the SRB assay. Bars represent mean \pm SD. (D) Effects of FS-108 on the degradation of the indicated kinases. HCC827, HCC827/GR6, NCI-H1650, and NCI-H1975 cells were treated with gefitinib (0.01, 0.1, and 1 $\mu\text{mol/L}$) or FS-108 (0.05, 0.1, and 0.2 $\mu\text{mol/L}$) for 24 h. Cell lysates were prepared and analyzed by Western blotting.

blotting. As expected, only FS-108 treatment induced a dose-dependent cleavage of PARP, which is a hallmark of apoptosis, in gefitinib-resistant cells. Meanwhile, activation of caspase-3 and caspase-7 was observed in a dose-dependent manner (Figure 4B).

FS-108 alone exerts a superior effect compared to combined kinase inhibition in gefitinib-resistant cells

New generation kinase inhibitors and a combined inhibition

of bypass survival kinases are routine approaches to overcome drug resistance. For example, co-treatment by gefitinib with the addition of c-Met and IGF-1R inhibitors was reported to suppress the viability of HCC827/GR6 and NCI-H1650 cells, respectively, whereas the third generation EGFR inhibitor AZD9291 had a significant inhibitory effect in NCI-H1975 cells^[28, 40, 41]. We thus asked whether inhibition of Hsp90 activity alone can lead to the identical effect of attacking resistance to these strategies. To test this possibility, we compared the

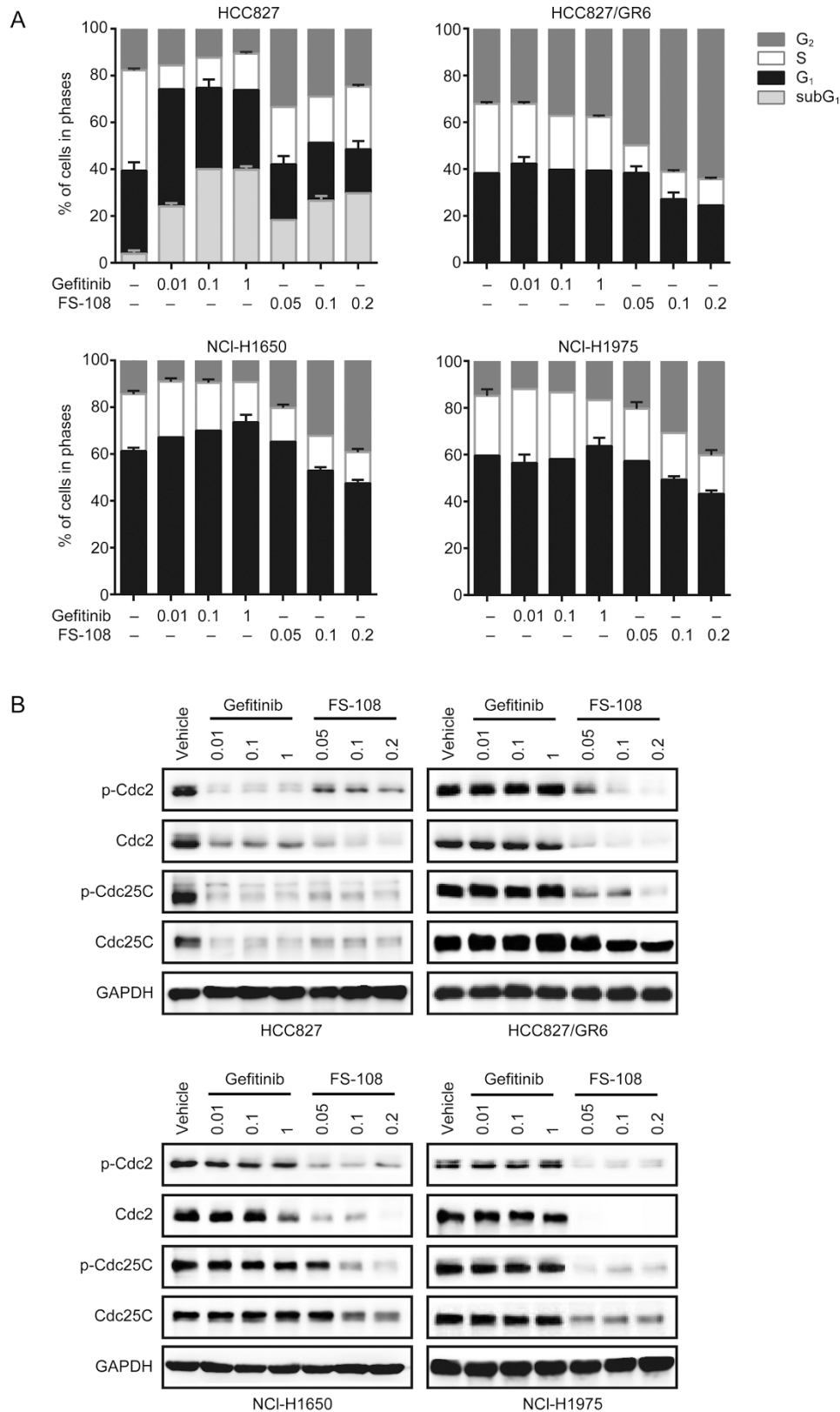


Figure 2. FS-108 induces G_2/M cell cycle arrest in gefitinib-resistant cells. (A) Effects of FS-108 on cell cycle distribution. HCC827, HCC827/GR6, NCI-H1650 and NCI-H1975 cells were treated with gefitinib or FS-108 at the indicated concentrations for 24 h. Cell cycle distribution was analyzed by FACS after propidium iodide staining. The quantified results are presented. Bars represent mean \pm SD. (B) Impact of FS-108 on G_2/M transition regulators. HCC827, HCC827/GR6, NCI-H1650, and NCI-H1975 cells were treated with gefitinib or FS-108 at the indicated concentrations for 24 h and the cell lysates were immunoblotted with the indicated antibodies.

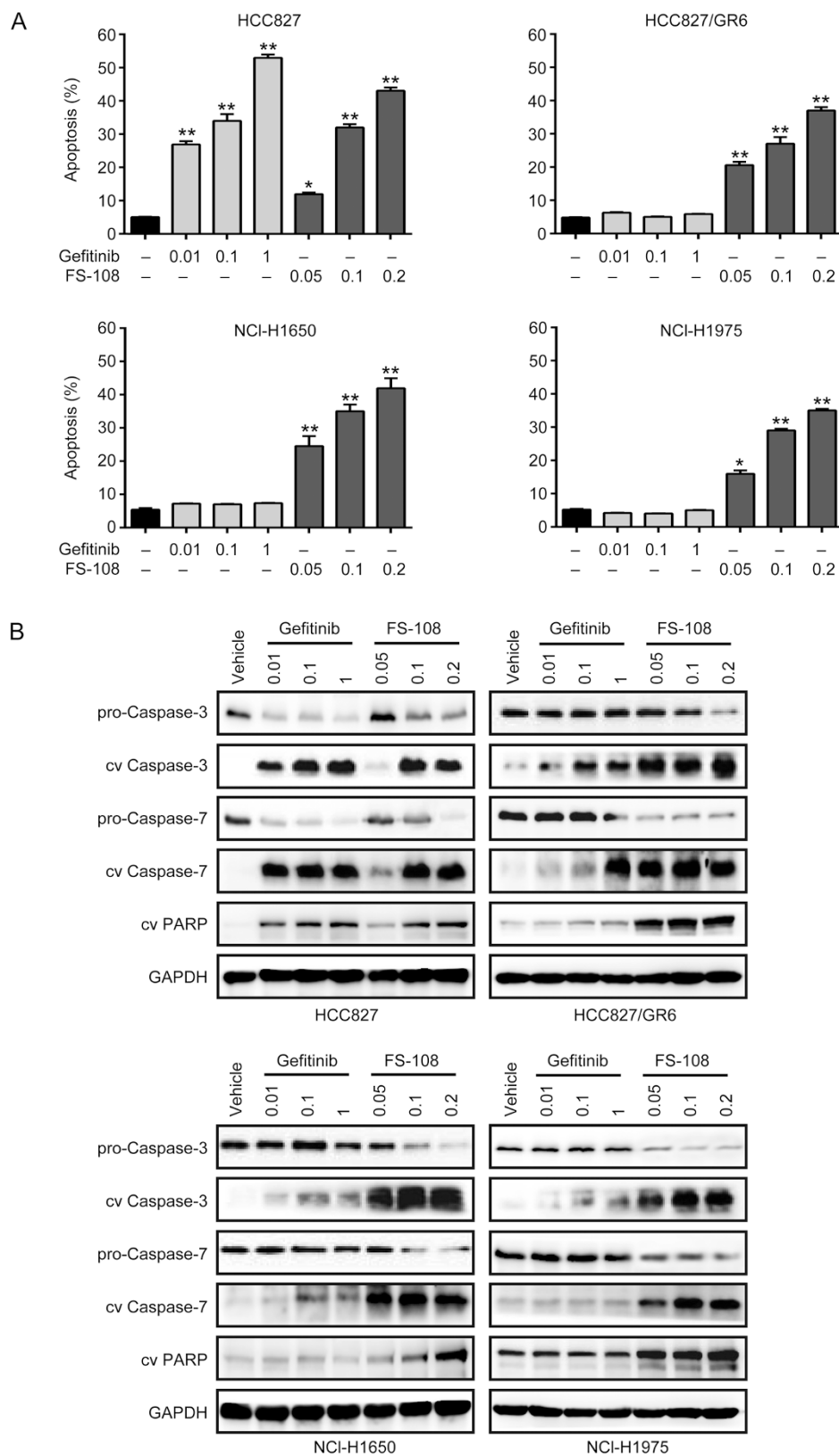


Figure 3. FS-108 induces apoptosis in gefitinib-resistant cells. (A) Effects of FS-108 on apoptosis induction. HCC827, HCC827/GR6, NCI-H1650, and NCI-H1975 cells were treated with gefitinib or FS-108 at the indicated concentrations for 72 h. Apoptosis was determined by the annexin-V/PI assay. Bars represent mean \pm SD. * P <0.05, ** P <0.01 vs vehicle control. (B) Modulation of FS-108 on apoptotic proteins. HCC827, HCC827/GR6, NCI-H1650, and NCI-H1975 cells were treated with gefitinib or FS-108 at the indicated concentrations for 72 h and analyzed by immunoblotting with the indicated antibodies.

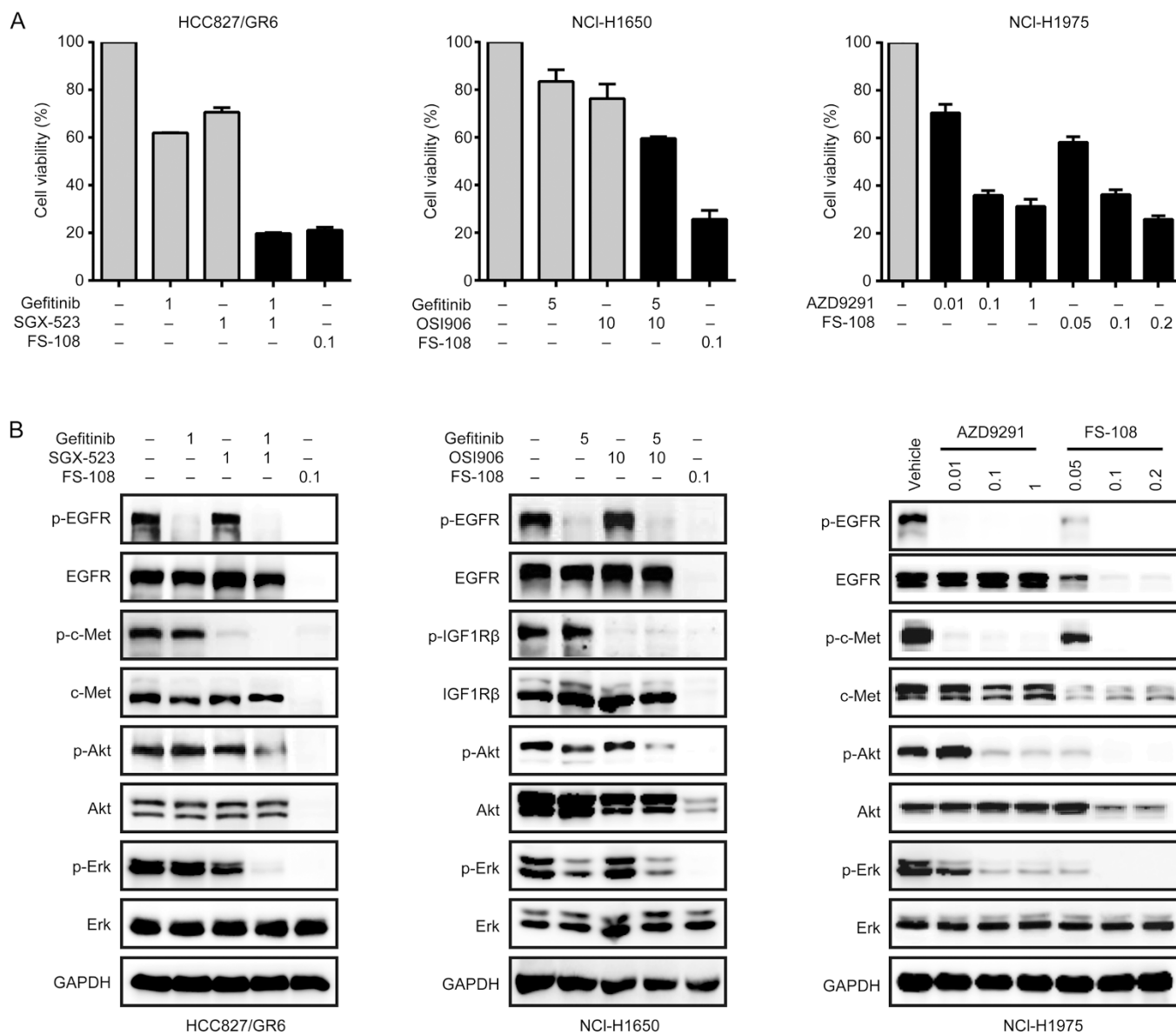


Figure 4. FS-108 alone has a larger effect compared to combined kinase inhibition. (A) Effects of the indicated compounds on cell growth. HCC827/GR6, NCI-H1650, and NCI-H1975 cells were treated with the indicated compounds for 72 h. Cell viability was determined with the SRB assay. Bars represent mean±SD. (B) Effects of indicated compounds on modulation of protein expression. HCC827/GR6, NCI-H1650, and NCI-H1975 cells were treated with the indicated compounds for 24 h and analyzed by Western blotting.

effect of FS-108 treatment with combined therapies co-treated with the indicated kinase inhibitors in gefitinib-resistant cells. Of note, FS-108 alone apparently inhibited the proliferation of resistant cells to the same level as the combination of gefitinib and the c-Met kinase inhibitor SGX-523 in HCC827/GR6 cells and AZD9291 in NCI-H1975 cells (Figure 4A). Interestingly, FS-108 alone demonstrated a larger suppression of the viability of NCI-H1650 cells compared to the combined treatment of EGFR and IGF-1R inhibition (Figure 4A). Next, we treated gefitinib-resistant cells with the indicated compounds for 24 h and examined their ability to shut down signal transduction. As expected, FS-108 alone significantly suppressed the phos-

phorylation and basal expression of kinases involved in resistance and the downstream signaling pathways (Figure 4B).

Collectively, these data implied that FS-108 alone overrides gefitinib resistance effectively to a similar or improved extent compared with conventional combined kinase inhibition strategies.

FS-108 suppresses the growth of NCI-H1975 tumors *in vivo*

Our results demonstrated the therapeutic potential of targeting Hsp90 with FS-108 to thwart gefitinib resistance. We sought to confirm this finding by testing the ability of FS-108 to decrease the growth of gefitinib-resistant tumors *in vivo*. To this end,

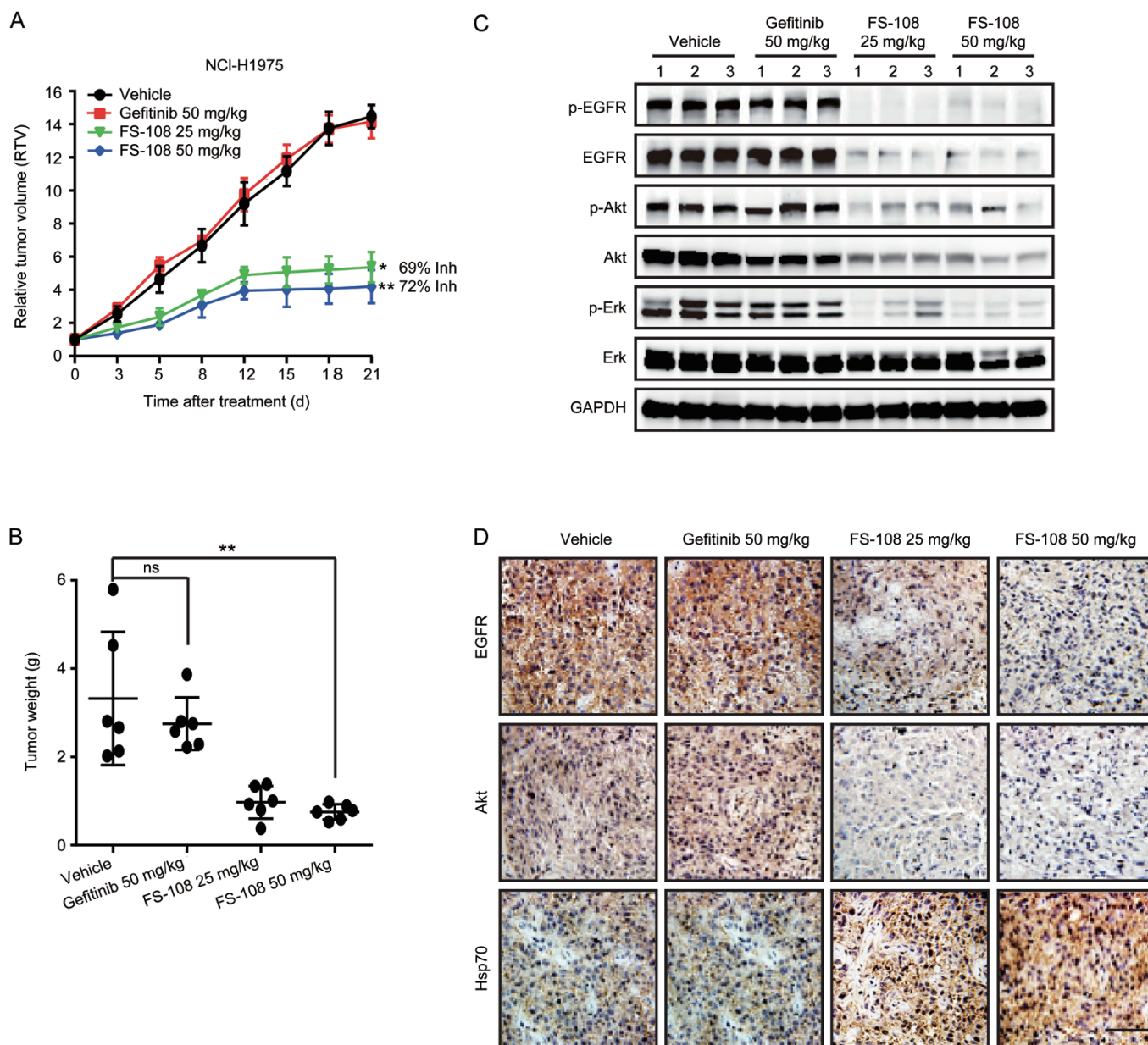


Figure 5. FS-108 inhibits the tumor growth of NCI-H1975 *in vivo*. (A, B) Tumor growth inhibition in FS-108-treated NCI-H1975 xenografts. Mice bearing NCI-H1975 cells were administered gefitinib (orally, 50 mg/kg, once a day) or FS-108 (intraperitoneally, 25 mg/kg or 50 mg/kg, every other day) for 21 d after the tumor volume reached 100–150 mm³. The results are expressed as the mean±SD. The percentage of tumor volume inhibition values (Inh) (A) and tumor weight (B) were measured on the final day of the study for the FS-108-treated mice compared to the vehicle group. * $P < 0.05$, ** $P < 0.01$; ns, not significant. (C) Effects of FS-108 on the modulation of protein expression *in vivo*. Mice were humanely euthanized and protein extracts from tumor tissues were subjected to a Western blot analysis with the indicated antibodies. (D) Effects of FS-108 on the modulation of protein expression *in vivo*. The immunohistochemical evaluation of the basal expression of EGFR, Akt and Hsp70 is presented. Representative images are shown (scale bar, 1 mm).

mice bearing NCI-H1975 xenograft models were treated with gefitinib (50 mg/kg, once a day) or FS-108 (25 or 50 mg/kg, every other day) for 21 consecutive days, and their ability to suppress tumor growth was assessed. As expected, marked tumor growth inhibition was observed in the FS-108-treated groups, with inhibitory rates of 69% ($P < 0.05$) and 72% ($P < 0.01$) at doses of 25 mg/kg and 50 mg/kg, respectively (Figure 5A

and 5B). Meanwhile, no obvious change in body weight was observed in the FS-108-treated groups. A Western blot analysis demonstrated that FS-108 treatment significantly promoted the degradation of mutated EGFR accompanied by reduced levels of the Akt and Erk pathways (Figure 5C). In agreement, the immunohistochemical staining results demonstrated a remarkably increased expression of Hsp70 and a reduced

expression of EGFR and Akt in the FS-108 treated groups (Figure 5D).

Discussion

Targeted therapies with kinase inhibitors have achieved amazing breakthroughs and clinical success in recent decades^[42, 43]. However, the overall benefit of kinase inhibition is limited, stemming from the quick acquisition of innate and acquired resistance observed clinically^[44, 45]. The current strategies to circumvent resistance lie in the development of next generation kinase inhibitors and the combination of drugs targeting both the bypass track and the original oncogenic pathways^[46]. However, the clinical application of these strategies is more complicated than we believe. Only limited targets for overcoming resistance, such as the EGFR T790M mutation and MET amplification, can be detected at the genetic level in patients, while a host of other activated kinases, such as reactivation of IGF1R and AXL, cannot be identified by genomic examination^[47]. Thus, the intrinsic feature of Hsp90 inhibition attacking multiple oncogenic kinases simultaneously has been considered as another useful solution to combat resistance of kinase inhibitors.

In this study, we investigated the mechanisms of a novel Hsp90 inhibitor, FS-108, in overcoming primary and acquired resistance to gefitinib promoted by MET amplification, PTEN loss and EGFR T790M mutation. We discovered that FS-108 profoundly suppressed the proliferation of gefitinib-resistant cells, irrespective of different resistance mechanisms. Mechanistically, FS-108 simultaneously degraded the kinases that are responsible for resistance and abolished the downstream Akt and Erk signaling pathways in resistant cells. The findings in this study suggest that targeting Hsp90 by FS-108 is a powerful tactic to overcome gefitinib resistance in EGFR mutant non-small cell lung cancer, which should be translated into further clinical investigations in subgroups of patients who acquire resistance after gefitinib treatment.

In addition, consistent with our previous observations in U-87MG models^[37], FS-108 treatment had a larger inhibitory effect on tumor growth compared with NVP-AUY922 in NCI-H1975 xenograft models (data not shown). Meanwhile, FS-108 treatment did not lead to an obvious decrease in body weight in either model. These results suggested that FS-108 is a more potent and well tolerated Hsp90 inhibitor compared with NVP-AUY922, which suggests that FS-108 should be further developed for use in the clinic.

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Author contribution

Ai-jun SHEN and Yue-qin WANG designed the research; Yue-qin WANG, Jing-ya SUN, Xin WANG, Hong-chun LIU, and Min-min ZHANG performed the research and analyzed the data; Dan-qi CHEN, Bing XIONG, and Jing-kang SHEN synthesized and purified the compound FS-108; Yue-qin WANG, Ai-jun SHEN, and Mei-yu GENG drafted the manuscript; and Mei-yu GENG, Min ZHENG, and Jian DING provided supervision.

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