Hsp90 inhibition by WK88-1 potently suppresses the growth of gefitinib-resistant H1975 cells harboring the T790M mutation in EGFR

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Abstract. Heat shock protein 90 (Hsp90) is a molecular chaperone for numerous client proteins, many of which are crucial for the pathogenesis of non-small cell lung cancers (NSCLCs). To date, therapeutic approaches using epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) such as gefitinib or erlotinib for the treatment of NSCLCs have been limited due to the emergence of acquired drug resistance mainly mediated by a secondary T790M mutation in EGFR. Considering this, Hsp90 inhibition seems promising as it leads to overall degradation of the oncogenic EGFR family proteins. In this regard, the present study provides the preclinical basis for a new Hsp90 inhibitor, WK88-1, for the treatment of NSCLCs harboring the T790M mutation in EGFR. Our data revealed that inhibition of Hsp90 by WK88-1 induced overall degradation of multiple oncogenic signaling molecules including EGFR, ErbB2 and ErbB3, leading to subsequent growth arrest and apoptosis in the gefitinib-resistant H1975 cell line. In addition, treatment with WK88-1 markedly inhibited proliferation, migration and invasion in H1975 cells. Moreover, an *in vivo* xenograft assay indicated that WK88-1 markedly suppressed tumor growth in the H1975 xenografts, highlighting the potential efficacy of WK88-1 for overcoming gefitinib resistance in NSCLCs harboring the T790M mutation in EGFR.

Introduction

Lung cancer continues to be one of the most prevalent malignancies and a leading cause of cancer-related mortality worldwide. Non-small cell lung cancer (NSCLC) constituting nearly 85% of all cases of lung cancer has been treated by drugs targeting receptor tyrosine kinases (RTKs) (1-4). Of these RTKs, the epidermal growth factor receptor (EGFR) family, whose members include EGFR, ErbB2, ErbB3 and ErbB4 has been widely recognized. Therefore, it has been accepted that EGFR signaling may be crucial in cellular oncogenic transformation, and dysregulation of EGFR has been implicated in pathogenesis of various types of human cancers (5). EGFR-TKIs such as erlotinib (Tarceva) and gefitinib (Iressa) have been evaluated in patients with NSCLCs or other human cancers (6,7). Of note, activating mutations within the EGFR tyrosine kinase domain including an amino acid substitution at exon 21 (L858R) and in-frame deletions in exon 19 were found to be predictors of clinical response to EGFR-TKIs (8). Initially, these competitive and reversible EGFR-TKIs have been effective in patients with NSCLCs. However, acquired resistance universally develops in many patients responding to these drugs (9,10). In the past several years, studies have revealed the two molecular mechanisms for acquired resistance: a secondary T790M mutation in EGFR and Met amplification (11-13). To overcome the acquired resistance to EGFR-TKIs, conventional approaches have focused on inhibiting the kinase activity of EGFR with mutated T790M through selective inhibitors (14,15). However, these approaches have shown that tumor cells may escape such solitary inhibition through bypassing pathways that renew drug resistance.

In this regard, a highly promising rational multi-target drug may be an agent that targets heat shock protein 90 (Hsp90). Hsp90 is an ATP-dependent molecular chaperone which modulates the stability of a large number of oncogenic proteins including EGFR, c-Met, ErbB2, Raf1 and Akt (16-19). Therefore, Hsp90 inhibition appears to be beneficial for anticancer treatment as disruption of Hsp90 activity can induce the degradation of oncogenic client proteins including mutant T790M-EGFR in NSCLCs. Practically, geldanamycin ansamycin (GA) and its derivatives have been reported to possess strong antitumor effect on the growth of NSCLCs with T790M-EGFR (17,20,21). However, the clinical use of Hsp90-targeting drugs including GA and its derivatives are challenged by their intrinsic toxicity in spite of their effective preclinical

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antitumor activities (22,23). Therefore, these drawbacks have prompted the development of new GA derivatives with less toxic outcomes. Consistent with this, we previously reported the development of non-benzoquinone GA derivatives, produced by a mutasynthetic approach and directed biosynthetic method using genetically engineered *Streptomyces hygroscopicus* (24,25). Here, the present study analyzes the antitumor effects of WK88-1, a non-benzoquinone GA derivative, in the gefitinib-resistant NSCLC H1975 cell line, with a secondary T790M mutation in EGFR.

Materials and methods

Materials. Antibodies for phospho-EGFR (Tyr1068), ErbB2, ErbB3, phospho-ErbB3, Met, phospho-Met (Tyr1234/1235), Akt, phospho-Akt (Ser473), Hsp90, Hsp70, Erk1/2, phospho-Erk1/2 (Thr202/Tyr204), caspase-3, cleaved caspase-3, cleaved PARP and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies specific for EGFR, Bcl-2 and p53 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Gefitinib was purchased from LC Laboratories (Woburn, MA, USA). Fetal bovine serum (FBS), streptomycin and penicillin were obtained from Thermo Scientific (South Logan, UT, USA). Halt™ Protease and Phosphatase Inhibitor Cocktail (100X) and EDTA (100X) were purchased from Thermo Fisher Scientific (Rockford, IL, USA). WK88-1 was purified from a culture of *S. hygroscopicus* AC2, in which the AHBA synthase gene was disrupted by the kanamycin-resistance gene, supplemented with 3-aminobenzoic acid (24).

Cell culture. The human NSCLC cell line H1975 (EGFR L858R/T790M) was maintained in RPMI-1640 with L-glutamine containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were cultured as a monolayer at 37˚C with 5% CO_2 in a humidified incubator.

Cell proliferation assay. Cell proliferation was determined by MTS assay with CellTiter96® Aqueous One Solution Reagent (Promega, Madison, WI, USA). H1975 cells were plated in a 96-well plate at $2.5x10^3$ cells/well. Following 24 h of incubation, the cells were treated with the indicated concentration of the compounds or DMSO and subsequently incubated for 24, 48 and 72 h at 37˚C. After being incubated with compounds, 20 μl of CellTiter96® Aqueous One Solution reagent was added to the wells, and the plate was incubated at 37˚C for an additional 1 h. Absorbance at 490 nm was then read on a Tecan Infinite F200 Pro plate reader (Promega, Madison, WI, USA) and values were expressed as percent of absorbance from cells incubated in DMSO alone.

Western blot analysis. H1975 cells were seeded in a 100-mm culture dish at $2x10^6$ cells and allowed to attach. The cells were treated with various concentrations of compound or DMSO and incubated for 24 h. Cells were harvested in ice-cold lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40) with Halt Protease-phosphatase inhibitor cocktail and EDTA. The cells were incubated on ice for 30 min and centrifuged at 16,000 rcf for 20 min at 4˚C. Subsequently, 20-30 μg of lysate/lane was separated by SDS-PAGE followed by transfer to a PVDF membrane (Bio-Rad, Hercules, NJ, USA). The membrane was blocked with 5% skim milk in 0.1% TBS-T for 2 h and then probed with the corresponding primary antibodies overnight at 4˚C. The membranes were washed with 0.1% TBS-T and incubated with secondary antibodies for 1 h. The membranes were developed by horseradish peroxidase-conjugated secondary antibody, and proteins were visualized by SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Waltham, MA, USA). The membranes were imaged with LAS-3000 (Fuji, Japan) according to the instructions of the manufacturer.

Migration and invasion assay. Assessment of cell migration and Matrigel invasion capacity in H1975 cells was performed using BD cell-culture inserts and/or BD BioCoat Matrigel invasion chamber (8-μm pore size). The cells ($5x10^4$) were added into the upper chambers, followed by a 24- (for migration) or 48-h (for invasion) incubation at 37˚C in 5% CO_2. Culture media in inserts were carefully removed, and the membrane containing the cells on the lower surface of the inserts was fixed and stained with hematoxylin and eosin staining (H&E). The cells that migrated to the lower surface were quantified under a light microscope.

Flow cytometry. For determination of apoptotic cells, H1975 cells were seeded at $5x10^5$ cells/60-mm dish. Cell apoptosis was measured using FITC Annexin V Apoptosis Detection kit I (BD Biosciences Pharmingen) in accordance with the supplied protocols. After exposure to the indicated compounds for 24 h, cells were detached. The cells were washed twice with cold PBS, suspended in 1X binding buffer at a concentration of $5x10^5$ cells/ml. And then FITC Annexin V and propidium iodide (5 μl stock/100 μl buffer) were added. After incubation for 15 min at RT in the dark, 400 μl of 1X binding buffer was added to each tube. The cells were analyzed with a BD FACSVerse flow cytometer and BD FACSuite software. The fraction of cell population in different quadrants was analyzed using quadrant statistics.

Animals. For the *in vivo* xenograft assay, male athymic nude mice (5 weeks of age) were obtained from Orient (Seoul, Korea) and maintained under specific pathogen-free conditions based on the guidelines established by the Seoul National University. Animals were acclimated for 1 week before the study and housed in climate-controlled quarters with a 12-h light/dark cycle.

Xenograft mouse model. Mice were divided into 2 groups for each cell line: i) vehicle group (n=10); ii) 1 mg/kg of WK88-1 (n=10). H1975 cells ($1x10^6$/100 μl) were suspended in RPMI-1640 medium and inoculated with 100 μl Matrigel subcutaneously into the right flank of each mouse. Vehicle or WK88-1 was injected 3 times/week. Tumor volume was calculated from measurements of two diameters of the individual tumor base using the following formula: Tumor volume (mm^3) = length x width x height x 0.52. Tumor volume was measured every 3 or 4 days and the tumor weight was determined after excision on the final day of the experiment. Mice were monitored until tumors reached 1 cm^3 total volume, at which time mice were euthanized and tumors were extracted.

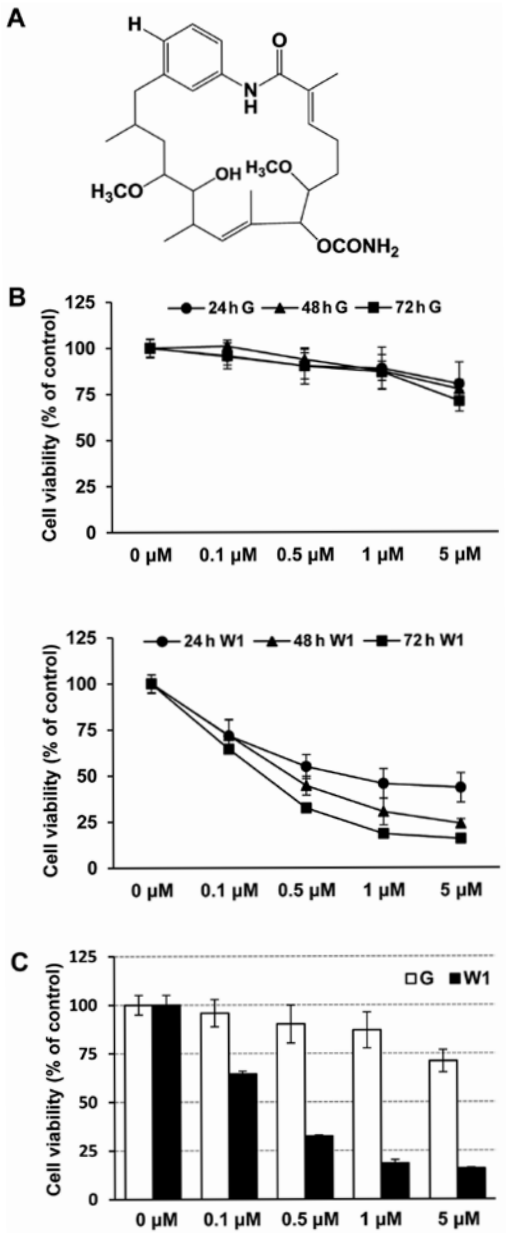

Figure 1. WK88-1 suppresses the proliferation of gefitinib-resistant NSCLC, H1975 cell line. (A) Chemical structure of WK88-1. (B and C) Gefitinib-resistant H1975 cells were treated with various concentrations of gefitinib or WK88-1 for 3 days and cell proliferation was determined using the MTS assay. Cell viability relative to the controls was determined after 3 days. Data shown are the representative of 3 independent experiments. Error bars represent the mean ± SD. C, control; G, gefitinib; W1, WK88-1; NSCLCs, non-small cell lung cancers.

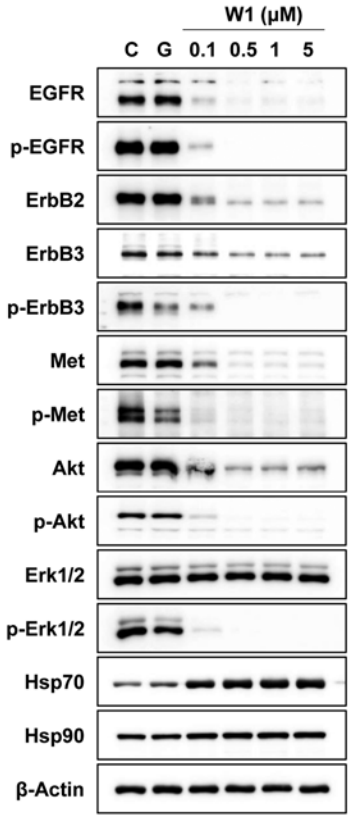

Figure 2. WK88-1 induces degrdation oncogenic RTKs and inhibits downstream signaling in gefitinib-resistant H1975 cell line. H1975 cells were treated with 1 μM gefitinib or the indicated concentrations of WK88-1 for 24 h. Whole cell lysates were assayed by western blot analysis using antibodies against total or phosphorylation form of EGFR, ErbB2, ErbB3, Met and downstream proteins. β-Actin was used as a loading control. C, control; G, gefitinib; W1, WK88-1; RTKs, receptor tyrosine kinases.

Statistical analysis. Quantitative data are presented as means value ± SD unless indicated otherwise. The statistical significance of compared measurements was measured using the Student's t-test, and $P<0.05$ was considered to indicate statistically significant results.

Results

WK88-1, a non-benzoquinone GA derivative, suppresses the proliferation of gefitinib-resistant H1975 cells. To date, one of the major obstacles to the development of Hsp90 inhibitors concerns the issue of toxicity. Our previous data revealed that a non-benzoquinone GA derivative, WK88-1, exhibited little hepatotoxicity compared with GA (data not shown), indicating that WK88-1 could be a potential alternative to GA. WK88-1, a 18-dehydroxyl-17-demethoxyreblastatin (Fig. 1A) was synthesized through mutasynthetic and directed biosynthetic approaches (24,25). To investigate the effect of WK88-1, we used H1975, a human lung adenocarcinoma cell line with the T790M/L858R mutation in EGFR. We first assessed the anti-proliferative effects of gefitinib or WK88-1 in H1975 cells. The cells were treated with the indicated concentration of gefitinib or WK88-1 up to 72 h, and cell proliferation was determined by MTS assay. Expectedly, our data showed that H1975 cells were relatively resistant to gefitinib treatment (Fig. 1B and C). However, a potent growth-inhibitory effect was evidently observed in the gefitinib-resistant H1975 cells which were treated with increasing concentrations of WK88-1 (Fig. 1B and C).

WK88-1 induces the degradation of RTKs and inhibits downstream signaling in H1975 cells. To investigate whether WK88-1 inhibits cancer cell growth by Hsp90 inhibition, we examined the protein expression and phosphorylation status of the client proteins of Hsp90 including EGFR, Met and downstream signaling molecules in H1975 cells. The cells were treated with 1 μM of gefitinib or various concentrations of WK88-1 at 0.1, 0.5, 1 and 5 μM for 24 h. As expected, treatment with 1 μM gefitinib did not show any inhibitory effect on

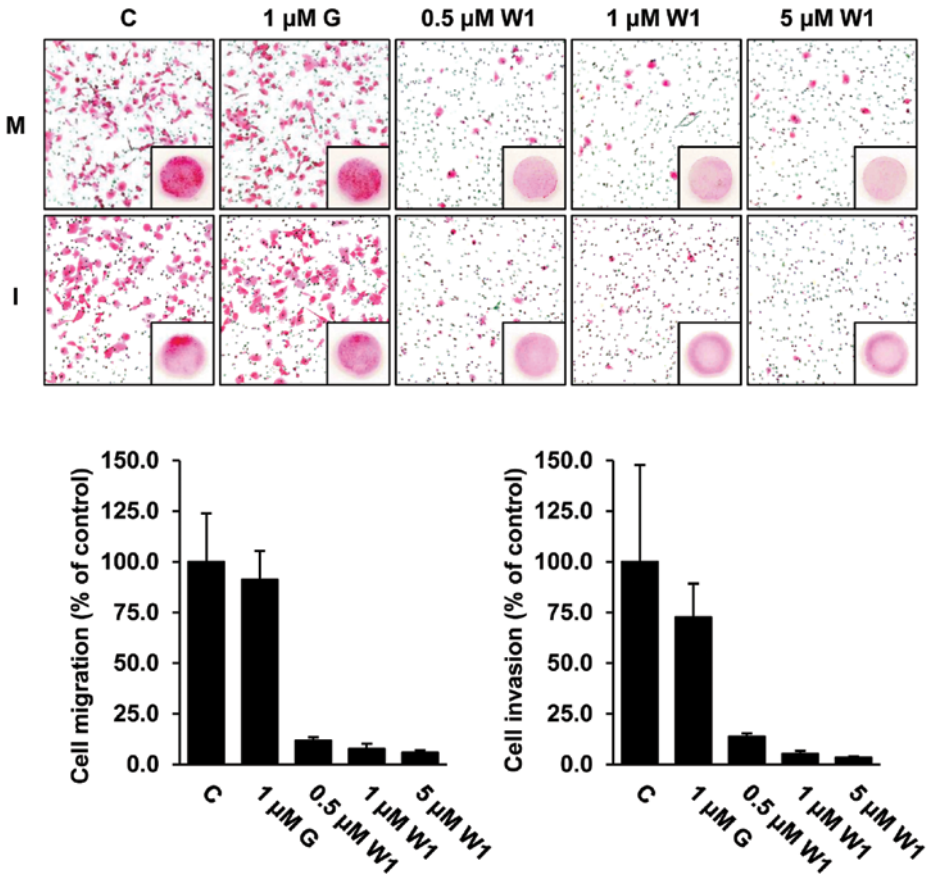

Figure 3. WK88-1 suppresses migration and invasion in H1975 cells. After incubation for 24 h (migration) or 48 h (invasion) with the indicated concentration of WK88-1, live cells that migrated or invaded to the lower surface were fixed, stained and counted using light microscopy. Random areas were scanned (five areas/membrane of the well) in cells on the lower surface of the membrane in both the Boyden (migration) and Matrigel (invasion) chamber. Error bars represent the mean ± SD of 5 areas. Representative images for migration (M) and invasion (I) of H1975 cells are shown. C, control; G, gefitinib; WI, WK88-1.

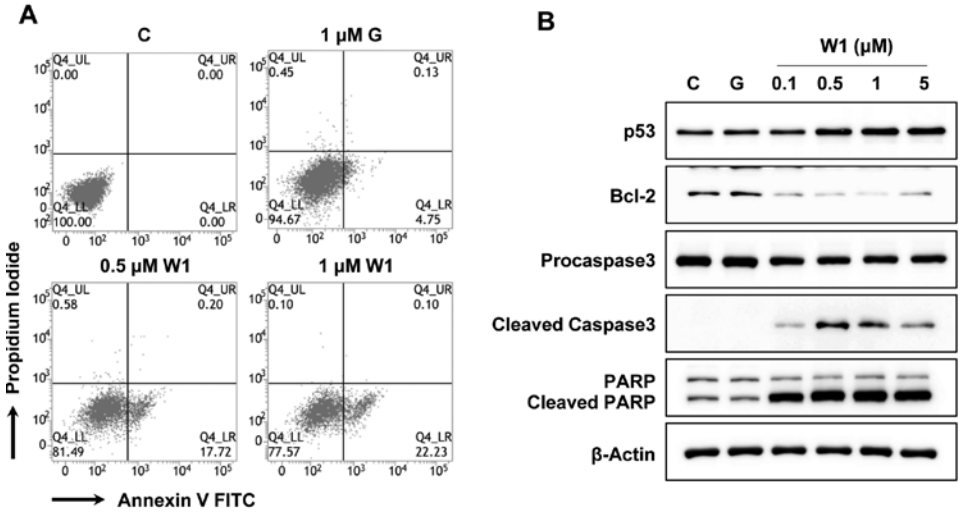

Figure 4. WK88-1 induces apoptosis in H1975 cells. (A) Flow cytometric analysis with Annexin V staining was performed in gefitinib- or WK88-1-treated H1975 cells. (B) Whole cell lysates were assayed by western blot analysis after exposure to the indicated concentrations of WK88-1 in the H1975 cell lines. β-actin was used as a loading control. C, control; G, gefitinib; W1, WK88-1.

the phosphorylation of EGFR or activation of Akt and Erk1/2 in the gefitinib-resistant H1975 cells (Fig. 2). In contrast, a robust decrease of total levels of EGFR, ErbB2, ErbB3, Met and Akt proteins was observed in the WK88-1-treated H1975 cells accompanied by upregulation of Hsp70 (Fig. 2). In addition, treatment with WK88-1 inhibited the activity of downstream Akt and Erk1/2 by inducing upstream protein degradation. Therefore, these findings indicate that WK88-1

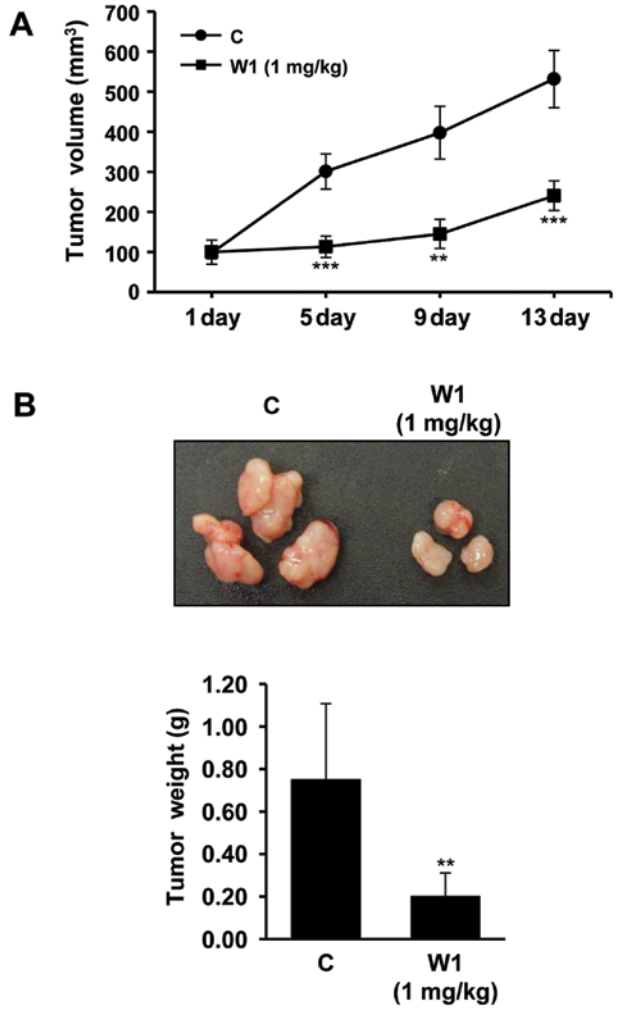

Figure 5. Antitumor activity of WK88-1 in a mouse xenograft model. (A) The average volumes of the H1975-derived tumors from the control and WK88-1-treated mice were plotted 15 days after inoculation. Statistical significance was determined by the Student's t-test ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$). (B) Representative images and tumor weights of the H1975-derived tumors formed in nude mice. Tumors were weighed after sacrificing the mice on the final day of the study. Significant decrease in tumor weight between the control group and the group treated with WK88-1 ($^{**}P<0.01$).

may circumvent gefitinib-resistance in H1975 cells through the inhibition of Hsp90 governing the stability of ErbB3 as well as EGFR, ErbB2 and Met.

Effects of WK88-1 on the migration and invasion in gefitinib-resistant H1975 cells. To assess whether WK88-1 has antitumor activity in detail, we next performed *in vitro* migration and invasion assays in the H1975 cells. As expected, our data revealed that treatment with 1 μM gefitinib did not exhibit any significant effect on the migratory and invasive capacities, whereas treatment with WK88-1 strongly abrogated the migratory and invasive capacities of the gefitinib-resistant H1975 cells (Fig. 3). Therefore, we conclude that a significant inhibition of migration and invasion was observed in the cells following treatment with WK88-1.

WK88-1 induces apoptosis in the gefitinib-resistant H1975 cells. Notably, proliferation of tumor cells may be suppressed by the induction of apoptosis. To ascertain whether the anti-proliferative activity of WK88-1 in the H1975 cells may be related to the induction of apoptosis, flow-cytometric analyses with Annexin V were performed. As a result, our data showed that treatment with WK88-1 facilitated early apoptosis in the gefitinib-resistant H1975 cells. As shown in Fig. 4A, dose-dependent treatment with 0.5-1 μM WK88-1 in the gefitinib-resistant H1975 cells markedly induced apoptosis at a level of 17.72 and 22.23%, respectively, but not in the cells treated with 1 μM gefitinib (4.75%). We next investigated the effects of WK88-1 on apoptotic markers under the same culture conditions. The p53 protein level was slightly increased in the WK88-1-treated cells, whereas an anti-apoptotic protein, Bcl-2, was markedly downregulated under the same conditions (Fig. 4B). Notably, treatment with WK88-1 resulted in the induction of cleaved caspase-3 which mediates PARP cleavage (Fig. 4B). These findings clearly suggest that treatment with WK88-1 is sufficient for inducing apoptosis through the extrinsic apoptotic pathway in gefitinib-resistant NSCLC with T790M mutation in EGFR.

WK88-1 inhibits gefitinib-resistant NSCLC tumor growth in a xenograft model. We next examined whether treatment with WK88-1 suppresses gefitinib-resistant tumor growth *in vivo*. Effects of WK88-1 were assessed in xenograft models. To accomplish this, gefitinib-resistant H1975 cells were injected subcutaneously in nude mice. As expected, the H1975 cell line which harbors a mutant T790M-EGFR showed rapid growth rate. Our data revealed that a significant reduction in tumor growth rate (Fig. 5A) and tumor weight (Fig. 5B) was observed following 1 mg/kg WK88-1 treatment. Therefore, we reasoned that WK88-1 has significant antitumor efficacy in nude mice bearing H1975 tumor xenografts, suggesting that WK88-1 could overcome gefitinib resistance in NSCLCs.

Discussion

It has been suggested that Hsp90 contributes to the six basic hallmarks of cancers such as escape from apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, sustained angiogenesis, limitless replicative potential and tissue invasion and metastasis (26,27). Consistently, pharmacological inhibition of Hsp90 activity has emerged as a promising therapeutic strategy for antitumor treatment. Hsp90 comprising ~2% of the total cellular protein is routinely expressed in malignant cells, and mutated oncogenic proteins are more reliant on Hsp90 function (28). Notably, tumor cells have been reported to exhibit greater dependence on Hsp90's chaperoning function to restructure numerous unfolded and mutated proteins found in tumors which are surrounded by a hypoxic, acidotic and nutrient-starved microenvironment (29,30). A previous report suggested that increased expression of Hsp90 is linked to worse prognosis in NSCLC patients (31). Therefore, targeting Hsp90 has been regarded as an attractive strategy for the treatment of NSCLCs. For this reason, diverse naturally occurring Hsp90 inhibitors including GA, 17-AAG and 17-DMAG have been extensively evaluated; however, many of these drugs still have toxicity issues in clinical trials despite their preclinical efficacy. Therefore, our research has focused on developing novel GA derivatives targeting Hsp90 in tumors with less toxicity. Previously, we

reported diverse non-benzoquinone GA derivatives produced by a mutasynthetic approach (24,25).

In the present study, we evaluated the antitumor activity of a non-benzoquinone derivative, WK88-1, in a gefitinib-resistant H1975 cell line harboring the T790M mutation in EGFR. Our data revealed that treatment with WK88-1 in the H1975 cells induced simultaneous degradation of oncogenic RTKs including EGFR, ErbB2 and ErbB3, and subsequently inhibited downstream signaling such as Akt phosphorylation. Treatment with WK88-1 inhibited ATP-binding activity and dissociated mutant T790M-EGFR in the H1975 cells, resulting in destabilization of multiple proteins. Of note, destabilization of mutant T790M-EGFR by WK88-1 may disrupt EGFR-ErbB3 heterodimers, resulting in rapid depletion of phspho-Akt considering that ErbB3 is used to couple EGFR to the PI3K-Akt pathway (11,32). As many Hsp90 client proteins including EGFR have been shown to be implicated in cancer cell growth and survival (33), these findings provide insight as to how treatment with WK88-1 abrogates gefitinib-resistance in NSCLCs harboring mutant T790M-EGFR.

In conclusion, our findings revealed that WK88-1 has significant antitumor efficacy, as confirmed by the significant reduction in *in vivo* tumor growth in a mouse xenograft model as well as decreased proliferation, migration and invasion in gefitinib-resistant H1975 cells. Therefore, our data suggest that WK88-1 may be a potential Hsp90 inhibitor for overcoming acquired resistance to gefitinib in NSCLCs with T790M-EGFR.

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