



Research update

Suppression of Stat3 activity sensitizes gefitinib-resistant non small cell lung cancer cells

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ABSTRACT

Epidermal growth factor receptor (EGFR) is a proven therapeutic target to treat a small subset of non small cell lung cancer (NSCLC) harboring activating mutations within the EGFR gene. However, many NSCLC patients are not sensitive to EGFR inhibitors, suggesting that other factors are implicated in survival of NSCLC cells. Signal transducers and activators of transcription 3 (Stat3) function as transcription factor to mediate cell survival and differentiation and the dysregulation of Stat3 has been discovered in a number of cancers. In this study, we found that a small molecule, reactivation of p53 and induction of tumor cell apoptosis (RITA), showed anti-cancer activity against gefitinib-resistant H1650 cells through a p53-independent pathway. Stat3 suppression by RITA attracted our attention to investigate the role of Stat3 in sustaining survival of H1650 cells. Pharmacological and genetic approaches were employed to down-regulate Stat3 in H1650 cells. WP1066, a known Stat3 inhibitor, was shown to exhibit inhibitory effect on the growth of H1650 cells. Meanwhile, apoptosis activation by siRNA-mediated down-regulation of Stat3 in H1650 cells provides more direct evidence for the involvement of Stat3 in viability maintenance of H1650 cells. Moreover, as a novel identified Stat3 inhibitor, RITA increased doxorubicin sensitivity of H1650 cells *in vitro* and *in vivo*, suggesting that doxorubicin accompanied with Stat3 inhibitors may be considered as an alternative strategy to treat NSCLC patients who have inherent resistance to doxorubicin. Overall, our observations reveal that targeting Stat3 may be an effective treatment for certain NSCLC cells with oncogenic addition to Stat3.

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1. Introduction

Lung cancer is the leading cause of cancer-related death worldwide. More effective therapies are needed due to the poor survival rates in patients of lung cancer. Lung cancer is broadly classified into two major categories: small cell lung cancer (SCLC) and NSCLC. Clinical reports have shown that 80% of lung cancers are diagnosed with NSCLC [1]. Platinum-based chemotherapy has become the standard treatment for NSCLC based on the results from numbers of clinical trials [2–4]. Nowadays, target therapies causing no or minor side effects may compensate the incompleteness of conventional chemotherapies. A strong correlation is revealed between the expression levels of ErbB family and the malignant proliferation [5]. EGFR mutations are identified in 10–

15% of Caucasian patients with higher percentage in Asian patients and can result in lung cancer pathogenesis [6]. EGFR has thus become a promising therapeutic target for the treatment of NSCLC.

EGFR is a member of the ErbB family that is classified as receptor tyrosine kinase (RTK). Activated EGFR phosphorylates downstream targets, including phosphoinositide 3-kinase (PI-3K), phospholipase C- γ (PLC- γ), extracellular signal regulated kinase (Erk), and Stat3/Stat5 to promote cell proliferation and survival. The majority of NSCLC-associated mutations occur as an in-frame deletion of exon 19 (DelE746–A750) or a point mutation in exon 21 (L858R), leading to constitutively activated EGFR [7]. Patients with certain EGFR mutations have a higher response rate to an EGFR targeted drug, gefitinib (ZD1839, Iressa), than those with wild type EGFR [8–11]. However, carrying EGFR mutations does not assure NSCLC patients of the sensitivity to EGFR inhibitors [12–14]. Similar outcome was observed in cell-based studies as well. H1650, a NSCLC cell line carrying mutant EGFR (DelE746–A750), exhibits much greater resistance to gefitinib compared to other cell lines which carry drug-sensitive mutations within EGFR [15]. Thus, it is important to develop more effective therapeutic strategies to treat NSCLC with the resistance to current therapies.

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Stat3, a member of STAT family, is activated mainly through the phosphorylation at Tyr705 by receptor or non-receptor tyrosine kinases including EGFR, Janus activated kinase (JAK), and c-Src [16]. Following activation, Stat3 dimerizes via its SH2 domain, translocates to the nucleus, and functions as a transcription factor to induce the expression of target genes. Stat3 activation is accompanied by the upregulation of cyclin D1, c-Myc, and Bcl-X_L to promote cell survival and proliferation [17]. Dysregulation of Stat3 activity is associated with the pathogenesis of numerous types of cancers including breast, colon, cervical, and prostate cancers [18]. Several lines of evidence have also indicated the correlation between Stat3 activity and pathogenesis of lung cancer [19,20].

Due to the strong correlation between Stat3 activation and tumorigenesis, suppression of Stat3 by genetic or pharmaceutical modalities has been shown to have anti-tumor effects *in vivo* and *in vitro* [21]. Phosphotyrosyl peptides with the Stat3 SH2 domain-binding activity were shown to inhibit Stat3 activation [22]. Gao et al. demonstrated the effectiveness of vector based RNA interference to suppress Stat3 and its downstream effectors [23]. In their study, inhibition of Stat3 activity using the same approach reduces the survival of PC3, a prostate cell line, *in vivo* and *in vitro*. Recently, small molecule inhibitors, including WP1066, FLLL31, and FLLL32, for upstream effectors of Stat3 have also shown their anti-tumor activity in pancreatic, breast, and malignant glioma cancer cells [24,25]. These observations strongly suggest that Stat3 may be a promising molecular target for drug development.

Doxorubicin is widely employed to treat a variety of cancers such as lymphoma, leukemia, breast, lung, ovarian, gastric, and thyroid malignancies [26]. Doxorubicin induces DNA damage through the generation of free radicals and inhibits topoisomerase II in cancer cells [27]. Although doxorubicin is very potent to treat cancers, resistance to doxorubicin and its toxicity, e.g., heart damage, have restricted its applications. Once a cumulative dose of doxorubicin exceeds the safety threshold, doxorubicin is generally excluded from the regimen to reduce the incidence of doxorubicin-induced cardiotoxicity [28]. Unfortunately, this implies that patients may be forced to give up such an effective therapeutic strategy to avoid the severe side effects. Thus, it will be important to assess whether the chemosensitivity to doxorubicin in cancers could be enhanced by combination with other agents.

In this study, we found that RITA could sensitize gefitinib-resistant H1650 cells probably through its suppression of Stat3 activity. The role of Stat3 in maintaining the survival of H1650 cells was further evaluated using WP1066, a known Stat3 inhibitor, and small interfering RNA (siRNA) technology. Results from the pharmacological and genetic characterizations indicate that Stat3 is essential to maintain cell viability of H1650 cells. Moreover, the functional significance of RITA-mediated Stat3 inhibition in H1650 cells was determined by examining its effects on the sensitivity of H1650 cells to doxorubicin, a relatively ineffective agent for NSCLC [29,30]. Altogether, our studies suggest that the development of Stat3 targeting drugs may be beneficial for NSCLC patients who do not respond to current therapeutics.

2. Materials and methods

2.1. Cell lines and chemicals

H322 (gifts from Dr. Jeou-Yuan Chen), H1650, and H1975 cells (purchased from American Type Culture Collection, Manassas, VA) were maintained in RPMI1640 (Invitrogen, Carlsbad, CA) containing 10% FBS (Sigma–Aldrich, St. Louis, MO) and penicillin/streptomycin (Invitrogen, Carlsbad, CA) at 37 °C. RITA, CP-31398 (Tocris, St. Louis, MO), gefitinib (Ryss Lab, Inc., Union City, CA), doxorubicin (EMD Chemicals, Gibbstown, NJ), and WP1066

(CalBiochem, San Diego, CA) were commercially obtained. All chemicals were dissolved in DMSO to make a final concentration, 10 μM.

2.2. MTS assay

Cells were plated in 96-well plates overnight and incubated with the indicated compounds. Individual compound was administered once and left for 3 days without the change of medium. The cell viability was determined by the MTS assay. RPMI1640/MTS (Promega, Madison, WI)/PMS (Sigma–Aldrich, St. Louis, MO) (8:2:0.1) medium was added to each well and incubated for 1.5 h. The absorbance was measured at 490 nm by PowerWavex (BioTEK instruments, Winooski, VT). The percentage of cell viability was calculated as the absorbance ratio of treated to vehicle (DMSO)-treated cells.

2.3. Flow cytometric analysis

Indicated compounds were administered once and present in the same medium for 16 or 72 h. Cells receiving the treatment were harvested by trypsin–EDTA and stained with Annexin V-FITC (BD Pharmingen, San Diego, CA) and propidium iodide (PI) (Sigma–Aldrich, St. Louis, MO) in binding buffer containing 2.5 mM of Ca²⁺. After 10 min, samples were subjected to flow cytometry FACSCalibur (BD Bioscience, Franklin Lakes, NJ) to analyze the apoptotic population using CellQuest Pro software (BD Bioscience, Franklin Lakes, NJ). Cells in early apoptotic stage exhibited Annexin V-FITC positive and PI negative, whereas cells in late apoptotic stage showed positive on both stains.

2.4. Western blotting

Cells were lysed in lysis buffer (150 mM of NaCl, 1% of Triton X-100, and 50 mM of Tris, pH 8.0). Cell extracts were resolved in 10% SDS–PAGE and transferred to PVDF membrane (PerkinElmer, Waltham, MA). The membranes were blocked by 5% skim milk in TBS with 0.1% of Tween-20 (TBST) for 0.5 h at room temperature prior to be incubated overnight with primary antibodies recognizing p53 (Millipore, Billerica, MA), β-tubulin (Sigma–Aldrich, St. Louis, MO), pStat3 (Tyr705), and Stat3 (both from Cell Signaling, Danvers, MA). The membranes were then washed thrice in TBST and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. After consecutive washes, the membranes were visualized using a chemiluminescence kit (PerkinElmer, Waltham, MA).

2.5. Clonogenic survival assay

125 of H1650 cells plated in 6-well plate were incubated with vehicle (DMSO), 0.01 μM, or 0.1 μM of RITA. RITA was administered once and left in the same medium during the assay. After 14 days, cells were washed once in PBS and then stained with crystal violet (Sigma–Aldrich, St. Louis, MO) for 1 h at room temperature. Colonies including more than 50 cells were counted. The percentage of clonogenic survival was calculated by dividing the number of colonies from treated cells by the number of colonies from untreated cells.

2.6. *In vivo* evaluations of anticancer activity in a H1650 xenograft model

All animal protocols were proved by the institutional animal care and use committee (IACUC) of National Health Research Institutes in Taiwan. 6–8 week-old male BALB/c AnN nude mice were purchased from National Laboratory Animal Center in

Taiwan. In the xenograft model, 5×10^6 cells of H1650 cells suspended in PBS containing 50% Matrigel™ (BD Bioscience, Franklin Lakes, NJ) was subcutaneously injected into the right rear flank of each mouse. 28 mice were randomly stratified into 4 groups and treatments were initiated when all mice had established mean tumor size about 150 mm³. Group 1, 2, 3, and 4 were administered with vehicle control, RITA, doxorubicin, and the combination of two agents, respectively. The PBS and RITA (10 mg/kg) were administered subcutaneously q.d. and the doxorubicin (1 mg/kg) was administered intraperitoneally q.2d. The treatments lasted 16 days. The body weights and tumor sizes of individual mice were recorded twice weekly. The sizes of tumors were measured in two dimensions with a caliper. The tumor volume was calculated using the equation $(l \times w^2)/2$ where l and w represent the largest and smallest dimensions in each measurement.

2.7. RNA interference

H1650 cells were transfected with control and Stat3 siRNA using Lipofectamin RNAiMAX (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Stat3 (VHS40491) and negative universal control (46-2001) siRNA were purchased from Invitrogen (Carlsbad, CA). Expression levels of Stat3 were analyzed by Western blotting as the indicated time.

2.8. Statistical analysis

All data are expressed as means \pm standard deviation (SD) or standard error of the mean (SEM). Mean values in xenograft studies were compared by one way analysis of variance (ANOVA) to determine the significance.

3. Results

3.1. Inhibition of gefitinib-resistant H1650 cells by RITA

Dysregulation of p53 has been found in numerous types of cancers including lung cancer. To determine whether restoration of p53 function affects the viability of NSCLC cell lines, RITA, a p53 stabilizing agent, was selected to examine its cytotoxicity in H322 (p53 mutant), H1975 (p53, ++), and H1650 (p53, ++) cells. RITA was first identified as a suppressor of the growth of HCT116, a colon carcinoma cell line expressing wild type p53 [31]. RITA binds to p53 to prevent the association between p53 and human double minute-2 (HDM-2), leading to the prolonged half life of p53. Cell viability of each cell line in response to RITA was determined by the MTS assay. As shown in Fig. 1A, RITA showed moderate cytotoxicity against H322 cells with a CC₅₀ (the concentration required to cause 50% of cytotoxicity) value of $>5 \mu\text{M}$ and profound cytotoxicity against H1650 cells. Meanwhile, H1975 cells were relatively resistant to RITA compared to H1650 cells. Results from at least 3 independent experiments showed that the CC₅₀ value of RITA against H1650 cells was $\sim 0.1 \mu\text{M}$ which was much more effective than gefitinib in that the CC₅₀ value of gefitinib against H1650 cells was $>15 \mu\text{M}$ under the same cell-based assay (Fig. 1B).

3.2. RITA sensitizes H1650 cells through a p53-independent pathway

Our results indicated that p53 status was not completely correlated with the sensitivity to RITA, implying that p53 reactivation may not be sufficient to cause cytotoxicity in H1650 cells. In order to verify this hypothesis, CP-31398, a p53 modulator which restores p53 function in both p53 wild type and mutant cells, was selected to test its efficacy in H1650 cells [32,33].

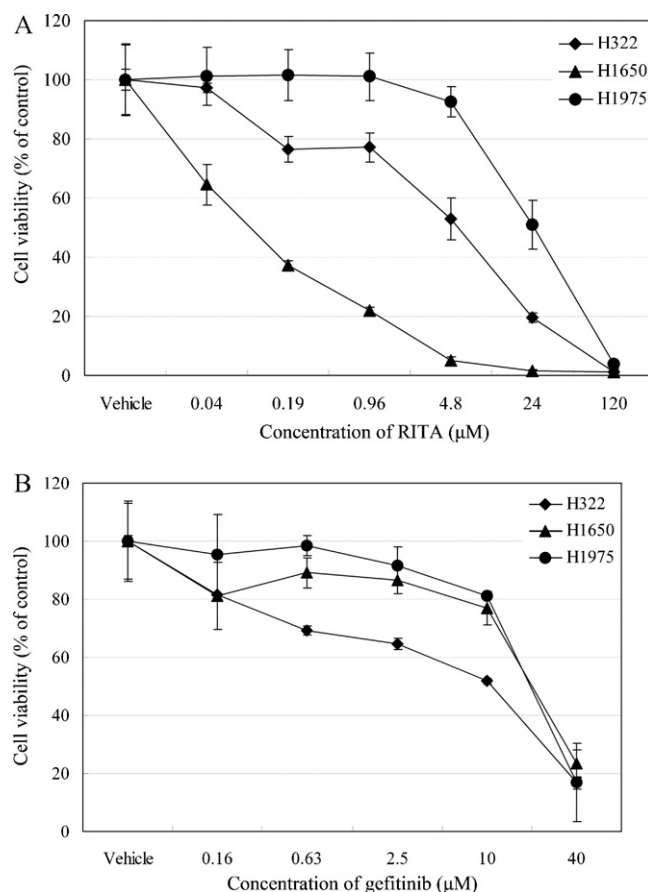


Fig. 1. RITA induces cell death in H1650, a gefitinib-resistant NSCLC cell line. H322, H1650, and H1975 cells were seeded into 96-well plates, followed by a 3-day treatment of (A) RITA and (B) gefitinib with diverse concentrations. Cell viability was determined by the MTS assay. Cell viability of vehicle (DMSO)-treated cells was considered as 100%. Data in the diagram represent means \pm SD from 3 experiments.

Lack of efficacy of CP-31398 in H1650 cells suggests a p53-independent pathway involved in the sensitivity of cells to RITA (Fig. 2A). Despite the absence of a correlation between p53 status and the efficacy of RITA, we still tended to investigate whether RITA stabilizes p53 in H1650 cells as it does in HCT116 cells. Consistent with previous studies, low dose of RITA (0.2 μM) increased the expression of p53 and the increase of RITA to 2 μM led to higher expression of p53 in HCT116 cells (Fig. 2B) [31]. Surprisingly, p53 was not even detectable in H1650 cells although H1650 cell line is previously described as the cell line carrying wild type p53 [34]. Moreover, RITA was not capable of inducing the expressions of p53 after 8 h of incubation (Fig. 2B). The failure of detecting p53 expressions was also observed in H1650 cells treated with extended incubation time and increased dose of RITA (data not shown).

3.3. Suppression of Stat3 activity by RITA induces apoptosis in H1650 cells

Along with testing the effects of RITA on p53 stability in H1650 cells, we also determined whether downstream effectors of p53 were affected by RITA. HDM-2, a downstream target and a main negative regulator of p53, which has been reported to be induced by RITA-mediated p53 activation in HCT116 cells [31] remained unchanged in H1650 cells treated with RITA (data not shown). Of note, 0.2 μM of RITA resulted in the suppression of Stat3 phosphorylation at Tyr705 in H1650 cells which is one of the phenomena observed in prostate and breast cancer cells with over-produced p53; meanwhile, RITA did not have effects on Stat3

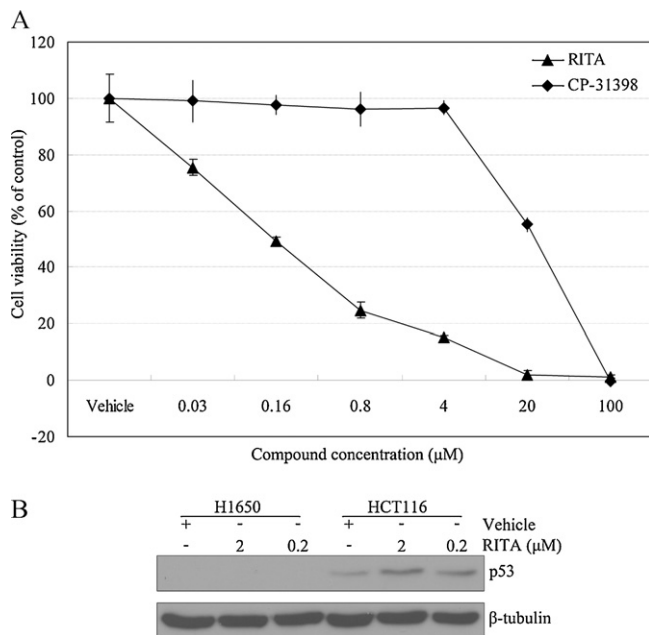


Fig. 2. RITA-mediated cytotoxicity is independent of p53 in H1650 cells. (A) Cell viability of H1650 cells treated with CP-31398 or RITA for 3 days was determined by MTS assay. Cell viability of vehicle-treated cells was considered as 100%. Data in the diagram represent means \pm SD from 3 experiments. (B) Lysates from H1650 and HCT116 cells treated with 0.2 μ M or 2 μ M of RITA for 8 h were analyzed by Western blotting using antibodies recognizing p53 and β -tubulin.

phosphorylation in both H322 and H1975 cells (Fig. 3A) [35,36]. Expression levels of Stat3 were served as the loading control in Western blotting since RITA had no effects on the total proteins of Stat3.

In order to demonstrate if apoptosis was triggered in RITA-treated H1650 cells, apoptosis signals were examined by flow cytometry analysis 3 days after RITA treatment. Compared to the vehicle (DMSO)-treated control, the percentage of apoptotic cells was increased with the increase of RITA concentration (Fig. 3B). Moreover, a clonogenic assay was conducted to confirm its effect on cell proliferation. Clonogenic assay has been commonly employed to determine the efficacy of certain agent to inhibit cell survival and proliferation. In our studies, RITA was present during the assay and clonogenic survival was determined 14 days after RITA treatment. The vehicle treatment did not cause significant effects on the proliferation of H1650 cells compared to the untreated control (Fig. 3C). RITA at 0.01 μ M, however, was effective to suppress the proliferation of H1650 cells and the increase of concentration of RITA to 0.1 μ M contributed to further inhibition on cell proliferation ($p < 0.05$, Student's unpaired t -test) (Fig. 3C).

3.4. WP1066-mediated Stat3 suppression sensitizes H1650 and H1975 cells

Given the observation of RITA-induced Stat3 suppression in H1650 cells, we hypothesized that Stat3 inhibition may be one of the contributable factors to sensitize H1650 cells. To ascertain this hypothesis, genetic and pharmacological approaches were employed to suppress Stat3. Stat3 was down-regulated by Stat3 siRNA in H1650 cells. A significant decline in the Stat3 protein levels was evident 48 h after transfection of the Stat3 siRNA into H1650 cells (Fig. 4A). More, importantly, the siRNA of Stat3 induced apoptosis signals in H1650 cells as evidenced by the increase of apoptotic cells determined by flow cytometry 72 h after transfection (Fig. 4B). Altogether, these results suggest that Stat3 activity is required to maintain the viability of H1650 cells.

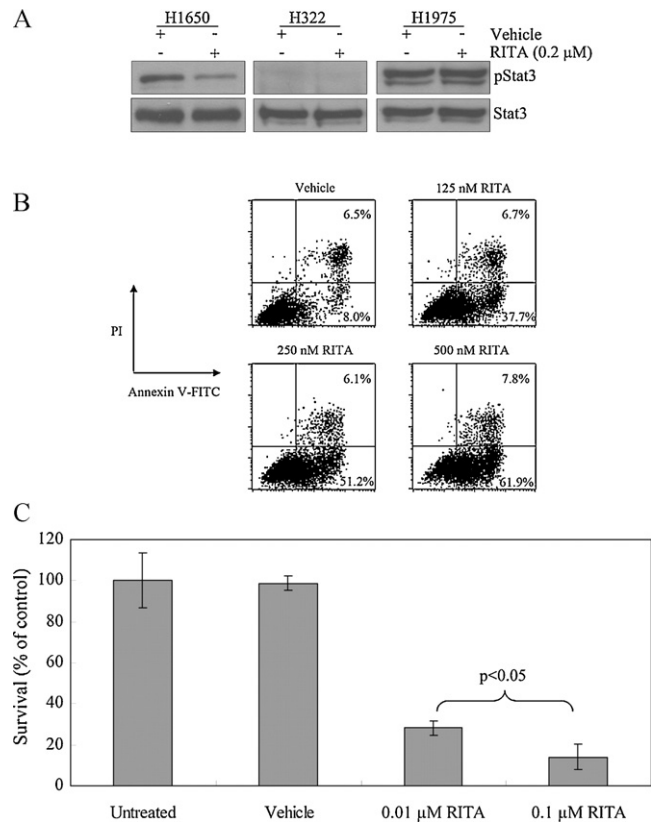


Fig. 3. Stat3 suppression and apoptosis induction were observed in RITA-treated H1650 cells. (A) H1650, H322, and H1975 cells were treated with 0.2 μ M of RITA for 8 h. Cells were lysed and subjected to Western blotting to analyze the expression levels of pStat3 and Stat3. (B) H1650 cells were seeded into 6-well plates 1 day prior to the RITA treatment. Cells were treated with vehicle (DMSO), 125, 250, or 500 nM of RITA for 3 days. Apoptosis was analyzed by the flow cytometry. Data represent one of the 3 sets of experiments. (C) H1650 cells were treated with vehicle, 0.01 μ M or 0.1 μ M of RITA for 14 days. After 14 days, cells were stained with crystal violet to determine the presence of colonies. The percentage of clonogenic survival was calculated by dividing the number of colonies from treated cells by the number of colonies from untreated cells. The percentage of untreated cells was considered as 100%. Data in the histogram represent means \pm SD from 3 experiments. Student's unpaired t -test was used to evaluate the difference between 0.01 μ M and 0.1 μ M of RITA-treated cells.

To further confirm the importance of Stat3 for survival, WP1066 which has been shown to inhibit JAK2 and its downstream PI3K and STAT pathways in acute myelogenous leukemia (AML) cells was selected to inhibit Stat3 activity [25,37]. When H1650 cells were treated with WP1066 at 10 μ M for 8 h, Stat3 phosphorylation was effectively suppressed (Fig. 5A). More importantly, WP1066 was effective in sensitizing H1650 cells with a CC_{50} value of 2–3 μ M (Fig. 5B). WP1066-induced Stat3 suppression and cytotoxicity were also observed in H1975 cells (Fig. 5A and B). In order to determine whether apoptosis were triggered by WP1066, apoptosis signals were analyzed by flow cytometry. Given the observation that longer exposure of cells to WP1066 caused severe cytotoxicity which impedes sample collection for flow cytometry analysis, samples were analyzed after 16 h exposure to WP1066. The increase of apoptotic cells demonstrates that apoptosis was induced by WP1066 in both H1650 and H1975 cells (Fig. 5C).

3.5. Enhancement of doxorubicin efficacy by RITA in H1650 cells in vitro and in vivo

Inhibition of Stat3 signaling pathway has been shown to result in enhanced sensitivity to doxorubicin in a number of metastatic breast and hepatocellular cancer cell lines [38,39]. It is interesting

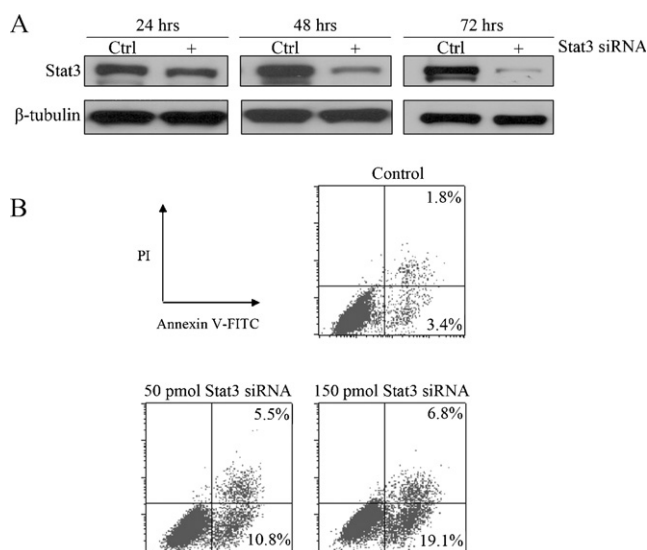


Fig. 4. siRNA mediated down-regulation of Stat3 sensitizes H1650 cells. (A) H1650 cells were transfected with the negative control or Stat3 siRNA. Lysates collected at the indicated time were subjected to Western blotting to analyze expression levels of Stat3. (B) Apoptosis signals of H1650 cells transfected with the negative control or Stat3 siRNA (50 or 150 pmol) were determined by the flow cytometry 3 days after transfection. Data represent one of the 3 sets of experiments. Ctrl, control.

to know if suppression of Stat3 activity may render NSCLC cells to be more sensitive to doxorubicin which is relatively ineffective in NSCLC cells [29]. To determine the combined effects of RITA and doxorubicin on Stat3 activity, moderate doses of RITA (0.2 μ M) and doxorubicin (4 μ M) were selected for treatments. As shown in Fig. 6A, when H1650 cells were treated with RITA (0.2 μ M) or doxorubicin (4 μ M) alone, there were only slight declines in the levels of pStat3 (lanes 2 and 3). However, a more pronounced reduction in pStat3 was observed when cells were treated with the combination of these 2 agents (lane 4). We then examined whether the Stat3 suppression was accompanied by the induction of apoptosis in H1650 cells treated with the combination of RITA and doxorubicin [40]. We found that the combination treatment resulted in enhanced accumulation of apoptotic cells (Fig. 6B). Approximately 18% and 22% of apoptotic cells were detected when cells were treated with RITA (0.2 μ M) and doxorubicin (4 μ M), respectively. When cells were treated with the combination of these two agents, nearly 70% of cells were in the apoptotic stage. Such results suggest that RITA and doxorubicin exert strong synergistic anti-cancer effects in H1650 cells. To give an insight into the pharmacological interaction between RITA and doxorubicin, combination index (CI) values were determined. Results from the MTS assay were analyzed by the median effect principle (CalcuSyn analysis) to provide a CI value [41]. As shown in Table 1 which represents one of the 3 sets of data, all CI values under different dilutions of doxorubicin and RITA were much less than 1, suggesting that a strong synergism exists between these agents in suppressing H1650 cells.

Consequently, we were interested in understanding the anti-tumor activity of such a combination treatment under *in vivo* conditions. BALB/c AnN nude mice bearing H1650 NSCLC xenografts were stratified into 4 treatment groups. After the sizes of tumors reached approximate 150 mm³, the mice were then administered with PBS control, RITA (10 mg/kg, q.d. \times 16), doxorubicin (1 mg/kg, q.2d. \times 8), or a combination of these two agents (RITA; 10 mg/kg, q.d. \times 16; doxorubicin: 1 mg/kg, q.2d. \times 8). In all treatments, there was no or minor weight loss in mice. The sizes of tumors were slightly reduced in mice treated RITA or doxorubicin alone than those in mice treated with the

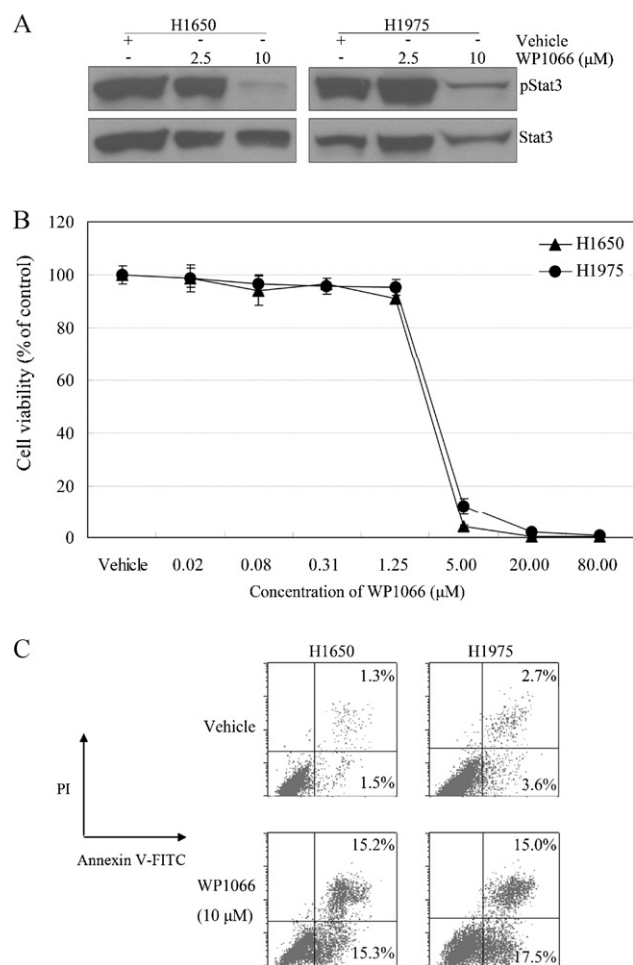


Fig. 5. WP1066 induces apoptosis in H1650 and H1975 cells. (A) Cell lysates from H1650 and H1975 cells treated with 2.5 μ M or 10 μ M of WP1066 for 8 h were analyzed by Western blotting. (B) Cell viability of H1650 and H1975 cells treated with different concentrations of WP1066 was determined by MTS assay. Viability of the vehicle control was considered as 100%. Data in the diagram represent means \pm SD from 3 independent experiments. (C) H1650 and H1975 cells were treated with vehicle or 10 μ M of WP1066 for 16 h. Apoptosis was analyzed by the flow cytometry. Data represent one of the 3 sets of experiments.

vehicle (Fig. 6C and D). However, the mice receiving the combination of RITA and doxorubicin treatment showed a more pronounced decrease ($p = 0.0038$ by one way ANOVA) in tumor sizes compared to the PBS control. Furthermore, the tumors showed a growth delay (T – C) value with an average of 12.2 days to reach 250 mm³ compared to that of the PBS control (Table 2). Taken together, the *in vivo* studies show that RITA increases doxorubicin sensitivity in the xenografts of mice inoculated with H1650 cells.

4. Discussion

Lung cancer including NSCLC claims approximately 1.2 million lives annually [42]. NSCLC treatment with conventional chemotherapies is accompanied with severe side effects and poor prognosis. Although target therapies have been successfully developed, only a small subset of NSCLC patients benefits from the EGFR inhibitors and patients who initially respond to the treatments with EGFR inhibitors would eventually develop acquired resistance to these target therapies. Hence, development of drugs with other molecular targets is in urgent need. Results from our studies suggest that it might be worthy to further evaluate Stat3 as a valid target in clinical setting of NSCLC.

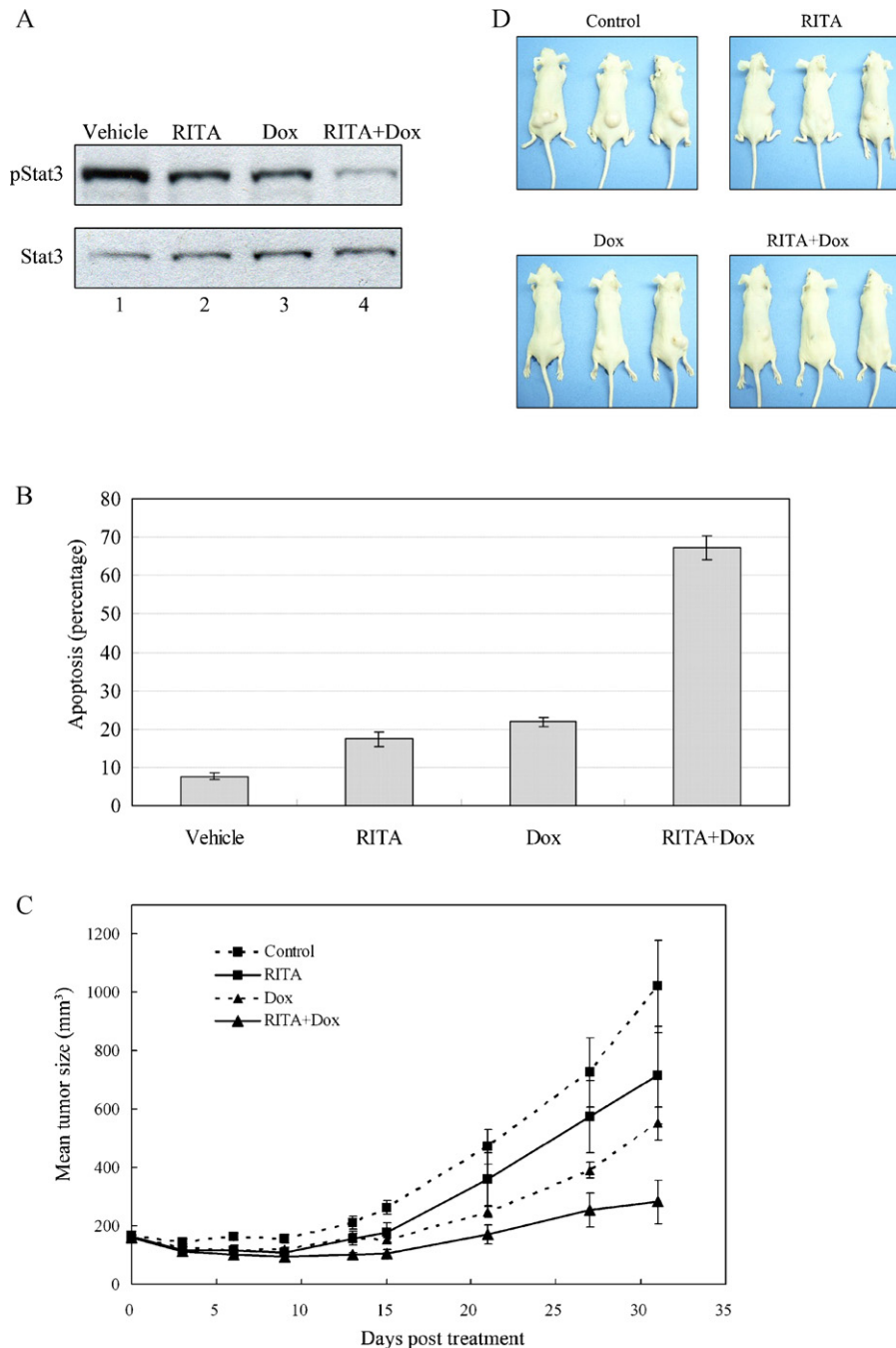


Fig. 6. RITA enhances the sensitivity of H1650 cells to doxorubicin *in vitro* and *in vivo*. (A) Lysates from H1650 cells treated with 0.2 μ M of RITA, 4 μ M of doxorubicin, or a combined treatment for 8 h were analyzed by Western blotting. (B) Apoptosis of H1650 cells treated with vehicle, 0.2 μ M of RITA, 4 μ M of doxorubicin, or a combination of RITA and doxorubicin for 3 days was determined by the flow cytometry. Data in the histogram represent means \pm SD from 3 experiments. (C) After the tumor sizes reached approximately 150 mm³, BALB/c AnN nude mice bearing the H1650 xenografts received PBS control, RITA (10 mg/kg, q.d. \times 16), doxorubicin (1 mg/kg, q.2d. \times 8), or a combination of these two agents. Values represent means \pm SEM from 7 mice in each group. (D) Tumor morphology of mice received the indicated treatments as described in (C). Dox, doxorubicin.

Table 1

Combination index value suggests the synergism between RITA and doxorubicin in suppressing H1650 cells.

Dilution	IC ₅₀	IC ₇₅	IC ₉₀
RITA:Dox (1:1)	0.28	0.20	0.17
RITA:Dox (1:5)	0.16	0.11	0.10
RITA:Dox (1:25)	<0.01	<0.01	<0.01

CI < 1, CI = 1, or CI > 1 suggests that a synergistic, additive, or antagonistic effect, respectively, exists between 2 chemicals. Dox, doxorubicin.

Stat3 activation was shown to be necessary for the oncogenic effects of NSCLC cell lines harboring EGFR somatic mutations [43,44]. Phosphorylated Stat3 and EGFR are frequently observed in NSCLC specimens [20,45]. Stat3 is overexpressed in the cytoplasm of 52.1% of NSCLC specimens with higher percentage (60.8%) in the nucleus [19]. Cell lines with EGFR mutations have higher levels of pStat3 than those with wild type EGFR [45]. Furthermore, synergistic anti-proliferative effects of EGFR and Stat3 inhibitors have been observed in a variety of NSCLC cells [46–48]. Altogether, these studies suggest that Stat3 activity is vital to maintain survival of NSCLC cells with oncogenic addition to EGFR. In our studies, we

Table 2
Tumor growth delay of mice treated with the indicated regimens.

Group	Mean time required for tumors to reach 250 mm ³ (d)	Tumor growth delay (d) ^a
PBS control	14.5	–
RITA (10 mg/kg)	17.4	2.9
Dox (1 mg/kg)	21.1	6.6
RITA + Dox	26.7	12.2

^a Tumor growth delay is the difference in days between treated and control groups to reach 250 mm³.

found that disruption of Stat3 activity alone by genetic or pharmacological approaches was sufficient to cause different degrees of cytotoxicity in gefitinib-resistant H1650 cells. Likewise, similar results were also observed in another gefitinib-resistant cell line, H1975. These data raise a possibility that Stat3 inhibitors may be considered as an alternative treatment for NSCLC patients that are insensitive to current EGFR inhibitors.

Pharmacological administrations, such as RITA with partial inhibition on Stat3 activity, generated higher degrees of cytotoxicity than Stat3 siRNA which actually suppressed Stat3 activity much greater than RITA. These results suggest that RITA may cause cytotoxicity through other signaling pathways in addition to Stat3 involved pathways. One of the possibilities is through Erk-mediated pathways. Erk, one of the members in MAPK family, phosphorylates more than 100 substrates to affect cellular activities such as cell proliferation, survival, differentiation, and apoptosis as well [49]. The importance of Erk activation is emphasized in apoptosis caused by DNA damaging agents such as doxorubicin and cisplatin [50,51]. Our findings showed that Erk was activated as well in RITA-treated H1650 cells (data not shown). That the inhibition of Erk activity by PD98059 reduced the sensitivity of H1650 cells to RITA suggests that Erk activation is essential for maintaining viability of H1650 cells along with Stat3 (data not shown).

Currently, treatments with a combination of drugs are commonly employed to improve the efficacy of NSCLC treatment. Doxorubicin is an effective chemotherapy drug that has been widely used to treat a variety of solid and hematologic malignancies [30]. Combination of doxorubicin with other anti-tumor agents, e.g., gemcitabine or bortezomib, has also been shown to be effective in treating metastatic breast cancer or refractory multiple myeloma, respectively [52,53]. However, NSCLCs were shown to bear some inherent resistance to doxorubicin [29]. Meanwhile, Stat3 activation was known to be closely related to the resistance to chemotherapeutic drugs, including doxorubicin, in cancer cell lines [54–56]. The anti-cancer effects of doxorubicin can be enhanced by Stat3 inhibitors such as andrographolide, diosgenin, and AG490 [38,39,55].

In this study, we for the first time show that doxorubicin with the combination of Stat3 inhibitors would give rise to strong synergistic anti-cancer effects in certain NSCLC cells. RITA-mediated Stat3 suppression caused an increase in the efficacy of doxorubicin in H1650 cells under *in vivo* and *in vitro* conditions. In addition to RITA, another Stat3 inhibitor, WP1066, also functions to enhance the sensitivity of H1650 cells to doxorubicin *in vitro* (Table 3). More importantly, the synergism between doxorubicin and the Stat3 inhibitors was evident in other NSCLC cell lines such as H1975 and A549 as well (Table 3). The combination strategy may reduce the needed doxorubicin dosage so that the doxorubicin-induced severe heart damages may be minimized. Although cautious considerations should be taken in extrapolating the *in vitro* data to the clinical setting, it is worthy of prospective clinical investigations to evaluate if the combination of doxorubicin and

Table 3
Combination index values suggest the synergism between WP1066 and doxorubicin in suppressing H1650, H1975, and A549 cells.

Dilution	IC ₅₀	IC ₇₅	IC ₉₀
H1650			
WP:Dox (1:1)	0.14	0.10	0.07
WP:Dox (1:5)	0.12	0.06	0.03
WP:Dox (1:25)	0.14	0.12	0.10
H1975			
WP:Dox (1:1)	0.40	0.48	0.59
WP:Dox (1:5)	0.34	0.32	0.30
WP:Dox (1:25)	0.36	0.35	0.35
A549			
WP:Dox (1:1)	0.56	0.62	0.70
WP:Dox (1:5)	0.34	0.38	0.44
WP:Dox (1:25)	0.38	0.26	0.18

CI < 1, CI = 1, or CI > 1 suggests that a synergistic, additive, or antagonistic effect, respectively, exists between 2 chemicals. WP, WP1066; Dox, doxorubicin.

Stat3 inhibitors may be beneficial for NSCLC patients that respond poorly to current therapies.

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