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Salinomycin acts through reducing AKT-dependent thymidylate synthase expression to enhance erlotinib-induced cytotoxicity in human lung cancer cells

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ABSTRACT

Erlotinib (Tarceva[R]) is a selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor in the treatment of human non-small cell lung cancer (NSCLC). Salinomycin, a polyether antibiotic, has been promising a novel therapeutic agent for lung cancer, and down-regulated the expression of thymidylate synthase (TS) in NSCLC cell lines. Previous study showed that against EGFR and TS was strongly synergistic cytotoxicity in NSCLC cells. In this study, we showed that erlotinib (1.25–10 μM) treatment down-regulating of TS expression in an AKT inactivation manner in two NSCLC cell lines, human lung squamous cell carcinoma H1703 and adenocarcinoma H1975 cells. Knockdown of TS using small interfering RNA (siRNA) or inhibiting AKT activity with PI3K inhibitor LY294002 enhanced the cytotoxicity and cell growth inhibition of erlotinib. A combination of erlotinib and salinomycin resulted in synergistic enhancement of cytotoxicity and cell growth inhibition in NSCLC cells, accompanied with reduced protein levels of phospho-AKT(Ser473), phospho-AKT(Thr308), and TS. Overexpression of a constitutive active AKT (AKT-CA) or Flag-TS expression vector reversed the salinomycin and erlotinib-induced synergistic cytotoxicity. Our findings suggested that the down-regulation of AKT-mediated TS expression by salinomycin enhanced the erlotinib-induced cytotoxicity in NSCLC cells. These results may provide a rationale to combine salinomycin with erlotinib for lung cancer treatment.

1. Introduction

Lung cancer is the leading cause of cancer death and accounts for the most common malignancy in the world. Epidermal growth factor receptor (EGFR) is highly expressed in non-small cell lung cancer (NSCLC) [1,2] and is most commonly reported in squamous cell carcinoma, followed by adenocarcinoma and large cell carcinoma [3]. The classic EGFR mutations, including the L858R point substitution in exon 21 and in-frame deletions in exon 19, are demonstrating about 85–90% of all EGFR mutations and associated with affected response to the EGFR tyrosine kinase inhibitors (TKIs) [4]. EGFR-TKIs can block the lipid kinase phosphatidyl inositol 3-kinase (PI3K)-AKT and Ras-Raf-MKK1/2-ERK1/2 pathways [5], which have been implicated in the promotion of cell growth and survival and the inhibition of cell apoptosis [6]. Erlotinib is an EGFR-TKI targeting the ATP-binding site of the EGFR, leading to inhibition of tyrosine kinase activity [7]. Erlotinib has been used in prolong survival in patients with advanced NSCLC after first-line and second-line chemotherapy [8]. However, enhancement of PI3K-AKT and MKK1/2-ERK1/2 signals have been found in the EGFR-TKI-resistant NSCLC [9,10].

Thymidylate synthase (TS) is an essential enzyme for the *de novo* synthesis of dTMP; block EGFR by erlotinib and the cytotoxic agent pemetrexed against TS, was strongly synergistic in NSCLC cell lines [11]. Moreover, dasatinib, a Src inhibitor, enhances the sensitivity to pemetrexed in mesothelioma cells through TS downregulation [12]. In colorectal cancer, B7-H3, a member of the B7 family, induces the chemoresistance to 5-fluorouracil (5-FU) through the upregulation of TS via the PI3K/AKT signaling pathway [13]. Inhibition of the mammalian target of rapamycin (mTOR) showed growth inhibitory activity against gastric cancer cells and acted synergistically with 5-FU

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by down-regulation of TS expression [14]. Enzastaurin (a PKCbeta-inhibitor) and pemetrexed has synergistic cytotoxicity effect in the NSCLC cells SW1573 and A549, and this combination reduced AKT phosphorylation and decreased pemetrexed-induced TS expression [15]. However, whether erlotinib could affect TS expression through AKT signal in NSCLC cell lines is unclear.

Salinomycin, a polyether ionophore antibiotic isolated from *Streptomyces albus*, was originally used to eliminate bacteria, fungi, and parasites [16]. Moreover, salinomycin improves the efficacy of gemcitabine in pancreatic cancer [17], and sensitizes breast cancer cells to the effects of doxorubicin and etoposide treatment by increasing DNA damage [18]. In Parajuli et al. study, salinomycin treatment inhibited AKT/NF-kB activation and induced apoptosis in cisplatin-resistant ovarian cancer cells [19]. Our previous study showed that salinomycin enhances cisplatin-induced cytotoxicity in human lung cancer cells via down-regulation of AKT-dependent TS expression [20]. However, the sensitization of lung cancer cells to an EGFR-TKI using salinomycin has yet to be determined.

Previous studies have showed that PI3K-AKT pathway is one of most promising targets of anticancer agents. PI3Ks are activated by EGFR and convert PIP2 (phosphatidylinositol-4,5-bisphosphate) to PIP3 (phosphatidylinositol-3,4,5-trisphosphate) [21,22]. When AKT kinase is recruited to the plasma membrane by PIP3, it is activated by specific phosphorylation at residues Ser473 and Thr308. Activated PI3K-AKT signal pathway controls cell survival, cell growth, and proliferation [21,22]. In the present study, we investigated whether cotreatment with salinomycin could sensitize NSCLC cancer cells to erlotinib. We further analyzed whether salinomycin and erlotinib cotreatment influenced the activation status of AKT or expression levels of TS mRNA and protein in NSCLC cells.

2. Materials and methods

2.1. Cell lines, cell cultures, and chemicals

Human lung adenocarcinoma H1975 cells (CRL-5908), lung squamous cell carcinoma H1703 (CRL-5889), bronchioloalveolar carcinoma A549 cells (CCL-185), and lung adenocarcinoma H1650 cells (CRL-5883) were obtained from the American Type Culture Collection (Manassas, VA) and the cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI-1640 complete medium supplemented with sodium bicarbonate (2.2%, w/v), L-glutamine (0.03%, w/v), penicillin (100 units/ml), streptomycin (100 μg/ml), and fetal calf serum (10%). The cell lines were routinely tested to confirm that they were free of *Mycoplasma*. Salinomycin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Erlotinib was purchased from Genentech (South San Francisco, CA, USA). LY294002 and wortmannin were purchased from Calbiochem-Novabiochem (San Diego, CA, USA). Erlotinib, LY294002, and wortmannin were dissolved in DMSO.

2.2. Western blot analysis

After different treatments, equal amounts of proteins from each set of experiments were subjected to Western blot analysis as previously described [23]. The specific phospho-AKT(Ser473) and phospho-AKT(Thr308) antibodies were purchased from Cell Signaling (Beverly, MA, USA). Rabbit polyclonal antibodies against TS(TS-106) (sc-33679), AKT(H-136) (sc-8312), and Actin(I-19) (sc-1616) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.3. Plasmid and transfection

Exponentially growing human lung cancer cells (10^6) were plated for 18 h, and then constitutively active AKT (AKT-CA), which harbored a consensus myristylation domain that replaced the 4–129 amino acids of wild-type AKT, were transfected into H1703 or H1975 cells using Lipofectamine (Invitrogen). The sense-strand sequences of siRNA duplexes were as follows: TS: 5′-GCACAUAUUUACCUGAAUC-3′, and scrambled (as a control): 5′-GCG CGC UUU GUA GGA TTC G-3′ (Dharmacon Research, Lafayette, CO). The AKT1 siRNA (sc-29195) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cells were transfected with siRNA duplexes (200 nM) using Lipofectamine 2000 (Invitrogen) for 24 h.

2.4. Quantitative real-time polymerase chain reaction (PCR)

PCRs were performed using an ABI Prism 7900HT, in accordance with the manufacturer's instructions. Amplification of specific PCR products was performed using the SYBR Green PCR Master Mix (Applied Biosystems). For each sample, the data was normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The designed primers in this study were: TS forward primer, 5′- ACTGCAAAGAGTGATTGACACC -3′, TS reverse primer, 5′-CACTGTTCACCACATAGAACTGG -3′; GAPDH forward primer, 5′-CATGAGAAGTATGACAACAGCCT -3′; GAPDH reverse primer, 5′-AGTCCTTCCACGATACCAAAGT -3′. Analysis was performed using the comparative Ct value method. For each sample, the data was normalized to the housekeeping gene GAPDH.

2.5. MTS assay

In vitro 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4- sulfophenyl)-2H-tetrazolium (MTS) assay was performed. Cells were cultured at 5000 per well in 96-well tissue culture plates. To assess cell viability, drugs were added after plating. At the end of the culture period, 20 μL of MTS solution (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA) was added, the cells were incubated for a further 2 h, and the absorbance was measured at 490 nm using an ELISA plate reader (Biorad Technologies, Hercules, CA).

2.6. Combination index analysis of drug interactions

Erlotinib and salinomycin were combined at a ratio of 1:1 or 1:2, and the effect of combined treatment on cell viability was examined by MTS assay. To calculate a combination index (CI), the computer software Calcusyn (Biosoft, Oxford, UK) was used, taking the entire shape of the cell viability curve into account to calculate whether a combination is synergistic (CI < 0.9), additive (CI=0.9–1.1), or antagonistic (CI > 1.1) [24]. The mean of CI values at a fraction affected (FA) of 0.90, 0.75, 0.50 were used to calculate between the three independent experiments.

2.7. Trypan blue dye exclusion assay

Cells were treated with salinomycin and/or erlotinib for 24, 48, and 72 h. After treatment, 500 cells were harvested, and the proportion of dead cells was determined by hemocytometer, counting the number of cells stained with trypan blue. Trypan blue dye can be excluded from living cells, but is able to penetrate dead cells. The dead cells were calculated as follow: trypan blue (+) cells ratio (%)=(stained cell number/total cell number)x100.

2.8. Statistical analyses

For each protocol, three or four independent experiments were performed. Results were expressed as the mean ± SEM. Statistical calculations were performed using SigmaPlot 2000 software (Systat Software, San Jose, CA). Differences in measured variables between the experimental and control groups were assessed via unpaired *t*-test. $P < 0.05$ was considered statistically significant.

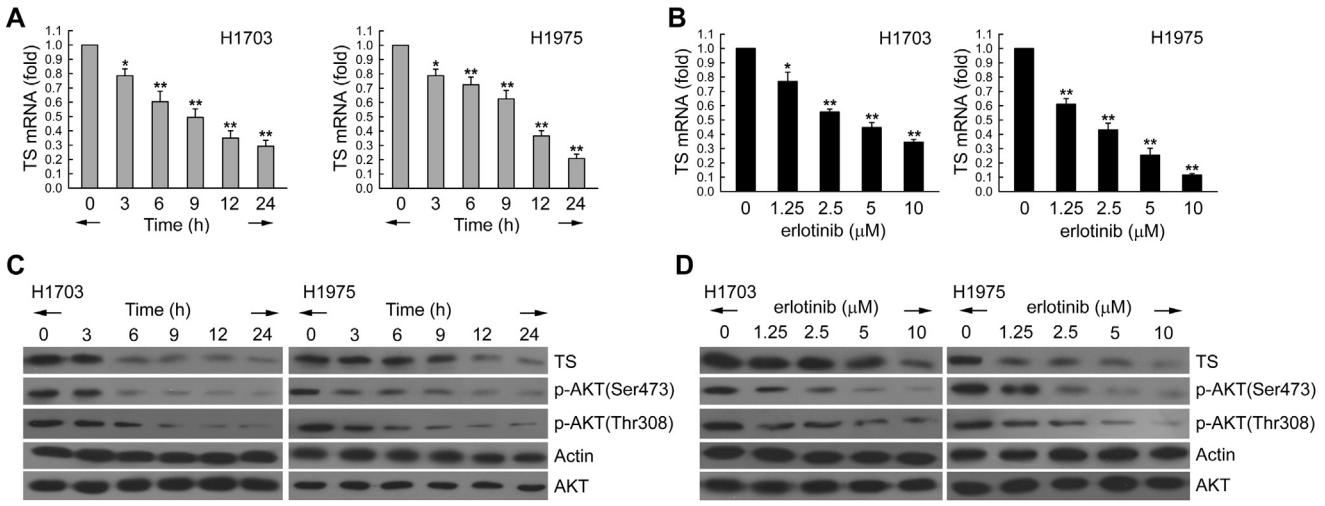

Fig. 1. Erlotinib decreased TS expression in a dose and time-dependent manner. (A) H1703 or H1975 cells (10^6) were cultured in complete medium for 18 h and then exposed to erlotinib (5 μM) for 3, 6, 9, 12, or 24 h. (B) Various concentrations of erlotinib (1.25–10 μM) for 24 h in complete medium. The total RNA was isolated and subjected to real-time PCR for TS mRNA expression. The results (mean ± SEM) were from three independent experiments. (C and D) After treatment as above, the cell extracts were examined by Western blot for determination of TS, phospho-AKT(Ser473), phospho-AKT(Thr308), actin, and AKT protein levels. $^{**}p < 0.01$, $^{*}p < 0.05$ using Student's *t*-test for comparison between the cells treated with or without erlotinib.

3. Results

3.1. TS mRNA and protein levels were decreased after erlotinib exposure

To determine whether TS expression was associated with the effects of erlotinib, we first assessed H1703 or H1975 cells, treated with erlotinib (5 μM) for 3–24 h or various concentrations of erlotinib (1.25, 2.5, 5, 10 μM) for 24 h. The real-time PCR and Western blot analysis revealed that erlotinib reduced TS mRNA and protein expression in a time and dose-dependent manner; this was accompanied with a decrease in phospho-AKT(Ser473) and phospho-AKT(Thr308) protein levels (Fig. 1A-D). Next, to determine whether AKT inactivation was involved in down-regulation of TS by erlotinib, these cell lines were transiently transfected with AKT-CA plasmids, a constitutively active form of AKT. Overexpression of AKT-CA rescued TS mRNA and protein expression in H1703 and H1975 cells inhibited by erlotinib (Fig. 2A and B). However, once these cells were pretreated with PI3K inhibitors (wortmannin or LY294002) (Fig. 2C and D) or transfected with si-AKT RNA (Fig. 2E and F), the TS mRNA and protein levels in erlotinib-exposed H1703 or H1975 cells would further decrease. As expected, the addition of wortmannin or LY294002 further decreased phospho-AKT(Ser473) and phospho-AKT(Thr308) protein levels in erlotinib-exposed NSCLC cell lines (Fig. 2D). Therefore, we concluded that erlotinib decreased TS expression in a PI3K-AKT inactivation manner.

3.2. Knockdown of TS increased erlotinib-induced cytotoxicity and growth inhibition in NSCLC cells

We next examined the effect of siRNA-mediated TS knockdown on erlotinib-induced cytotoxicity and cell growth inhibition in NSCLC cells. At 24 h post-transfection, real-time PCR and Western blot analysis showed a further decrease in TS mRNA and protein in erlotinib-treated H1703 and H1975 cells (Fig. 3A and B). Furthermore, suppression of TS expression by si-TS RNA resulted in increased sensitivity to erlotinib compared to si-control transfected cells (Fig. 3C and D). We also conducted a cell growth inhibition assay to evaluate the synergistic effects of TS knockdown with erlotinib treatment. In Fig. 3E, more inhibition of cell growth was induced by the combination of TS siRNA and erlotinib than by erlotinib alone in H1703 or H1975 cells. Therefore, down-regulation of TS expression enhanced erlotinib-induced cytotoxicity and growth inhibition in NSCLC cells.

3.3. AKT inactivation and TS downregulation were associated with erlotinib-induced cytotoxicity and growth inhibition

Next, the role of AKT inactivation and decrease of TS expression in the cytotoxic effect of erlotinib was examined. In Fig. 3F and G, enforced expression of the AKT-CA or Flag-TS expression vector rescued H1703 and H1975 cell viability after being decreased by erlotinib, and also reverse the growth inhibition effect. In contrast, co-treatment with PI3K inhibitor LY294002 caused further significant decreases in cell viability in erlotinib-exposed H1703 or H1975 cells, compared with erlotinib treatment alone (Fig. 3H). On the other hand, LY294002 could more effectively inhibit cell growth than either drug alone after erlotinib treatment (Fig. 3I). Taken together, inactivation of the AKT-TS signal enhanced erlotinib-induced cytotoxicity and growth inhibition in NSCLC cells.

3.4. Salinomycin synergized erlotinib-induced cytotoxicity and cell growth inhibition in H1703 and H1975 cells

In our previous study demonstrated that salinomycin inhibits the AKT-TS pathway, leading to enhance cytotoxicity in cisplatin-treated NSCLC cells [20]; therefore, we attempted to determine whether salinomycin could enhance the cytotoxic effects of erlotinib through down-regulating TS expression in NSCLC cells. First, we used different NSCLC cell lines, including adenocarcinoma (H1975 and H1650), bronchioloalveolar cell carcinoma (A549), and squamous cell carcinoma (H1703). The H1975 cell line contains erlotinib resistance EGFR L858R mutation plus T790M mutation. The H1703 and A549 cell line contains wild-type EGFR. The H1650 cell line contains a deletion mutation (delE746-A750) of EGFR. Erlotinib and salinomycin were combined at a ratio of 1:1 or 1:2, and the effect of combined treatment on cell viability was examined by MTS assay (Fig. 4A; Supplementary Fig. S1A). Combined treatment with salinomycin and erlotinib for 24 h resulted in a greater loss of cell viability in these four NSCLC cells lines than treatment with either erlotinib or salinomycin alone (Fig. 4A; Supplementary Fig. S1A). As the CI values are below 1, the results demonstrate that both combination treatments synergistically enhance cytotoxicity in these four NSCLC cells (Fig. 4B; Supplementary Fig. S1B). In addition, H1703 and H1975 cells were exposed to salinomycin

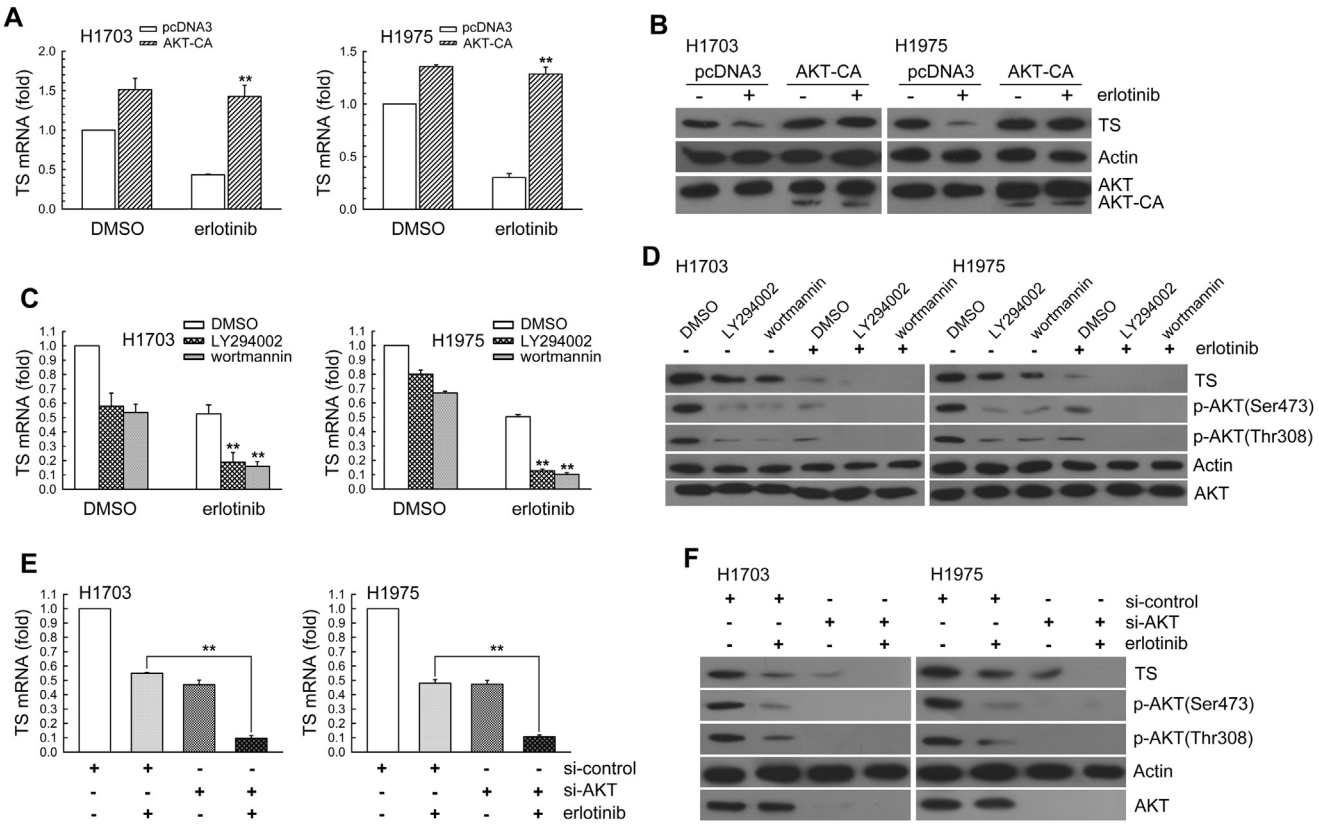

Fig. 2. Erlotinib decreased TS expression via PI3K-AKT inactivation in NSCLC cells. (A and B) H1703 or H1975 cells (5×10^5) were transfected with AKT-CA expression vector. After incubation for 24 h, the cells were treated with erlotinib (5 μM) for 24 h. The results (mean ± SEM) were from three independent experiments. $^{**}p < 0.01$ using Student's *t*-test for comparison between the cells treated with erlotinib in pcDNA3 or AKT-CA vector-transfected cells. (C and D) LY294002 (5 μM) or wortmannin (5 μM) was added to H1703 or H1975 cells for 1 h before erlotinib (2.5 μM) treatment for 24 h. The results (mean ± SEM) were from three independent experiments. $^{**}p < 0.01$ using Student's *t*-test for comparison between the cells treated with erlotinib–DMSO or an erlotinib–LY294002/wortmannin combination. (E and F) H1703 or H1975 cells were transfected with siRNA duplexes (200 nM) specific to AKT or scrambled (control) in complete medium for 24 h prior to treatment with erlotinib (2.5 μM) in complete medium for 24 h. The results (mean ± SEM) were from three independent experiments. $^{**}p < 0.01$, using Student's *t*-test for comparison between the cells treated with erlotinib in si-AKT RNA or si-scrambled RNA-transfected cells. After treatment, the cell extracts were examined via real-time PCR (A, C, E) and Western blot (B, D, F) for determination of TS mRNA and protein levels, respectively.

and/or erlotinib, and cell proliferation was determined 1–3 days after exposure. Salinomycin and erlotinib co-treatment had a greater cell growth inhibition effect than either treatment alone (Fig. 4C). The results showed that combined salinomycin and erlotinib had a synergistic cytotoxic effect on human NSCLC cells.

3.5. Salinomycin down-regulated TS protein and mRNA level in erlotinib-treated human lung cancer cells

In order to assess the mechanism of the synergistic effects, we hypothesized that salinomycin would affect TS expression in erlotinib-treated NSCLC cells. To test this hypothesis, H1703 and H1975 cells were exposed to erlotinib (1.25, 2.5, 5 μM) and salinomycin (1.25 μM) for 24 h. The results from real-time PCR analysis showed that salinomycin further decreased erlotinib-induced TS mRNA levels in H1703 and H1975 cells (Fig. 4D). Moreover, salinomycin suppressed the phospho-AKT(Ser473), phospho-AKT(Thr308), and TS protein levels in erlotinib-treated NSCLC cells (Fig. 4E).

3.6. Transfection with AKT-CA or Flag-TS vectors enhanced the cell survival suppressed by erlotinib and salinomycin

Next, we investigated whether salinomycin and erlotinib combination-mediated TS downregulation was correlated with AKT inactivation in NSCLC cells. In Fig. 5A and B, enforced AKT-CA vector expression could rescue TS mRNA and protein levels that were suppressed by salinomycin and erlotinib. In Fig. 5C and D, enforced expression of the AKT-CA vector rescued H1703 and H1975 cell viability after being decreased by salinomycin and erlotinib. Moreover, enforced TS expression by Flag-TS vector transfection enhanced the TS protein level as well as the cell viability in salinomycin and erlotinib cotreated cells (Fig. 6A-C). Taken together, the downregulation of AKT-mediated TS expression by salinomycin is an important regulator to enhance the erlotinib-induced cytotoxicity in NSCLC cells.

4. Discussion

Salinomycin is an antibiotic and known to act as an ionophore on cell membranes [16,25]. Salinomycin was initially used to kill bacteria, fungi and parasites [16,26]. In past years, the polyether salinomycin has emerged from a high-throughput screen of substances that selectively deplete breast cancer stem cells [27]. More recently, salinomycin been shown to kill cancer stem cells and inhibit the growth of chemoresistant cancer cells [27–29]. Moreover, salinomycin can sensitize cancer cells to various anticancer drugs and radiation [18,30,31]. It has been reported that salinomycin was able to potentiate the anticancer effects of 4-Hydroxytamoxifen and frondoside A on breast cancer cells MCF-7 and MDA-MB-231, respectively [32]. Similarly, the combination of trastuzumab, an antibody that blocks the activity of HER2, and salinomycin leads to enhance cell death in MDA-MB-231 and MCF-7 mammospheres [33]. Here, we showed that salinomycin sensitized lung cancer cells to the effects of erlotinib.

Several studies have discovered anti-cancer effects of salinomycin in various malignancies including inhibition of cancer cell proliferation and migration, and induction of apoptosis and autophagy [29,34–36]. Our study results showed that downregulation of AKT-mediated TS

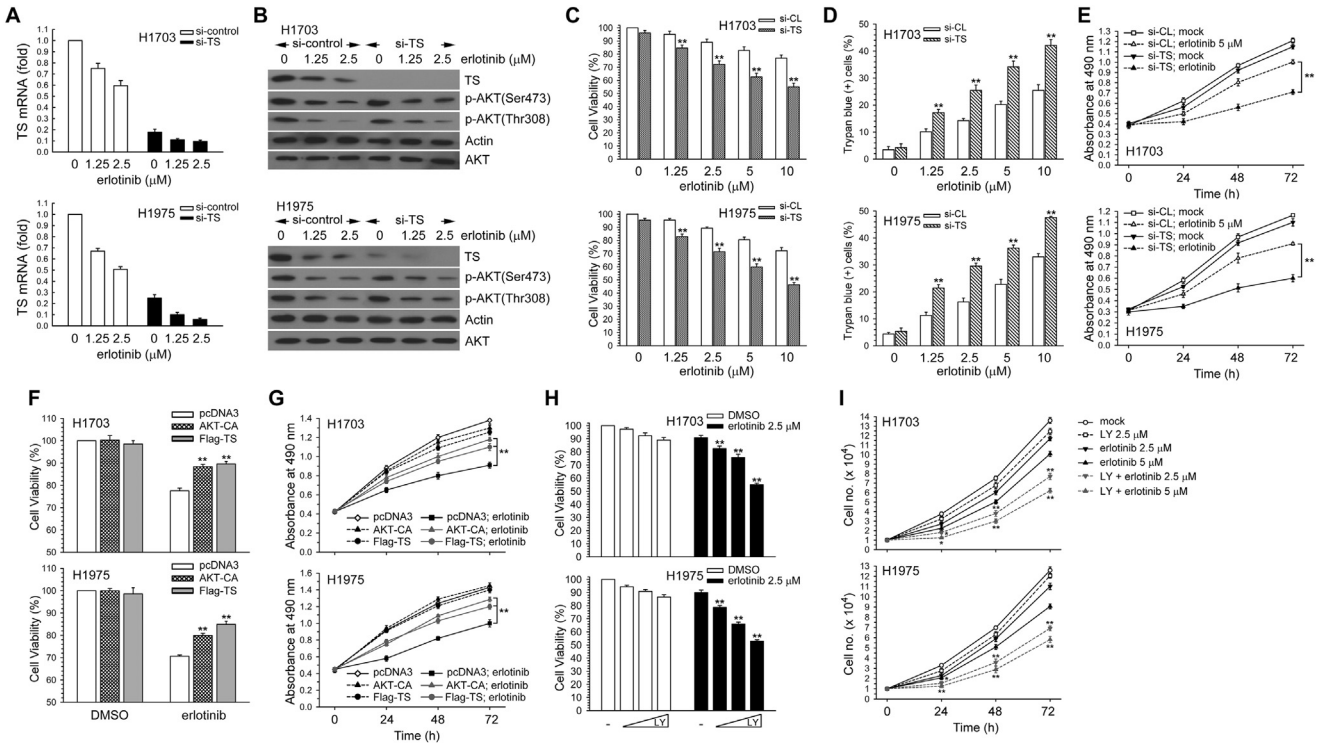

Fig. 3. Knockdown of TS expression by si-RNA transfection enhanced the cytotoxicity induced by erlotinib. (A and B) H1703 or H1975 cells were transfected with siRNA duplexes (200 nM) specific to TS or scrambled (control) in complete medium for 24 h prior to treatment with erlotinib (1.25, 2.5 μM) in complete medium for 24 h; the cell extracts were examined by real-time PCR (A) and Western blot (B) for determination of TS mRNA and protein levels, respectively. (C and D) After the above-mentioned treatment, cytotoxicity was determined by MTS assay and trypan blue dye exclusion assay. (E) After the cells were transfected with si-TS or si-scrambled RNA, the cells were treated with erlotinib (5 μM) for 24, 48, and 72 h, after which living cells were determined by MTS assay. The results (mean ± SEM) were from three independent experiments. $^{**}p < 0.01$ using Student's *t*-test for comparison between the cells treated with erlotinib in si-TS RNA or si-scrambled RNA-transfected cells. (F) After the cells were transfected with pcDNA3, AKT-CA or Flag-TS expression vector, the cells were treated with erlotinib (10 μM) for 24 h, cytotoxicity was determined by MTS assay. The results (mean ± SEM) were from three independent experiments. (G) After the cells were transfected with pcDNA3 or AKT-CA/Flag-TS expression vector, the cells were treated with erlotinib (10 μM) for 24, 48, and 72 h, and cytotoxicity was determined by MTS assay. $^{**}p < 0.01$ using Student's *t*-test for comparison between the cells treated with erlotinib in AKT-CA/Flag-TS or pcDNA3 vector-transfected cells. Inhibition of AKT activation enhanced the cytotoxicity induced by erlotinib. (H) H1703 or H1975 cells were pretreated with LY294002 (1.25, 2.5, 5 μM) for 1 h and then co-treated with erlotinib (2.5 μM) for 24 h. Cytotoxicity was determined by MTS assay. $^{**}p < 0.01$ using Student's *t*-test for comparison between the cells pretreated with or without LY294002 in erlotinib exposed cells. (I) Cells were treated with erlotinib (2.5, 5 μM) and/or LY294002 (2.5 μM) for 1–3 days after which living cells were determined by trypan blue dye exclusion assay. $^{**}p < 0.01$ using Student's *t*-test for comparison between cells treated with erlotinib alone or with an erlotinib and LY294002 combination.

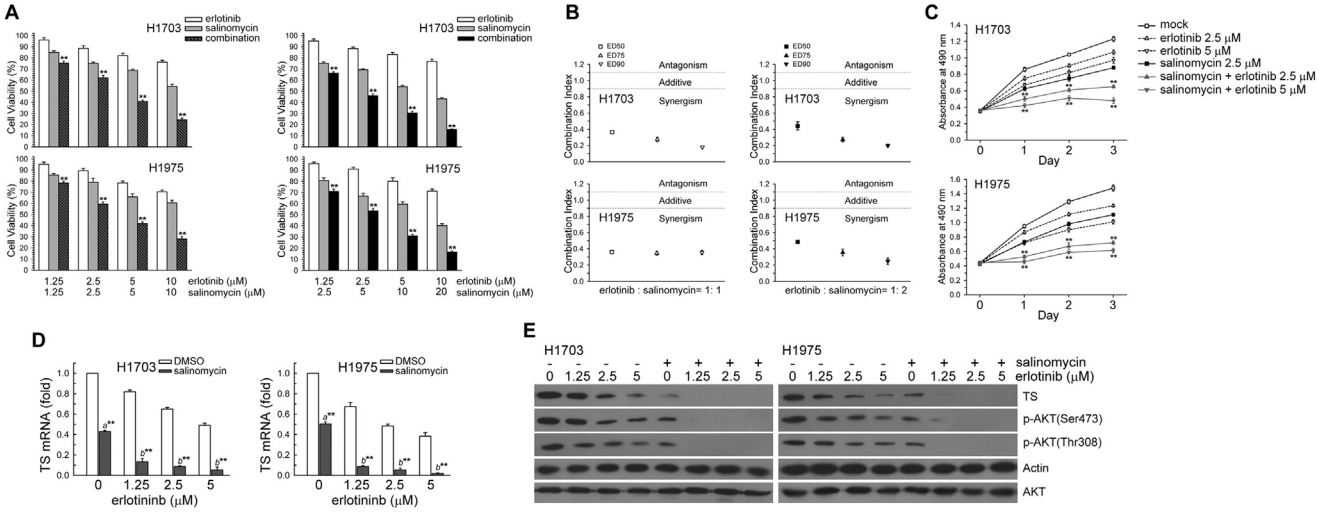

Fig. 4. Salinomycin co-treatment with erlotinib synergistically enhanced cytotoxicity. (A) Erlotinib and salinomycin were combined at a ratio of 1:1 (left panel) or 1:2 (right panel) and the MTS assay was used to analyze cell viability. (B) The mean CI values at a fraction affected (FA) of 0.50, 0.75, 0.90 for erlotinib and salinomycin combined treatment were averaged for each experiment and used to calculate the mean between experiments. (C) Cells were treated with salinomycin (2.5 μM) and/or erlotinib (2.5 or 5 μM) for 1–3 days after which living cells were determined by MTS assay. $^{**}p < 0.01$ using Student's *t*-test for comparison between cells treated with a drug alone or with a salinomycin/erlotinib combination. (D) Salinomycin decreased TS protein and mRNA levels in erlotinib-exposed NSCLC cells. H1703 or H1975 cells (10^6) were cultured in complete medium for 18 h and then were exposed to erlotinib and salinomycin (1.25 μM) for 24 h. After treatment, total RNA was isolated and subjected to real-time PCR for TS mRNA expression. Means ± standard deviation (SD) from four independent experiments. a^{**} denotes $p < 0.01$, respectively, using Student's *t*-test for comparison between the cells treated with or without salinomycin. b^{**} denotes $p < 0.01$, respectively, using Student's *t*-test for comparison between the cells treated with salinomycin/erlotinib alone or combined. (E) After treatment as above, cell extracts were examined by Western blot for determination of TS, phospho-AKT(Ser473), phospho-AKT(Thr308), actin, and AKT protein levels.

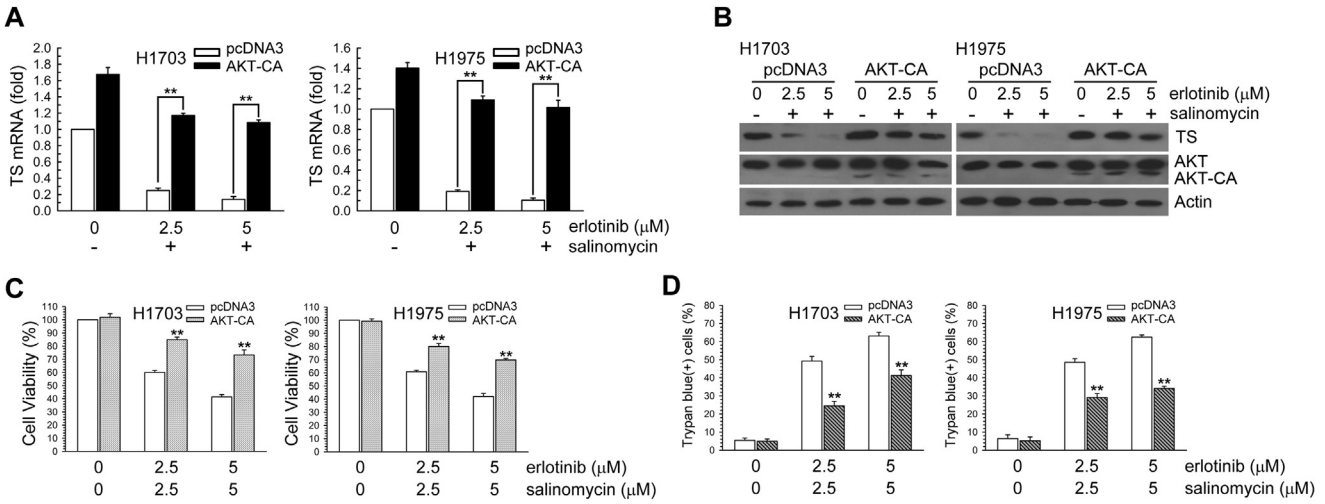

Fig. 5. Overexpression of AKT-CA restored the suppressed TS protein expression and cell survival in salinomycin and erlotinib-exposed H1703 and H1975 cells. (A) AKT-CA (5 μg) or pcDNA3 (5 μg) expression plasmids were transfected into cells using lipofectamine. After expression for 24 h, the cells were treated with salinomycin (1.25 μM) and erlotinib (2.5, 5 μM) for an additional 24 h, and total RNA was isolated and subjected to real-time PCR for TS mRNA expression. Means ± standard deviation (SD) from four independent experiments. ** denotes $p < 0.01$, respectively, using Student's *t*-test to compare cells treated with salinomycin and erlotinib in AKT-CA vs. pcDNA3-transfected cells. (B) After treatment as above, whole-cell extracts were collected for Western blot analysis. (C and D) After pcDNA3 control vector (5 μg) or AKT-CA (5 μg) expression plasmids transfection, cells were treated with salinomycin and erlotinib for 24 h. Cytotoxicity was determined by assessment with the MTS assay and trypan blue dye exclusion assay. **$p < 0.01$ by Student's *t*-test to compare cells treated with salinomycin and erlotinib in AKT-CA vs. pcDNA3-transfected H1703 or H1975 cells.

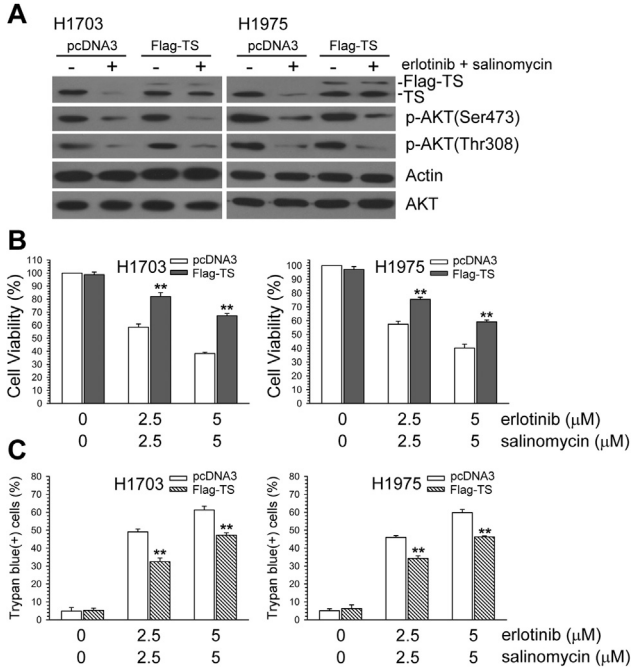

Fig. 6. Overexpression of Flag-TS expression vector restored the cell viability decreased by salinomycin and erlotinib in H1703 and H1975 cells. (A) Flag-TS (5 μg) or pcDNA3 (5 μg) expression plasmids were transfected into cells using lipofectamine. After expression for 24 h, the cells were treated with salinomycin (1.25 μM) and erlotinib (1.25 μM) for an additional 24 h, and the whole-cell extracts were collected for Western blot analysis. (B and C) After treatment as above, Cytotoxicity was determined by assessment with the MTS assay and trypan blue dye exclusion assay. ** denotes $p < 0.01$, respectively, using Student's *t*-test to compare cells treated with salinomycin and erlotinib in Flag-TS vs. pcDNA3-transfected cells.

expression by salinomycin enhanced the erlotinib–induced cell death and growth inhibition effect. Compared to previous studies, Zhang et al. indicated that the pemetrexed combined with EGFR-TKI gefitinib decreased TS mRNA expression. Additionally, gefitinib inhibited the pemetrexed induced AKT and ERK1/2 activation, and pemetrexed combined with gefitinib has a significant synergistic effect on colorectal cancer cells [37]. Previous study showed that the addition of TS-targeting drugs to irreversible EGFR-TKI BIBW2992 overcome EGFR-TKI resistance and synergistically inhibit the proliferation of NSCLC cells with the T790M mutation *in vitro* [38]. Moreover, BIBW2992 induced down-regulation of TS in the gefitinib-resistant NSCLC cells, implicating depletion of TS in the enhanced antitumor effect of the combination therapy [38]. In this study, salinomycin combined treatment with erlotinib could further down-regulate AKT-mediated TS expression, and subsequently result in synergistic cytotoxic effects in NSCLC cells.

In human and rat cells, there are two E2F consensus binding sites in the inverted repeat of the TS promoter [39,40]. Moreover, the transcription of TS is regulated by the transcription factor E2F1 in the S phase of the cell cycle [41]. It has demonstrated that EGFR-TKI gefitinib inhibited the expression of the transcription factor E2F1, thereby decreasing the TS expression and activity, and mediating the synergistic interaction with oral fluorouracil S-1 [42]. The PI3K-AKT signaling activation has been found to accumulate E2F1 [43], and it has shown that E2F is involved in TS regulation [44–46]. However, whether E2F1 was involved in regulating TS expression in salinomycin and erlotinib-exposed NSCLC cells were under our investigation.

In this study, we compared the cytotoxic effect of elrotinib/salinomycin alone or two drug combination on different NSCLC cell lines, including adenocarcinoma (H1975 and H1650), bronchioloalveolar cell carcinoma (A549), and squamous cell carcinoma (H1703). The results showed that the combination of elrotinib with salinomycin synergistically inhibited the cell viability of erlotinib resistant H1975 cells with L858R and T790M mutations of EGFR and also the H1703 and A549 cells with wild-type EGFR. In the Supplementary Fig. 1, this combination treatment also manifested a synergistic cytotoxicity effect on the elrotinib sensitive H1650 cell line contains an EGFR exon 19 deletion mutation. To the best of our knowledge, our work is the first report of new insight into the mechanism of salinomycin in down-regulating the expression of TS to enhance the cytotoxic effect of erlotinib in NSCLC cells. These results may provide for the rational design of future drug regimens incorporating salinomycin and erlotinib for the treatment of NSCLC. Though further *in vivo* study is required to evaluate the effects of salinomycin, erlotinib, and their combination, and the side effects from this regimen, the concepts of erlotinib combined with salinomycin can be considered as a baseline to develop a novel treatment strategy for optimizing response to EGFR-TKIs for

NSCLC.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2017.04.026.

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