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ORIGINAL ARTICLE

Activation of ER stress and inhibition of EGFR N-glycosylation by tunicamycin enhances susceptibility of human non-small cell lung cancer cells to erlotinib

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Abstract
Purpose The epidermal growth factor receptor (EGFR), an N-glycosylated transmembrane protein, is the target of erlotinib, an orally bioavailable agent approved for treatment of patients with non-small cell lung cancer (NSCLC). In this study, we examined whether inhibition of EGFR N-glycosylation and stimulation of endoplasmic reticulum (ER) stress by tunicamycin enhances erlotinib-induced growth inhibition in NSCLC cell lines.
Methods We examined the effects of tunicamycin and erlotinib on cytotoxicity of erlotinib-sensitive and resistant NSCLC cell lines, as well its effects on apoptotic pathways and on EGFR activation and subcellular localization.
Results A minimally cytotoxic concentration of tunicamycin (1 μM) resulted in~2.6–2.9 fold and~6.8–13.5 fold increase in erlotinib-induced antiproliferative effects in sensitive (H322 and H358) and resistant cell lines (A549 and H1650), respectively. We found that tunicamycin generated an aglycosylated form of 130 kDa EGFR. Tunicamycin additionally affected EGFR activation and subcellular localization. Interestingly, the combination of tunicamycin and erlotinib caused more inhibitory effect on EGFR phosphorylation than that of erlotinib alone. Moreover, the combination induced apoptosis in H1650 cells through induction of CHOP expression, activation of caspase-12 and caspase-3, cleavage of PARP and bak, and down-regulation of anti-apoptotic proteins bcl-xL and survivin.
Conclusions Overall, our data demonstrate that tunicamycin significantly enhances the susceptibility of lung cancer cells to erlotinib, particularly sensitizing resistant cell lines to erlotinib, and that such sensitization may be associated with activation of the ER stress pathway and with inhibition of EGFR N-glycosylation.

Keywords Tunicamycin · Erlotinib · EGFR TKI · ER stress · Human NSCLC cells

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Introduction

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein with a cysteine-rich extracellular region and intracellular domain containing an uninterrupted kinase site and multiple autophosphorylation sites clustered at the C-terminal [1]. Upon binding to ligands, the EGFR forms either a homodimer or a heterodimer with other family members, resulting in EGFR autophosphorylation and in recruitment of cascades of signaling molecules to transmit potent mitogenic signals [2, 3]. Increased expression of EGFR family members has been noted in several human malignancies—including cancers of the lung, head and neck, brain, bladder and breast [4, 5]. As increased EGFR expression is associated with poor clinical outcome in patients with NSCLC, agents that are able to interfere with the EGFR pathway have been considered to be of potential therapeutic benefit [6].

Erlotinib (Tarceva™, OSI-774), an orally bioavailable quinazoline derivative, selectively inhibits the EGFR tyrosine kinase by competitively inhibiting the intracellular ATP binding domain and blocking signal transduction pathways implicated in cell proliferation and survival of cancers [7, 8]. Preclinical studies demonstrated erlotinib's

potent activity against tumor cell growth accompanied by suppression of EGFR activation. Erlotinib as a single agent has demonstrated significant clinical benefit even in previously treated patients and has received FDA approval as either second or third line treatment for patients with non-small-cell lung cancer (NSCLC) [9]. Although therapeutic effectiveness has been verified in NSCLC tumors, objective response to erlotinib occurs in only a fraction of NSCLC patients. Efforts to improve the efficacy of this targeted therapy to date have primarily centered on identification of patient subsets most likely to respond to this therapy. While combinations of erlotinib with cytotoxic chemotherapies have not improved clinical outcomes in NSCLC [10, 11], rational, mechanistic combinations with biological therapies may improve the therapeutic efficacy and clinical utility of this agent.

Tunicamycin is a naturally occurring antibiotic which inhibits protein glycosylation by blocking the first step in the biosynthesis of N-linked oligosaccharides in the endoplasmic reticulum (ER) and Golgi [12]. The EGFR is a glycoprotein containing 11 potential N-linked glycosylation sites [13]. Previous reports have demonstrated that inhibition of EGFR glycosylation by tunicamycin alters EGFR conformation and phosphorylation [14]. In addition, tunicamycin induces ER stress by blocking protein glycosylation and further triggers ER-related apoptotic pathways [15]. Co-treatment with tunicamycin was found to increase cisplatin-induced cytotoxicity in human cell lines [16], and recent studies demonstrated that tunicamycin sensitizes human prostate cancer cells to TNF-induced apoptosis by increased expression of DR5 protein [17].

In the present study, we determined whether the interruption of EGFR glycosylation and stimulation of ER stress by tunicamycin enhances the cell response to erlotinib in human NSCLC cell lines. Our results demonstrate that tunicamycin at minimally cytotoxic concentration (1 μM) significantly enhances erlotinib-induced cell growth inhibition in both erlotinib-sensitive and resistant cell lines. Furthermore, we utilized H322 and H1650 cell lines as models to investigate the cellular and molecular basis of the observed synergistic effects of tunicamycin and erlotinib. These findings provide insights into the unique mechanisms of cell growth-inhibitory effects of erlotinib and tunicamycin in human NSCLC cell lines which can be exploited in clinical studies of novel drug combinations with erlotinib.

Materials and methods

Chemicals and antibodies

Erlotinib was obtained from OSI pharmaceuticals (Melville, NY, USA). Erlotinib was dissolved in DMSO at a stock concentration of 10 mM and diluted to the indicated concentrations with medium. Tunicamycin was purchased from Sigma-Aldrich Co. (St Louis, MO, USA), dissolved in DMSO at stock solution of 1 mM, and diluted to the indicated concentrations with medium. The polyclonal antibodies of EGFR, p-EGFR, caspase-3, PARP, bid, and monoclonal antibody of survivin (6E4) were purchased from Cell Signaling (Beverly, MA); the polyclonal antibody of CHOP (GADD153, F-168, SC-575), monoclonal antibodies of bax (B-9, sc-7480), bcl-xL (H-5, SC-8392) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal antibodies of bak (Ab-1) and caspase-12 (Ab-1) were obtained from Calbiochem (La Jolla, CA, USA). Other chemicals were purchased from Sigma-Aldrich Co.

Cell Lines

Human NSCLC H322, H358, A549 and H1650 cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained in 75 cm^2 flask in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 Units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

Assay of cell growth inhibition

Cells (1×10^5/ml) were plated in 96-well sterile plastic plates and were allowed to attach overnight. Cells were exposed to varying concentrations of tunicamycin alone or to varying concentrations of erlotinib with or without 1 μM tunicamycin at 37°C for 72 h. Following exposure, the cell survival fraction was measured by colorimetric assay based on the reduction of 3-(4,5-dimethylthioiazol-2-yl)-2,5-diphnyltetrazolium bromide (MTT).

Apoptosis assay

Cells were exposed to 1 μM erlotinib, 1 μM tunicamycin, the combination of both agents, or to the same volume of medium containing 0.1% DMSO as a control. Following exposure, cell morphological changes were observed with a Nikon phase-contrast microscope. To assay sub-G0/G1 cells, cells (0.5×10^6 cells/well) were grown in 6-well plates and exposed to 1 μM erlotinib, 1 μM tunicamycin, the combination of both agents, or to the same volume of medium containing 0.1% DMSO as described above. After exposure, cells were harvested and fixed with 75% cold ethanol at 4°C overnight, and then incubated at room temperature for 3 h with 1 μg/ml propidium iodide and 5 μg/ml RNase I (Roche Molecular Biochemicals, Indianapolis, IN). The numbers of cells at apoptosis (sub-G0/G1) were

measured by a Becton Dickinson FACScan flow cytometer (BD Biosciences, San Jose, CA, USA).

Immunoblot analysis

Following exposure, cells were scraped from the culture, washed twice with cold PBS solution, and then suspended in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMFS, 1 mM DTT, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 1% Triton X-100, and 1% SDS at 0–4°C for 15 min. After centrifugation at 15,000*g* for 10 min at 0°C, the supernatants were collected and protein concentration of cell lysate was measured with a Bio-Rad protein DC assay kit (Bio-Rad, Hercules, CA, USA). In some experiments, cells were separated into Triton X-100 soluble and insoluble fractions as described by Lampugnami et al. [18]. An equal amount of cell lysate (30 μg of protein) was subjected to electrophoresis on either a 12 or 15% SDS-polyacrylamide gel. Following electrophoresis, protein blots were transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in TBST solution and incubated at 4°C overnight with the corresponding primary antibodies in the blocking solution. After washing three times with TBST solution, the membrane was incubated at room temperature for 1 h with horseradish peroxidase-conjugated secondary antibody diluted with TBST solution (1:1,000). The detected protein signals were visualized by an enhancement chemiluminescence reaction system as recommended by the manufacturer (Amersham, Arlington Heights, IL).

Immunofluorescence staining

Cells were plated on glass coverslips in 6-well plates and exposed to 1 μM erlotinib, 1 μM tunicamycin, to the combination of both agents, or to the same volume of medium containing 0.1% DMSO as a control for 24 h. After exposure, cells were washed twice with cold PBS solution, fixed with 4% paraformaldehyde in PBS solution at room temperature for 15 min, and then treated with 1% Triton X-100 in PBS solution for 10 min. After blocking with 5% bovine serum albumin in PBS solution for 30 min, cells were incubated with polyclonal anti-EGFR antibodies (1:400) at room temperature for 1 h. After washing three times with PBS solution, cells were incubated with fluorescence FITC-conjugated secondary antibodies (1:500) for 30 min in a dark room. The immunofluorescence signals were examined with a Nikon fuorescence microscope.

Statistical analysis

Data are presented as mean ± SD of the number of experiments indicated. The comparisons were made with a *t* test and the difference was considered to be statistically significant if the *P* value was <0.05.

Results

Tunicamycin enhances erlotinib-induced cell growth inhibition in human NSCLC cell lines

In previous work, we demonstrated that human NSCLC cell lines H322 and H358 are sensitive to erlotinib, but A549 and H1650 cell lines are resistant to erlotinib [19]. In this work, we employed these erlotinib-sensitive and resistant cell lines as models to determine whether tunicamycin enhances erlotinib-induced cell growth inhibition. We first determined the sensitivities of these cell lines to tunicamycin as a single agent. As shown in Fig. 1, exposure to tunicamycin at various concentrations ranging from 0.125 μM to 8 μM for 72 h results in a concentration-dependent inhibition of cell growth in all tested cell lines. For example, tunicamycin at 1 μM resulted in 18–25% cell growth inhibition, and high concentration (8 μM) induced 45–56% cell growth inhibition. Next, we utilized a minimally cytotoxic concentration of tunicamycin (1 μM) to determine its effect on erlotinib-induced cell growth inhibition. All tested cells were plated in a 96-well plate and exposed to various concentrations of erlotinib alone or plus 1 μM tunicamycin at 37°C for 72 h. Following exposure, cell survival fractions were assessed by MTT assay and evaluated in comparison to control cell survival. The results presented in Fig. 2 illustrate that erlotinib as a single agent caused cell growth inhibition in H322 and H358 cells with IC_{50} of 0.83 and 1.63 μM, respectively; A549 and H1650 cells were more resistant, with IC_{50} of 12.3 and 8.5 μM, respectively. Interestingly, the combination with 1 μM tunicamycin resulted in a significant increase in erlotinib-induced cell growth inhibition, i.e. growth-inhibitory effects of erlotinib increased by 2.6–2.9 fold in H322 and H358 cells and 6.8–13.5 fold in A549 and H1650 cells, respectively. The combination reduced IC_{50} values for erlotinib from 12.3 ± 1.7 to 1.82 ± 0.75 μM in A549 cells, and the IC_{50} value from 8.5 ± 2.7 to 0.63 ± 0.09 μM in H1650 cells. All reduced IC_{50} values (<2 μM) are close to clinically achievable concentrations of erlotinib [20]. All data suggest that tunicamycin strongly enhances NSCLC cell susceptibility to erlotinib.

Effects of tunicamycin and erlotinib on the expression and activation of EGFR in human NSCLC cells

Given that tunicamycin inhibits EGFR glycosylation and activation in A431 cells [15], and that erlotinib-induced cell growth inhibition is associated with suppression of EGFR

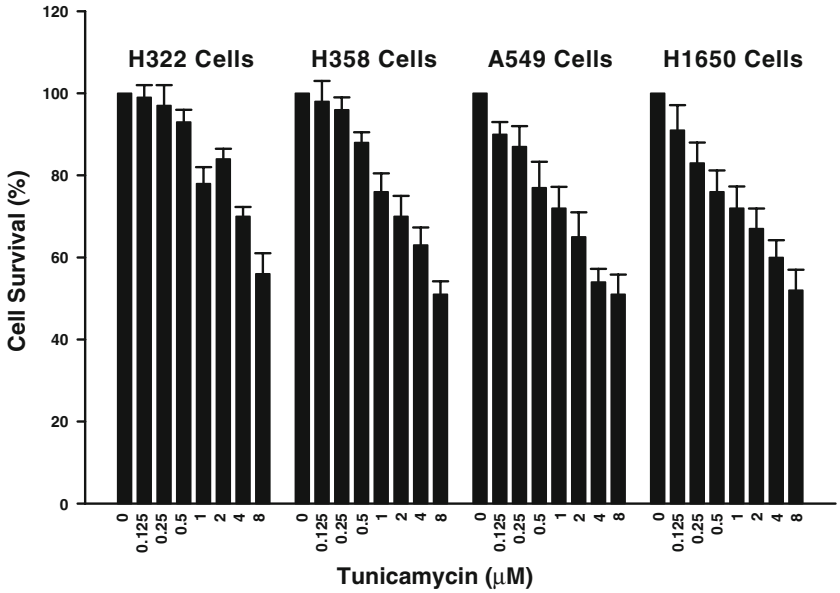

Fig. 1 Tunicamycin induces cytotoxicity in human non-small cell lung cancer cell lines. All tested cells were plated in a 96-well plate and exposed to varying concentrations of tunicamycin for 72 h. After exposure, cell survival fractions were measured by MTT assay as described in "Materials and methods". Each column represents the mean ± SD of three independent experiments

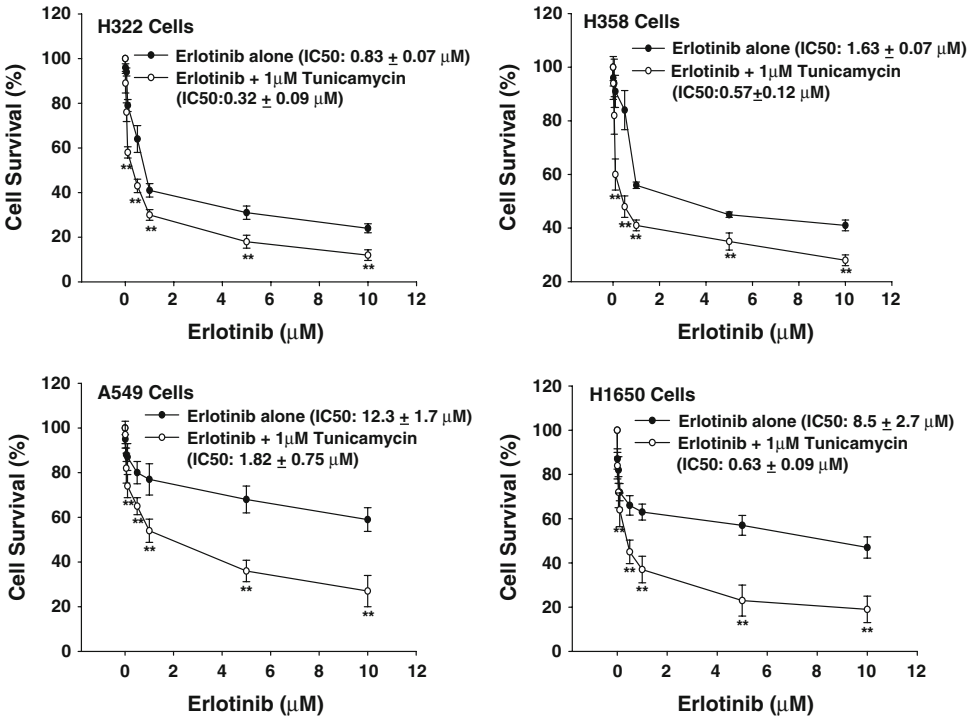

Fig. 2 Tunicamycin enhances erlotinib-induced cell growth inhibition in human non-small cell lung cancer cell lines. All tested cells were plated in a 96-well plate and exposed to various concentrations of erlotinib alone or plus 1 μM tunicamycin at 37°C for 72 h. Following exposure, cell survival fractions were measured by MTT assay and evaluated in comparison with control cell survival as 100%. Each point represents the mean ± SD of three independent experiments. $^{**}P < 0.01$

activation [7, 8], we determined whether tunicamycin enhanced cell growth inhibition by erlotinib could be associated with interruption of EGFR expression and phosphorylation. As shown in Fig. 3, 1 μM erlotinib treatment for 24 h results in suppression of EGFR phosphorylation but does not affect the expression of EGFR in H322 and H1650 cells. However, exposure of H322 and H1650 cells to 1 μM tunicamycin alone or to the combination of both agents results in accumulation of a small molecule EGFR (130 kDa) with faster electrophoresis mobility accompanied by decreased amounts of full size EGFR (170 kDa). Interestingly, the amounts of phosphorylated full size EGFR were markedly decreased in tunicamycin-treated H322 and H1650 cells, and phosphorylation of small molecule EGFR was not much different as compared with controls. Importantly, the inhibitory extent of EGFR phosphorylation by the combination treatment was significantly higher than that for erlotinib or tunicamycin alone in H322 and H1650 cells, suggesting that the enhanced cell growth inhibition by the combination may be at least in part associated with the interruption of EGFR glycosylation and phosphorylation.

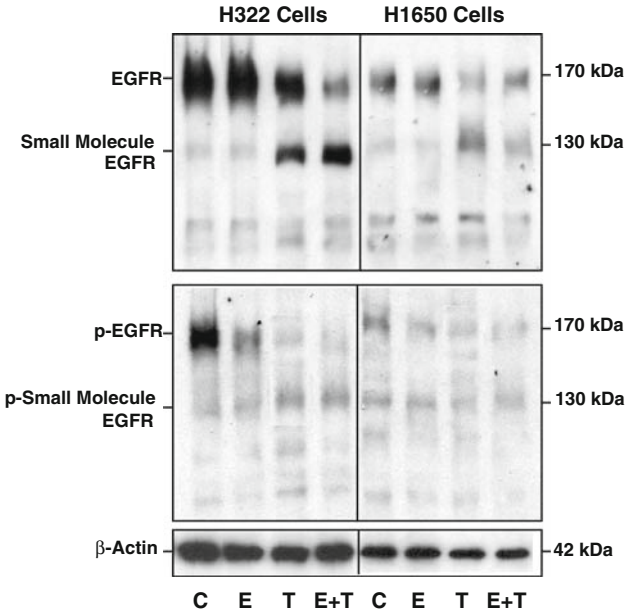

Fig. 3 Effects of erlotinib, tunicamycin, and the combination of both agents on EGFR expression and phosphorylation in H322 and H1650 cells. Cells were exposed to 1 μM erlotinib (E), 1 μM tunicamycin (T), to the combination of erlotinib and tunicamycin (E + T), or to the same volume of medium containing 0.1% DMSO as a control (C). After exposure for 24 h, cells were harvested and suspended in lysis buffer for preparation of cell lysates. Equal amounts of cell lysate (30 μg of protein) were subjected to a 7.5% SDS-polyacrylamide gel. After electrophoresis, protein blots were transferred to a nitrocellulose membrane. The levels of total EGFR and p-EGFR were detected by immunoblot analysis using corresponding antibodies. β-actin was used as sample loading control

Effects of the combination of tunicamycin and erlotinib on subcellular localization of EGFR

Reports have demonstrated that tunicamycin treatment leads to interference with EGFR maturation and its transportation from the ER to the cell membrane [21]. We therefore determined the effects of tunicamycin and the combination of erlotinib and tunicamycin on EGFR subcellular localization. We first employed immunocytochemical techniques to detect alteration in subcellular localization of EGFR in H1650 cells following 24 h of drug treatment. As shown in Fig. 4a, EGFR in control and erlotinib treated cells localized predominantly around the cell membrane; treatment with tunicamycin or the combination led to EGFR localization in the cytoplasm. Next, we separated the cells into detergent-soluble and insoluble fractions and found that the while full sized EGFR in control and erlotinib-treated cells was mainly detected in the Triton X-100 insoluble fraction, the small molecule EGFR was predominantly detected in the Triton X-100 soluble fraction in tunicamycin and combination-treated cells (Fig. 4b). The results suggest that treatment with tunicamycin and with both agents may block EGFR maturation and its transportation from the ER to the cell membrane.

Effects of the combination of tunicamycin and erlotinib on apoptosis

To determine whether the observed tunicamycin enhanced cytotoxicity of erlotinib could be associated with apoptosis, we treated H1650 cells with either single agent or the combination of both compounds for 48 h as described above and then detected cell morphological changes with a phase-contrast microscope. As shown in Fig. 5a, the combination treatment induced cell death with apoptotic features, including the generation of shrunken cells, nuclear condensation, and apoptotic bodies, whereas tunicamycin or erlotinib alone had lesser extents of cell death. Quantitative analysis of apoptotic cells (sub-G0/G1) measured by flow cytometric analysis revealed that combination treatment led to a significant increase in apoptotic death (68%) as compared to either tunicamycin (~23%) or erlotinib (~11%) in H1650 cells (Fig. 5b).

Given evidence that blocking protein glycosylation by tunicamycin can trigger ER stress-related apoptotic signal pathways to induce cell death [22], we explored the role of this pathway in the induction of apoptosis by the combination of tunicamycin and erlotinib. We treated H1650 cells with tunicamycin or erlotinib alone or in combination for 24 and 48 h as described above and then prepared cell extracts for determination of ER stress related signal molecules. As induction of C/EBP homologous protein (CHOP) and activation of caspase-12 have been shown to be involved in ER stress-mediated signaling [23], we examined whether the combination of erlotinib and tunicamycin could induce the expression of CHOP and activation of caspase-12. Western blot analysis revealed that expression of CHOP in cells treated with erlotinib or medium containing 0.1% DMSO was barely detectable at 24 h and seen to an even lesser extent at 48 h. However, CHOP protein was markedly induced in cells treated with either tunicamycin alone or in combination with erlotinib at both 24 and 48 h. The amounts of procaspase-12 (52 kDa) were strongly decreased in a time dependent manner in cells following treatment with either tunicamycin alone or the combination, but did not change in cells treated with either erlotinib or with 0.1% DMSO, suggesting that treatment with tunicamycin or the combination led to the cleavage of procaspase-12 to form the active cleaved caspase-12. Furthermore, we found that the combination treatment led to generation of the active form of cleaved caspase-3 (32, 19, and 17 kDa) and increased amounts of cleaved PARP protein (89 kDa), the hallmark of apoptosis, as compared to either erlotinib or tunicamycin alone (Fig. 5c). Taken together, the data suggest that the synergistic effect of

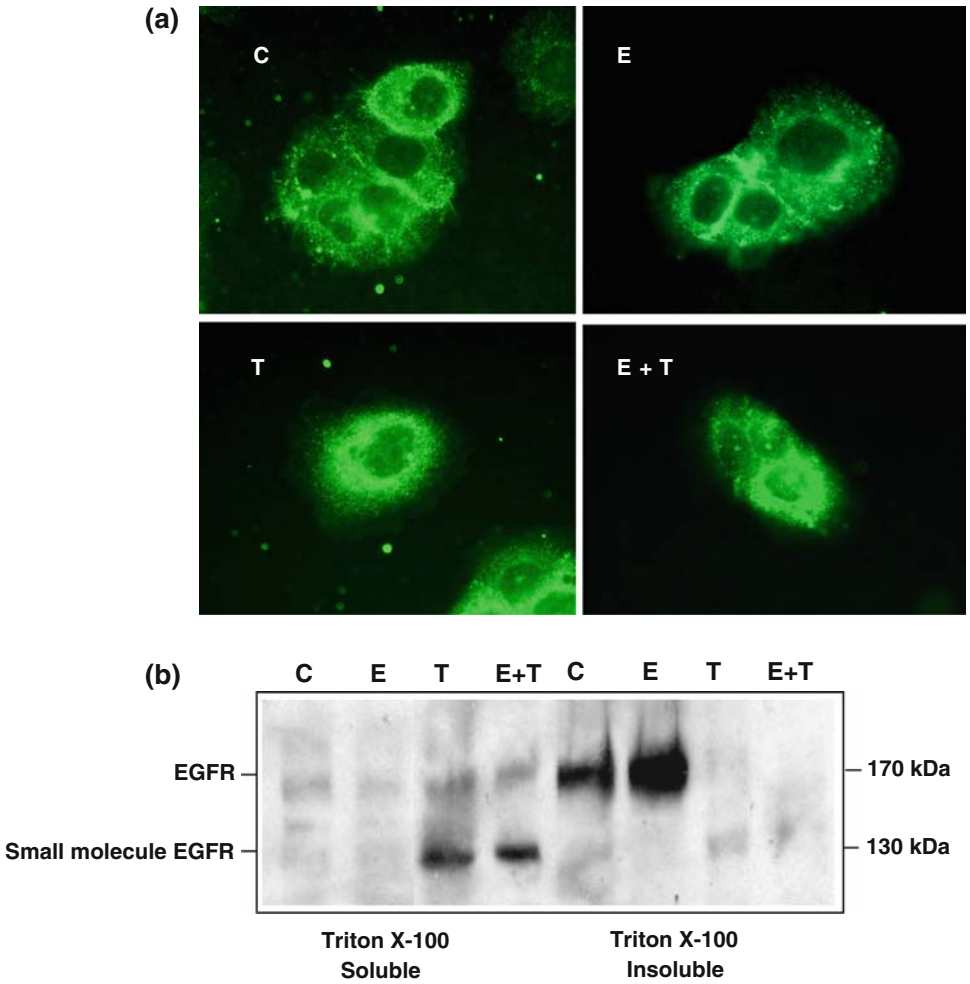

Fig. 4 Effects of erlotinib, tunicamycin, and the combination of both agents on EGFR subcellular localization in H1650 cells. **a** Cells were plated on glass coverslips and treated with 1 μM erlotinib (E), 1 μM tunicamycin (T), the combination of both agents (E + T), or with the same volume of medium containing 0.1% DMSO as a control (C). After a 24 h of treatment, cells were washed with cold PBS solution and fixed with 4% paraformaldehyde. The subcellular localization of EGFR was detected by immunofluorescence microscopy. **b** H1650 cells were plated on a 6-well plate and treated with erlotinib (E), tunicamycin (T), the combination of both agents (E + T), or with the same volume of medium containing 0.1% DMSO as a control (C) as described above. After treatment, cells were harvested and Triton X-100 soluble and insoluble fractions were prepared as described in "Materials and methods". The levels of EGFR in Triton X-100 soluble and insoluble fractions were analyzed by immunoblots

tunicamycin on erlotinib-induced cytotoxicity may at least in part be associated with the activation of ER stress-related apoptotic pathways.

Effects of the combination of tunicamycin and erlotinib on expression of apoptotic components

It has been known that altered expression of a number of apoptosis-related components, including bcl-2 family proteins, play a critical role in regulating apoptotic signals [24]. The western blot analysis presented in Fig. 6 reveals that the combination treatment leads to down-regulation of anti-apoptosis proteins including bcl-xL and survivin in a time-dependent manner, but failed to change the expression of bcl-2, bax, and XIAP proteins. As shown in Fig. 6b and c, combination treatment with tunicamycin and erlotinib for 48 h resulted in nearly 80% decrease in bcl-xL expression and approximately 90% decrease in survivin protein expression as compared with controls. Moreover, it was found that the combination treatment for 48 h caused cleavage of bid protein in H1650 cells, suggesting activation of the caspase-8 related pathway. Interestingly, we found that treatment with tunicamycin or the combination led to the cleavage of pro-apoptotic bak protein to form a 28 kDa small fragment over the experimental time. To our knowledge, this is the first demonstration of bak protein cleavage by tunicamycin alone or in combination with erlotinib. The molecular and biochemical significance of bak cleavage for apoptosis remains to be elucidated.

Discussion

Erlotinib, a quinazoline derivative, is a specific inhibitor of the EGFR tyrosine kinase. Preclinical studies have shown that erlotinib effectively inhibits human NSCLC cell growth, and clinical trials have shown that it is safe and effective against human NSCLC, resulting in FDA approval. However, the clinical objective response rate by in several trials was approximately 15%, merely a fraction of NSCLC patients. Ongoing research has centered on identifying patient subsets who most benefit from erlotinib treatment. The known clinical and molecular predictors of clinical response to erlotinib include never smokers, asian

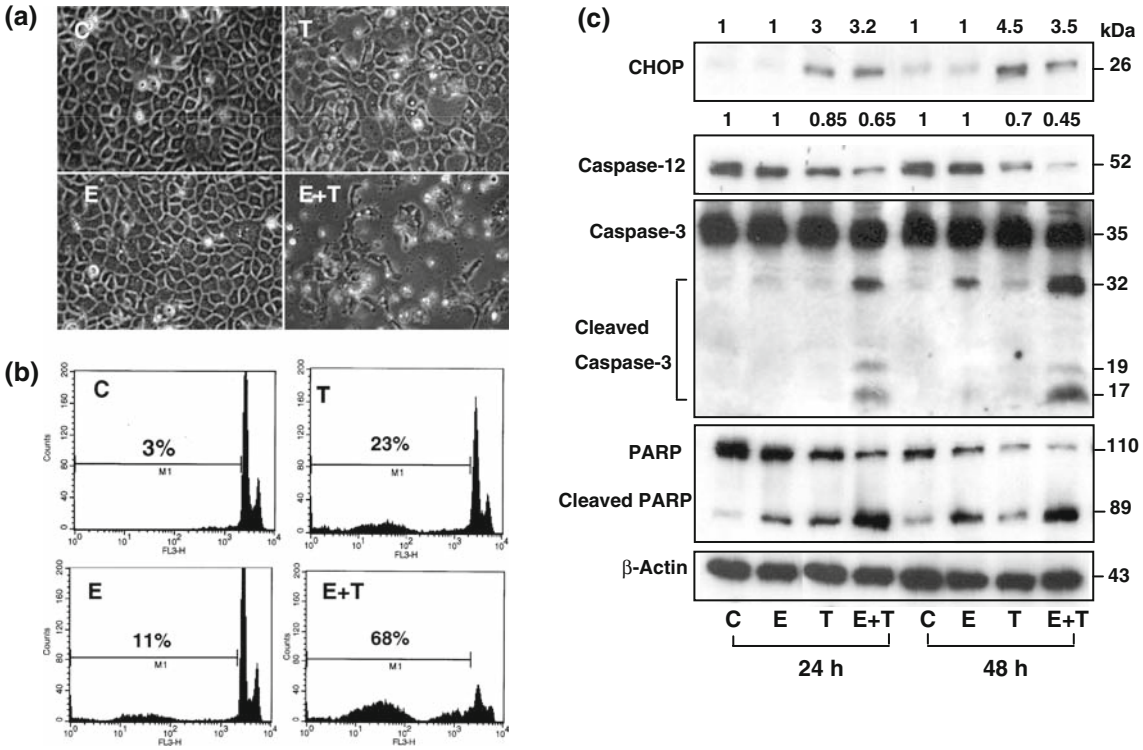

Fig. 5 Effects of erlotinib, tunicamycin, and the combination of both agents on apoptosis in H1650 cells. **a** Cells were treated with 1 μM erlotinib (E), 1 μM tunicamycin (T), the combination of both agents (E + T), or with the same of medium containing 0.1% DMSO as a control (C). The morphological changes in cells following 48 h of treatment were observed with a Nikon phase-contrast microscope. **b** Cells were treated as described above, and the apoptotic cells (sub-G0/G1) were measured by flow cytometric analysis after staining with propidium iodide as described in "Materials and methods". **c** Cells were treated with erlotinib (E), tunicamycin (T), the combination of both agents (E + T), or with medium containing 0.1% DMSO as a control (C) for 24 and 48 h. The expression of CHOP and cleavage of procaspase-12, caspase-3, and PARP protein were detected by immunoblot analysis using corresponding antibodies. β-actin was used as a sample loading control. The values on the top of CHOP and caspase-12 represent the relative densities normalized to β-actin

origin, adenocarcinomas, development of skin rash during therapy, and tumors harboring activating mutations in the EGFR tyrosine kinase domain [25]. Although second-generation EGFR tyrosine kinase inhibitors are in clinical development with the hope of circumventing primary and acquired resistance to erlotinib [26], additional efforts are needed to improve the range of patients who can benefit from erlotinib therapy.

In search for a new approach, we have evaluated the combinative effects of tunicamycin and erlotinib in a panel of human NSCLC cell lines and found that tunicamycin is a potent enhancer of erlotinib-induced cell growth inhibition. In this communication, we have shown that cotreatment with a minimally cytotoxic concentration of tunicamycin significantly enhances NSCLC cell sensitivity to erlotinib. Of particular interest is the observation that tunicamycin sensitizes resistant cell lines to erlotinib. These results raise the intriguing possibility that such a combination may benefit patients whose tumors are resistant or refractory to erlotinib.

Possible mechanisms behind tunicamycin-induced sensitization of erlotinib-resistant cell lines are under investigation. A number of reports have shown that NSCLC cell susceptibility to EGFR TK inhibitors is strongly correlated to presence of somatic mutations of the EGFR gene [27]. Controversially, our results show that NSCLC cells with EGFR gene mutations may not always correlate with sensitivity to erlotinib. In this study, H1650 cells which harbor DelE746-A750 were not sensitive to erlotinib, whereas another report demonstrated sensitivity to gefitinib [28]. Interestingly, we found that the combination with tunicamycin increased sensitization of H1650 cells to erlotinib by 13.5-fold, suggesting that the EGFR gene mutation could contribute to tunicamycin-induced cell sensitization. To address this possibility, we treated H3255 cells harboring the identical EGFR gene mutation with tunicamycin and erlotinib and found that the combination with tunicamycin produced only a 1.2-fold increase in erlotinib-induced cell growth inhibition, indicating that the EGFR gene mutation might not be involved in such a synergistic mechanism (data not shown).

It has been known that N-glycosylation, but not O-glycosylation, is a characteristic of the EGFR protein [29]. Oligosaccharides of EGFR contribute about 40 kDa of the

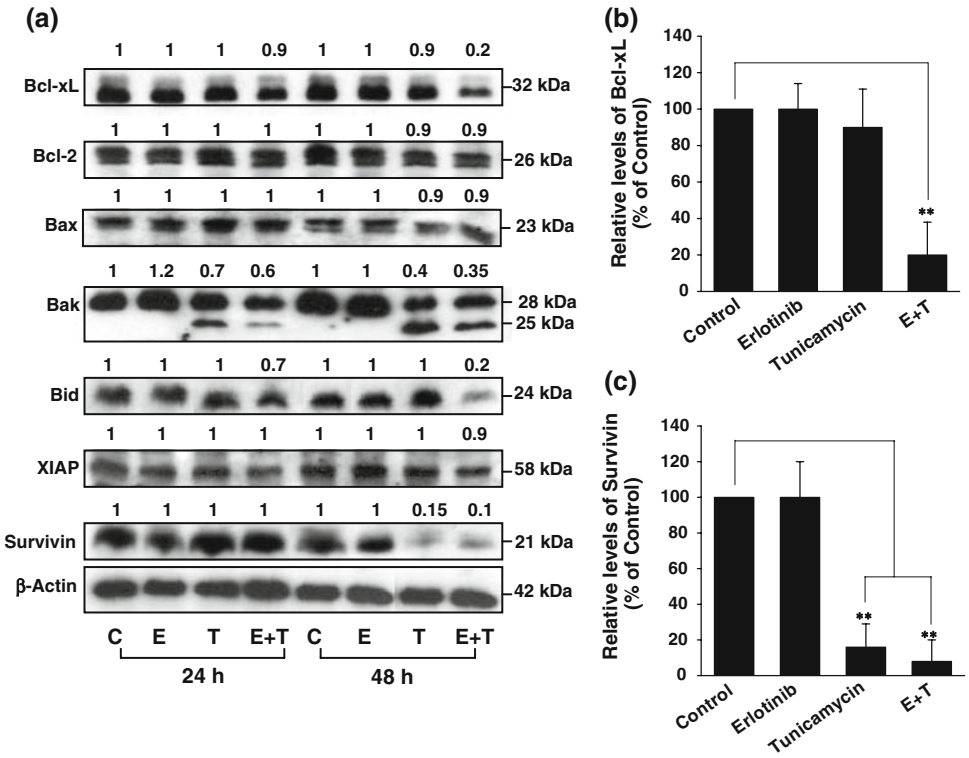

Fig. 6 Effects of erlotinib, tunicamycin, and the combination of both agents on the expression of apoptotic components in H1650 cells. **a** Cells were treated with 1 μM erlotinib (E), 1 μM tunicamycin (T), the combination of both agents (E + T) or with the same volume of medium containing 0.1% DMSO as a control (C). After treatment for the indicated time point, cells were harvested and cell lysates were prepared for immunoblot analysis. The levels of apoptotic components were detected by immunoblot analysis using corresponding antibodies. β-actin was used as a sample loading control. The values on top of bands represent the relative densities normalized to β-actin. **b** and **c** The effect of combination treatment with tunicamycin and erlotinib on expression of Bcl-xL and survivin in H1650 cells. Cells were treated with erlotinib or tunicamycin or with combination of both agents for 48 h as described above. Cells were harvested and cell extracts were prepared for determination of expression of bcl-xL and survivin by immunoblots. The relative levels of bcl-xL and survivin were evaluated in comparison with that in the control. Data represent the mean ± SD of three independent experiments, $^{**}P < 0.01$

170 kDa mature protein [30, 31]. Indeed, our results showed that N-glycosylation of EGFR was blocked by tunicamycin and resulted in a smaller molecule of EGFR in H322 and H1650 cells. Although the functions of EGFR glycosylation are not fully elucidated, some investigations have shown that EGFR glycosylation may be related to its conformation, binding to ligands, and tyrosine kinase activity [14, 21]. In this study, we found that tunicamycin treatment caused a significant accumulation of small size (130 kDa) EGFR and an associated decrease in full size (170 kDa) EGFR expression by blocking glycosylation. Additionally, tunicamycin treatment resulted in a remarkable suppression of EGFR phosphorylation, suggesting that blockage of EGFR glycosylation by tunicamycin could affect EGFR autophosphorylation and activation. Moreover, the preliminary results showed that tunicamycin alone led to a marked reduction of EGF-stimulated phosphorylation of EGFR and the combination of tunicamycin and erlotinib caused an almost abolishment of EGF-induced EGFR phosphorylation (data not shown), suggesting that the aglycosylated form of EGFR induced by tunicamycin may lose its ability to bind to ligands and further interrupt its related signal pathway. In addition, our results from immunofluorescence microscopy and cell fraction studies showed that tunicamycin treatment caused EGFR localization to the cytoplasm, suggesting that the inhibition of glycosylation resulted in blockade of EGFR maturation and its translocation from the ER to cell membrane. Interestingly, the combination of tunicamycin and erlotinib produced more inhibition of EGFR phosphorylation than erlotinib or tunicamycin alone (Fig. 3). These results suggest that disruption of EGFR glycosylation by tunicamycin could synergistically enhance erlotinib-induced suppression of EGFR phosphorylation.

Tunicamycin induces ER stress-mediated apoptosis by blocking protein glycosylation in the ER [12, 15]. Although the ER stress-mediated apoptotic pathway is not fully understood, two specific hallmarks of this process have been identified. The induction of pro-apoptotic transcription factor CHOP and activation of ER-localized caspase-12 are thought to be involved in ER stress-mediated apoptosis [23]. Our results show that treatment with

tunicamycin alone or in combination with erlotinib resulted in induction of CHOP expression and cleavage of procaspase-12 in H1650 cells. Tunicamcyin alone only produced 23% apoptotic cells compared with 68% in combination treatment, suggesting that the other apoptotic factors could be involved in cell death resulting from the combination. For instance, we found the combination treatment led to more activation of caspase-3 and cleavage of PARP protein. Aside from activation of apoptotic pathways, ER stress is known to induce a cell survival response [32]. Our preliminary results showed that exposure to tunicamycin alone actually resulted in activation of AKT and ERK1/2 in H1650 cells. However, co-treatment with erlotinib resulted in successful inhibition of AKT and ERK1/2 phosphorylation, suggesting that inhibition of survival pathways by erlotinib may be involved in the enhancement of tunicamycin-induced cell death (data not shown). In addition, Oda et al. recently reported that the induction of GADD153/CHOP protein and cleavage of caspase-4 by activation of calpain may play the crucial roles in tunicamycin-induced ER stress and apoptosis in SK-N-SH and SH-SY5Y cells [33], and we found that erlotinib-induced apoptosis was mediated by activation of mitochondrial pathways through the activation of BAX and BAK proteins in human non-small-cell lung cancer cells [34]. The results suggest that activation of multiple apoptotic pathways is involved in the observed enhancement of cytotoxic effects by the combination of erlotinib and tunicamycin.

Our results raise the possibility that combinations of erlotinib with tunicamycin or other N-glycosylation inhibitors may be a promising approach for improving the clinical activity of erlotinib. Of note, inhibitors of the HMG-CoA reductase enzyme (statins) have demonstrated synergistic effects in preclinical combination with EGFR tyrosine kinase inhibitors [35], and such combinations are being examined in current clinical trials; these molecules have known inhibitory effects on tyrosine kinase receptor signaling via inhibition of both receptor N-glycosylation as well as effects on Ras prenylation [36]. Our observations provide mechanistic support for the clinical development of combinations of nontoxic inhibitors of N-glycosylation with erlotinib.

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