Tamoxifen enhances erlotinib-induced cytotoxicity through down-regulating AKT-mediated thymidine phosphorylase expression in human non-small-cell lung cancer cells

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**A B S T R A C T**

Tamoxifen is a triphenylethylene nonsteroidal estrogen receptor (ER) antagonist used worldwide as an adjuvant hormone therapeutic agent in the treatment of breast cancer. However, the molecular mechanism of tamoxifen-induced cytotoxicity in non-small cell lung cancer (NSCLC) cells has not been identified. Thymidine phosphorylase (TP) is an enzyme of the pyrimidine salvage pathway which is upregulated in cancers. In this study, tamoxifen treatment inhibited cell survival in two NSCLC cells, H520 and H1975. Treatment with tamoxifen decreased TP mRNA and protein levels through AKT inactivation. Furthermore, expression of constitutively active AKT (AKT-CA) vectors significantly rescued the decreased TP protein and mRNA levels in tamoxifen-treated NSCLC cells. In contrast, combination treatment with PI3K inhibitors (LY294002 or wortmannin) and tamoxifen further decreased the TP expression and cell viability of NSCLC cells. Knocking down TP expression by transfection with small interfering RNA of TP enhanced the cytotoxicity and cell growth inhibition of tamoxifen. Erlotinib (Tarceva, OSI-774), an orally available small molecular inhibitor of epidermal growth factor receptor (EGFR) tyrosine kinase, is approved for clinical treatment of NSCLC. Compared to a single agent alone, tamoxifen combined with erlotinib resulted in cytotoxicity and cell growth inhibition synergistically in NSCLC cells, accompanied with reduced activation of phospho-AKT and phospho-ERK1/2, and reduced TP protein levels. These findings may have implications for the rational design of future drug regimens incorporating tamoxifen and erlotinib for the treatment of NSCLC.

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**1. Introduction**

Lung cancer is the leading cancer death in the world, and non-small–cell lung cancer (NSCLC) is the most frequently seen type of lung cancer, accounting for approximately 85% of all cases [1,2]. Epidermal growth factor receptor (EGFR), also known as HER-1 and erbB-1, is overexpressed in NSCLC [3] and activation of the EGFR signaling pathway in cancer cells can enhance apoptosis, cell proliferation, angiogenesis, and metastasis, leading to a poor disease prognosis [4,5]. Efforts to develop novel target therapeutic strategies for lung cancer have led to the approval of erlotinib, a small molecular EGFR tyrosine kinase inhibitor (TKI), to prolong survival in patients with advanced NSCLC after first-line and second-line chemotherapy [6]. EGFR-TKIs can block
the Ras-Raf-MKK-ERK and lipid kinase phosphatidyl inositol 3-kinase (PI3K)-AKT pathways [7], which have been implicated in the inhibition of cell apoptosis and the promotion of cell growth and motility [8,9].

Tamoxifen, an estrogen receptor (ER) antagonist, is widely used as an adjuvant chemotherapeutic agent in the treatment of breast cancer [10,11]. Since the first clinical trial of tamoxifen for breast cancer treatment, it has been found to induce apoptosis in colorectal, ovarian, prostate, head and neck cancer cells [12–15]. Moreover, tamoxifen has shown clinical benefit as a chemotherapeutic agent in the treatment of ER-negative glioma tumors [16]. However, few studies have explored the potential effect of tamoxifen on NSCLC.

Thymidine phosphatase (TP) is identical to platelet-derived endothelial cell growth factor, and catalyzes the reversible conversion of thymidine to thymine and 2-deoxy-β-ribose-1-phosphate [17,18]. Immunohistochemical studies have shown that TP expression in various kinds of tumors is higher than that in the adjacent non-neoplastic tissues, and TP is found to protect cells from apoptosis induced by cisplatin, Fas, and microtubule-interfering agents [19–25]. Elevated TP levels in cancer cells are related to a poor prognosis for patients [26–29]. A recent study demonstrated that the combination of TP inhibitor and irradiation is effective in colon cancer [30]. However, whether TP is involved in tamoxifen or erlotinib-induced cytotoxicity in NSCLC cells is still unclear.

In this study, we wanted to explore the molecular mechanism of tamoxifen in downregulating TP expression to enhance the cytotoxic effect of erlotinib in human lung cancer cells. These results may provide a rationale to combine erlotinib with anti-estrogen therapy for lung cancer treatment, especially in women with lung adenocarcinoma and a sensitive mutation of EGFR.

2. Methods

2.1. Chemicals

Tamoxifen, actinomycin D, and cycloheximide were purchased from Sigma Chemical (St. Louis, MO, USA). Erlotinib was purchased from Genentech (South San Francisco, CA, USA), LY294002, wortmannin, and U0126 were purchased from Calbiochem–Novabiochem (San Diego, CA, USA).

2.2. Cell culture

Human lung carcinoma cells H520 and H1975 were obtained from the American Type Culture Collection (Manassas, VA, USA) and the cells were cultured at 37 °C in a humidified atmosphere containing 5% CO2 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.

2.3. Western blot analysis

After different treatments, equal amounts of proteins from each set of experiments were subjected to Western blot analysis as previously described [31]. The specific phospho-AKT (Ser473) and phospho-ERK1/2 (Thr180/Tyr182) antibodies were purchased from Cell Signaling (Beverly, MA, USA). Rabbit polyclonal antibodies against PD-ECGF (PGF-44C) (sc-47702), AKT (H-136) (sc-8312), ERK2 (C-14) (sc-154), HA (F-7) (sc-7392), and actin (1-19) (sc-1616) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.4. Transfection with AKT-CA, MKK1/2-CA vectors, small interfering RNA

Exponentially growing human lung cancer cells (10^6) were plated for 18 h, and then constitutively active AKT expression plasmid (AKT-CA), which harbored a consensus myristylation domain that replaced the 4–129 amino acids of wild-type AKT, and MKK1/2-CA (a constitutively active form of MKK1/2) expression vectors were transfected into H520 or H1975 cells using Lipofectamine (Invitrogen) [32]. The sense-strand sequences of siRNA duplexes were as follows: TP: 5′-AUA GAC UCC AGA UUA UCC A-3′, AKT: 5′-UGC AGC AUC GCU UCU UUG CCG GUU U-3′, and scrambled (as a control): 5′-GGC CGC UUU GUA GGA TTC G-3′ (Dharmacon Research, Lafayette, CO). Cells were transfected with siRNA duplexes (200 nM) by using Lipofectamine 2000 (Invitrogen) for 24 h.

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

PCRs were performed using an ABI Prism 7900HT according to the manufacturer’s instructions. Amplification of specific PCRs products was performed using the SYBR Green PCR Master Mix (Applied Biosystems). For each sample, the data were normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (gapdh). The designed primers in this study were: TP forward primer, 5′-AGCTGGAGCTATCATTCCTGATT-3′; TP reverse primer, 5′-GGCTGATATAGGATCCGTC-3′; gapdh forward primer, 5′-CATGAGAATGATGACACGT-3′; gapdh reverse primer, 5′-AGTCCTTCCAGATACCAAAGT-3′. Analysis was performed using the comparative Ct value method. For each sample, the data were normalized to the housekeeping gene gapdh.

2.6. Reverse transcription-PCR (RT-PCR)

RNA was isolated from cultured cells using TRIzol (Invitrogen) according to the manufacturer’s instructions. RT-PCR was performed with 2 μg of total RNA using random hexamers following the Moloney murine leukemia virus reverse transcriptase cDNA synthesis system (Invitrogen). The final cDNA was used for subsequent PCRs [32].

2.7. 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).

2.8. Combination index analysis

The cytotoxicity induced by the combined treatment with tamoxifen and/or erlotinib was compared with the cytotoxicity induced by each drug using the combination index (CI), where CI < 0.9, CI = 0.9–1.1, and CI > 1.1 indicate synergistic, additive, and antagonistic effects, respectively. The combination index analysis was performed using CalcuSyn software (Biosoft, Oxford, UK). The mean of CI values at a fraction affected (FA) of 0.90, 0.75, 0.50 were averaged for each experiment, and the values were used to calculate the mean between the three independent experiments.
2.9. Colony-forming ability assay

Immediately after drug treatment, the cells were washed with phosphate-buffered saline and trypsinized to determine the cell numbers. The cells were plated at a density of 500–1000 cells on a 60 mm-diameter Petri dish in triplicate for each treatment and cultured for 12–14 days. The cell colonies were stained with 1% crystal violet solution in 30% ethanol. Cytotoxicity was determined by the number of colonies in the treated cells divided by the number of colonies in the untreated control.

2.10. Trypan blue dye exclusion assay

Cells were treated with tamoxifen and/or erlotinib for 24 h. Trypan blue dye can be excluded from living cells, but is able to penetrate dead cells. The proportion of dead cells was determined by hemocytometer, counting the number of cells stained with trypan blue.

2.11. 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 by unpaired t-test. p < 0.05 was considered statistically significant.

3. Results

3.1. Tamoxifen inhibited cell survival of NSCLC cells

To investigate if tamoxifen had any growth inhibitory activity against human NSCLC cells, cells were treated with 5, 10, 20, 30, and 40 μM of tamoxifen for 24, 48, and 72 h. The cell proliferation rate and cell viability were assessed by MTS and trypan blue exclusion assays. In Fig. 1A and B, tamoxifen induced a concentration- and time-dependent reduction in cell viability and proliferation in H520 and H1975 cells. Colony-forming assays were conducted to investigate whether tamoxifen affected long-term clonogenic cell survival. As shown in Fig. 1C, tamoxifen treatment significantly suppressed the cell colony forming ability in H520 and H1975 cells. At 40 μM of tamoxifen treatment, there were 70.77% and 38.22% reductions in cell colony forming ability in H520 cell and H1975 cells, respectively (Fig. 1C).

3.2. Tamoxifen decreased TP protein and mRNA expression and suppressed AKT activation

Next, to determine whether TP expression was associated with the effects of tamoxifen, we first assessed H520 or H1975 cells treated with various concentrations of tamoxifen for 24 h, and the real-time PCR and RT-PCR was used for determination of the TP mRNA level. The protein levels of TP were determined by Western blot analysis. In Fig. 2, tamoxifen reduced TP mRNA and protein expression in a time and dose-dependent manner; this was accompanied with a decrease in phospho-AKT protein levels (Fig. 2). However, tamoxifen treatment did not significantly affect the phospho-ERK1/2 protein levels.

In addition, we proposed that the inactivation of AKT was involved in the down-regulation of TP expression. To determine whether the PI3K-AKT signaling inactivation was involved in down-regulation of TP by tamoxifen, these cell lines were transiently transfected with AKT-CA plasmids, a constitutively active form of AKT, harboring a consensus myristylation domain that replaces the amino acids 4–129 of wild-type AKT. Overexpression of AKT-CA could rescue TP protein and mRNA expression in H520 and H1975 cells inhibited by tamoxifen (Fig. 3A and B).

However, once these cells were pretreated with PI3K inhibitors (LY294002 or wortmannin), the TP protein and mRNA levels in tamoxifen-exposed H520 or H1975 cells would further decrease (Fig. 3C and D). In Fig. 3C, as expected, the addition of wortmannin or LY294002 decreased cellular phospho-AKT protein levels, without affecting the cellular phospho-ERK1/2 levels in tamoxifen-exposed NSCLC cell lines. Also, knockdown of AKT expression by specific si-AKT RNA could also promote reduced TP protein and mRNA levels after a decrease by tamoxifen (Fig. 3E and F). Therefore, we concluded that tamoxifen decreased TP expression in an AKT inactivation manner.

Fig. 1. Dose and time-response curves of tamoxifen for cell survival in H520 or H1975 cells. (A) H520 or H1975 cells were treated with various concentrations of tamoxifen (5–40 μM) for 24, 48, and 72 h. Cell survival was determined by MTS assay. (B) After cells had been treated with various concentrations of tamoxifen for 24 h, both unattached and attached cells were collected and stained with trypan blue dye, and the numbers of dead cells were manually counted. Columns, percentage of trypan blue-positive cells, representing a population of dead cells; Bar, standard error (SE) from three independent experiments. (C) Tamoxifen was added to cells for 24 h, cytotoxicity was determined by colony-forming ability assay.
3.3. Down-regulation of TP expression by tamoxifen was through increased mRNA and protein instability in NSCLC cells

Next, we examined the possible mechanisms for post-transcriptional regulation of TP transcripts under tamoxifen treatment. To evaluate the stability of TP mRNA in tamoxifen-exposed H520 or H1975 cells, we treated these cells with actinomycin D to block de novo RNA synthesis and then measured the levels of existing TP mRNA using quantitative real-time PCR at 3, 6, and 9 h after treatment. After actinomycin D co-exposure, lower levels of TP mRNA were observed after tamoxifen treatment than in untreated cells (Fig. 3G). Then, cycloheximide (an inhibitor of de novo protein synthesis) was added to tamoxifen treatment for 3, 6, and 9 h, and the remaining TP protein was analyzed by Western blot. Tamoxifen treatment triggered TP degradation after cycloheximide treatment, compared to untreated cells (Fig. 3H). It was of interest that enforced expression of the AKT-CA vector significantly reduced tamoxifen-induced TP mRNA and protein instability, compared with pcDNA3 control vector transfection (Fig. 3G and H). These results indicated that tamoxifen decreased TP mRNA and protein levels by augmentation of mRNA and protein instability through AKT inactivation.

3.4. Knockdown of TP enhanced tamoxifen-induced cytotoxicity and growth inhibition in NSCLC cells

We next examined the effect of siRNA-mediated TP knockdown on tamoxifen-induced cytotoxicity and cell growth inhibition in NSCLC cells. At 24 h post-transfection, real-time RT-PCR and Western blot analysis showed a decrease in TP mRNA and protein in tamoxifen-treated H520 and H1975 cells; however, the tamoxifen-induced AKT inactivation was not affected (Fig. 4A and B). Furthermore, suppression of TP protein expression by si-TP RNA resulted in increased sensitivity to tamoxifen compared to si-control transfected cells (Fig. 4C). We also conducted a cell growth inhibition assay to evaluate the synergistic effects of TP knockdown with tamoxifen treatment. More inhibition of cell growth was induced by the combination of TP siRNA and tamoxifen than by tamoxifen alone in H520 or H1975 cells (Fig. 4D). Therefore, down-regulation of TP expression could enhance tamoxifen-induced cytotoxicity and growth inhibition in NSCLC cells.

3.5. Blocking AKT and ERK1/2 activation enhanced tamoxifen-induced cytotoxicity and growth inhibition

Next, the role of AKT and ERK1/2 in the cytotoxic effect of tamoxifen was examined; the PI3K inhibitor LY294002 and MKK1/2 inhibitor U0126 were added to block AKT and ERK1/2 activation, respectively. Co-treatment with LY294002 or U0126 further decreased significantly cell viability in tamoxifen-exposed H520 or H1975 cells, compared with tamoxifen treatment alone (Fig. 4E and F). Either LY294002 or U0126 could enhance growth inhibition after treatment with tamoxifen (Fig. 4G). Blocking both ERK1/2 and AKT activity could more effectively inhibit cell growth than either drug alone after tamoxifen treatment (Fig. 4G). Taken together, inactivation of the PI3K-AKT and MKK1/2-ERK1/2 signals could enhance tamoxifen-induced cytotoxicity and growth inhibition in NSCLC cells.

3.6. Combination treatment with erlotinib enhanced the cytotoxic effect and growth inhibition of tamoxifen

Preclinical studies suggested that erlotinib can block tyrosine kinase activity, leading to inactivation of the Ras-Raf-MKK-ERK and lipid kinase phosphatidyl inositol 3-kinase (PI3K)-AKT pathways [9]; therefore, we attempted to determine whether erlotinib could enhance the cytotoxic effects of tamoxifen in NSCLC cells. The effect of combined treatment with erlotinib and tamoxifen on cell viability was examined by MTS and trypan blue exclusion.
assays. Combined treatment with erlotinib and tamoxifen for 24 h resulted in a greater loss of cell viability in H520 and H1975 cells than treatment with either erlotinib or tamoxifen alone (Fig. 5A and B). Erlotinib and tamoxifen were combined at a ratio of 1:2 and MTS assay was used to analyze cell viability. The CI values for erlotinib and tamoxifen were <1, indicating the combination treatment had a synergistic effect (Fig. 5C). In addition, H520 and H1975 cells were exposed to erlotinib and/or tamoxifen, and cell proliferation was determined 1–4 days after exposure to the drugs. Erlotinib and tamoxifen co-treatment had a greater cell growth inhibition effect than either treatment alone (Fig. 5D). Furthermore, erlotinib also significantly decreased the cell forming ability in tamoxifen-exposed H520 and H1975 cells (Fig. 5E). The results showed that erlotinib and tamoxifen combination have synergistic cytotoxic effect for human NSCLC cells.

3.7. Erlotinib enhanced down-regulation of TP protein and the mRNA level in tamoxifen-treated human lung cancer cells

In order to assess the mechanism of the synergistic effects, we hypothesized that erlotinib would affect TP expression. To test this hypothesis, H520 and H1975 cells were exposed to various concentrations of tamoxifen (10, 20, and 30 μM) and erlotinib (10 μM) for 24 h. Erlotinib suppressed the phospho-AKT, phospho-ERK1/2, and TP protein levels in tamoxifen-exposed NSCLC cells (Fig. 6A). Moreover, the results from real-time PCR analysis showed that erlotinib further decreased tamoxifen-reduced TP mRNA levels in H520 and H1975 cells (Fig. 6B).

3.8. Transfection with AKT-CA or MKK1-CA vectors enhanced the TP protein level as well as the cell survival suppressed by erlotinib and tamoxifen

We investigated whether erlotinib-mediated TP down-regulation was correlated with MKK1/2-ERK1/2 and PI3K-AKT down-regulation in tamoxifen and erlotinib-exposed NSCLC cells. Over-expression of AKT-CA or MKK1/2-CA could rescue the cellular TP protein and mRNA levels which were suppressed by erlotinib and tamoxifen (Fig. 6C and D). Both AKT-CA and MKK1-CA vector transfection could rescue H520 and H1975 cell viability after being decreased by erlotinib and tamoxifen (Fig. 6E).

4. Discussion

A close relationship between estrogen and the risk of NSCLC cancers has been verified recently in a series of prospective
studies [33–35]. This study provides new insight into the mechanism of tamoxifen in down-regulating the expression of TP to enhance the cytotoxic effect of TKIs in NSCLC cells. The results showed that phospho-AKT significantly decreased in a dose and time-dependent manner after tamoxifen treatment in H520 and H1975 cells. The PI3K inhibitor, LY294002 or wortmannin, augmented the tamoxifen-induced cytotoxicity in H520 and H1975 cells. In C6 glioma cells, tamoxifen decreased the activation of AKT significantly in a time-dependent manner; however the ERK signal showed sustained activation in response to tamoxifen treatment. The inhibition of PI3K/AKT enhanced tamoxifen-induced cell apoptosis in malignant gliomas [36]. Based on our results, we proposed a novel mechanism of tamoxifen and TKIs combination have synergistic cytotoxic effect on human lung cancer cells—this mechanism involves down-regulation of PI3K-AKT-mediated TP expression. The PI3K signal also has been proved to be a therapeutic target for tamoxifen-resistant breast cancer cells [37]. It has been reported that LY294002 or wortmannin treatment significantly attenuated the protective effect of TP on cell apoptosis in TP-overexpressed human epidermoid carcinoma and colorectal cancer cell lines after doxorubicin or VP-16 treatment [22]. In addition, we have shown in vitro that the combination of tamoxifen and erlotinib in NSCLC can synergistically inhibit cell proliferation and reduce cell viability, both of which were correlated with down-regulation of AKT and ERK1/2 activity and TP expression in H520 and H1975 cells. Therefore, the PI3K-AKT-TP pathway might be implicated in the cytotoxicity of cisplatin against cell death induced by tamoxifen.

Tamoxifen could enhance the antitumor activity of cisplatin in a preclinical model [38]. In addition, TP plays a protective role in cisplatin-induced cytotoxicity in NSCLC cells [39]. This study showed that tamoxifen decreased TP expression in a dose and time-dependent manner. Therefore, it would be of value to understand whether down-regulation of TP expression can enhance the cytotoxicity of cisplatin by tamoxifen in NSCLC cells. In endometrial carcinoma, TP, an angiogenic factor, can activate by hypoxia-inducible factor 2alpha (HIF-2alpha) [40]. In the monocytic cell line (THP-1), tumor necrosis factor-alpha (TNF-alpha) could up-regulate the expression of TP through TNF-alpha receptor binding and NF-kB activation [41]. TNF-alpha could increase the TP mRNA level in human colon carcinoma cells via the activation of SPI transcription factor [42]. In this study, erlotinib treatment further decreased TP expression in tamoxifen-treated NSCLC cells via the down-regulation of AKT and ERK1/2 activity. However, in epidermal tumor xenografts, combined treatment with erlotinib and capecitabine achieved significantly increased tumor inhibition compared with either capecitabine or erlotinib administered as single-[Image 42x433 to 546x739]
agents, but the TP expression was significantly up-regulated by the administration of erlotinib [43].

The presence of ERs in human lung tumor cells has been controversial. However, instead of ERalpha, it is ERbeta is expressed in the major part of NSCLC cells [44]. In the Shen et al. study, real-time PCR and Western blot results showed that no ERbeta mRNA and protein were expressed in H520 cells [45]. However, in our study, knocking down TP expression could enhance the cytotoxicity and cell growth inhibition of tamoxifen in H520 cells. In addition, no correlation was found between TP and ER expression in breast cancer [46]. Therefore, raised a possibility that tamoxifen affected the TP expression could through non-ER-mediated mechanism. A previous study indicated that treatment of endometrial cultures with tamoxifen resulted in the largest number of gene changes relative to control cultures and a high proportion of genes associated with regulation of gene transcription, signal transduction and cell-cycle control. Tamoxifen-specific changes that might point toward mechanisms for its proliferative response in the endometrium included changes in retinoblastoma and c-myc binding proteins, dihydrofolate reductase (DHFR) and E2F1 genes and other transcription factors [47]. However, so far, we could not find a direct or indirect link between the action of tamoxifen and the expressions of TP. In this study, at least, the AKT signaling protein involved in TP induction after tamoxifen treatment. Moreover, tamoxifen combined with erlotinib resulted in cytotoxicity and cell growth inhibition synergistically in NSCLC cells, accompanied with reduced activation of phospho-AKT and phospho-ERK1/2, and reduced TP protein levels. We believe that this study contributes to expanses the application of tamoxifen and erlotinib in NSCLC cancer therapy.

Some studies indicated that estrogen contributes greatly to both the genesis and development of NSCLC by either triggering cell proliferation or inhibiting apoptosis; this is generally similar to the finding in breast cancers [48,49]. In this study, combination treatment with erlotinib significantly decreased the expression of TP, thereby enhancing the tamoxifen-induced cytotoxic effect on NSCLC cells. Taken together, these results may have implications for the rational design of future drug regimens incorporating tamoxifen and erlotinib for the treatment of NSCLC, especially in women with lung adenocarcinoma with an EGFR mutation that is sensitive to TKI treatment.

Fig. 5. Erlotinib co-treatment with tamoxifen synergistically enhanced cytotoxicity. (A and B) Left panel, tamoxifen (5, 10, 20, 30, and 40 μM) and/or erlotinib (10 μM) were added to H520 or H1975 cells for 24 h. Right panel, tamoxifen (30 μM) and/or erlotinib (1, 2, 5 and 10 μM) were added to H520 or H1975 cells for 24 h. Cytotoxicity was determined by assessment of the MTS and trypan blue exclusion assay. (C) Left panel, erlotinib and tamoxifen were combined at a ratio of 1:2 and the MTS assay was used to analyze cell viability. Right panel, the mean CI values at a fraction affected (FA) of 0.50, 0.75, 0.90 for tamoxifen and erlotinib combined treatment were averaged for each experiment and used to calculate the mean between experiments. Points and columns, mean values obtained from three independent experiments; bars, standard error (SE). (D) Cells were treated with tamoxifen (10 μM) and/or erlotinib (10 μM) for 1–4 days after which living cells were determined by the trypan blue dye exclusion assay. ** denotes p < 0.01 using Student’s t-test for comparison between cells treated with a drug alone or with a tamoxifen/erlotinib combination. (E) Tamoxifen (20 μM) and erlotinib (10 μM) were added to cells for 24 h, and cytotoxicity was determined by colony-forming ability assay. * denotes p < 0.05, using Student’s t-test for the comparison between the cells treated with tamoxifen alone or in combination with erlotinib.
Fig. 6. Erlotinib further decreased TP protein and mRNA levels in tamoxifen-exposed NSCLC cells. (A) H520 or H1975 cells (10^5) were cultured in complete medium for 18 h and then were exposed to tamoxifen (5, 10, 20 μM) and erlotinib (10 μM) for 24 h. After treatment, cell extracts were examined by Western blot for determination of TP, phospho-AKT, phospho-ERK1/2, and AKT, ERK1/2, and actin protein levels. (B) After treatment as the above, total RNA was isolated and subjected to real-time PCR for TP mRNA expression. The means ± standard deviation (SD) from four independent experiments. * denotes p < 0.01, respectively, using Student's t-test for comparison between the cells treated with tamoxifen/erlotinib alone or combined. Overexpression of AKT-CA or MKK1-CA restored erlotinib-suppressed TP protein expression and cell survival in tamoxifen-exposed H520 and H1975 cells. (C) AKT-CA (5 μg) or MKK1-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 tamoxifen (30 μM) and erlotinib (10 μM) for an additional 24 h, and whole-cell extracts were collected for Western blot analysis. (D) After treatment as the above, total RNA was isolated and subjected to real-time PCR for TP mRNA expression. The means ± standard deviation (SD) from four independent experiments. * denotes p < 0.01, respectively, using Student's t-test to compare cells treated with tamoxifen and erlotinib in AKT-CA or MKK1-CA vs. pcDNA3-transfected cells. (E) After AKT-CA (1, 3, 5 μg) or MKK1-CA (1, 3, 5 μg) expression plasmids transfection, cells were treated with tamoxifen (30 μM) and erlotinib (10 μM) for 24 h. Cytotoxicity was determined by assessment with the MTS assay. ** p < 0.01, ** p < 0.05 by Student's t-test to compare cells treated with tamoxifen and erlotinib in AKT-CA or MKK1-CA vs. pcDNA3-transfected H520 or H1975 cells.

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References

Platelet-derived inhibitor induced endothelial carcinogenesis

Olivo-Marston Relf Tominaga


