TD-19, an Erlotinib Derivative, Induces Epidermal Growth Factor Receptor Wild-Type Nonsmall-Cell Lung Cancer Apoptosis through CIP2A-Mediated Pathway

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Received April 16, 2014; accepted August 29, 2014

ABSTRACT

Some patients with nonsmall-cell lung cancer (NSCLC) without epidermal growth factor receptor (EGFR) mutations still respond to gefitinib and erlotinib, suggesting that there may be a mechanism(s) other than the EGFR pathway that mediates the tumoricidal effects. In the current study, we tested the efficacy of TD-19, a novel compound chemically modified from erlotinib, which has more potent apoptotic effects than erlotinib in EGFR wild-type NSCLC cell lines. TD-19 induced significant cell death and apoptosis in H358, H441, H460, and A549 cells, as evidenced by increased caspase-3 activity and cleavage of procaspase-9 and poly (ADP-ribose) polymerase. The apoptotic effect of TD-19 in H460 cells, which were resistant to erlotinib, was associated with downregulation of cancerous inhibitor of protein phosphatase 2A (CIP2A), increased protein phosphatase 2A (PP2A) activity, and decreased AKT phosphorylation, but minimal effects on EGFR phosphorylation. Overexpression of CIP2A partially protected the H460 cells from TD-19–induced apoptosis. Okadaic acid, a known PP2A inhibitor, significantly reduced TD-19–induced apoptosis, while forskolin, which increased PP2A activity, increased the apoptotic effect of TD-19. TD-19 inhibited the growth of H460 xenograft tumors by ∼80%. We conclude that TD-19 exerted tumoricidal effects on NSCLC cells. TD-19 provides proof that the CIP2A pathway may be a novel target for the treatment of EGFR wild-type NSCLC.

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, and 80% of lung cancers are diagnosed as nonsmall-cell lung cancer (NSCLC) (Jemal et al., 2008). Epidermal growth factor receptor (EGFR) gene mutations are identified in 10–15% of Caucasian NSCLC patients and in even higher percentages in Asian patients (Shigematsu et al., 2005). Patients with certain EGFR mutations, such as L858R and exon 19 deletion, have a higher response rate to the EGFR-targeted drugs, such as gefitinib and erlotinib (Huang et al., 2004; Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004). Some NSCLC patients without EGFR mutations, however, still respond to gefitinib and erlotinib (Cappuzzo et al., 2010; Ciuleanu et al., 2012), suggesting that there may be a mechanism(s) other than the EGFR pathway that mediates the tumoricidal effects of gefitinib and erlotinib.

Cancerous inhibitor of protein phosphatase 2A (CIP2A) is a cellular protein phosphatase 2A (PP2A) inhibitor that inhibits proteolytic degradation of c-Myc (Junttila et al., 2007) and is overexpressed in several human epithelial malignancies, including NSCLC (Soo Hoo et al., 2002; Junttila et al., 2007; Côme et al. 2009; Khanna et al., 2009; Katz et al., 2010; Vaarala et al., 2010; Dong et al., 2011; Ma et al., 2011; Xu et al., 2012). Overexpression of CIP2A in NSCLC correlates with poor prognosis (Dong et al., 2011; Ma et al., 2011; Xu et al., 2012). Decrease in CIP2A expression inhibits proliferation and induces apoptosis in a variety of lung cancer cells (Ma et al., 2011).

In our previous study, we found that erlotinib could also suppress CIP2A and that it induced apoptosis in vivo and in vitro in hepatocellular carcinoma (Yu et al., 2013a) and lung
cancer (Wang et al., 2014). Erlotinib is a quinazoline derivative with amino substitu-tes at position 4 (Hennequin et al., 1996; van Muijlwijk-Koezen et al., 2000; Shreder et al., 2004; Morphy, 2010). With various chemical modifications, we have previously developed a series of erlotinib analogs that had stronger CIP2A suppression effects (Chen et al., 2012). TD-19 (Fig. 1) is one such compound modified from erlotinib that has 4-phenoxylaniline added at the 2-position of quinazoline. The changes in the chemical structure impede the hydrogen bond interaction between erlotinib and the ATP-binding site of EGFR. Without an amide functional group or a pyridine ring, TD-19 exhibits low binding affinity to the ATP-binding site of the EGFR tyrosine kinase domain. These changes, however, increased the compound's potency in suppressing CIP2A compared with erlotinib. In this study, we tested the efficacy of TD-19 in EGFR wild-type NSCLC cells and verified that the antitumor activity of TD-19 was mediated by attenuating CIP2A.

Materials and Methods

Cell Culture. Four NSCLC cell lines were used in this study. H358 (bronchioloalveolar carcinoma, mutant KRAS), H441 (papillary adenocarcinoma, mutant KRAS, and tumor protein p53), and A549 (bronchioloalveolar carcinoma, mutant KRAS, cyclin-dependent kinase inhibitor 2A, and serine/threonine kinase 11) cell lines were obtained from the American Type Culture Collection (Manassas, VA), and H460 (large-cell lung cancer, mutant KRAS, phosphatidylinositol-4,5-bisphosphate 3-kinase, serine/threonine kinase 11, and cyclin-dependent kinase inhibitor 2A) cell line was from the Bioresource Collection and Research Center (Hsinchu, Taiwan). The NSCLC cell lines were kept in RPMI 1640 (Invitrogen/Life Technologies, Saint Louis, MO) supplemented with 10% fetal bovine serum (Gibco/Life Technologies, Grand Island, NY), 100 units/ml of penicillin G, and 100 μg/ml of streptomycin sulfate in a 37°C humidified incubator with 5% CO2 in air.

Reagents and Antibodies. Erlotinib was purchased from Selleck Chemicals (Houston, TX). TD-19 was synthesized by C.-W.S. For in vitro studies, erlotinib and TD-19 at various concentrations were dissolved in dimethylsulfoxide and then added to the cells in serum-free RPMI 1640. PP2A inhibitor and activator were purchased from Sigma-Aldrich (St. Louis, MO) and Merck Millipore (Billerica, MA), respectively. Antibodies for immunoblotting, such as those targeting CIP2A, AKT, and poly (ADP-ribose) polymerase, were purchased from Santa Cruz Biotechnology (San Diego, CA). Other antibodies, such as those directed at PP2A, EGFR, p-EGFR, and p-AKT (Ser473), were from Cell Signaling Technology (Danvers, MA).

Cell Viability Assay and Apoptosis Analysis. Four NSCLC cell lines were seeded in 96-well plates (5 × 104 cells/well), and 10% water-soluble tetrazolium monosodium salt (Cell Proliferation Reagent WST-1; Roche Applied Science, Indianapolis, IN) was added to the cell suspension in each well. Cells were then incubated for 1–2 hours, and cell viability and proliferation were quantified by measuring the absorbance at 450 nm using a BioTek Synergy HT enzyme-linked immunosorbent assay reader (BioTek, Winooski, VT). Apoptotic cells were measured by flow cytometry (sub-G1), and cell death was detected by Western blot.

Overexpression of CIP2A. CIP2A cDNA (KIAA1524) was purchased from Origene (Rockville, MD). Briefly, following transfection, H460 cells were incubated in the presence of G418 (0.78 mg/ml; Sigma-Aldrich). After 8 weeks of selection, surviving colonies, i.e., those arising from stably transfected cells, were selected and individually amplified. H460 cells with stable overexpression of CIP2A were then treated with erlotinib or TD-19, harvested, and processed for Western blot analysis.

PP2A Activity. PP2A activity was measured in fresh cells as described previously (Yu et al., 2013a) using PP2A DuoSet IC activity assay kit (R&D Systems, Minneapolis, MN), according to the manufacturer's description. Briefly, an immobilized capture antibody specific for the catalytic subunit of PP2A that binds both active and inactive PP2A was used. After washing, a substrate was added that was dephosphorylated by active PP2A to generate free phosphate, which was detected by a sensitive dye-binding assay using malachite green and molybdic acid.

Quantification of CIP2A Gene Expression. Total RNA was extracted from TD-19–treated H460 cells (~5 × 105) using RNeasy Mini Kit (Qiagen, Gaithersburg, MD) and then reversely transcribed using QuantiTect Reverse Transcription Kit (Qiagen). The real-time quantitative polymerase chain reaction (PCR) was performed on an Applied Rotor-Gene 3000 detector (Qiagen) with a specific primer set for each target gene and SYBR Green dye (Qiagen) for detection, as described in the manufacturer’s guidelines. The PCR primer sets for target genes were as follows: human CIP2A [Hs_KIAA1524 QuantiTect Primer Assay (NM_020890)] and human actin [Hs_ACTB QuantiTect Primer Assay (NM_001101)]. An aliquot of each sample was analyzed by quantitative PCR for β-actin to normalize for inefficiencies in cDNA synthesis and RNA input amounts. For each sample, the average

Fig. 1. (A) Chemical structures of erlotinib (left) and TD-19 (right). (B) EGFR phosphorylation activity of TD-19. H358, H441, H460, and A549 cells were exposed to TD-19 at 10 μM for 24 hours, and cell lysates were analyzed for EGFR phosphorylation.
cycle threshold value (Ct) was determined from quadruplicate assays, and the ΔCt value was determined by subtracting the average β-actin Ct value from the average CIP2A Ct value. Three independent experiments were performed to measure the levels of CIP2A in H460 cells.

Xenograft Tumor Growth. Male NCr nude mice (5–7 weeks of age) were used. All experimental procedures were performed according to protocols approved by the Institutional Laboratory Animal Care and Use Committee of Cardinal Tien Hospital (New Taipai City, Taiwan). Each mouse was inoculated subcutaneously in the dorsal flank with 1 × 10^5 H460 cells suspended in 0.1 ml of serum-free medium containing 50% Matrigel (BD Biosciences, Bedford, MA). When tumors reached 100–200 mm³, the mice received erlotinib (10 mg/kg p.o.) once daily or TD-19 (10 mg/kg p.o.) once daily. The controls received vehicle. The tumors were measured twice weekly using calipers, and their volumes were calculated using the following standard formula: width × length × height × 0.523 (Yu et al., 2013a,b).

Immunohistochemistry and Quantitative Histological Measurement. Immunohistochemistry (IHC) stains were performed using the Ventana BenchMark XT automated stainer (Ventana, Tucson, AZ). Briefly, 4-µm-thick sections were cut consecutively from formalin-fixed, paraffin-embedded human tissues. Sections were mounted and allowed to dry overnight at 37°C. After deparaffinization and rehydration, slides were incubated with 3% hydrogen peroxide solution for 5 minutes. After washing with buffer, tissue sections were repaired for 40 minutes with EDTA. The slides were incubated with the primary antibody overnight at 4°C. The primary antibodies used in the study were anti-β-actin (1:50; Novus Biologicals, Littleton, CO), anti-CIP2A (1:50; Novus Biologicals, Littleton, CO), and anti-AKT (1:25; Santa Cruz Biotechnology). After three rinses in buffers, the slides were incubated with a secondary antibody (unbiotinylated antibody, EnVision System, Denmark). Tissue staining was visualized with a 3,3’-diaminobenzidine substrate chromogen solution (DakoCytomation). Slides were counterstained with hematoxylin, dehydrated, and mounted. Each run included phosphate-buffered saline as the negative control and samples known to express these markers strongly as the positive controls. The quantitative protein expression level by IHC stain was determined using the NIH ImageJ (National Institutes of Health, Bethesda, MD) program to obtain the mean expression level from 10 random fields (400×) of each sample.

Statistical Analysis. Statistical analysis was performed using analysis of variance followed by the Tukey’s subtest. The results were expressed as mean ± S.D. Differences were considered significant at P < 0.05.

Results

TD-19, an Erlotinib Derivative Lacking the Inhibitory Function of EGFR. The chemical structure of TD-19 is shown in Fig. 1A. It is modified from erlotinib by adding 4-phenoxyaniline at the 2-position of quinazoline, which impedes the hydrogen bond interaction between erlotinib and the ATP-binding site of the EGFR (Hennequin et al., 1996). TD-19 does not change the phosphorylation status of EGFR in the EGFR wild-type H358, H441, H460, and A549 cells (Fig. 1B). In contrast to erlotinib, TD-19 had no effect on different EGFR phosphorylation sites in EGFR mutation PC9 cells and EGFR wild-type H358 and H460 cells (Supplemental Fig. 1).

TD-19 Showed Cell Death Effect in NSCLC Cell Lines. TD-19 decreased the viability of H358, H441, H460, and A549 cells in a dose-dependent (Fig. 2A) and a time-dependent manner (Fig. 2B). Because H460 cells are resistant to erlotinib, we further tested the effects of TD-19 on this cell line. TD-19 treatment for 24 hours increased the sub-G1–phase population in H460 cells (Fig. 3A). TD-19 decreased CIP2A and p-AKT protein levels and induced apoptosis in H460 cells in a dose-dependent (Fig. 3B) and a time-dependent manner (Fig. 3C; Supplemental Fig. 2). The data indicate that TD-19 exhibited more potent antitumor activity than erlotinib in association with CIP2A and p-AKT downregulation in NSCLC cells independent of EGFR activation.

Sensitization by TD-19 in NSCLC Cell Lines via the CIP2A/PP2A/AKT Pathway. To confirm the role of the CIP2A signaling reduction as a determinant molecular mechanism mediated by TD-19–induced apoptosis, we overexpressed CIP2A in H460 cells (CIP2A-myc in Fig. 4A). Overexpression of CIP2A partially protected the cells from apoptosis induced by TD-19.

Fig. 2. Comparison of the effects of TD-19 and erlotinib on cell death in the four human NSCLC cell lines. (A) Dose-dependent effects of TD-19 and erlotinib on cell viability in the four human NSCLC cell lines. Data are shown as mean ± S.D.; n = 3. **P < 0.01; ***P < 0.001 for each concentration for 48 hours, TD-19 versus erlotinib. (B) Time-dependent effects of TD-19 and erlotinib on cell viability in the four human NSCLC cell lines. Data are shown as mean ± S.D.; n = 3. **P < 0.01 for 24, 48, and 72 hours at 5 µM, TD-19 versus erlotinib.
Addition of okadaic acid, a known PP2A inhibitor, also significantly reduced TD-19–induced apoptosis in H460 cells (Fig. 4B). Forskolin activates a variety of adenylyl cyclases and increases cAMP production (Tang and Hurley, 1998), which results in activation of protein kinase A and increased PP2A activity. Forskolin also has other cAMP-independent effects, including inhibition of the Hedgehog signaling pathway (Yamnaka et al., 2010); inhibition of the binding of platelet-activating factor (Wong et al., 1993); and inhibition of glucose transport in erythrocytes, adipocytes, platelets, and other cells (Mills et al., 1984). In Fig. 4C, downregulating of p-AKT and promoting apoptosis has synergistic effects in combination with TD-19 and forskolin. These results indicate that the CIP2A/PP2A/p-AKT pathway plays a role in mediating the apoptotic effect of TD-19 in erlotinib-resistant H460 cells.

To examine the mechanisms by which TD-19 inhibited CIP2A protein expression, we investigated whether TD-19 affected CIP2A protein degradation. After protein translation was blocked by cycloheximide, the rate of CIP2A degradation did not change significantly with or without TD-19 treatment in H460 cells (Fig. 5A). We next investigated whether TD-19 affected CIP2A transcription. Figure 5B shows that the mRNA levels of CIP2A decreased in a time-dependent and dose-dependent manner in H460 cells. Because this finding suggests that TD-19 suppressed transcription of CIP2A, we further investigated whether TD-19 affected CIP2A promoter activity. TD-19 significantly downregulated the activity of CIP2A promoters in a dose-dependent manner in H460 cells (Fig. 5C, right), while erlotinib had little effect (Fig. 5C, left). From these results, we concluded that TD-19 treatment, by
diminishing the transcription of CIP2A, enhances PP2A activity and downregulates p-AKT, leading to NSCLC cell apoptosis.

Evaluation of the Therapeutic Effect of TD-19 on H460-Bearing Mice. To determine whether the in vitro effects of TD-19 on H460 cells could be reproduced in vivo, mice were implanted with H460 xenografts. Treatment with TD-19 decreased H460 xenograft tumor growth by ~80% compared with the control (Fig. 6A, left). TD-19 was more potent than erlotinib in inhibiting H460 xenograft tumor growth. No apparent differences in body weight or toxicity were found in any mice (Fig. 6A, right).

To determine whether the antitumor effect of TD-19 correlated with the CIP2A-dependent pathway in vivo, tumor extracts from vehicle-, TD-19-, and erlotinib-treated mice were immunoblotted for CIP2A, AKT, and p-AKT. PP2A activity in TD-19- and erlotinib-treated H460 xenografts was also examined. TD-19-treated tumors showed downregulation of CIP2A and p-AKT expression (Fig. 6B, top) compared with vehicle- and erlotinib-treated tumors. TD-19-treated tumors also showed significant increases in PP2A activity (Fig. 6C). To assess the expression level of CIP2A, p-AKT, and AKT, IHC staining was performed in H460 xenograft tumor specimens. All the tumor specimens showed a cytoplasmic staining pattern in CIP2A, p-AKT, or total AKT. The CIP2A and p-AKT expression levels were significantly decreased in TD-19-treated samples compared with vehicle- and erlotinib-treated samples. Moreover, there were no significant differences between vehicle- and erlotinib-treated samples. The CIP2A expression level was 62.8% ± 7.2% in the TD-19-treated samples and 96% ± 8.7% in the erlotinib-treated samples. There was an ~33% decrease in the expression level. Similarly, the p-AKT expression level was 71.2% ± 5.9% in TD-19-treated samples and 91% ± 11.3% in erlotinib-treated samples. There was an ~19% decrease in the expression level. In contrast, total AKT expression level did not show significant changes between vehicle-, erlotinib-, and TD-19-treated samples (Fig. 6D).

Discussion

Most NSCLC patients with various EGFR mutations respond to EGFR inhibitors, and many quinazoline derivatives are strong inhibitors of EGFR. TD-19 is a compound modified from erlotinib, a quinazoline derivative, that has 4-phenoxyaniline added at the 2-position of quinazoline. These modifications minimize the effects of erlotinib against EGFR while increasing the potency against the CIP2A-dependent pathway (Chen et al., 2012). In this study, we showed that TD-19 induced cell death in a lung cancer cell line (H460) that was resistant to erlotinib. This inhibitory effect in vitro could be reproduced in vivo. Because TD-19 had minimal effect against EGFR (Fig. 1B; Supplemental Fig. 1), the antitumor effects of TD-19 were likely mediated by its attenuation of the CIP2A/PP2A/AKT pathway. That the TD-19-induced cell death was mediated by the CIP2A pathway was supported by the following results. First, TD-19 inhibited the RNA and protein expression of CIP2A. Second, overexpression of CIP2A, which upregulated p-AKT, partially protected the H460 cells against TD-19-induced apoptosis. Third, a PP2A inhibitor, okadaic acid, significantly reduced the TD-19-induced apoptosis, and a PP2A enhancer, forskolin, increased the apoptotic effect of TD-19 in H460 cells. Taken together, these results indicate that the inhibition of CIP2A and downstream activation of PP2A and inhibition of p-AKT mediated the antitumor effects of TD-19 (Chen et al., 2010, 2011; Huang et al., 2012; Lin et al., 2012; Tseng et al., 2012, 2013; Liu et al., 2013; Yu et al., 2013a,b).

Whether TD-19 acts directly on CIP2A remains unclear. Although we showed that TD-19 inhibited CIP2A RNA and protein levels, we did not measure its direct effect on CIP2A activity. The precise molecular target of TD-19 remains unclear. Future studies are needed to elucidate the exact mechanisms. CIP2A is overexpressed in NSCLC, and its expression correlates with poor prognosis (Dong et al., 2011; Ma et al., 2011; Xu et al., 2012) and TNM stage (Ma et al., 2011). Besides being a prognostic biomarker, CIP2A may also act as a novel therapeutic target (Yu et al., 2013a,b). Ma et al. (2011) demonstrated that
Rabdocoetin B, a diterpenoid isolated from *Isodon coetsa*, inhibited proliferation and induced apoptosis in a variety of lung cancer cells by downregulating CIP2A and inactivating the AKT pathway. In this study, we used a new erlotinib derivative, TD-19, to demonstrate its potent antitumor efficacy on EGFR wild-type NSCLC cells. We discovered that TD-19 enhanced PP2A activity by suppressing CIP2A and subsequently reduced p-AKT expression through depletion of CIP2A transcriptional activity. Several reports previously demonstrated that there are different ways to modulate CIP2A expression: e.g., upregulation of CIP2A by the Src and Ras/mitogen-activated protein kinase/extracellular signal–regulated kinase pathways (Jung et al., 2013), repression of CIP2A by microRNA that binds to the coding region of CIP2A (Zhao et al., 2010), and use of transcription factors that interact with CIP2A proximal promoters to regulate CIP2A expression (Khanna et al., 2011; Pallai et al., 2012). Our study demonstrated that TD-19 induced cell death and apoptosis by attenuation of CIP2A signaling through decreased transcription of CIP2A. However, exactly how TD-19 actually modulates CIP2A transcription will require further elucidation.

In conclusion, TD-19 induced apoptotic cell death in NSCLC cells that were resistant to the EGFR inhibitor erlotinib. The antitumor effects were mediated by enhancing PP2A-mediated p-AKT downregulation by inhibition of CIP2A. Thus, this compound may be a novel therapy for patients who have NSCLC without EGFR mutations. The therapeutic efficacy of TD-19 needs to be tested and examined more closely in future clinical trials in patients with NSCLC.

**Authorship Contributions**

*Participated in research design:* Chao, Wang, Lai, Y.-L. Chen, Lin.

*Conducted experiments:* Chao, Y.-L. Chen, Tsai, P.-T. Chen.

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