Cancer Therapy: Preclinical

Caspase-Independent Cell Death Is Involved in the Negative Effect of EGF Receptor Inhibitors on Cisplatin in Non–Small Cell Lung Cancer Cells

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Abstract

**Purpose:** Results of multiple clinical trials suggest that EGF receptor (EGFR) tyrosine kinase inhibitors (TKI) exhibit negative effects on platinum-based chemotherapy in patients with lung cancer with wild-type (WT) EGFR, but the underlying molecular mechanisms are still uncertain. Studies that identify the mechanism of how TKIs negatively affect patients with WT EGFR are important for future development of effective strategies to target lung cancer. Thus, we returned to in vitro study to investigate and determine a possible explanation for this phenomenon.

**Experimental Design:** We investigated the effects of TKIs and cisplatin on caspase-independent cell death (CID) and the role of CID in the efficacy of each drug and the combination. Furthermore, we studied the mechanism by which EGFR signaling pathway is involved in CID. Finally, on the basis of the identified mechanism, we tested the combinational effects of cisplatin plus suberoylanilide hydroxamic acid (SAHA) or erastin on CID.

**Results:** We found that gefitinib inhibited cisplatin-induced CID but not caspase-dependent apoptotic cell death. In WT EGFR cells, gefitinib not only inhibited CID but also failed to induce apoptosis, therefore compromising the efficacy of cisplatin. Inhibition of EGFR-ERK/AKT by gefitinib activates FOXO3a, which in turn reduces reactive oxygen species (ROS) and ROS-mediated CID. To overcome this, we showed that SAHA and erastin, the inducers of ROS-mediated CID, strongly enhanced the effect of cisplatin in WT EGFR cells.

**Conclusion:** TKI-mediated inhibition of CID plays an important role in the efficacy of chemotherapy. Moreover, FOXO3a is a key factor in the negative effects of TKI by eliminating cisplatin-induced ROS. *Clin Cancer Res; 19(4); 845–54.* ©2012 AACR.

Introduction

Lung cancer is the leading cause of cancer-related death in the United States. More than 70% of patients with lung cancer diagnosed at advanced stage, and those patients are treated primarily with platinum-based chemotherapy (1). Recently, the EGF receptor (EGFR) tyrosine kinase inhibitors (TKI) such as gefitinib or erlotinib have shown effectiveness in blocking tumor growth and increased survival rate. Preclinical studies showed that gefitinib enhances the efficacy of cytotoxic drugs (2, 3). However, several large-scale phase III clinical trials that were conducted in the United States to test the combination of TKIs and chemotherapy in randomly selected patients with lung cancer (4–6) failed when patient groups that received TKIs and chemotherapy did not show any benefit in the overall survival rate compared with chemotherapy alone (7). Surprisingly, several studies showed that sensitivity of patients with lung cancer to gefitinib correlated with EGFR mutations in which patients who had mutant (mt) but not those with wild-type (WT) EGFR showed response to gefitinib (8, 9). Subsequently, data analysis of EGFR mutation status from clinical trials indicated that TKIs might even induce a negative or antagonistic effect when administered with chemotherapeutic drugs in patients with WT EGFR, whereas additive effects were observed in patients with mt EGFR (7).

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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In memoriam of Mr. Nan-Tu Haung for his courageous fight against lung cancer.

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doi: 10.1158/1078-0432.CCR-12-2621

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Studies that identify the mechanism of how TKIs negatively affect patients with WT EGFR will likely be important for future development of effective strategies to target lung cancer. Thus, we return to in vitro study to investigate and determine a possible explanation for this phenomenon. EGFR TKIs exhibit distinct responses in WT EGFR and mt EGFR lung cancer cells: they induce apoptotic (caspase-dependent) cell death in lung cancer cells expressing mt EGFR (10) but not in those expressing WT EGFR (11). Cisplatin, a commonly used drug for treating lung cancer, can induce cell death via caspase-dependent (CDD; apoptosis) or caspase-independent cell death (CID) pathway (12, 13) regardless of EGFR mutation status. Because we discovered that gefitinib actually inhibits CID independently of EGFR mutation, we hypothesized that the absence of active TKI-induced apoptosis in WT EGFR cells concurrent with gefitinib-induced inhibition of CID might negatively impact the therapeutic benefit of cisplatin. Here, we identified a potential mechanism for TKI-mediated inhibition of CID and provided, at least in part, an explanation to why the clinical trials of combination of TKIs and chemotherapeutic drugs have failed in lung cancer patients with WT EGFR.

Materials and Methods

Detection of cell death

To determine viability, we stained the cells with Trypan blue dye (Fig. 1D and Supplementary Figs. S1B and S2A) and counted at least 200 to 300 cells under microscope. All experiments were carried out in triplicate and repeated several times. To determine the long-term viability, the cells were seeded in 6-well plates at about 50% confluency and treated with the indicated reagents. The medium was changed 4 to 5 days later and further cultured for 10 days. The living cells were then stained with crystal violet.

Reagents

Caspase inhibitor z-VAD-fmk was purchased from Axrra. Cisplatin, N-acetyl-l-cysteine (NAC), U0126, and erastin were obtained from Sigma. Suberoylanilide hydroxamic acid (SAHA) was synthesized as described previously (14). AKT inhibitor (AKTii), MK2206, was obtained from Selleck Chemicals. The anti-Bax 6A7, phospho-ERK, extracellular signal–regulated kinase (ERK), Mn-superoxide dismutase (SOD), and catalase antibodies were purchased from Santa Cruz Biotechnology and anti-Bax, AKT substrate, AKT, FOXO3a, phospho-AKT (Thr32), GSK3β, phosphor-GSK3β (Ser9) antibodies from Cell Signaling Technology.

Cell culture

All cell lines used in this research were obtained from American Type Culture Collection and maintained in MDEM/F112 medium supplemented with 10% FBS and antibiotics. No further authentication was conducted. Transient and stable transfections were conducted by electroporation.

Plasmid and siRNA

Myc-tagged Bcl-XL expression plasmid, pcDNA3-myc-Bcl-XL, was described previously (14). The active form of AKT and mitogen-activated protein/extracellular signal-regulated kinase (MEK), FOXO3a mutant, WT, and GFP-FOXO3a expression plasmid were described previously (15). siRNAs against FOXO3a were purchased from Sigma, and siRNAs for apoptosis inducing factor (AIF), calpain, Mn-SOD, and catalase were purchased from Dharmaco.

Animal model

H1299 or A549 cells stably expressing Bcl-XL and luciferase was used for xenograft experiment. In brief, 5 × 10^6 (H1299) or 2 × 10^6 (A549) cells were intrathoracically injected into the chest of nude mice. Drug treatments were started on day 29. Cisplatin (1 mg/kg) was injected intraperitoneally once per week, whereas gefitinib (100 mg/kg) was injected by oral injection 5 times per week. Total 2 treatment cycles were conducted. Luciferase activity, which represents tumor volume, was monitored by the In Vivo Imaging System. Each treatment group contains 10 (for H1299) or 20 (for A549) mice.

ROS measurement

Cellular reactive oxygen species (ROS) levels were determined by using H2DCFDA (Molecular Probe). Briefly, cells treated or untreated with drugs were trypsinized and washed with PBS and then incubated in PBS containing 10 ng/mL of H2DCFDA for 10 minutes at room temperature. Fluorescence signal were measured using flow cytometry.

Results

Antagonistic effect of TKI on cisplatin in WT EGFR lung cancer cells

To determine the combinational effects of EGFR TKIs and chemotherapeutic drugs in vitro, we first treated lung cancer
there is no signi-
ificant difference between Cis and Cis + Gef. B, the same cells used in A were treated with 50 μmol/L of cisplatin (Cis) and/or 5 μmol/L of gefitinib (Gef) for 24 hours and subjected to caspase assay (n = 3; *, P < 0.01; #, P < 0.05). In A549 and H1299 cells, there is no significant difference between Cis and Cis + Gef, and the long-term effects of drug combination were determined as described in Materials and Methods. The relative densities of cells (from the left) treated with cisplatin (Cis) or cisplatin plus gefitinib (Cis + Gef) are shown on the right (n = 3). C, A549 cells were treated with 50 μmol/L of cisplatin in the presence or absence of 50 μmol/L of z-VAD-fmk, and the viability was determined by Trypan blue dye exclusion assay (left, n = 3). The cellular morphology of A549 cells treated for 72 hours (right). Right, the cells stained with Trypan blue dye. Arrows indicate typical apoptotic cell death, whereas arrowheads indicate CID. D, A549 control (Neo) or Bcl-XL stable (Bcl-XL) cells were treated with 50 μmol/L of cisplatin. The viability was determined by Trypan blue dye exclusion assay (top, n = 3). Caspase activity was determined by caspase assay (bottom, n = 3).

Figure 1. Gefitinib induces apoptotic cell death (CDD) in only mt EGFR lung cancer cells, whereas cisplatin induces both CDD and CID in WT EGFR and mt EGFR lung cancer cells. A, H3255 (mt EGFR), PC9 (mt EGFR), H1299 (WT EGFR), and A549 (WT EGFR) lung cancer cells were treated with 50 μmol/L of cisplatin (Cis) and/or 5 μmol/L of gefitinib (Gef) for 24 hours and subjected to caspase assay (n = 3; *, P < 0.01; #, P < 0.05). In A549 and H1299 cells, there is no significant difference between Cis and Cis + Gef, and the long-term effects of drug combination were determined as described in Materials and Methods. The relative densities of cells (from the left) treated with cisplatin (Cis) or cisplatin plus gefitinib (Cis + Gef) are shown on the right (n = 3). C, A549 cells were treated with 50 μmol/L of cisplatin in the presence or absence of 50 μmol/L of z-VAD-fmk, and the viability was determined by Trypan blue dye exclusion assay (left, n = 3). The cellular morphology of A549 cells treated for 72 hours (right). Right, the cells stained with Trypan blue dye. Arrows indicate typical apoptotic cell death, whereas arrowheads indicate CID. D, A549 control (Neo) or Bcl-XL stable (Bcl-XL) cells were treated with 50 μmol/L of cisplatin. The viability was determined by Trypan blue dye exclusion assay (top, n = 3). Caspase activity was determined by caspase assay (bottom, n = 3).

The role of CID in cisplatin-induced cell death

Our data from above indicate that gefitinib reduces the killing effect of cisplatin without any significant effects on cells harboring either mt EGFR (H3255, PC9) or WT EGFR (A549, H1299) with cisplatin, gefitinib, or the combination and measured the caspase-3 activity, which is indicative of apoptosis. As shown in Fig. 1A, cisplatin induced apoptosis in both mt EGFR and WT EGFR cells, whereas gefitinib induced apoptosis only in mt EGFR cells. As expected, cell growth of WT EGFR cells was not significantly affected under gefitinib treatment (Supplementary Fig. S1). The differential response to cisplatin and gefitinib was further validated by long-term drug treatment (Fig. 1B). Cisplatin nearly eliminated viable cells as effectively as gefitinib in mt EGFR cells; however, only cisplatin treatment eliminated the majority of cells with WT EGFR but not gefitinib. Interestingly, we observed residual cells after the combinational treatment only in WT EGFR cells (Fig. 1B), suggesting that the TKI might have also reversed the cell-killing effect of cisplatin in WT EGFR lung cancer cells. These results are consistent with the data from the previous clinical trials in which addition of EGFR TKI to chemotherapy yielded disappointing outcomes in patient with WT EGFR (7), and thus prompted us to further investigate the underlying mechanisms.

The role of CID in cisplatin-induced cell death

Our data from above indicate that gefitinib reduces the killing effect of cisplatin without any significant effects on
the caspase activity, suggesting that other caspase-independent mechanism may be involved in the antagonistic effect of TKI on cisplatin in WT EGFR cells. Because it has been shown that chemotherapeutic drugs induce CID (16–19), we hypothesized that cisplatin also induces CID in addition to apoptosis in lung cancer cells, whereas gefitinib inhibits CID. To test this hypothesis, we first measured cisplatin-induced CID in WT EGFR cells by blocking apoptosis with a caspase inhibitor (z-VAD-fmk). When we treated A549 and H1299 cells with cisplatin (Fig. 1C and Supplementary Fig. S2A), a majority of the cells exhibited typical apoptosis morphology including membrane blebbing, apoptotic body formation, and cell shrinkage (Fig. 1C, right, arrows). However, in the presence of z-VAD-fmk, which inhibits apoptosis, cells treated with cisplatin were eventually killed without any apoptotic morphologic changes as shown by Trypan blue dye, which stains all dead cells, suggesting CID had occurred (Fig. 1C, right). In some cells treated with cisplatin alone, we observed similar CID characteristics without any apoptotic morphologic changes (Fig. 1C, right, arrowheads), indicating both apoptosis and CID had occurred.

To mimic the effect of z-VAD-fmk, we also established Bcl-XL overexpressing stable clones in WT EGFR cells to block cisplatin-induced apoptotic signaling. Indeed, cisplatin-induced caspase activation was completely inhibited in A549 Bcl-XL stable line (Fig. 1D, bottom). After cisplatin treatment, the Bcl-XL stable cells eventually underwent CID, which is similar to the treatment of cisplatin plus z-VAD-fmk (Fig. 1D, top and Supplementary Fig. S2B). We also obtained similar results using H1299 cells (Supplementary Fig. S2C). Furthermore, we confirmed the HMGB1 was released from the cells to extracellular environment after cisplatin treatment (data not shown), which is the characteristic of caspase-independent necrosis (19). Therefore, the CID induced by cisplatin is likely necrosis. Collectively, these results indicate that cisplatin induces both apoptosis and CID in lung cancer cells and that CID remains intact when apoptosis is compromised.

The effects of TKI on cisplatin-induced CID

Next, we examined the effects of TKIs on CID induced by cisplatin. We treated H1299 and A549 cells with cisplatin or cisplatin plus gefitinib in the presence or absence of z-VAD-fmk and compared the effects of gefitinib on cisplatin-induced cell death. We did not observe any effects of gefitinib alone on cell viability and caspase activation in these cells (data not shown). However, when we added gefitinib to cells pretreated with cisplatin and z-VAD-fmk, we observed increased cell viability (Fig. 2A). When apoptosis is inhibited by z-VAD-fmk in mt EGFR cells, CID was also attenuated by gefitinib (Supplementary Fig. S3A). Likewise, when we overexpressed Bcl-XL to block apoptosis, we found that gefitinib inhibited cisplatin-induced cell death more strongly in Bcl-XL stable cells than control cells (Fig. 2B). Similar results were observed when we examined the long-term effect of these drugs (Fig. 2C) and when we used erlotinib, another EGFR TKI, instead of gefitinib (Supplementary Fig. S3B and S3C). To further validate the negative effects of TKI on cisplatin in vivo, we established H1299 and A549 Bcl-XL/luciferase stable cell lines and inoculated the cells into the lung of nude mice. The animals were then treated with cisplatin or gefitinib or the combination, and then the tumor volumes were monitored by a bioluminescent imaging system. As shown in Fig. 2D, gefitinib alone did not inhibit tumor growth in H1299-Bcl-XL or in A549-Bcl-XL (Supplementary Fig. S4) tumors compared with cisplatin. These results are consistent with that from in vitro study (Fig. 1). Importantly, the effects of cisplatin on these tumors growth were significantly attenuated by the addition of gefitinib (Fig. 2D and Supplementary Fig. S4), further indicating that EGFR TKIs have negative effects on cisplatin-induced antitumor activity.

The molecular mechanisms of TKI-mediated inhibition of CID

Next, to explain the underlying molecular mechanisms in TKI-mediated inhibition of CID, we first analyzed the activity of 2 major EGFR downstream molecules, ERK and AKT, in the presence of cisplatin. Consistent with the previous study (20), our data also showed that EGFR, ERK, and AKT were activated by cisplatin (Fig. 3A and Supplementary Fig. S5). The addition of TKIs could reverse the cisplatin-enhanced AKT/ERK activation (Fig. 3A and Supplementary Fig. S5), which suggests that activation of EGFR-AKT/ERK pathway plays a role in cisplatin-induced CID. To validate this, we treated the cells with cisplatin in the presence of MEK or AKT. Inhibition of either MEK/ERK or AKT-attenuated CID induced by cisplatin in Bcl-XL stable A549 (Fig. 3B) or H1299 cells (Fig. 3C and Supplementary Fig. S6). In contrast, overexpression of active form of MEK or AKT promoted CID induced by cisplatin (Fig. 3D). Thus, these results indicate that EGFR downstream ERK/AKT signaling increased cisplatin-induced CID.

Next, we investigated which downstream signaling molecules of ERK/AKT are involved in cisplatin-induced CID. Previously, it has been shown that FOXO3a is a common target of AKT and ERK (15, 21) and that FOXO3a protects cells from oxidative stress (22, 23). Phosphorylation of FOXO3a by AKT induces the nuclear exclusion of FOXO3a and inhibits its transcriptional activity (21). Our data indicated that the basal level of AKT was unable to phosphorylate FOXO3a or induce FOXO3a nuclear exclusion (Fig. 4A). However, we observed increased phosphorylation and nuclear exclusion of FOXO3a under cisplatin treatment (Fig. 4A) that is most likely through cisplatin-enhanced EGFR and AKT activation (Fig. 3A). In contrast, cisplatin-induced FOXO3a nuclear exclusion was reversed by gefitinib treatment (Fig. 4A). Thus, to determine if FOXO3a plays a role in cisplatin-induced CID, we established H1299 GFP-FOXO3a stable cell lines and observed their response to cisplatin in the presence of z-VAD-fmk (Fig. 4B). As shown in Fig. 4B, CID was attenuated by
overexpression of GFP-FOXO3a. We also observed nuclear exclusion of GFP-FOXO3a after cisplatin treatment, which was reversed by gefitinib (Fig. 4B, right). In contrast, when FOXO3a was knocked down using 2 specific siRNAs for FOXO3a, cisplatin-induced CID was enhanced (Fig. 4C). The mutant FOXO3a (TM) in which 3 phosphorylation sites (21) by AKT were substituted with alanine protected cells from cisplatin-induced CID more effectively than did WT FOXO3a. However, in the presence of TKI, the protective effect of both WT FOXO3a and mutant FOXO3a are similar, suggesting that TKI-mediated dephosphorylation of FOXO3a is critical for the negative effect of TKI on cisplatin (Fig. 4D). Collectively, the results suggest that cisplatin enhances EGFR/AKT kinase activity and that the activated EGFR/AKT further inactivates FOXO3a via phosphorylation.

The role of ROS in cisplatin-induced CID

As mentioned earlier, it has been shown that FOXO3a protects cells from oxidative stress. Both catalase and Mn-SOD, which are known to reduce cellular ROS level, are critical FOXO3a targets for FOXO3a-mediated cytoprotective effect from ROS (17, 18). Because cisplatin is also known to induce ROS (24, 25), which is induced by variety of stress such as etoposide, arsenic trioxide, TNF, and histone deacetylase inhibitor (HDACi; refs. 16, 26–28), we hypothesized that ROS might be involved in cisplatin-induced CID. Indeed, ROS was upregulated in response to cisplatin (Fig. 5A), and the addition of NAC, an antioxidant, inhibited CID but not apoptosis induced by cisplatin (Fig. 5B and C and Supplementary Fig. S7). Moreover, we found that gefitinib decreased cisplatin-induced ROS (Fig. 5A), indicating that TKI inhibits cisplatin-induced CID by...
blocking ROS production. As mentioned earlier, activation of FOXO3a by gefitinib inhibited CID. Two FOXO3a targets, Mn-SOD and catalase (22, 23), which are known to reduce cellular ROS level, were upregulated by inhibiting EGFR-ERK/AKT signaling (Fig. 5D), and knockdown of these FOXO3a targets sensitized the cells to cisplatin-induced CID in the presence of gefitinib (Supplementary Fig. S8). Moreover, knockdown of FOXO3a enhanced cellular ROS level in response to cisplatin (Fig. 5E). In H1299 Bcl-XL xenograft tumors, cisplatin induced FOXO3a phosphorylation and decreased the expression of Mn-SOD, which was reversed by gefitinib (Supplementary Fig. S9). Thus, EGFR signaling contributed to cisplatin-induced CID, at least in part, through ROS upregulation by the inhibition of FOXO3a.

In addition to FOXO3a, we also tested the role of AIF and calpain-1, which are known to function in CID (12, 13, 29, 30), in cisplatin-induced CID by using siRNA. However, knockdown of either AIF or calpain-1 did not attenuate cisplatin-induced CID (data not shown).

Finally, as the above molecular pathway provides a rationale to propose a combination treatment to sensitize cell-killing activity of cisplatin, we tested reagents that enhance CID through upregulation of ROS in combination with cisplatin. For example, both HDACi and erastin have been reported to induce CID through upregulation of ROS (27, 31). We confirmed that SAHA, an HDACi, and erastin indeed increased cellular ROS and enhanced cisplatin-induced CID (Fig. 6A and B). These results further support our model that CID plays a critical role in the efficacy of cisplatin in lung cancer cells (Fig. 6C).

**Discussion**

EGFR TKIs have been shown distinct responses in WT EGFR and mt EGFR lung cancer cells. In WT EGFR lung cancer cells, EGFR TKIs have almost no effects on cell viability. In contrast, mt EGFR lung cancer cells are quite sensitive to EGFR TKIs and frequently undergo apoptosis in response to TKI treatment (11). One potential mechanism by which mt EGFR lung cancer cells undergo apoptosis is
through upregulation of BH3-only proapoptotic Bcl-2 family protein Bim and downregulation of antiapoptotic Bcl-2 family protein Mcl-1 as they have been shown to be critical for TKI-induced apoptosis in mt EGFR lung cancer cells (10). Both WT EGFR and mt EGFR are known to transduce qualitatively distinct signals, such as the AKT and STAT signaling pathways, possibly due to the structural alterations within the catalytic pocket affecting substrate specificity or altered interactions with accessory proteins that modulate EGFR signaling (11). Therefore, the initial mechanism of how TKIs affect patients between WT EGFR and mt EGFR lung cancer may be EGF-mediated autophosphorylation of multiple tyrosine residues linked to activation of distinct downstream effectors. These effectors may regulate Bim and Mcl-1 levels in mt EGFR cells but not in WT EGFR cells, and thus, the inhibition of EGFR in mt EGFR cells may initiate the apoptotic program via Bim/Mcl-1 alteration. Here, we showed EGFR TKI has a negative effect on cisplatin regardless of EGFR status. We have shown that in both WT and mt EGFR cells, gefitinib inhibited cisplatin-induced cell death, while caspase activity was blocked (Fig. 2A and Supplementary Fig. S3A). Therefore, in mt EGFR cells, TKIs inhibited CID and induced apoptosis, whereas in WT EGFR cells, TKIs only inhibited CID in WT EGFR cells without inducing apoptosis (Fig. 6C).

Although we showed here that AKT/ERK-FOXO3a pathways has negative effects on CID, it is quite possible that other downstream target of this signaling are also involved in the negative effects on CID. For example, AKT is the negative regulator of autophagy, via several pathways such...
cells and showed that TKI inhibited CID in this cell line, signaling pathways. However, we used p53-null H1299 study, we did not examine the effect of TKI on these (which are activated by DNA damage, phosphorylates H2AX and cathepsin, respectively (16, 35). Also, ATM/DNA-PK, can induce apoptosis and necrosis by inducing Bax/puma program. For example, DNA damage activates p53, which induces DNA damage, which links to various cell death effects of chemotherapy (34). However, in this study, gefitinib had minimal effect on cell-cycle arrest in the WT EGFR cells used. Thus, cell cycle effects may not cause cells to arrest at G1-phase may explain the mechanism by which TKI negatively affect cisplatin and reduce the cell-cycle-dependent toxicity of chemotherapy (34). However, in this study, gefitinib had minimal effect on cell-cycle arrest in the WT EGFR cells used. Thus, cell cycle effects may not play a major role in the negative effects observed in our experimental condition. It is well known that cisplatin induces DNA damage, which links to various cell death program. For example, DNA damage activates p53, which can induce apoptosis and necrosis by inducing Bax/puma and cathepsin, respectively (16, 35). Also, ATM/DNA-PK, which are activated by DNA damage, phosphorylates H2AX in response to DNA damage, and phosphorylated H2AX (γ-H2AX) interacts with AIF and induces CID (30). In this study, we did not examine the effect of TKI on these signaling pathways. However, we used p53-null H1299 cells and showed that TKI inhibited CID in this cell line, indicating that p53 may not be involved in cisplatin-induced CID. Moreover, we did not observe any significant effects of AIF knockdown on cisplatin-induced CID (data not shown). Although these factors may not be involved in cisplatin-induced CID or TKI-mediated negative effect on CID, it is still plausible that DNA damage response plays a role in these events. Further systematic studies will be required to explore these possibilities.

In this study, we focused on EGFR TKIs that are used for treatment of patients with NSCLC. However, EGFR antibodies, for example, cetuximab and panitumumab, which also inhibit and downregulate EGFR, have been used for patients with colon cancer (cetuximab and panitumumab) or head and neck cancer (cetuximab; refs. 36, 37). Therefore, it would be important to further investigate the effects of EGFR antibodies to chemotherapeutic drugs in these cancer types.

Identification of the underlying mechanisms in EGFR mutation status-dependent response to TKIs as well as the impact of CID in chemotherapy led us to 2 drug candidates, SAHA and erastin, that actually maximized the effect of cisplatin. As mTOR (32), and autophagy has been shown to have negative effects on chemotherapy and protect cells from oxidative stress (33). Thus, the inhibition of AKT by gefitinib activates autophagy, which could be involved in the negative effects on cisplatin. The cytostatic effect of TKIs that cause cells to arrest at G1-phase may explain the mechanism by which TKI negatively affect cisplatin and reduce the cell-cycle-dependent toxicity of chemotherapy (34). However, in this study, gefitinib had minimal effect on cell-cycle arrest in the WT EGFR cells used. Thus, cell cycle effects may not play a major role in the negative effects observed in our experimental condition. It is well known that cisplatin induces DNA damage, which links to various cell death program. For example, DNA damage activates p53, which can induce apoptosis and necrosis by inducing Bax/puma and cathepsin, respectively (16, 35). Also, ATM/DNA-PK, which are activated by DNA damage, phosphorylates H2AX in response to DNA damage, and phosphorylated H2AX (γ-H2AX) interacts with AIF and induces CID (30). In this study, we did not examine the effect of TKI on these signaling pathways. However, we used p53-null H1299 cells and showed that TKI inhibited CID in this cell line, indicating that p53 may not be involved in cisplatin-induced CID. Moreover, we did not observe any significant effects of AIF knockdown on cisplatin-induced CID (data not shown). Although these factors may not be involved in cisplatin-induced CID or TKI-mediated negative effect on CID, it is still plausible that DNA damage response plays a role in these events. Further systematic studies will be required to explore these possibilities.

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Identification of the underlying mechanisms in EGFR mutation status-dependent response to TKIs as well as the impact of CID in chemotherapy led us to 2 drug candidates, SAHA and erastin, that actually maximized the effect of cisplatin on the basis of the pathways discovered in this

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Figure 5. ROS induced by cisplatin plays a critical role in CID and the negative effects of gefitinib. A, A549 Bcl-XL stable cells were treated with cisplatin (Cis) or cisplatin plus gefitinib (Cis + Gef) for 24 hours, and the cellular ROS levels were determined using ROS sensitive fluorescence dye (n = 3). B, A549 Bcl-XL stable cells were treated with 50 μmol/L of cisplatin or cisplatin plus 0.5 mmol/L of NAC for 72 hours, and the viability was determined by Trypan blue dye exclusion assay (n = 3). The cellular morphology is shown on the right. C, A549 or H1299 Bcl-XL stable cells were treated with cisplatin or cisplatin plus NAC, and the long-term effects of the drug were determined as described in Materials and Methods. The relative densities of cells are shown on the right (n = 3). D, A549 Bcl-XL stable cells were treated with cisplatin in the presence of gefitinib (Gef), MEKi, or AKTi and subjected to Western blot analysis with the indicated antibodies. E, A549 Bcl-XL stable cells were transfected with control siRNA (siCont) or siRNA for FOXO3a (siFOXO #1) and then treated with cisplatin. After treating cells for 24 hours, the cellular ROS levels were determined using ROS sensitive fluorescence dye (n = 3).
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Figure 6. SAHA and erastin effectively enhance cisplatin-induced CID. A and B, A549 or H1299 Bcl-XL stable cells were treated with cisplatin (25 μmol/L), SAHA (HDACi; 2.5 μmol/L), erastin (5 μmol/L), or the combination of SAHA plus cisplatin or erastin plus cisplatin. After 48 hours, the cellular morphology was observed and recorded under microscope (A, left, SAHA + cisplatin; right, erastin + cisplatin). The viability was determined by Trypan blue dye exclusion assay (B, n = 3). C, a model of TKI-induced inhibition of killing effect of cisplatin. In both WT and mt EGFR cells, cisplatin induces both CDD (apoptosis) and CID, although CID is dominant (top). In WT EGFR cells, TKI inhibit cisplatin-induced CID but do not affect CDD. Therefore, TKI exhibit minor negative effect on the killing effect of cisplatin. In mt EGFR cells, both TKI and cisplatin induce CDD (apoptosis). Therefore, the combination of TKI and cisplatin exhibits an additive effect that masks TKI-mediated inhibition of CID. SAHA/erastin induces CID, thus they enhance the killing effect of cisplatin in WT EGFR cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: H. Yamaguchi, C.-T. Chen, R. Herbst, M.-C. Hung
Development of methodology: H. Yamaguchi, C.-T. Chen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Yamaguchi, C.-T. Chen, Y.-N. Wang, M.-C. Hsu, S.-S. Chang, H.-W. Ko, R. Herbst
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Yamaguchi, M.-C. Hsu, R. Herbst, M.-C. Hung
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www.aacjrournals.org

Published OnlineFirst January 23, 2013; DOI: 10.1158/1078-0432.CCR-12-2621

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