

Role of Epidermal Growth Factor Receptor Degradation in Cisplatin-Induced Cytotoxicity in Head and Neck Cancer

Aarif Ahsan, Susan M. Hiniker, Susmita G. Ramanand, Shyam Nyati, Ashok Hegde, Abigail Helman, Radhika Menawat, Mahaveer S. Bhojani, Theodore S. Lawrence, and Mukesh K. Nyati

Abstract

Cisplatin and its analogues are the most commonly used agents in the treatment of head and neck squamous cell carcinoma. In this study, we investigated a possible role of epidermal growth factor (EGF) receptor (EGFR) phosphorylation and degradation in cisplatin-induced cytotoxicity. Cisplatin treatment led to an increase in initial EGFR phosphorylation at Y1045, the binding site of ubiquitin ligase, Casitas B-lineage lymphoma (c-Cbl), followed by ubiquitination in the relatively cisplatin-sensitive cell lines. However, cisplatin-resistant cell lines underwent minimal EGFR phosphorylation at the Y1045 site and minimal ubiquitination. We found that EGFR degradation in response to cisplatin was highly correlated with cytotoxicity in seven head and neck cancer cell lines. Pretreatment with EGF enhanced cisplatin-induced EGFR degradation and cytotoxicity, whereas erlotinib pretreatment blocked EGFR phosphorylation, degradation, and cisplatin-induced cytotoxicity. Expression of a mutant Y1045F EGFR, which is relatively resistant to c-Cbl-mediated degradation, in Chinese hamster ovary cells and the UMSCC11B human head and neck cancer cell line protected EGFR from cisplatin-induced degradation and enhanced cell survival compared with wild-type (WT) EGFR. Transfection of WT c-Cbl enhanced EGFR degradation and cisplatin-induced cytotoxicity compared with control vector. These results show that cisplatin-induced EGFR phosphorylation and subsequent ubiquitination and degradation is an important determinant of cisplatin sensitivity. Our findings suggest that treatment with an EGFR inhibitor before cisplatin would be antagonistic, as EGFR inhibition would protect EGFR from cisplatin-mediated phosphorylation and subsequent ubiquitination and degradation, which may explain the negative results of several recent clinical trials. Furthermore, they suggest that EGFR degradation is worth exploring as an early biomarker of response and as a target to improve outcome. *Cancer Res*; 70(7): 2862–9. ©2010 AACR.

Introduction

Cisplatin and its analogues are among the most commonly used and effective agents in the treatment of head and neck cancers (1–4), as well as several other solid tumors, including those of the lung, testis, bladder, and ovary (5–7). Cisplatin induces cytotoxicity through the production of DNA damage caused by the formation of cisplatin-DNA adducts (8), which leads to irreparable DNA damage and, ultimately, cell death.

Cisplatin-DNA adduct formation might trigger downstream effects that could potentiate cisplatin toxicity. One such potential downstream effect of cisplatin-induced

DNA damage that could affect cell survival occurs through the degradation of the epidermal growth factor (EGF) receptor (EGFR). Cancer cell survival is maintained by EGFR independent of its kinase activity. Weihua and colleagues (9) found that the knockdown of EGFR with small interfering RNA led to cell death in an autophagic process. Nishikawa and Donato (10, 11) reported a central role for EGFR expression in cisplatin-mediated cytotoxicity. Furthermore, Niwa and colleagues (12) used an antisense approach to target EGFR to inhibit the growth of head and neck squamous cell carcinoma (HNSCC), finding that treatment with a combination of docetaxel and EGFR antisense oligonucleotides resulted in increased cytotoxicity and reduced tumor volumes compared with monotherapy. Using this approach, Lai and colleagues (13) showed that intratumoral EGFR oligonucleotide injection is safe and resulted in anti-tumor activity in patients with advanced HNSCC. Therefore, it is hypothesized that agents which could induce or potentiate EGFR degradation would sensitize the cells driven by EGFR.

We recently found that treatment of head and neck cancer cells with gemcitabine caused EGFR phosphorylation and subsequent degradation, and that inhibition of EGFR

Authors' Affiliation: Department of Radiation Oncology, University of Michigan, Ann Arbor, Michigan

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

A. Ahsan and S.M. Hiniker contributed equally to this work.

Corresponding Author: Mukesh K. Nyati, Department of Radiation Oncology, University of Michigan, 1301 Catherine Street, 4326C Med Sci I, SPC 5637, Ann Arbor, MI 48109. Phone: 734-936-9163; Fax: 734-763-1581; E-mail: nyati@umich.edu.

doi: 10.1158/0008-5472.CAN-09-4294

©2010 American Association for Cancer Research.

degradation using the proteasome inhibitor MG-132 increased cell survival (14). As cisplatin is the most commonly used chemotherapeutic agent in the treatment of HNSCC, we sought to understand the possible role of EGFR phosphorylation and degradation in cisplatin-induced cytotoxicity using specific genetic techniques rather than potentially nonspecific proteasomal inhibitors. We found that treatment with cisplatin, like gemcitabine, led to an increase in EGFR phosphorylation followed by degradation in sensitive cell lines, and that this degradation strongly correlates with cytotoxicity. We then investigated the implications of cisplatin-induced EGFR degradation in the rational design of combination therapy involving EGFR inhibitors and chemotherapy.

Materials and Methods

Reagents. Cisplatin was acquired from Bedford Laboratories. Phosphorylated EGFR (pY845EGFR), c-Cbl, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Cell Signaling Technology. Phosphorylated Y1045 (pY1045EGFR) antibody was obtained from Millipore. EGFR (Sc-03) and ubiquitin antibodies were acquired from Santa Cruz Biotechnology. Erlotinib was kindly provided by Genentech. EGF was obtained from Sigma.

Cell culture. The Chinese hamster ovary (CHO) cell line was purchased from the American Type Culture Collection. The human HNSCC cell lines UMSSC1, 10B, 11B, 12, 17B, 29, and 33, and cervical squamous cell carcinoma ME-180Pt cells were kindly provided by Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI). All cell lines were grown in RPMI 1640 supplemented with 10% cosmic calf serum (Hyclone). For all *in vitro* experiments, cells were released from flasks using PBS containing 0.01% trypsin and 0.20 mmol/L of EDTA, and 6×10^5 cells were plated onto 100-mm culture dishes 2 d before any treatment.

Immunoblotting. Cells were scraped into PBS containing a sodium orthovanadate and protease inhibitor mixture (Roche Diagnostic, Co.). Cells were incubated for 15 min on ice in Laemmli buffer [63 mmol/L Tris-HCl, 2% (w/v) SDS, 10% (v/v) glycerol, and 0.005% (w/v) bromphenol blue] containing 100 mmol/L of NaF, 1 mmol/L of Na_3VO_4 , 1 mmol/L of phenylmethylsulfonyl fluoride, and 1 $\mu\text{g}/\text{mL}$ of aprotinin. After sonication, particulate material was removed by centrifugation at 13,000 rpm for 15 min at 4°C. The soluble protein fraction was heated to 95°C for 5 min, then applied to a 4% to 12% bis-tris precast gel (Invitrogen) and transferred onto a polyvinylidene difluoride membrane. Membranes were incubated for 1 h at room temperature in blocking buffer consisting of 3% bovine serum albumin and 1% normal goat serum in TBS [137 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.6), and 0.1% (v/v) Tween 20]. Membranes were subsequently incubated overnight at 4°C with 1 $\mu\text{g}/\text{mL}$ of primary antibody in blocking buffer, washed, and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (Cell Signaling). After three additional washes in TBS, bound antibody was detected by enhanced chemiluminescence plus

reagent (Amersham Biosciences). For quantification of relative protein levels, immunoblot films were scanned and analyzed using the ImageJ 1.32j software (NIH). Unless otherwise indicated, the relative protein levels shown represent a comparison with nontreated controls.

Immunoprecipitation. Following treatments, cells were trypsinized, washed twice with $1 \times$ PBS, and cell lysates were prepared by incubation for 30 min on ice in fresh lysis buffer [1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.15 mol/L sodium chloride, 0.01 mol/L sodium phosphate (pH 7.2), 1 mmol/L phenylmethylsulfonyl fluoride, 2 $\mu\text{g}/\text{mL}$ aprotinin, 0.2 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, 2 mmol/L EDTA, and 20 mmol/L ammonium molybdate]. Immunoprecipitation of EGFR was performed as previously described (14).

Measurement of mRNA levels of EGFR in cells treated with cisplatin. Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen) according to the instructions of the manufacturer. RNA (1 μg) was reverse transcribed to cDNA using the High Capacity cDNA Archive kit (Applied Biosystems) and purified (Millipore Centrifugal Filter Units). Diluted cDNA was used to amplify GAPDH (F, 5'-GAG TCA ACG GAT TTG GTC GT-3'; and R, 5'-TTG ATT TTG GAG GGA TCT CG-3') and EGFR (F, 5'-CTC AGC CAC CCA TAT GTA CC-3'; and R, 5'-CGT CCA TGT CTT CTT CAT CC-3') by quantitative reverse-transcription PCR using SYBR green chemistry (Applied Biosystems). The PCR products were resolved by electrophoresis on 1.5% agarose gels and melting curve analysis was carried out to confirm the specificity of the product. The $\delta\delta\text{Ct}$ method was used to analyze the data as described (15, 16) and GAPDH was used as control.

Clonogenic cell survival assay. Clonogenic assays were performed using standard techniques (17). The fraction surviving each treatment was normalized to the survival of the control cells. Cisplatin cell survival curves were fitted using the equation $\text{SF} = (C_{50})^m / [(C_{50})^m + C^m]$, in which SF is the surviving fraction, C is the cisplatin concentration, C_{50} is the concentration of cisplatin that produces a 50% cell survival, and m is the slope of the sigmoid curve. The effects of EGF, or erlotinib, on cisplatin-induced clonogenic death were calculated by comparing the ratio of the areas under the respective cell survival curves.

Plasmid constructions and transfections. A site-directed mutagenesis approach was used to create the Y1045F substitution in the pEYFP-N1 vector. The mutation was confirmed by sequencing the plasmid in the DNA sequencing core at the University of Michigan. Wild-type (WT) EGFR in the pEYFP-N1 vector was provided by Dr. Zhixiang Wang (University of Alberta, Edmonton, Alberta, Canada). The WT c-Cbl construct was provided by Dr. Nancy Lill (University of Iowa, Iowa City, Iowa). UMSSC11B and CHO cells were transiently transfected with the constructs using Lipofectamine (Invitrogen) according to the instructions of the manufacturer.

Live cell imaging. Live cell imaging of CHO and UMSSC11B cells transfected with WT EGFR and Y1045F EGFR constructs was performed using an Olympus DP70 camera fitted in an Olympus IX-71 microscope. All the images were captured at $\times 60$ magnification and processed

similarly in Adobe Photoshop by removing the unsharp mask.

Statistics. Results are presented as mean \pm SEM of at least three experiments. Student's *t* test was used to assess the statistical significance of differences. A significance level threshold of $P < 0.05$ was used.

Results

EGFR degradation in HNSCC cell lines correlates with cisplatin sensitivity. Our initial goal was to determine if there was a correlation between cisplatin-induced cytotoxicity and EGFR degradation. Cisplatin cytotoxicity was assessed by clonogenic survival analysis in seven HNSCC cell lines, UMSCC1, 10B, 11B, 12, 17B, 29, 33, and in the cisplatin-resistant cervical carcinoma ME-180Pt cell line. We selected ME-180Pt cells for use along with the head and neck cancer cell lines because platinum resistance in this cervical cancer line has previously been reported to be associated with higher expression of EGFR (10, 11). Our results revealed that the UMSCC17B and 11B lines were the most sensitive,

and UMSCC1 and ME-180Pt were the most resistant, with the other cell lines displaying intermediate sensitivity (Fig. 1A). Based on these results and clinically achievable levels of cisplatin (18), we selected a 2-hour exposure to 10 μ mol/L of cisplatin to assess the effects of cisplatin treatment on EGFR protein level by immunoblotting relative to nontreated controls in each of the eight cell lines. We found a direct relationship between relative EGFR levels at 72 hours and surviving fraction after cisplatin treatment ($R^2 = 0.97$; Fig. 1A). For example, in response to 10 μ mol/L of cisplatin, the relatively cisplatin-sensitive UMSCC11B cell line showed a 95% decrease in EGFR levels at 72 hours, whereas the relatively cisplatin-resistant ME-180Pt line showed almost no change in EGFR levels at 72 hours (Fig. 1B).

We then sought to determine whether cisplatin, like gemcitabine, caused initial EGFR phosphorylation followed by subsequent degradation in head and neck cancer cell lines. We chose UMSCC11B as a representative cisplatin-sensitive and UMSCC1 and ME-180Pt as cisplatin-resistant cell lines for further studies. We observed increased phosphorylation of EGFR at both the Y845 and Y1045 positions in (sensitive)

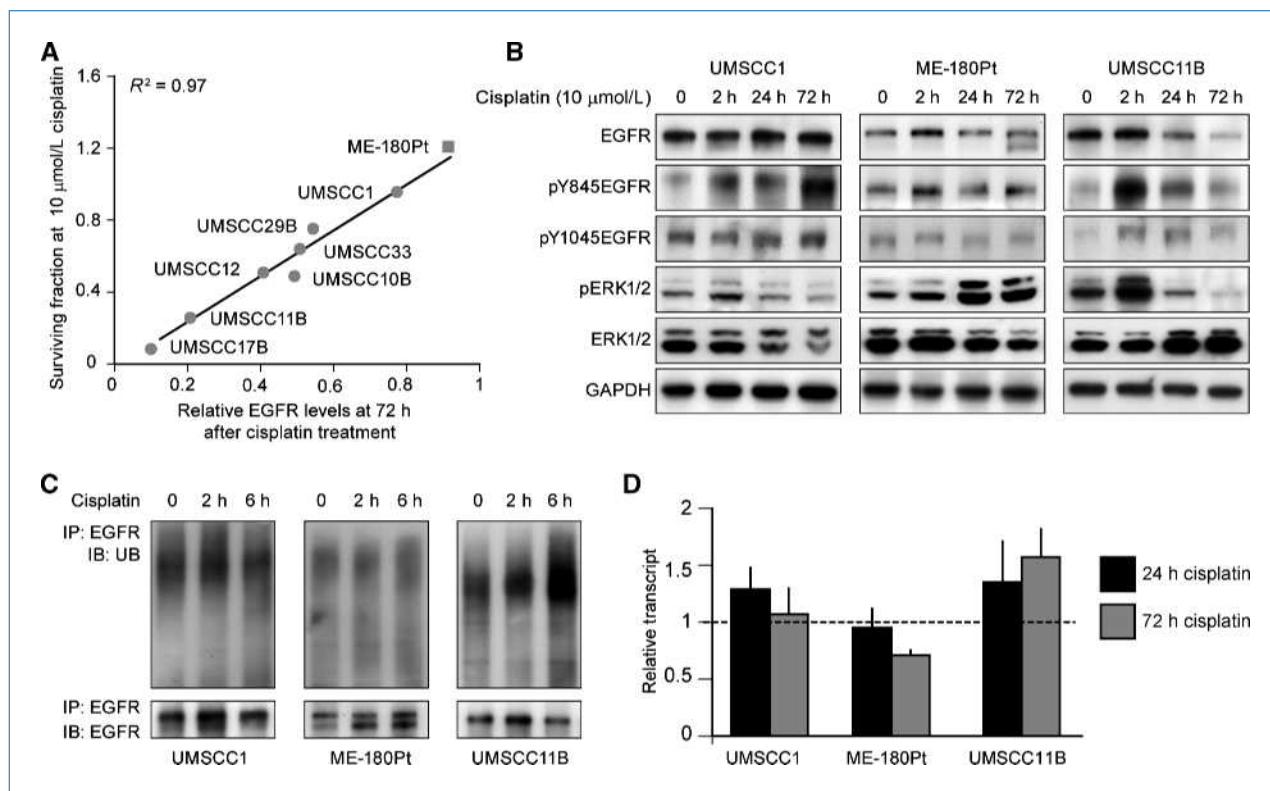


Figure 1. Effect of cisplatin on cell survival and EGFR degradation in various cell lines. Clonogenic survival assays were performed on eight cell lines (UMSCC1, 10B, 11B, 12, 17B, 29B, 33, and ME-180Pt) to assess cisplatin-induced cytotoxicity. Each cell line was exposed to 10 μ mol/L of cisplatin for 2 h, then either plated for clonogenic survival or harvested at the indicated time points and immunoblotted for various total and phosphorylated proteins and GAPDH (as a loading control). A, densitometric analysis was performed for total EGFR levels; ratios of EGFR protein levels at 72 h compared with nontreated controls were plotted against survival fractions as assessed by colony formation assay. B, representative immunoblots of EGFR, pY845EGFR, pY1045EGFR, pERK1/2, tERK1/2, and GAPDH from UMSCC1, ME-180Pt, and UMSCC11B cell lines. C, UMSCC1, ME-180Pt, and UMSCC11B cells were treated with 10 μ mol/L of cisplatin for 2 h and were immunoprecipitated (IP) for EGFR, then immunoblotted (IB) with ubiquitin at the indicated times. D, after treatment with 10 μ mol/L of cisplatin for 2 h, RNA was extracted at 24 and 72 h from UMSCC1, ME-180Pt, and UMSCC11B cells and real-time PCR analysis for EGFR and GAPDH was performed. EGFR transcript levels were normalized to GAPDH levels at the same time point.

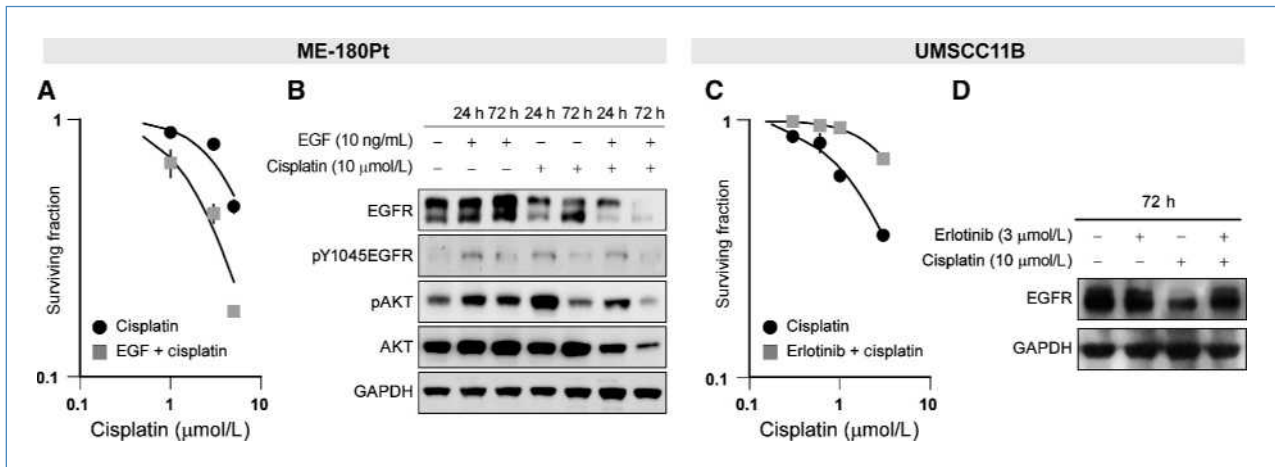


Figure 2. Promotion of EGFR phosphorylation facilitates cisplatin-induced EGFR degradation, whereas inhibition of phosphorylation protects against degradation. ME-180Pt cells were stimulated with EGF (10 ng/mL) for 30 min and then treated with various doses of cisplatin for 2 h; medium was replaced with drug-free medium and cells were plated for clonogenic survival assays (A) or harvested for immunoblotting at the indicated times (B). UMSSC11B cells were treated with erlotinib (3 μ mol/L) for 2 h and then treated with various doses of cisplatin for 2 h. Medium was replaced with drug-free medium, and cells were plated for clonogenic survival assays (C), or harvested at the indicated time points and immunoblotted for EGFR and GAPDH (D).

UMSSC11B cells. We also noted a moderate increase in the phosphorylation of EGFR at the Y845 site, but little to no increase in phosphorylation of the Y1045 site in (resistant) UMSSC1 and ME-180Pt cells. The pY845 and pY1045 EGFR levels decreased by 72 hours in the cisplatin-sensitive UMSSC11B cell line. However, in cisplatin-resistant UMSSC1 and ME-180Pt, pY845 EGFR levels remained high at 72 hours. These results showed that the phosphorylation of EGFR at Y1045 was more strongly correlated with cisplatin cytotoxicity than the Y845 site and suggested to us that the Y1045 site might be critical for the eventual degradation of EGFR in response to cisplatin. To determine if this observed EGFR phosphorylation affected signaling, we analyzed the downstream mediators of EGFR signaling in these cell lines. As anticipated, pERK levels were significantly increased in UMSSC11B by 2 hours and then decreased at 72 hours in a similar manner to pEGFR levels. In cisplatin-resistant UMSSC1 and ME-180Pt, we noticed an initial moderate increase in pERK levels, which did not decrease appreciably by 72 hours, also consistent with pEGFR levels.

Role of EGFR ubiquitination in cisplatin-induced EGFR degradation. To determine whether the decrease in EGFR levels was due to decreased production or increased degradation, we assessed ubiquitination of EGFR at 2 and 6 hours after treatment with cisplatin in cisplatin-sensitive and cisplatin-resistant cell lines. In the sensitive cell line UMSSC11B, we observed EGFR binding to ubiquitin in response to cisplatin (Fig. 1C), whereas in the relatively resistant cell line ME-180Pt, there was no increase in ubiquitination of EGFR at 2 and 6 hours after cisplatin treatment. This lack of ubiquitination was congruent with the lack of EGFR degradation in these cells. UMSSC1 cells showed a slight increase in ubiquitinated EGFR at 6 hours, but not as substantial as was observed in UMSSC11B cells. Therefore, ubiquitination of EGFR in the three cell lines was directly

correlated with levels of Y1045 phosphorylation of EGFR in response to cisplatin. We further carried out EGFR transcript analysis in UMSSC1, ME-180Pt, and UMSSC11B cells. Real-time PCR results showed an increase in EGFR transcript levels in response to cisplatin at both 24 and 72 hours compared with controls in UMSSC11B (Fig. 1D). These results suggested that the decrease in EGFR levels was occurring at the protein level, and its transcript level might be undergoing a partially compensatory upregulation. In the case of UMSSC1 and ME-180Pt, EGFR transcript levels did not increase at 24 and 72 hours but in fact slightly decreased for ME-180Pt (Fig. 1D). We also analyzed the effects of cisplatin on EGFR degradation after blocking protein synthesis using cyclohexamide (50 μ g/mL) in UMSSC11B cells. EGFR degradation was more rapid in the case of cells treated with the combination of cisplatin and cyclohexamide compared with cyclohexamide alone (Supplementary Fig. S1), confirming that EGFR degradation was occurring earlier than it was detected through immunoblotting, and suggesting that the increase in transcription of EGFR was partially compensating for the loss of protein.

Effects of EGF and erlotinib on cisplatin-induced EGFR degradation. We next hypothesized that if EGFR phosphorylation were critical for its eventual degradation, then pretreatment with EGF would enhance cisplatin-induced EGFR degradation whereas erlotinib would protect EGFR from degradation. For these experiments, ME-180Pt (resistant) cells were treated with EGF (10 ng/mL) for 1 hour before cisplatin was added to the cells. As we anticipated, EGF induced rapid EGFR degradation by 24 hours after treatment with cisplatin (Fig. 2A). Cell survival data for EGF and cisplatin combination showed a significant decrease in clonogenic survival with combined cisplatin and EGF compared with cisplatin alone in ME-180Pt cells (Fig. 2B). Conversely, pretreatment of UMSSC11B (sensitive) cells with erlotinib (3 μ mol/L) for

2 hours before the addition of cisplatin protected EGFR from cisplatin-induced degradation at 72 hours, supporting a critical role for EGFR phosphorylation in cisplatin-induced EGFR degradation (Fig. 2C). Likewise, pretreatment with erlotinib significantly protected UMSCC11B cells from cisplatin-induced cytotoxicity (Fig. 2D). Erlotinib pretreatment also protected UMSCC1 cells from cisplatin-induced cytotoxicity (Supplementary Fig. S2). These findings emphasize the important role of initial EGFR phosphorylation followed by degradation in cisplatin cytotoxicity.

Effects of Y1045F EGFR construct on cisplatin-induced EGFR degradation and cytotoxicity in CHO cells. We next hypothesized that blocking EGFR phosphorylation at the Y1045 residue, which is necessary for binding with the ubiquitin ligase c-Cbl and thus required for EGFR degradation, would protect cells from cisplatin-induced toxicity. We initially tested our hypothesis in a system with no endogenous

expression of EGFR to avoid the confounding effects of native EGFR. We selected CHO cells that were EGFR negative (19) but upon transfection with EGFR constructs are able to not only express EGFR but also to activate normal downstream signaling events (20, 21). We used WT EGFR and Y1045F EGFR constructs in the NI-EYFP vector backbone to monitor changes in EGFR levels and localization in response to cisplatin in live cells. CHO cells were transfected with either WT EGFR or Y1045F EGFR constructs, treated with cisplatin, and assessed for EGFR levels at 24 and 72 hours after treatment (Fig. 3A). We detected a more rapid disappearance of WT EGFR when compared with Y1045F EGFR, verifying that the Y1045 site is important for EGFR degradation. Immunoblotting data also confirmed these results (Fig. 3A, middle). We then assessed the role of EGFR degradation in cisplatin toxicity. As expected, expression of the Y1045F EGFR construct in CHO cells induced significant

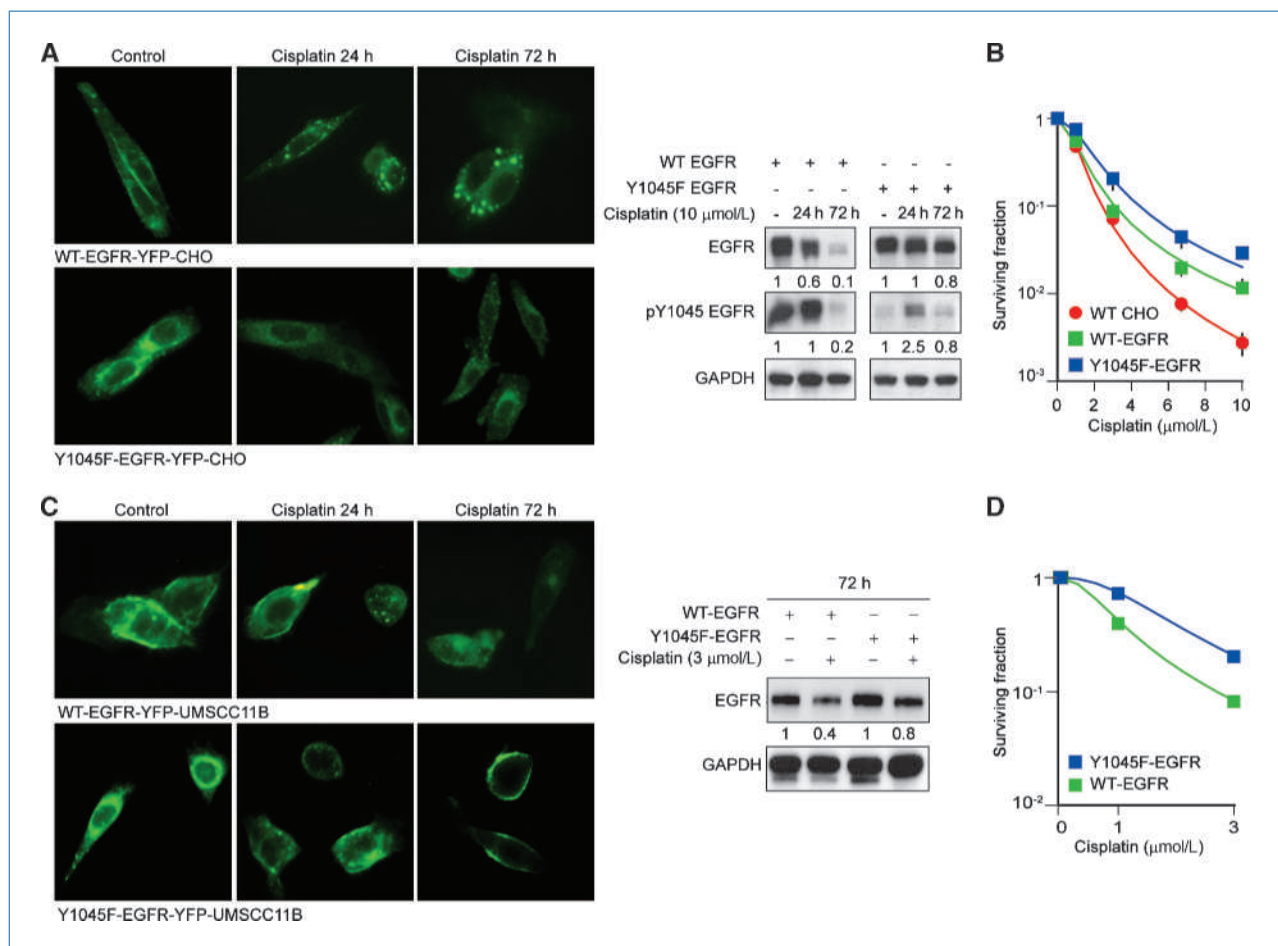
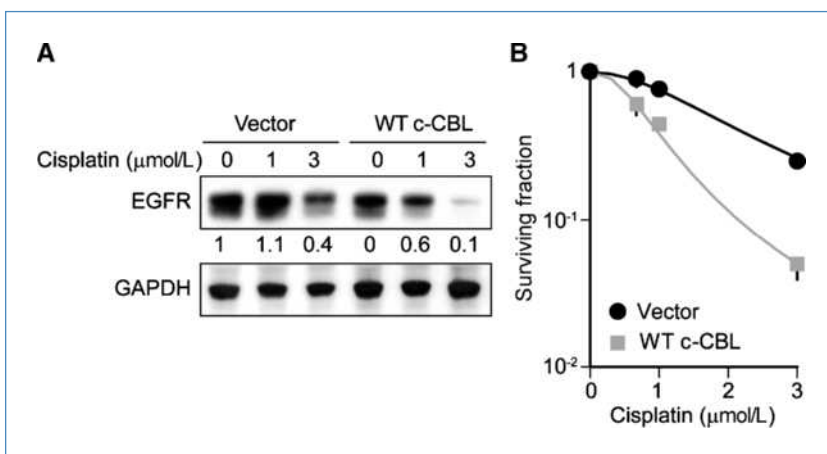


Figure 3. Effect of Y1045F expression on cisplatin-induced EGFR degradation and cytotoxicity in CHO and UMSCC11B cells. Full-length WT and Y1045F mutant forms of EGFR were transfected into CHO or UMSCC11B cells. A, CHO cells were treated with cisplatin (10 $\mu\text{mol/L}$) for 2 h and representative images were taken at 2, 24, and 72 h or harvested on either day 1 or day 3 for immunoblotting. B, CHO cells were treated with various doses of cisplatin, harvested on day 1, and plated for colony formation assay. The dose-modifying factor is defined as the area under the curve of mutant over WT EGFR-transfected cells. C, UMSCC11B cells were transfected with the Y1045F and WT EGFR constructs. Cells were treated with cisplatin (3 $\mu\text{mol/L}$) for 2 h and images were taken at 2, 24, and 72 h or harvested for immunoblotting at 72 h. D, UMSCC11B cells were treated with various doses of cisplatin, harvested on day 1, and plated for colony formation assay.

Figure 4. Effect of ectopic overexpression of c-Cbl on EGFR degradation and cytotoxicity. UMSCC11B cells were transfected with either control vector or WT c-Cbl. Twenty-four hours after transfection, cells were treated with 1 or 3 $\mu\text{mol/L}$ doses of cisplatin and (A) processed for immunoblotting with EGFR and GAPDH or (B) 24 h posttransfection with these two vectors; UMSCC11B cells were treated with various doses of cisplatin, and 24 h after treatment, cells were plated for clonogenic survival assay.



protection from cisplatin cytotoxicity compared with WT EGFR-expressing cells (dose modifying factor, 1.5; Fig. 3B).

Effects of Y1045F EGFR construct on cisplatin-induced EGFR degradation and cytotoxicity in UMSCC11B cells. Next, we sought to extend our findings on the critical role of the 1045 phosphorylation of EGFR to a head and neck cancer cell line. We transfected UMSCC11B cells with WT or Y1045F EGFR constructs and treated cells with cisplatin to monitor the effects on transfected EGFR in these cells. Similar to our results in CHO cells, we found a more rapid decrease in WT EGFR compared with Y1045F EGFR in response to cisplatin (Fig. 3C). In addition, although the high endogenous levels of EGFR make it more difficult to detect a difference in EGFR levels after transfection of the mutant, we found that Y1045F-transfected cells were protected from cisplatin-induced EGFR degradation (Fig. 3C). Importantly, cells expressing the Y1045F EGFR construct were significantly protected from cisplatin-induced cytotoxicity (Fig. 3D). These results, especially when combined with those obtained in the CHO system described above, strengthen the importance of Y1045 phosphorylation of EGFR in cisplatin-induced EGFR degradation and resulting cytotoxicity.

Effects of WT c-Cbl overexpression on cisplatin-induced EGFR degradation and cytotoxicity in UMSCC11B cells. To further assess the role of EGFR degradation in cisplatin-induced cytotoxicity in head and neck cancer, we decided to accelerate ubiquitination by enhancing the ability of c-Cbl to bind EGFR. Therefore, we transfected UMSCC11B cells with a control vector and WT c-Cbl constructs and assessed the effects on EGFR degradation and cytotoxicity. We found that cells transfected with WT c-Cbl showed enhanced EGFR degradation compared with control vector-transfected cells (Fig. 4A). WT c-Cbl-transfected cells had a greater sensitivity to cisplatin than cells transfected with control vector construct (Fig. 4B). We further found that after cisplatin treatment, WT c-Cbl-expressing cells underwent increased ubiquitination of EGFR compared with cells expressing the control vector (data not shown). These results confirm that a sufficient degree of EGFR degradation (in this case, achieved by enhancing c-Cbl activity to degrade ~90% of EGFR) increases cisplatin-mediated cytotoxicity.

Discussion

In this study, we show that cisplatin-induced cytotoxicity in head and neck cancer is critically dependent on the phosphorylation and subsequent degradation of EGFR. This conclusion is supported by our findings that (a) decreased EGFR levels after cisplatin treatment correlate with decreased clonogenic survival; (b) stimulation of EGFR phosphorylation and degradation (by treatment with EGF) before cisplatin treatment increases cytotoxicity; and (c) inhibition of EGFR phosphorylation and degradation (by pretreatment with erlotinib) before cisplatin diminishes cytotoxicity. Using distinct genetic methods of preventing and accelerating EGFR degradation, we then confirmed that preventing degradation decreases cisplatin cytotoxicity, whereas promoting EGFR degradation increases cisplatin cytotoxicity. These findings elucidate a new step in the process by which cisplatin produces cell death and suggest that enhancing EGFR degradation may be a novel way of overcoming cisplatin resistance in head and neck cancer cells. Furthermore, our findings provide insight into maximizing the therapeutic benefit of EGFR inhibitors when used in combination with chemotherapy.

Substantial evidence now shows that radiation and chemotherapy induce EGFR phosphorylation and activation of downstream survival pathways. For example, Benhar and colleagues (22) reported that cisplatin-induced EGFR phosphorylation coincides with DNA adduct formation, suggesting that a DNA damage sensor activates (directly or indirectly) EGFR and downstream cell survival pathways. Others have suggested that cisplatin induces EGFR activation through a p38 mitogen-activated kinase and that this activation leads to downstream effectors such as protein kinase B/AKT (23). In addition, phosphorylated EGFR could undergo nuclear translocation and interact with DNA protein kinase (24) and could also mediate DNA repair (25). Likewise, ionizing radiation produces EGFR activation and nuclear translocation, which stimulates repair mechanisms (24). Nuclear localization of EGFR has also been found to be important for its role as a transcription factor that activates genes required for cell growth and proliferation (26). Inhibition of this activation of

downstream survival and repair signals by treatment with an EGFR inhibitor *after* chemotherapy causes dramatic potentiation of cell death (14, 27), which has clinical implications as described below.

In contrast to EGFR activation after chemotherapy and radiation, the role of treatment-induced EGFR degradation has been much less appreciated. Our prior finding that gemcitabine causes EGFR degradation and cell death (14) and the results of our current study show that radiation¹ and chemotherapy-induced degradation causes cell death downstream of the initial DNA damage. Our findings are in agreement with a recent study showing that the loss of EGFR could induce autophagic cell death (9) and that antisense oligonucleotides against EGFR could produce clinical responses (13). Thus, the degradation of EGFR might decrease the ability of cells to repair damaged DNA, leading to increased cytotoxicity.

Our studies are consistent with several preclinical studies that have shown a direct correlation between EGFR activation and sensitivity to cisplatin (28–31). In particular, Christen and colleagues (32) reported that the stimulation of ovarian carcinoma cells with EGF sensitized cells to cisplatin, independent of the mitogenic effects of EGF. Similarly, Nishikawa and colleagues (10) found that the cisplatin-resistant cervical carcinoma cell line ME-180Pt (used in our study) was sensitized to cisplatin by EGF pretreatment. Although the mechanism by which EGF sensitized cells to cisplatin was not elucidated, we would hypothesize that EGF increased EGFR activation and subsequent degradation.

As we now know that both cisplatin treatment and EGF stimulation could cause EGFR phosphorylation leading to receptor degradation, it is of interest to compare the mechanisms of these two processes. EGFR is internalized and degraded following stimulation with EGF through binding with c-Cbl at the phosphorylated Y1045-EGFR residue (33), which is a docking site for the ubiquitin ligase c-Cbl (33, 34). Similarly, in response to treatment with cisplatin, we found that EGFR is phosphorylated at the Y1045 site, ubiquitinated and degraded through c-Cbl, which occurs in degradation after ligand binding. Thus, it seems that EGFR phosphorylation, ubiquitination, and degradation after EGF stimulation resemble the results from cisplatin treatment, although differences might still emerge with further study.

Irrespective of the exact mechanism of cisplatin-induced EGFR degradation, our results suggest that the schedule of EGFR inhibition and chemotherapy could have a crucial effect on the effectiveness of therapy. We have found that the activation of EGFR is necessary for its subsequent degradation and resultant chemosensitization. Under these conditions, the addition of an EGFR inhibitor before cisplatin treatment *prevents* EGFR activation, thereby resulting in a cytoprotective effect. In contrast, treatment with chemotherapy or radiation first, leading to EGFR activation, followed by an EGFR inhibitor (depriving the cell of downstream “life” and DNA repair signaling) stimulates degradation and has a synergistic cytotoxic effect (27, 35). This is consistent with

previous studies examining combination treatment with EGFR inhibitors and chemotherapy, in which it has been suggested that EGFR phosphorylation in response to chemotherapeutic agents (29, 36) or antimetabolites (37, 38) is necessary for the chemosensitization benefit of added EGFR inhibitors. Thus, our findings might provide the underlying biological rationale to the paradoxical clinical observations that although EGFR inhibitors after chemotherapy could increase treatment efficacy, the converse schedule could actually be antagonistic (39).

It may be possible to develop new methods of potentiating cisplatin-induced cytotoxicity by discovering new ways of increasing cisplatin-induced EGFR degradation. Under conditions of cellular stress, the molecular chaperone heat shock protein 90 (Hsp90) regulates the stability of several oncoproteins (40). Yang and colleagues (41) have reported that the association of mutant EGFR with Hsp90 decreases its downregulation mediated by c-Cbl binding. It is also known that ErbB-2 can bind to both EGFR and Hsp90 directly. Whether an EGFR-Hsp90 interaction (directly or through ErbB-2) is involved in cisplatin-induced EGFR activation and degradation is not known at this time. In addition to EGFR and stress-related molecular interactions, it would also be interesting to explore if the interaction between EGFR and DNA-PK plays a role in cisplatin-induced cell death.

In summary, we show here that EGFR phosphorylation and degradation is a determinant of cisplatin cytotoxicity in head and neck cancer cells, and that this degradation is mediated by c-Cbl. At present, the mechanism of cisplatin-induced signaling in EGFR degradation is not known. Regardless of the mechanism of degradation, our results suggest that the incorporation of EGFR inhibitors into therapy may be most effective following cisplatin administration and may even be antagonistic if given before cisplatin. Thus, our results indicate that EGFR inhibitors should be used after chemotherapy to facilitate EGFR degradation and chemosensitization. These findings also indicate that agents which facilitate EGFR degradation should be explored in overcoming cisplatin resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dipankar Ray for helpful discussion, Mary Davis for her careful review of the manuscript, and Steven Kronenberg for assistance in making the figures.

Grant Support

NIH R01CA131290, NIH through the University of Michigan Head and Neck Specialized Program of Research Excellence grant P50 CA097248, Michigan Institute for Clinical and Health Research, University of Michigan Cancer Center support grant 5 P30 CA46592 (M.K. Nyati), and a Howard Hughes Medical Institute training fellowship (S.M. Hiniker).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 11/24/2009; revised 12/28/2009; accepted 01/07/2010; published OnlineFirst 03/09/2010.

¹ Unpublished data.

References

- The Department of Veterans Affairs Laryngeal Cancer Study Group. Induction chemotherapy plus radiation compared with surgery plus radiation in patients with advanced laryngeal cancer. *N Engl J Med* 1991;324:1685–90.
- Adelstein DJ, Li Y, Adams GL, et al. An intergroup phase III comparison of standard radiation therapy and two schedules of concurrent chemoradiotherapy in patients with unresectable squamous cell head and neck cancer. *J Clin Oncol* 2003;21:92–8.
- Glicksman AS, Slotman G, Doolittle C III, et al. Concurrent cis-platinum and radiation with or without surgery for advanced head and neck cancer. *Int J Radiat Oncol Biol Phys* 1994;30:1043–50.
- Samant S, Kumar P, Wan J, et al. Concomitant radiation therapy and targeted cisplatin chemotherapy for the treatment of advanced pyriform sinus carcinoma: disease control and preservation of organ function. *Head Neck* 1999;21:595–601.
- Zamble DB, Lippard SJ. Cisplatin and DNA repair in cancer chemotherapy. *Trends Biochem Sci* 1995;20:435–9.
- Wong E, Giandomenico CM. Current status of platinum-based antitumor drugs. *Chem Rev* 1999;99:2451–66.
- Kelland L. The resurgence of platinum-based cancer chemotherapy. *Nat Rev* 2007;7:573–84.
- Takahara PM, Rosenzweig AC, Frederick CA, Lippard SJ. Crystal structure of double-stranded DNA containing the major adduct of the anticancer drug cisplatin. *Nature* 1995;377:649–52.
- Weihua Z, Tsan R, Huang WC, et al. Survival of cancer cells is maintained by EGFR independent of its kinase activity. *Cancer Cell* 2008;13:385–93.
- Nishikawa K, Rosenblum MG, Newman RA, Pandita TK, Hittelman WN, Donato NJ. Resistance of human cervical carcinoma cells to tumor necrosis factor correlates with their increased sensitivity to cisplatin: evidence of a role for DNA repair and epidermal growth factor receptor. *Cancer Res* 1992;52:4758–65.
- Donato NJ, Perez M, Kang H, Siddik ZH, Ling YH, Perez-Soler R. EGF receptor and p21WAF1 expression are reciprocally altered as ME-180 cervical carcinoma cells progress from high to low cisplatin sensitivity. *Clin Cancer Res* 2000;6:193–202.
- Niwa H, Wentzel AL, Li M, Gooding WE, Lui VW, Grandis JR. Antitumor effects of epidermal growth factor receptor antisense oligonucleotides in combination with docetaxel in squamous cell carcinoma of the head and neck. *Clin Cancer Res* 2003;9:5028–35.
- Lai SY, Koppikar P, Thomas SM, et al. Intratumoral epidermal growth factor receptor antisense DNA therapy in head and neck cancer: first human application and potential antitumor mechanisms. *J Clin Oncol* 2009;27:1235–42.
- Feng FY, Varambally S, Tomlins SA, et al. Role of epidermal growth factor receptor degradation in gemcitabine-mediated cytotoxicity. *Oncogene* 2007;26:3431–9.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-ΔΔC(T)) method. *Methods* 2001;25:402–8.
- Tomlins SA, Rhodes DR, Perner S, et al. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science* 2005;310:644–8.
- Nyati MK, Maheshwari D, Hanasoge S, et al. Radiosensitization by pan ErbB inhibitor CI-1033 *in vitro* and *in vivo*. *Clin Cancer Res* 2004;10:691–700.
- Himmelstein KJ, Patton TF, Belt RJ, Taylor S, Repta AJ, Sternson LA. Clinical kinetics on intact cisplatin and some related species. *Clin Pharmacol Ther* 1981;29:658–64.
- Krug AW, Schuster C, Gassner B, et al. Human epidermal growth factor receptor-1 expression renders Chinese hamster ovary cells sensitive to alternative aldosterone signaling. *J Biol Chem* 2002;277:45892–7.
- Ullrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. *Cell* 1990;61:203–12.
- Livneh E, Prywes R, Kashles O, et al. Reconstitution of human epidermal growth factor receptors and its deletion mutants in cultured hamster cells. *J Biol Chem* 1986;261:12490–7.
- Benhar M, Engelberg D, Levitzki A. Cisplatin-induced activation of the EGF receptor. *Oncogene* 2002;21:8723–31.
- Winograd-Katz SE, Levitzki A. Cisplatin induces PKB/Akt activation and p38(MAPK) phosphorylation of the EGF receptor. *Oncogene* 2006;25:7381–90.
- Dittmann K, Mayer C, Fehrenbacher B, et al. Radiation-induced epidermal growth factor receptor nuclear import is linked to activation of DNA-dependent protein kinase. *J Biol Chem* 2005;280:31182–9.
- Li L, Wang H, Yang ES, Arteaga CL, Xia F. Erlotinib attenuates homologous recombinational repair of chromosomal breaks in human breast cancer cells. *Cancer Res* 2008;68:9141–6.
- Lin SY, Makino K, Xia W, et al. Nuclear localization of EGF receptor and its potential new role as a transcription factor. *Nat Cell Biol* 2001;3:802–8.
- Chun PY, Feng FY, Scheurer AM, Davis MA, Lawrence TS, Nyati MK. Synergistic effects of gemcitabine and gefitinib in the treatment of head and neck carcinoma. *Cancer Res* 2006;66:981–8.
- Nouri AM, Zubairi ST, Russell MV, et al. Concordance between tumor cell activation by epidermal growth factor and alteration of cell sensitivity to cisplatin and lymphokine-activated killer cell activity. *Oncol Rep* 2000;7:197–201.
- Van Schaeybroeck S, Kyula J, Kelly DM, et al. Chemotherapy-induced epidermal growth factor receptor activation determines response to combined gefitinib/chemotherapy treatment in non-small cell lung cancer cells. *Mol Cancer Ther* 2006;5:1154–65.
- Benedetti V, Perego P, Luca Beretta G, et al. Modulation of survival pathways in ovarian carcinoma cell lines resistant to platinum compounds. *Mol Cancer Ther* 2008;7:679–87.
- Muscella A, Urso L, Calabriso N, et al. Functions of epidermal growth factor receptor in cisplatin response of thyroid cells. *Biochem Pharmacol* 2009;77:979–92.
- Christen RD, Hom DK, Porter DC, et al. Epidermal growth factor regulates the *in vitro* sensitivity of human ovarian carcinoma cells to cisplatin. *J Clin Invest* 1990;86:1632–40.
- Levkowitz G, Waterman H, Ettenberg SA, et al. Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. *Mol Cell* 1999;4:1029–40.
- Levkowitz G, Waterman H, Zamir E, et al. c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev* 1998;12:3663–74.
- Azzariti A, Xu JM, Porcelli L, Paradiso A. The schedule-dependent enhanced cytotoxic activity of 7-ethyl-10-hydroxy-camptothecin (SN-38) in combination with Gefitinib (Iressa, ZD1839). *Biochem Pharmacol* 2004;68:135–44.
- Van Schaeybroeck S, Karaiskou-McCaul A, Kelly D, et al. Epidermal growth factor receptor activity determines response of colorectal cancer cells to gefitinib alone and in combination with chemotherapy. *Clin Cancer Res* 2005;11:7480–9.
- Li T, Ling YH, Goldman ID, Perez-Soler R. Schedule-dependent cytotoxic synergism of pemetrexed and erlotinib in human non-small cell lung cancer cells. *Clin Cancer Res* 2007;13:3413–22.
- Giovannetti E, Lemos C, Tekle C, et al. Molecular mechanisms underlying the synergistic interaction of erlotinib, an epidermal growth factor receptor tyrosine kinase inhibitor, with the multitargeted anti-folate pemetrexed in non-small-cell lung cancer cells. *Mol Pharmacol* 2008;73:1290–300.
- Langer CJ. The “lazarus response” in treatment-naive, poor performance status patients with non-small-cell lung cancer and epidermal growth factor receptor mutation. *J Clin Oncol* 2009;27:1350–4.
- Pratt WB, Morishima Y, Osawa Y. The Hsp90 chaperone machinery regulates signaling by modulating ligand binding clefts. *J Biol Chem* 2008;283:22885–9.
- Yang S, Qu S, Perez-Tores M, et al. Association with HSP90 inhibits Cbl-mediated down-regulation of mutant epidermal growth factor receptors. *Cancer Res* 2006;66:6990–7.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Role of Epidermal Growth Factor Receptor Degradation in Cisplatin-Induced Cytotoxicity in Head and Neck Cancer

Aarif Ahsan, Susan M. Hiniker, Susmita G. Ramanand, et al.

Cancer Res 2010;70:2862-2869. Published OnlineFirst March 9, 2010.

Updated version	Access the most recent version of this article at: doi: 10.1158/0008-5472.CAN-09-4294
Supplementary Material	Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2010/03/08/0008-5472.CAN-09-4294.DC1

Cited articles	This article cites 41 articles, 21 of which you can access for free at: http://cancerres.aacrjournals.org/content/70/7/2862.full#ref-list-1
Citing articles	This article has been cited by 9 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/70/7/2862.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org .
Permissions	To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/70/7/2862 . Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.