ABSTRACT

Background

Mutations in the epidermal growth factor receptor (EGFR) gene are associated with increased sensitivity of lung cancers to kinase inhibitors like erlotinib. Mechanisms of cell death that occur after kinase inhibition in these oncogene-dependent tumors have not been well delineated. We sought to improve understanding of this process in order to provide insight into mechanisms of sensitivity and/or resistance to tyrosine kinase inhibitors and to uncover new targets for therapy.

Methods and Findings

Using a panel of human lung cancer cell lines that harbor EGFR mutations and a variety of biochemical, molecular, and cellular techniques, we show that EGFR kinase inhibition in drug-sensitive cells provokes apoptosis via the intrinsic pathway of caspase activation. The process requires induction of the proapoptotic BH3-only BCL2 family member BIM (i.e., BCL2-like 11, or BCL2L11); erlotinib dramatically induces BIM levels in sensitive but not in resistant cell lines, and knockdown of BIM expression by RNA interference virtually eliminates drug-induced cell killing in vitro. BIM status is regulated at both transcriptional and posttranscriptional levels and is influenced by the extracellular signal-regulated kinase (ERK) signaling cascade downstream of EGFR. Consistent with these findings, lung tumors and xenografts from mice bearing mutant EGFR-dependent lung adenocarcinomas display increased concentrations of Bim after erlotinib treatment. Moreover, an inhibitor of antiapoptotic proteins, ABT-737, enhances erlotinib-induced cell death in vitro.

Conclusions

In drug-sensitive EGFR mutant lung cancer cells, induction of BIM is essential for apoptosis triggered by EGFR kinase inhibitors. This finding implies that the intrinsic pathway of caspase activation may influence sensitivity and/or resistance of EGFR mutant lung tumor cells to EGFR kinase inhibition. Manipulation of the intrinsic pathway could be a therapeutic strategy to enhance further the clinical outcomes of patients with EGFR mutant lung tumors.

The Editors’ Summary of this article follows the references.
Introduction

Lung adenocarcinomas that harbor somatic kinase domain mutations in the epidermal growth factor receptor (EGFR) are highly likely to respond to the EGFR tyrosine kinase inhibitors gefitinib (Iressa) and erlotinib (Tarceva) [1–3]. One possible explanation for this phenomenon is that the cancer cells are “addicted” to signaling via the mutant EGFRs and die when the mutant oncoprotein is inactivated. However, specific mechanisms underlying erlotinib-induced cell death have not been well delineated.

To date, drug responses have been characterized in limited detail in a few drug-sensitive human lung adenocarcinoma cell lines that harbor EGFR mutations. These studies indicate that EGFR kinase inhibitors can induce the activation of cysteine aspartyl-specific proteases (caspases) in EGFR mutant cells [4–7]. Similarly, analyses of mouse models of drug-sensitive mutant EGFR-driven lung adenocarcinomas have demonstrated that erlotinib induces caspase activation [8,9]. None of these studies addressed the molecular mechanisms by which caspases are activated.

Two major pathways of caspase activation exist: the extrinsic pathway, triggered by “death receptor”-ligand binding (such as FASL to FAS), and the intrinsic pathway, triggered by cellular stress, such as occurs after cytokine deprivation, DNA damage, and anoikis (reviewed in [10]). BCL2 family proteins are a critical feature of the intrinsic pathway. Overexpression of some antiapoptotic family members, such as BCL2 and BCL-xL (also called BCL2-like ligand 1 [BCL2L1]), can prevent apoptosis induced by withdrawal of growth factor [11,12]. Control of stability of BCL2-related antiapoptotic myeloid cell leukemia sequence 1 (MCL1) protein is also an important mechanism for the regulation of apoptosis by growth factors [13]. BCL2-associated X protein (BAX) and BCL2 antagonist/killer (BAK), on the other hand, are proapoptotic BCL2 family members. Deletion of both renders cells highly resistant to apoptosis following growth factor withdrawal [14,15]. During apoptosis, BAX translocates to mitochondria, causing release of cytochrome c into the cytosol [16].

A third proapoptotic subgroup of the BCL2 family includes BIM (also known as BCL2L11 [BCL2-like 11]), BAD (BCL2-antagonist of cell death), and BBC3 (BCL2-binding component 3; also called PUMA), which all contain a “BH3” protein domain. These BH3-only members of the BCL2 family promote apoptosis when overexpressed [17,18] by inhibiting antiapoptotic members and/or directly activating BAX/BAK [19,20]. Bim is induced in interleukin 3 (IL3)-dependent murine hematopoietic cells by IL3 deprivation but not by other apoptotic triggers, such as DNA damage or Fas [21]. BIM is one of the most potent proapoptotic BH3-only proteins; unlike BAD, BIM binds to all prosurvival BCL2 family members with high affinity [19]. Recent analyses of IL3-dependent cell lines from mice lacking the genes for Bad, Bim, Puma, Bad and Bim, or Bax and Bak, showed that while Bad and Bim were not required for cell death following IL3 withdrawal, cell death did in part depend upon Puma [22]. Mechanisms of death in leukemic cells that depend upon signaling from the Bcr-Abl oncoprotein for survival may be different from those in cells that depend upon IL3, as studies using RNA interference and cells from gene-targeted mice revealed that the combined loss of Bim and Bad abrogates cell killing induced by the kinase inhibitor imatinib (Gleevec) [23].

In this study, we sought to determine the mode of cell death induced by EGFR kinase inhibitors in drug-sensitive human lung adenocarcinoma cells that harbor EGFR mutations. We hoped that an improved understanding of this process could provide insight into mechanisms of sensitivity and/or resistance to tyrosine kinase inhibitors (TKIs) and uncover new targets for therapy.

Methods

Cell Culture

The human lung adenocarcinoma cell lines H3255, PC-9, H1650, and H1975 were described previously [4–7,24]. Cells were maintained in a humidified incubator with 5% CO2 at 37 °C in RPMI 1640 medium (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum and pen-strep solution (both from Gemini Bio-Products, West Sacramento, CA; final concentrations of 100 U/ml penicillin G and 100 μg/ml streptomycin, respectively). NIH 3T3 cells stably transfected with various EGFR cDNAs were kindly provided by M. Meyerson [25]. BCL-xL-overexpressing PC-9 cells were generated by transfecting parental cells with pcDNA3.1(−) hygromycin vector carrying a BCL-xL cDNA [26] using Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN). Resistant clones were selected in 250 μg/ml hygromycin B (Invitrogen, Carlsbad, CA) for 12 d, isolated using cloning cylinders, and subsequently expanded in hygromycin B-containing medium. Control cells were transfected with empty pcDNA3.1(−) hygromycin vector.

Reagents

Erlotinib was synthesized by the Organic Synthesis Core Facility at Memorial Sloan-Kettering Cancer Center. Gefitinib was kindly provided by AstraZeneca (Wilmington, DE). ABT-737 and its inactive enantiomer were kindly provided by Abbott Laboratories (Abbott Park, IL). The Vybrant Apoptosis Assay Kit #2 was from Invitrogen. CellTiter Blue Reagent and APO-ONE Homogenous Caspase3/7 assay kits were from Promega (Madison, WI). Propidium iodide and actinomycin D were from Sigma-Aldrich (St. Louis, MO). CL-387,785, U0126, PD98059, LY294002, and wortmannin were from Calbiochem (San Diego, CA).

Antibodies

Cytochrome c-, MCL1-, and EGFR-specific antibodies were from BD Biosciences (San Jose, CA). Anti-actin antibody was from Sigma-Aldrich. Anti-BCL2, -BCL-xL, -BAX, -BAD, -BIM, -PUMA, -pERK (Thr202/Tyr204), -ERK, -pAKT (Ser473), -AKT, and -pEGFR (Tyr1092) antibodies, and secondary antibodies including HRP-conjugated anti-mouse and anti-rabbit antibodies, were purchased from Cell Signaling Technology (Danvers, MA). (Note that EGFR has two numbering systems. The first denotes the initiating methionine in the signal sequence as amino acid –24. The second denotes the methionine as amino acid +1. Commercial antibodies, such as the Y1068-specific anti-phospho-EGFR, use the first nomenclature. In the second nomenclature, which we use here, Y1068 is Y1092.) BAK-specific antibody was from Lab Vision Corporation (Fremont, CA). L858R EGFR antisera was previously described [9]. Anti-total EGFR
Cells were examined for mutations in EGFR by dideoxynucleotide sequencing of PCR products spanning individual exons (18–24). The IC_{50} values were determined using CellTiter Blue assays as described in Methods. Del, deletion; amino acids noted using single-letter abbreviations.
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Animal Husbandry and Genotyping
The generation, maintenance, genotyping, and drug treatment of bitransgenic lung tumor-bearing mice that express a tetracycline-inducible exon 19 deletion mutant (EGFR^1857G_746–A750del^) or the L858R mutant (EGFR^L858R^) in mouse lung epithelia have been previously described [9]. Animals were humanely killed with a lethal dose of CO_2 per institutional guidelines. The lungs were excised, flash-frozen in liquid nitrogen, and crushed for protein extraction in lysis buffer supplemented with protease and phosphatase inhibitors.

For xenograft studies, the hind flanks of 4- to 6-wk-old female athymic (nu/nu) nude mice (NCI-Frederick Cancer Center, Frederick, MD) were injected subcutaneously with 10^7 PC-9 cells resuspended in 100 μl of Matrigel (BD Biosciences). Tumor-bearing animals (12 d after injection) were administered either vehicle control or 25 mg/kg erlotinib intraperitoneally every 12 h for 3 doses and then humanely killed 24 h after the first injection. Xenografts were excised, flash-frozen in liquid nitrogen, and crushed for protein extraction in lysis buffer supplemented with protease and phosphatase inhibitors.

All animals were kept in specific pathogen-free housing with abundant food and water under guidelines approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee and Research Animal Resource Center.

Results
EGFR Kinase Inhibition Induces G1 Cell Cycle Arrest in Drug-Sensitive Lung Adenocarcinoma Cells with EGFR Mutations
A number of lung adenocarcinoma cell lines have been reported to harbor drug-sensitizing EGFR mutations [4–7,24]. To verify these findings, we obtained four of these cell lines and determined their mutation status by direct Sanger-based resequencing of EGFR exons 18–24 (Table 1). As reported, PC-9 and H1650 cells contained the in-frame exon 19 deletion that eliminates amino acids ELREA (del E746–A750). H3255 and H1975 harbored the exon 21 T→G point mutation at nucleotide position 2,573, which substitutes arginine for leucine at position 858 (L858R). H1975 cells also contained the exon 20 C→T mutation at nucleotide position 2,369. This change leads to substitution of methionine for threonine at position 790 (T790M) and is associated with acquired resistance to gefitinib and erlotinib [24].
We established the sensitivity of these cell lines to erlotinib using a cell viability assay that measures a colorimetric signal produced by conversion of resazurin to resorufin, which is directly proportional to the numbers of viable cells. Cells were grown in 10% serum for 72 h in different concentrations of erlotinib. As previously reported, the concentration of drug required for 50% growth inhibition (IC_{50}) of two lines—H3255 and PC-9—was in the nanomolar range (10 nM and 40 nM respectively) (Table 1). The IC_{50} values corresponded with the degrees of inhibition of phosphorylation of EGFR and key components of downstream signaling cascades (i.e., ERK and AKT), as seen by immunoblotting cytosolic fractions from drug-treated H3255 cells, even at higher doses of erlotinib (1 μM) (Figure 2B).

**Figure 1.** Effects of Erlotinib on EGFR Signaling Pathways in H3255, PC-9, H1650, and H1975 Cells

Cells were treated with the indicated concentrations of erlotinib for 6 h. Cell lysates were analyzed by immunoblotting, using the indicated antibodies. t, total protein; p, phospho-protein. doi:10.1371/journal.pmed.0040294.g001

EGFR Kinase Inhibition Induces Apoptosis in H3255 and PC-9 Cells

We next investigated whether erlotinib also induced apoptosis in the cell lines with nanomolar sensitivity. Cells treated with DMSO or different concentrations of erlotinib for 48 h were stained with FITC-conjugated annexin V and PI, and percentages of apoptotic cells were measured by FACS. The phospholipid-binding protein annexin V has a high affinity for phosphatidylserine, which is translocated from the inner to the outer leaflet of the plasma membrane during the early phases of apoptosis, and PI penetrates the plasma membrane of cells only when membrane integrity is breached, as occurs in the later stages of apoptosis. After exposure to escalating concentrations of drug, PC-9 and H3255 cells demonstrated dose-dependent increases in the percentages of annexin V- and PI-positive cells (Figure 2B). Consistent with these findings, the activity of downstream "executioner" caspases, caspases 3 and 7, in lysates from cells treated with erlotinib at various concentrations for various times, was also increased in both dose- and time-dependent fashion (Figure S1). By contrast, minimal increases in the percentage of annexin V- and PI-positive cells were observed in drug-treated H1650 and H1975 cells, even at higher doses of erlotinib (1 μM) (Figure 2B).

**Table 1.** IC_{50} Values for Erlotinib Treatment in H3255, PC-9, H1650, and H1975 Cells

| Cell Line  | IC_{50} (nM) |
|------------|-------------|
| H3255      | 10           |
| PC-9       | 40           |
| H1650      | 100          |
| H1975      | 1000         |

Mutant EGFR-Dependent Cells Die via the Intrinsic Pathway of Apoptosis

We subsequently examined mechanisms of apoptosis in the two cell lines that undergo apoptosis in response to erlotinib. Caspases can be activated via two major pathways: the extrinsic pathway, triggered by the binding of ligands to "death receptors," and the intrinsic pathway, triggered by cellular stress, such as occurs after cytokine deprivation, DNA damage, and anoikis [10]. Since inhibition of EGFR kinase activity by erlotinib is similar to growth factor withdrawal, we investigated whether erlotinib induced the intrinsic pathway of caspase activation in PC-9 and H3255 cells by a stress-related mechanism.

One hallmark of the intrinsic pathway of apoptosis is cytochrome c release from mitochondria [29]. We found by immunoblotting cytosolic fractions from H3255 cells treated with erlotinib that cytochrome c levels increased within the cytosol in both a time- and concentration-dependent manner (Figure 3A). Similar results were obtained with cytosolic fractions from drug-treated PC-9 cells (unpublished data).

Changes in the localization of proapoptotic BAX are another hallmark of the intrinsic pathway of apoptosis [29]. Immunostaining followed by confocal microscopy revealed that at baseline, BAX was found in the nuclei of both PC-9 and H3255 cells (Figure 3B and unpublished data); the
Figure 2. Erlotinib Induces G1 Cell Cycle Arrest and Apoptosis in Drug-Sensitive Lung Adenocarcinoma Cell Lines That Harbor EGFR Mutations

(A) Cell lines treated with either DMSO or indicated concentrations of erlotinib for 24 h were used for cell cycle analysis as described in the Methods. Data represent the mean ± standard deviation of three independent experiments.

(B) Cell lines treated with either DMSO or different concentrations of erlotinib for 48 h were used for annexin V/PI apoptosis assays as described in Methods.

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BIM and Erlotinib-Induced Apoptosis

**A**

| Erlotinib (nM) | 0 | 50 | 100 | 250 |
|---------------|---|----|-----|-----|
| CYTO C        |   |    |     |     |
| ACTIN         |   |    |     |     |

| Time (hrs)    | 0 | 12 | 24 | 36 |
|---------------|---|----|----|----|
| CYTO C        |   |    |    |    |
| ACTIN         |   |    |    |    |

**B**

**DMSO**

- BAX
- CYTO C
- DAPI
- merge

**Erlotinib (100nM, 48hrs)**

- BAX
- CYTO C
- DAPI
- merge

**C**

| V | B1 | B2 |
|---|----|----|
| BCL-xL |     |    |
| ACTIN  |     |    |

**D**

**DMSO**

- V
- B1
- B2

**Erlotinib (100 nM)**

- V
- B1
- B2

**E**

| V | B1 | B2 |
|---|----|----|
| 0 | 10 | 100 |
| 0 | 10 | 100 |
| 0 | 10 | 100 |

- Erlotinib (nM)
- pEGFR Y1092
- tEGFR
- pERK T202/Y204
- tERK
- pAKT S473
- tAKT
- ACTIN

**Annexin V-FITC Fluorescence**

- Cell number
- V
- B1
- B2

6%  26%  6%  9%  2%  7%
nuclear localization of BAX in human lung cancer cell lines has been previously reported [30,31]. Following erlotinib treatment, BAX was found in the cytoplasm in apoptotic cells. The distribution of cytochrome c changed from punctate to more diffuse, consistent with the release of cytochrome c from mitochondria. Merging of the two fluorescent signals revealed some overlap between BAX and cytochrome c staining. These observations suggest that BAX translocates to mitochondria during erlotinib-induced apoptosis in mutant EGFR-dependent lung cancer cells.

To verify further whether erlotinib-induced apoptosis is mediated through the intrinsic apoptotic pathway, we examined effects of erlotinib on PC-9 cells that stably overexpress the antiapoptotic BCL-2 family member BCL-xL. Two stable clones (B1 and B2) were derived, and both displayed significantly higher levels of BCL-xL than did cells transfected with vector alone (Figure 3C). Compared to vector-transfected cells, in which 100 nM erlotinib increased the fraction of annexin V-positive cells by 20% over DMSO-treated cells, drug treatment in cells overexpressing BCL-xL increased the fraction of apoptotic cells by only 3%–5% over DMSO-treated controls (Figure 3D). As expected, BCL-xL did not affect the inhibition of EGFR signaling by erlotinib. The drug still inhibited the autophosphorylation of EGFR as well as the phosphorylation of ERK and AKT (Figure 3E). These results collectively indicate that erlotinib-induced apoptosis in mutant EGFR-dependent lung adenocarcinoma cells is mediated predominantly by the intrinsic apoptotic pathway.

The Status of BIM Is Dramatically Altered by Inhibition of EGFR Kinase Activity in Cells Dependent upon Mutant EGFR Signaling for Survival

The intrinsic pathway of apoptosis is regulated by BCL2 family member proteins, some of which are antiapoptotic (e.g., BCL2, BCL-xL, MCL1) and some proapoptotic (e.g., the BAX family members BAX and BAK, and the BH3-only proteins BIM, BAD, and PUMA) [17,18]. To determine which BCL2 family members are potentially involved in the maintenance of tumors by mutant EGFR, we studied the effect of erlotinib treatment on the expression of BCL2, BCL-xL, MCL1, BAX, BAK, BIM, BAD, and PUMA in H3255 cells. We found that the expression levels of only the antiapoptotic protein MCL1 and the proapoptotic BH3-only protein BIM were significantly altered by the drug (Figure 4A).

MCL1 protein is rapidly degraded by the proteasome and its elimination is required for the initiation of apoptosis following ultraviolet radiation in HeLa cells [32]. To investigate if down-regulation of MCL1 is essential for erlotinib-induced apoptosis in lung adenocarcinoma cells, we blocked MCL1 degradation with the proteasome inhibitor MG132 in H3255 cells. Despite the efficient inhibition of MCL1 degradation by MG132, erlotinib was still able to induce apoptosis as measured by an increase in PARP (poly ADP ribose polymerase) cleavage (Figure S2). Moreover, MCL1 levels remained constant in erlotinib-treated PC-9 cells, which also undergo apoptosis in response to drug treatment (Figure 4B). These results collectively indicate that MCL1 elimination is not essential for erlotinib-induced apoptosis in mutant EGFR-dependent cells.

We next focused our attention on BIM. Immunoblotting analysis of the four cell lines revealed that the levels of all three major splicing isoforms—BIM-extra long (EL), BIM-long (L), and BIM-short (S)—were induced after erlotinib treatment in drug-sensitive H3255 and PC-9 cells but not in drug-resistant H1650 and H1975 cells (Figure 4A and 4B). Moreover, in the drug-sensitive but not in the drug-resistant lines, erlotinib appeared to induce significant dephosphorylation of BIM EL, as this isoform migrated faster in polyacrylamide gel electrophoresis after drug treatment (Figure 4A and 4B). This finding is notable because others have shown that dephosphorylation of BIM results in an increase in its proapoptotic function [33,34].

BIM has been shown to be regulated transcriptionally [35]. To investigate whether the induction of BIM protein by erlotinib occurred at the transcriptional level in H3255 cells, we measured BIM proteins in lysates from cells treated for varying amounts of time with either erlotinib alone or with erlotinib and the RNA polymerase II inhibitor, actinomycin D, which inhibits mRNA transcription (Figure 4C). BIM EL levels rose within 2 h after treatment with the kinase inhibitor alone and continued to increase up to at least 14 h. Cotreatment with actinomycin D efficiently blocked most of this increase. Consistent with this, transcriptional profiling of H3255 cells treated with erlotinib showed that levels of BIM mRNA increased significantly 2.5-fold by 12 h after treatment (unpublished data). These data suggest that augmented transcription of BIM can account for at least part of the increase in BIM protein after treatment of mutant EGFR-dependent cells with erlotinib.

Finally, to determine if induction of BIM was unique to erlotinib, we examined the status of BIM in H3255 and PC-9 cells treated with erlotinib (100 nM) or two other EGFR inhibitors: the related reversible EGFR inhibitor gefitinib (1 μM) and the irreversible EGFR inhibitor CL-387,785 (100 nM) [36]. All three drugs induced apoptosis in these cell lines, as indicated by the presence of PARP cleavage products in cell
lysates (Figure 4D). Consistent with these findings, all three EGFR inhibitors induced the expression and dephosphorylation of BIM EL. These data indicate that the induction of BIM is triggered by various EGFR inhibitors and is not a specific effect of erlotinib.

BIM Is Required for Erlotinib-Induced Apoptosis

To examine the role of BIM in erlotinib-induced cell killing in more detail, we used siRNAs to suppress BIM expression almost completely in both H3255 and PC-9 cells (Figure 5A). Two independent siRNAs were utilized to knock down BIM in PC-9 cells.

Knockdown effectively abolished erlotinib-induced killing in both cell lines (Figure 5B). By comparison, cells transfected with control siRNA against GFP continued to undergo apoptosis in response to erlotinib. In each of three independent experiments, the difference in percent of apoptotic cells induced by erlotinib in H3255 and PC-9 cells was statistically significant ($p = 0.001$ for H3255 with BIM

Figure 4. BIM Status Is Dramatically Altered by Inhibition of EGFR Kinase Activity in Cells Dependent upon Mutant EGFR Signaling for Survival

(A) Effects of erlotinib treatment on proapoptotic and antiapoptotic proteins in H3255 cells. H3255 cells were treated with different concentrations of erlotinib for 24 h. Cell lysates were examined by immunoblotting using antibodies specific for the indicated proteins.

(B) Erlotinib induces dephosphorylation and increased levels of BIM in drug-sensitive PC-9 cells but not in drug-resistant H1650 or H1975 cells. Cells were treated with different concentrations of erlotinib for 24 h. Cell lysates were analyzed by immunoblotting with antibodies that recognize the indicated proteins.

(C) Increased gene transcription contributes to the induction of BIM protein expression after erlotinib treatment. H3255 cells were treated with erlotinib (100 nM) in the presence or absence of actinomycin D (5 μg/ml) for the indicated times. Cell lysates were analyzed by immunoblotting using the indicated antibodies.

(D) Multiple EGFR kinase inhibitors affect BIM status. H3255 (left blot) and PC-9 (right blot) cells were treated with erlotinib (100 nM), gefitinib (1 μM), or CL-387,785 (100 nM) for 24 h and 48 h, respectively. Cell lysates were analyzed by immunoblotting with antibodies that recognize the indicated proteins.

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A significant difference in rates of apoptosis was observed (induced apoptosis in H3255 cells. Here, no statistically down of a different proapoptotic protein, BAD, on erlotinib—another comparison, we also examined the effect of knock-

multiple attempts with different siRNAs, we were unable to

0.13; Welch two-sample t-test) (Figure 5B). However, despite inclusion of both pathways is required for the increased expression and dephosphorylation of BIM. Thus, we examined the status of phospho-ERK, -AKT, and BIM in lysates from H3255 cells treated with erlotinib and compared results to those obtained from cells exposed to a mitogen-activated protein kinase kinase (MAP2K or MEK) inhibitor (PD98059 or U0126, which affect the ERK pathway), a PI3K inhibitor (wortmannin or LY294002), or both. We focused our attention primarily on the BIM EL isoform, because it is the major isoform detected in these cells, and the BIM EL isoform, but not BIM L and S, contains phosphorylation sites for ERK and AKT [33,34].

Treatment of H3255 cells with either MEK inhibitor led to an increase in BIM expression levels as well as the dephosphorylation of BIM (as indicated by the faster migrating form) (Figure 6A)—changes similar to those seen in erlotinib-treated cells. Treatment with the PI3K inhibitors alone had minimal effects on either BIM expression levels or phosphorylation status. Lysates from cells treated with the combination of MEK (U0126, Figure 6A) and PI3K inhibitors (wortmannin or LY294002, Figure 6A) did not show any additive effects on the induction of BIM EL, when compared with cells treated with the MEK inhibitor treatment alone. These data suggest that mutant EGFRs influence BIM expression and phosphorylation status mainly through the ERK signaling pathway. More precise mechanisms of BIM regulation remain to be elucidated.

Finally, to begin to determine how EGFR signaling affects BIM in an isolated cell system, we examined the effect of EGF stimulation on Bim in NIH 3T3 cells stably transfected with cDNAs encoding wild-type EGFR [25]. These mouse fibroblasts express very low levels of endogenous Egfr and its heterodimeric Erbb family members (i.e., Erbb2, Erbb3, and Erbb4). As expected, addition of exogenous EGF to serum-

diminished. Thus, Bim protein levels and phosphorylation are

These changes correlate with decreased Bim activity [34,38]. BIM levels were restored quickly as levels of phospho-EGFR decreased after EGF stimulation, presumably due to internalization and lysosomal degradation of the receptor. These changes were accompanied by transient increases in both Erk and Akt signaling. In parallel with these changes, EGF stimulation induced a rapid phosphorylation of Bim as well as a decrease in Bim protein levels; others have shown that these changes correlate with decreased Bim activity [34,38]. BIM levels were restored quickly as levels of phospho-EGFR diminished. Thus, Bim protein levels and phosphorylation are tightly regulated by the EGFR signaling pathway. Moreover,
EGFR stimulation by ligand can lead to changes suggestive of decreased BIM activity.

Bim Expression and Phospho-EGF Receptor Status Are Inversely Correlated in Mouse Models

We had hoped to extend our studies on the role of BIM in kinase inhibitor-induced apoptosis to human mutant EGFR tumor specimens. However, such studies would require precisely timed re-biopsies of tumors in patients just initiating treatment. Since such samples are not currently available, we took advantage of established mutant EGFR mouse lung tumor models. Specifically, we examined the relationship between levels of phospho-EGFR and Bim in tumors induced in transgenic mice that conditionally express mutant L858R EGFRs in lung epithelial cells under the control of tetracycline regulation [9]. We previously showed that treatment with erlotinib eliminated disease within lung tumor-bearing animals [9]. Here, tumor-bearing transgenic animals (as determined by magnetic resonance imaging) were treated with a single dose of vehicle (n = 5) or erlotinib (50 mg/kg; n = 5) intraperitoneally and humanely killed 13 h later. Lungs from all EGFR-transgenic animals expressed the induced oncoprotein, as measured by immunoreactivity of lysates with antisera against the specific L858R EGFR [9] (Figure 7A). At 13 h after treatment, lung tissues from control-treated animals expressed high levels of phosphorylated EGFR, while EGFR phosphorylation was abolished in lungs from erlotinib-treated mice.

We next determined corresponding Bim levels in the mouse lung lysates, using the same BIM antisera as used above to interrogate the human cell lines. Lungs from vehicle-treated L858R mice displayed low levels of Bim EL (Figure 7A). By contrast, corresponding tissue from erlotinib-treated animals displayed elevated levels of Bim. We detected only very low levels of Bim L and did not detect dephosphorylated Bim for unknown reasons (unpublished data). Similar results were obtained by parallel analyses of lung tumor nodules derived from mice expressing the EGFR L747–S752 deletion mutant (n = 4) (Figure 7B). By contrast, Bim levels remained constant in non-transgenic mice lacking tumors and treated with either vehicle (n = 2) or erlotinib (n = 3) in the same manner (Figure 7C).

To verify these results even further using homogenous tissue samples, we treated athymic nude mice bearing PC-9 xenografts with placebo (n = 4) or erlotinib (25 mg/kg × 2 doses; n = 4) and examined the status of EGFR phosphorylation and BIM in xenograft lysates. As expected, immunoblotting analyses demonstrated that while EGFR was dephosphorylated after erlotinib administration, BIM EL expression was induced (Figure 7D). Conversely, xenograft lysates from placebo-treated animals showed the opposite result—high levels of phospho-EGFR and low levels of BIM EL. Collectively, these studies with both transgenic and xenograft mouse models support our earlier conclusions from in vitro studies on human cells that erlotinib augments the concentration of Bim in lung adenocarcinomas dependent upon mutant EGFR for survival.

ABT-737 Significantly Enhances Erlotinib-Induced Apoptosis in Drug-Sensitive Lung Adenocarcinoma Cells

ABT-737 is a small-molecule BCL2 antagonist that has antitumor activity, most notably as a single agent against small cell lung cancers, lymphomas, and leukemias [39,40]. ABT-737 also displays synergistic cytotoxicity with chemotherapeutic agents (e.g., paclitaxel) against “less sensitive” cancer cell lines, including ones of non-small cell lung cancer origin (without EGFR mutations) [40]. The drug as a single agent has relatively little effect on imatinib-sensitive K562 cells, which harbor the BCR-ABL translocation; however, its addition to imatinib greatly enhances imatinib-induced cell death in vitro [23]. Taken together with our findings that mutant EGFR-dependent cells die via the intrinsic pathway of
apoptosis, we hypothesized that the intrinsic pathway could be manipulated by ABT-737 to enhance erlotinib-induced cell killing of EGFR mutant lung cancer cells. To verify this hypothesis, we treated H3255 cells for 6 h with different concentrations of ABT-737 alone or in combination with 50 nM erlotinib. Cells were then analyzed for the induction of apoptosis by annexin V/PI assays. Whereas erlotinib or ABT-737 alone induced minimal increases in the percent of apoptotic cells, the combination of the EGFR inhibitor and the BCL2 antagonist significantly increased the percentage of apoptotic cells, in a manner dependent upon the concentration of ABT-737 (Figure 8A). Similar results were obtained with PC-9 cells treated for 24 h (Figure 8B). Cotreatment with erlotinib and the inactive ABT-737 enantiomer, which served as a negative control, had no such effect in either set of cells. These data indicate that BCL2 antagonists such as ABT-737 can be combined with erlotinib to enhance cell death in mutant EGFR-dependent cells.

Discussion

Selective EGFR kinase inhibitors such as gefitinib and erlotinib induce tumor shrinkage in the majority of patients harboring mutant EGFR lung adenocarcinomas [41]. Studies with cell lines and mouse lung tumor models suggest that such responses involve cell death, but the underlying mechanisms have not been well elucidated. Here, we sought to characterize pathways of apoptosis induced by this class of agents in mutant EGFR-dependent lung adenocarcinoma cells. An
enhanced understanding of such processes could lead to the identification of even more effective therapeutic strategies. The studies were motivated by the fact that despite inducing dramatic clinical and radiographic tumor responses, gefitinib and erlotinib rarely induce complete responses and do not cure patients.

Using a panel of human lung cancer cell lines that harbor EGFR mutations, we show that mutant EGFR-dependent cells with nanomolar sensitivity to erlotinib undergo G1 cell cycle arrest and cell death in response to kinase inhibition. Cell death is mediated through the intrinsic apoptotic pathway: cells release cytochrome c from mitochondria, they display altered localization of the proapoptotic protein BAX, and apoptosis can be blocked by overexpression of the anti-apoptotic protein BCL-xL.

We also found that in cells dependent upon mutant EGFR signaling for survival, EGFR kinase inhibition leads to induced levels and dephosphorylation of BIM, a member of the proapoptotic BH3-only family of BCL2-related proteins. Importantly, these changes, which have been shown to enhance the proapoptotic activity of BIM [33,34], are required for erlotinib-induced cell death; knockdown of BIM expression by RNA interference completely eliminates drug-induced cell killing in vitro, and erlotinib dramatically induces BIM levels in cell lines that die but not in cell lines resistant to the drug. Consistent with these in vitro findings, lungs from transgenic mice bearing mutant EGFR-driven lung adenocarcinomas and xenografts of PC-9 cells display increased concentrations of Bim shortly after erlotinib treatment.

BIM has been previously implicated in signaling cell death following cytokine withdrawal [42]. This BH3-only protein has also been shown to be necessary for imatinib-induced killing of BCR-ABL-dependent leukemic cells in vitro (human cells) and in vivo (mice) [23]. The latter study found that only knockdown of Bim and Bad together by RNA interference could completely eliminate imatinib-induced killing. Here we found that knockdown of BIM alone by siRNAs was sufficient to prevent erlotinib-induced cell death in human lung cancer cells. Discrepancies between killing mechanisms in leukemic cells sensitive to imatinib and lung adenocarcinoma cells sensitive to EGFR inhibitors could be explained by different cell types of origin and/or different experimental procedures. Nevertheless, taken together, these findings suggest that the
induction of BIM is a common apoptotic mechanism shared by both hematopoietic and epithelial tumor cells that depend upon kinase signaling for survival.

The status of BIM appears to be regulated at multiple points. Transcriptional profiling of erlotinib-treated cells and immunoblotting analysis of cells co-treated with erlotinib and an RNA polymerase II inhibitor indicate that the induction of BIM occurs at least in part at the transcriptional level. Moreover, studies of Bim in NIH 3T3 (mouse) cells transfected with *EGFR* cDNAs and in human lung adenocarcinoma cell lines that harbor *EGFR* mutations indicate that Bim phosphorylation status is influenced by perturbation of EGF signaling. Use of specific PI3K/AKT and ERK pathway inhibitors on drug-sensitive *EGFR* mutant cells suggests that the ERK pathway predominantly influences BIM status downstream of EGF. However, others have shown that H3255 and PC-9 cells treated with a more potent and selective MEK1/2 inhibitor, PD0325901 (Pfizer), do not undergo apoptosis (Dr. David Solit, Memorial Sloan-Kettering Cancer Center, New York, personal communication). This finding suggests that (1) while BIM induction is required for erlotinib-induced apoptosis, it may not be sufficient to provoke cell death, and (2) other signaling components downstream of EGF are also likely to be involved in inducing apoptosis of these cells.

At the clinical level, our results have several implications. Since drug-sensitive *EGFR* mutant cells die via the intrinsic pathway of caspase activation, molecules within this cascade could influence both sensitivity and/or resistance of *EGFR* mutant lung tumor cells to EGF kinase inhibitors. Conversely, manipulation of this pathway could be a therapeutic strategy to enhance the death of cells that depend upon mutant EGF signaling for survival. Indeed, the fact that the BCL2 antagonist ABT-737 significantly enhances erlotinib-induced cell killing provides proof-of-principle that such an approach may achieve greater and more durable responses to EGFR inhibitors in patients whose lung tumors harbor *EGFR* kinase domain mutations.

**Supporting Information**

**Figure S1.** Erlotinib Induces Caspase 3/7 Activation in Drug-Sensitive Lung Adenocarcinoma Cell Lines That Harbor *EGFR* Mutations

Cell lines were treated with either DMSO or different concentrations of erlotinib in 96-well plates for the indicated times. Caspase 3/7 activity per well was measured as described in the Methods. Values represent the mean ± standard deviation of three independent experiments. Found at doi:10.1371/journal.pmed.0040294.sg001 (8.2 MB TIF).

**Figure S2.** MCL1 Elimination Is Not Required for Erlotinib-Induced Apoptosis in H3255 Cells

Cells were treated with erlotinib (100 nM) alone or with erlotinib (100 nM) and the proteasome inhibitor MG132 (10 μM) for the indicated times. Cell lysates were analyzed by immunoblotting with the indicated antibodies. Found at doi:10.1371/journal.pmed.0040294.sg002 (8.2 MB TIF).

**Accession Numbers**

The GenBank (http://www.ncbi.nlm.nih.gov/) accession numbers for the genes and proteins discussed in this article are BAD (NM_002089), BAK (also known as BAK1; NM_001188), BAX (NM_004324), BCL-xL (also known as BCL2L1; NM_138578), BIM (also known as BCL2L11; NM_138621), EGFR (NM_005228), MCL1 (NM_021960).

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**Author contributions.** YG, RS, KP, MB, JC, and WP designed and executed the study. YG, RS, KP, XJ, and WP contributed to writing the paper.

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Editors' Summary

Background. Lung cancer, a common type of cancer, has a very low cure rate. Like all cancers, it occurs when cells begin to divide uncontrollably because of changes (mutations) in their genes. Chemotherapy drugs kill these rapidly dividing cells but, because some normal tissues are sensitive to these agents, it is hard to destroy the cancer without causing serious side effects. Recently, “targeted” therapies have brought new hope to some patients with cancer. These therapies attack the changes in cancer cells that allow them to divide uncontrollably but leave normal cells unscathed. One of the first molecules for which a targeted therapy was developed was the epidermal growth factor receptor (EGFR). In normal cells, messenger proteins bind to EGFR and activate its “tyrosine kinase,” an enzyme that sticks phosphate groups on tyrosine (an amino acid) in other proteins. These proteins then tell the cell to divide. Alterations to this signaling system drive uncontrolled cell division in some cancers so blocking the EGFR signaling pathway should stop these cancers growing. Indeed, some lung cancers with mutations in the tyrosine kinase of EGFR shrink dramatically when treated with gefitinib or erlotinib, two tyrosine kinase inhibitors (TKIs).

Why Was This Study Done? TKI-sensitive lung cancers shrink when treated with TKIs because of drug-induced cell death, but what are the molecular mechanisms underlying this death? A better understanding of how TKIs kill cancer cells might provide new insights into why not all cancer cells with mutations in EGFR (the gene from which EGFR is made) are sensitive to TKIs. It might also uncover new targets for therapy. TKIs do not completely kill lung cancers, but if the mechanism of TKI-induced cell death was understood, it might be possible to enhance their effects. In this study, the researchers have investigated how cell death occurs after kinase inhibition in a panel of human lung cancer cell lines (cells isolated from human tumors that grow indefinitely in dishes) that carry EGFR mutations.

What Did the Researchers Do and Find? The researchers show, first, that erlotinib induces a type of cell death called apoptosis in erlotinib-sensitive cell lines but not in resistant cell lines. Apoptosis can be activated by two major pathways. In this instance, the researchers report that the so-called “intrinsic” pathway activates apoptosis. This pathway is stimulated by proapoptotic members of the BCL2 family of proteins and is blocked by antiapoptotic members, so the researchers examined the effect of erlotinib treatment on the expression of BCL2 family members in the EGFR mutant cell lines. Erlotinib treatment increased the expression of the proapoptotic protein BIM in sensitive but not in resistant cell lines. It also removed phosphate groups from BIM—dephosphorylated BIM is a more potent proapoptotic protein. Conversely, blocking BIM expression using a technique called RNA interference virtually eliminated the ability of erlotinib to kill EGFR mutant cell lines. The researchers also report that erlotinib treatment increased BIM expression in erlotinib-sensitive lung tumors growing in mice and that an inhibitor of the anti-apoptotic protein BCL2 enhanced erlotinib-induced death in drug-sensitive cells growing in dishes.

What Do These Findings Mean? These findings indicate that BIM activity is essential for the apoptosis triggered by TKIs in drug-sensitive lung cancer cells that carry EGFR mutations, and that treatment of these cells with TKIs induces both the expression and dephosphorylation of BIM. The finding that the intrinsic pathway of apoptosis activation is involved in TKI-induced cell death suggests that changes in this pathway (possibly mutations in some of its components) might influence the sensitivity of EGFR mutant lung cancers to TKIs. Finally, these findings suggest that giving drugs that affect the intrinsic pathway of apoptosis activation at the same time as TKIs might further improve the clinical outcome for patients with EGFR mutant tumors. Such combinations will have to be tested in clinical trials before being used routinely.

Additional Information. Please access these Web sites via the online version of this summary at http://dx.doi.org/10.1371/journal.pmed.0040294.

- US National Cancer Institute information for patients and professionals on lung cancer (in English and Spanish)
- Information for patients from Cancer Research UK on lung cancer including information on treatment with TKIs
- Wikipedia pages on apoptosis, epidermal growth factor receptor, and BCL-2 proteins (note that Wikipedia is a free online encyclopedia that anyone can edit; available in several languages)
- Information for patients from Cancerbackup on erlotinib and gefitinib