Proapoptotic BH3-Only BCL-2 Family Protein BIM Connects Death Signaling from Epidermal Growth Factor Receptor Inhibition to the Mitochondrion

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Abstract

A subset of lung cancers expresses mutant forms of epidermal growth factor receptor (EGFR) that are constitutively activated. Cancers bearing activated EGFR can be effectively targeted with EGFR inhibitors such as erlotinib. However, the death-signaling pathways engaged after EGFR inhibition are poorly understood. Here, we show that death after inhibition of EGFR uses the mitochondrial, or intrinsic, pathway of cell death controlled by the BCL-2 family of proteins. BCL-2 inhibits cell death induced by erlotinib, but BCL-2–protected cells are thus rendered BCL-2–dependent and sensitive to the BCL-2 antagonist ABT-737. BH3 profiling reveals that mitochondrial BCL-2 is primed by death signals after EGFR inhibition in these cells. As this result implies, key death-signaling proteins of the BCL-2 family, including BIM, were found to be up-regulated after erlotinib treatment and intercepted by over-expressed BCL-2. BIM is induced by lung cancer cell lines that are sensitive to erlotinib but not by those resistant. Reduction of BIM by siRNA induces resistance to erlotinib. We show that EGFR activity is inhibited by erlotinib in H1650, a lung cancer cell line that bears a sensitizing EGFR mutation, but that H1650 is not killed. We identify the block in apoptosis in this cell line, and show that a novel form of erlotinib resistance is present, a block in BIM up-regulation downstream of EGFR inhibition. This finding has clear implications for overcoming resistance to erlotinib. Resistance to EGFR inhibition can be modulated by alterations in the intrinsic apoptotic pathway controlled by the BCL-2 family of proteins. [Cancer Res 2007;67(24):11867–75]

Introduction

Lung cancer is the leading cancer-related cause of death in the United States. Reversible anilinoquinazoline tyrosine kinase inhibitors (TKI) targeting the epidermal growth factor receptor (EGFR), such as erlotinib and gefitinib, have shown clinical activity in patients with non–small cell lung cancer (NSCLC; refs. 1, 2). Activating point mutations and deletion mutations in the kinase domain of EGFR have been found to correlate with response to these TKIs (1–3). Mutations fall into four major classes: single-base substitutions in exon 18; deletions in exon 19; insertion/

duplications in exon 20; and a single-base substitution, L858R, in exon 21 (4–6). Although EGFR inhibitors produce dramatic responses in this population, acquired resistance to TKIs invariably emerges over time, in part mediated by a substitution mutation in exon 20 (T790M), known to confer resistance to gefitinib or erlotinib (7, 8). Among other mechanisms, this mutation can induce steric hindrance that limits access of drug to the ATP-binding pocket in the EGFR kinase domain. Recently, the emergence of c-Met amplification during the course of EGFR-target therapy has been identified as another novel resistance mechanism to reduce the efficacy of gefitinib (9). To overcome the resistance of cells expressing EGFRs harboring kinase domain mutations with the secondary T790M mutation, irreversible TKIs, such as CL-387,785 and HKI-272 have been used (10, 11), with variable potency in cell culture systems.

Most importantly, not all NSCLC patients with EGFR mutations initially respond to EGFR-targeting therapy, suggesting there are subsets of NSCLC cases where unknown underlying mechanisms are responsible for resistance under the complete inhibition of EGFR-signaling axis by kinase inhibitors. Furthermore, in the case of acquired resistance, after accounting for c-MET amplification and T790M EGFR mutations, there remains $\sim 30\%$ of resistant cases in which the molecular basis for resistance remains to be elucidated. It seems likely that many of these are resistant due to alterations in signaling pathways downstream of inhibition of EGFR activity, including whatever death-signaling and executing pathways are used. An important signaling pathway whose role is poorly understood in EGFR targeting is the programmed cell death (PCD) pathway. Therefore, we turned our attention to determining how EGFR inhibition was signaled to and executed by PCD pathways.

Apoptosis is the most thoroughly studied form of PCD; most chemotherapeutic agents can kill via apoptotic pathways. Apoptosis can progress along two distinct pathways, the extrinsic and the intrinsic (12). The extrinsic pathway is activated after ligation of any of several of the cell surface receptors in the TNFR family. After ligation, a death complex forms that results in the activation of the initiator caspase 8, which in turn can activate effector caspases, such as caspase 2 and caspase 7, resulting in widespread proteolysis, cellular dysfunction, and commitment to PCD.

The intrinsic pathway is also known as the mitochondrial apoptotic pathway because commitment to death via this pathway hinges on the control of permeabilization of the mitochondrial outer membrane. This pathway is controlled by interactions among the proapototic and antiapoptotic members of the BCL-2 family of proteins. In response to a wide range of signals of damage or dysfunction, including DNA damage, microtubule disruption, or growth factor withdrawal, certain of the proapoptotic BH3-only class of BCL-2 proteins are up-regulated. Up-regulation can occur

via an increase in protein levels, a change in subcellular localization, or acquisition of a posttranslational modification. These changes, in turn, can be caused by mechanisms including transcriptional means, phosphorylation, protease cleavage, or by protein stabilization (13, 14). The *activator* subtype of BH3-only proteins, which includes at least BID and BIM, can then proceed to activate proapoptotic BAX or BAK (15–18). Activated BAX and BAK undergo an allosteric change and oligomerize, and subsequently participate in the formation of a pore that permeabilizes the mitochondrial outer membrane (19–22). Proapoptotic contents, including cytochrome *c*, apoptosis-inducing factor, and SMAC/Diablo, are exposed to the cytoplasm where they engage various aspects of the downstream apoptotic machinery. For instance, cytochrome *c* forms a complex with caspase 9, apoptosis protease-activating factor 1, and ATP to form the apoptosome, which then activates effector caspase 3 (23). Permeabilization of the mitochondrial outer membrane may be considered the point of commitment to PCD, the point of no return. BCL-2, along with related cellular antiapoptotic proteins BCL-X_L, BCL-w, BFL-1, and MCL-1 interfere with the progression of death signals by binding and sequestering activator BH3-only proteins, preventing their activation of BAX and BAK (15, 24–26). These antiapoptotic proteins may also prevent death by binding BAX and BAK, particularly their activated forms (27). This system is further modulated by the *sensitizer* BH3-only proteins that lack the ability to activate BAX or BAK but nonetheless exert their prodeath function by competing for the BH3 domain–binding site in antiapoptotic proteins such as BCL-2 (15, 17). In doing so, they can displace activator proteins, facilitating the death cascade. Examples of sensitizer BH3-only proteins include BAD and NOXA. Notably, the sensitizer BH3-only proteins exhibit a selective pattern of interaction with antiapoptotic proteins, so that each antiapoptotic protein may be distinguished functionally based on its pattern of interaction with the BH3 domains of sensitizer BH3-only proteins (15, 16, 28, 29).

The intrinsic, or mitochondrial, pathway of PCD plays an important role in killing cancer cells in response to many types of therapies. Yet it is generally poorly understood how the initial event, drug reaching target, is connected molecularly to the proteins that control commitment to mitochondrial cell death, the BCL-2 family of proteins. We investigated whether the intrinsic pathway of PCD is essential for death after inhibition of mutated EGFR. We found that the intrinsic pathway was indeed essential and, furthermore, show that BIM is a key mediator of the death signal. This knowledge allows us to identify a novel mechanism for erlotinib resistance downstream of EGFR inhibition but upstream of BIM up-regulation. Such placement is the first step in investigation of strategies to overcome resistance to erlotinib.

Materials and Methods

Cell lines. Cell lines described in this paper (unless specified otherwise) were cultured in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (F2442-500ML; Sigma), L-glutamine, and penicillin/streptomycin (Invitrogen). NSCLC cell lines (PC9, HCC827, NCI-H1650, and NCI-H1975; hereafter PC9, HCC827, H1650, and H1975, respectively) and BA/F3 cells bearing EGFR mutation (L858R BA/F3 or Del 4 BA/F3) have been described previously (34, 11, 30). BCL-2 expressing stable clones of L858R BA/F3, Del 4 BA/F3, and HCC827 were obtained by electroporating (Gene Pulser II; Bio-Rad) or Fugene 6 (Roche) mediating transfection with pCI-Neo.FlagBcl2 plasmid into those cells and selecting them with G418 (1 mg/mL for L858R BA/F3 and HCC827; 0.5 mg/mL for Del

4 BA/F3; Sigma). Vector-only (pCI-Neo.Flag) was performed in parallel as control cells.

Cell viability assay. Cells were treated separately or in combination with erlotinib, ABT-737, or ABT-737 enantiomer (*Enant.*) as described in the figure legends (erlotinib was commercially obtained from American Custom Chemicals Corporation; ABT-737 and enantiomer were kindly provided by Abbott Laboratories). DMSO is a solvent-only negative control. After 48 h of incubation, cells were stained with fluorescent conjugates of Annexin V (BioVision or/and Roche) and analyzed on a FACSCalibur machine (Becton Dickinson). Viable cells are Annexin V-FITC–negative.

BH3 profiling. Mitochondria were purified from L858R BA/F3 or Del 4 BA/F3 cells (untreated or treated with erlotinib) by mechanical disruption followed by differential centrifugation, as previously described (17). Mitochondrial suspensions were made at 0.1 mg protein/mL in experimental buffer and exposed to BH3 domain peptides (100 μmol/L), ABT-737, or enantiomer (concentration as indicated) for 40 min at room temperature. Peptides used in this assay were synthesized by Tufts University Core Facility and purified by high performance liquid chromatography. Identity was confirmed by mass spectrometry. Stock solutions were made in DMSO. Peptide sequences are as previously reported (15). Release of cytochrome *c* was determined by a comparison of cytochrome *c* in the pellet and supernatant quantitated by ELISA (R&D systems).

Immunoblotting and immunoprecipitation. Protein lysates were obtained by cell lysis in Triton-x100 buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10% Glycerol, and 1% Triton-x100 (Sigma)]. Immunoprecipitation was performed in 500 μL lysates containing 100 μg proteins, which were precleared by centrifugation followed by exposure to 10 μL (50% slurry) protein A beads (Santa Cruz) at 4°C for 1 h. Cleared extracts were incubated overnight with protein A beads preexposed for 1 h to anti–BCL-2 antibody (6C8; PharMingen). Immunoprecipates were then washed thrice with Triton-x100 buffer and boiled in loading buffer (Invitrogen). Protein samples were electrophoretically separated on NuPAGE 10% Bis-Tris polyacrylamide gels (Invitrogen). Antibodies were used to detect the following proteins on membrane: human BCL-2 (6C8;/100; BD PharMingen); mouse BCL-2 (3F11; BD PharMingen); BCL-xL (kind gift from Larry Boise, University of Miami, Miami, Florida); human MCL-1 (S-19; Santa Cruz); mouse MCL-1 (600-401-394; Rockland); BIM (22-40; Calbiochem); BID (FL-195; Santa Cruz); PUMA (NT and P4743; Sigma); NOXA (114C307; Calbiochem); BMF (4692; Cell Signaling); BAX (N20; Santa Cruz); BAK (NT; Upstate Biotechnology); Actin (MAB1501; loading control; Chemicon); EGFR (SC-03; Santa Cruz); phospho-EGFR(Tyr[1068]), Akt, phospho-Akt(Ser[473]), Erk 1/2, phospho-Erk 1/2(Thr[202]/Tyr[204]) were purchased from Cell Signaling.

Caspase 9 activity assay. BCL-2 or vector expressing stable clones of HCC827 cells were subjected to erlotinib (0.1 μmol/L) treatment. At 6, 24, and 48 h time points after the addition of erlotinib or solvent-only control (DMSO), cells were lysed and analyzed for caspase 9 activity in ApoAlert Profiling Assay Plate (Clontech Laboratories, Inc.) according to manufacturer's protocol. Arbitrary units were derived by normalizing the fluorescent reading to the amount of proteins used in each sample.

BIM SiRNA knockdown. PC9 cells were transfected with either scramble or BIM SiRNA (Integrated DNA Technologies; Tri*FECTa* kit, cat. # HSC.RNAI.N006538.2. BIM SIRNA duplex1 was used in the study) using HiPerFect transfection Reagent (QIAGEN) according to manufacturer's protocols. Twenty-four hours after initial transfection, cells were treated with erlotinib and incubated further for 24 h before being harvested for Western blot analysis or for 48 h and being prepared for flow cytometry analysis with fluorescent conjugates of Annexin V.

Western blot protein quantification. Densitometry of protein bands were acquired using an AlphaImager EC gel documentation system (Alpha Innotec), and bands were analyzed with the spot densitometry analysis tool (Alpha Ease FC software, version 4.1.0).

Results

We initially used Ba/F3 cells as a pliable system in which to study the death signaling after EGFR inhibition. Interleukin-3

(IL-3)–dependent murine pro-B Ba/F3 cells were transduced with retrovirus containing the EGFR L858R mutant as described previously (30). This prior study had shown that Ba/F3 cells expressing the L858R mutant were able to grow independently of IL-3, but that treatment of such cells with an inhibitor of EGFR induced cell cycle arrest and apoptosis (30, 31). Other studies showed that in these Ba/F3 cells, erlotinib induced dephosphorylation of EGFR and of downstream targets of EGFR (32). To examine the molecular mechanisms involved in the apoptosis induced by this targeted therapy, we examined the effects of BCL-2 expression, as BCL-2 inhibits initiation of the mitochondrial, or intrinsic, pathway of apoptosis. We transfected Ba/F3 cells expressing L858R with BCL-2 or empty vector control (Fig. 1*A*).

Empty vector-transfected cells treated with erlotinib were killed as measured by Annexin V staining. Annexin V stains cells in which the phosphatidyl serine on the inner leaflet of the plasma membrane is externalized, a finding consistent with apoptotic death. BCL-2 inhibited cell death initiated by erlotinib, demonstrating that the mitochondrial apoptotic pathway is required for erlotinib-induced death (Fig. 1*B, top*).

An important mechanism by which BCL-2 inhibits cell death is by sequestering prodeath molecules of the BCL-2 family. This sequestration relies on binding of the BH3 domain of the prodeath proteins. ABT-737 is a small molecule mimetic of the proapoptotic BH3 domain that can be used as a competitive inhibitor of antiapoptotic BCL-2 function. Treatment with ABT-737 can displace prodeath BH3-containing molecules from BCL-2, allowing for the progression of death signaling. We hypothesized that if BCL-2 is protecting from cell death by binding proapoptotic BH3-containing proteins generated by EGFR inhibition, then ABT-737 should reverse the protection afforded by BCL-2. Vector or BCL-2–transfected cells were resistant to ABT-737 treatment in the absence of erlotinib (Fig. 1*B, middle*). However, ABT-737 treatment reversed the protection from erlotinib afforded by BCL-2 (Fig. 1*B, bottom*). Comparison with Fig. 1*B* (*top*) reveals that treatment with 100 nmol/L ABT-737 restored sensitivity to erlotinib in BCL-2–expressing cells to that of vector-transfected cells. Hence, we had found that treating a cell expressing an activating mutant of EGFR with erlotinib induced death via the mitochondrial apoptotic pathway using BH3 domain-containing proapoptotic proteins. BCL-2 could prevent this death, at the expense of rendering the cell newly dependent on BCL-2 function. Protection by BCL-2 could then be abrogated and dependence exploited by treatment with the BCL-2 antagonist ABT-737.

EGFR can be activated by several different mutations. To test whether this pathway of death signaling was specific to the L858R mutant, or rather more generalizable to EGFR activation in general, we turned to a functionally distinct activating mutant of EGFR containing a deletion. L747-P753del7insS (del4) is one of the most prevalent deletion mutations found in NSCLC patients. We transfected Ba/F3 cells previously transduced with the deletion with Flag-BCL-2 or empty vector control (Fig. 1*C*). Similar to cells

Figure 1. BCL-2 blocks erlotinib killing via mitochondrial apoptotic pathway; ABT-737 restores sensitivity to erlotinib. *A,* Western blot analysis of FlagBCL-2 and EGFR expression levels in L858R BA/F3 mutant clones transfected with control vector (*vec*) or pCI-Neo.FlagBCl-2 (*bcl-2*). Endogenous mouse BCL-2 was also shown. *B,* cell viability of L858R BA/F3 vec or bcl-2 cells treated with increasing doses in erlotinib (*top*), ABT-737 (*middle*), or the combination of erlotinib and ABT-737 (100 nmol/L; *bottom*) after 48-h incubation with compound(s). Cells were stained with fluorescent conjugates of Annexin V for flow cytometry analysis. Viability was shown as a percentage of control (DMSO treated) cells; *error bars,* SD of experiments done in triplicate. *C,* Western blot analysis of FlagBCL-2 and EGFR expression levels in Del4 BA/F3 mutant clones transfected with the following control vectors: *vec (3), vec (4)* or pCI-Neo.FlagBCl-2 [*bcl-2 (9)*], and *bcl-2 (13)*. Endogenous mouse BCL-2 in those cells was also shown. *D,* cell viability assays performed in Del 4 BA/F3 cells (clone types as indicated) as in *B* with increased doses in erlotinib (*top*), ABT737 (*middle*), or the combination of erlotinib (increased doses) and ABT-737 (100 nmol/L; *bottom*).
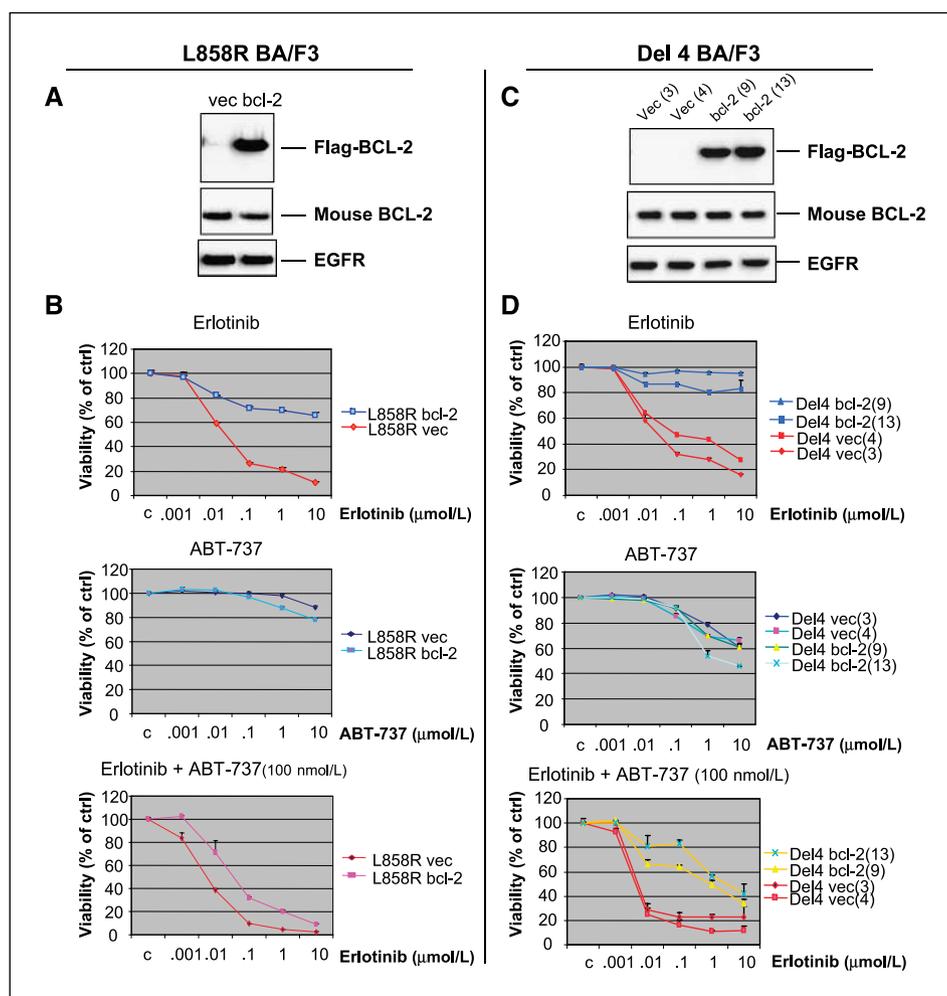
transduced with L858R, cells transduced with the del4 mutant were killed by erlotinib (Fig. 1D, top), a death that was in turn inhibited by BCL-2. Protection by BCL-2 was again abrogated by treatment with the BH3-mimetic ABT-737 (Fig. 1D, middle and bottom). Thus, we concluded that death signaling via the intrinsic apoptotic pathway is likely a general property of erlotinib inhibition of activated EGFR, not restricted to a specific activating mutation.

We hypothesized that BCL-2 was preventing death by sequestering prodeath signals at the mitochondrion. To test this hypothesis, we used a strategy called "BH3 profiling" that detects the priming of BCL-2 with death signals at the mitochondrion (15). We have previously shown that cells in which BCL-2 is actively intercepting death signaling by binding and sequestering prodeath BH3-only proteins may be considered "primed for death" (15, 33). Such cells are dependent on BCL-2 function for survival. This dependence can be detected using BH3 profiling.

BH3 profiling tests mitochondria from the cell of interest by exposing them to peptides derived from the BH3 domains of prodeath BH3-only molecules. Only mitochondria bearing antiapoptotic proteins "primed" with activator BH3 death signals respond to sensitizer BH3 peptides, as detected by cytochrome *c* release. Moreover, the pattern of response to sensitizer peptides may be compared with the binding pattern of the individual antiapoptotic proteins to determine exactly which antiapoptotic proteins are bearing the activator BH3-only proteins. This technique has been described in more detail elsewhere (15, 33). If our model is correct, then we would expect mitochondria to show limited dependence on BCL-2 when the cell was expressing activated forms of EGFR in the absence of erlotinib, with or without BCL-2. However, we would expect that cells expressing activated EGFR and BCL-2 would show increased dependence on BCL-2 after erlotinib treatment, as the mitochondrial BCL-2 would
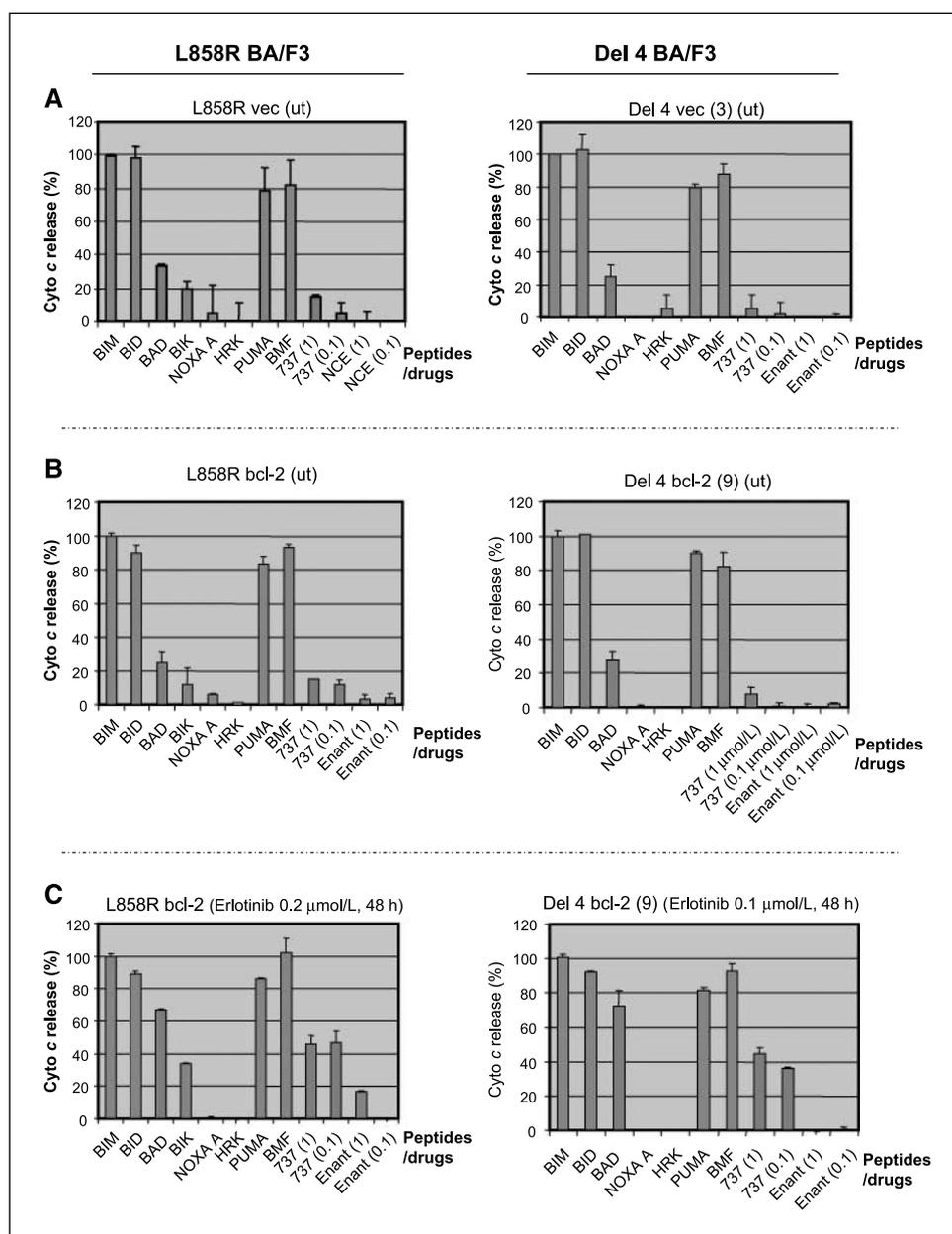

Figure 2. Cellular BCL-2 dependence after EGFR inhibition results from "priming" of BCL-2 at the mitochondrion. Mitochondria were isolated from different EGFR mutants cells and incubated with a panel of BH3 peptides (100 μmol/L), ABT-737, or enantiomer (concentrations as indicated). Release of cytochrome *c* was determined by a comparison of cytochrome *c* in the pellet and supernatant quantitated by ELISA. Values shown reflect subtraction of background determined from DMSO-treated samples and normalized to release by BIM; *error bars,* SD of duplicate experiments. *A, B,* and *C,* BH3 profiling of L858R BA/F3 cells (*left*) bearing with vector-only (*A, left*), bcl-2 (untreated; *B, left*), or bcl-2 treated with 0.2 μmol/L erlotinib for 48 h (*C, left*), respectively. *A, B,* and *C,* BH3 profiling of Del 4 BA/F3 cells (*right*) bearing with vector-only (*A, right*), bcl-2 (untreated; *B, right*), or bcl-2 treated with 0.1 μmol/L erlotinib for 48 h (*C, right*), respectively. Note that erlotinib treatment "primes" BCL-2 expressing cells, rendering them increasingly dependent on BCL-2 function. *Vec,* vector; *ut,* untreated.

now be primed with death signals requiring sequestration. This is what we found by BH3 profiling for both types of mutants (Fig. 2). Therefore, the experiments with ABT-737 and BH3 profiling agree that erlotinib treatment induces death signaling that converges on the mitochondrion and causes cell death via the intrinsic apoptotic pathway. This death signaling can be interrupted by mitochondrial BCL-2, keeping the cell alive, but causing priming of BCL-2 with death signals, and rendering it newly dependent on BCL-2 function for survival.

It is of obvious interest to determine which of the prodeath BH3-only proteins are dynamic participants in the death signaling after erlotinib's inhibition of EGFR. We first examined how abundance of likely candidate proteins varied with time after erlotinib treatment. We performed this assay using BCL-2–protected cells as this afforded us the possibility of examining death signaling without encountering the artifactual protein degradation encountered if cells are allowed to commit to PCD. The most striking increase we found was in the levels of BIM (Fig. 3A). Other proteins investigated showed more modest increases (PUMA, BAX, and BAK). Also notable was a modest reduction in levels of the antiapoptotic MCL-1 protein.

Because BCL-2 maintains survival by binding the prodeath-signaling molecules upstream of activation of BAX and BAK, it can also be considered for experimental purposes as a type of "bait" used to capture these death-signaling molecules. We would expect molecules important in death signaling to therefore be found in complex with BCL-2 after erlotinib treatment. Thus, we examined what prodeath BH3-only proteins were found in coimmunoprecipitation complexes with BCL-2. We found that both BIM and PUMA could be found at increasing levels in complex with BCL-2 after erlotinib signaling (Fig. 3B). Therefore, BIM and PUMA were candidates for signaling molecules downstream of EGFR inhibition that trigger apoptosis.

Currently, the key clinical application of EGFR inhibition in cancer is in NSCLC. Therefore, we next turned to NSCLC cell lines to study EGFR inhibition in a more clinically relevant model. We selected four NSCLC cell lines: PC9, which harbors a delE746_A750 activating mutation in EGFR; HCC827, which harbors a delE746_A750 activating mutation in EGFR; H3255, which harbors a L858R activating mutation in EGFR; H1650, which harbors a delE746_A750 activating mutation in EGFR; and H1975, which bears the activating L858R/T790M mutations in EGFR. Of note, the T790M mutation confers resistance to erlotinib by steric hindrance. PC9 and HCC827 are sensitive to erlotinib treatment. H1650, despite harboring an activating mutation in EGFR that is expected to foster erlotinib sensitivity, is resistant to erlotinib, as is H1975 (refs. 7, 8, 34; Fig. 4A). We next investigated the temporal pattern of BCL-2 family protein levels after erlotinib treatment in four of these cell lines. At 3 h after treatment, BIM levels increased strikingly in the two sensitive cell lines after erlotinib treatment, but much less so in the resistant cells (Fig. 4B). Also notable was a subtle but reproducible shift of BIM to a more rapidly migrating form, likely indicative of a change in a posttranslational modification. We examined a wider range of proteins at 24 h after treatment and found a similar pattern (Fig. 4C; see also Supplementary Figs. S1–4). Densitometric analysis suggests that higher basal BIM levels and posttreatment BIM levels correspond to erlotinib sensitivity (Fig. 4B and C). Of note, subtle PUMA up-regulation seems to correlate with sensitivity to erlotinib, but its increase is of lesser magnitude, and less consistent in repeated experiments (Supplementary Fig. S5; data not shown). Levels of
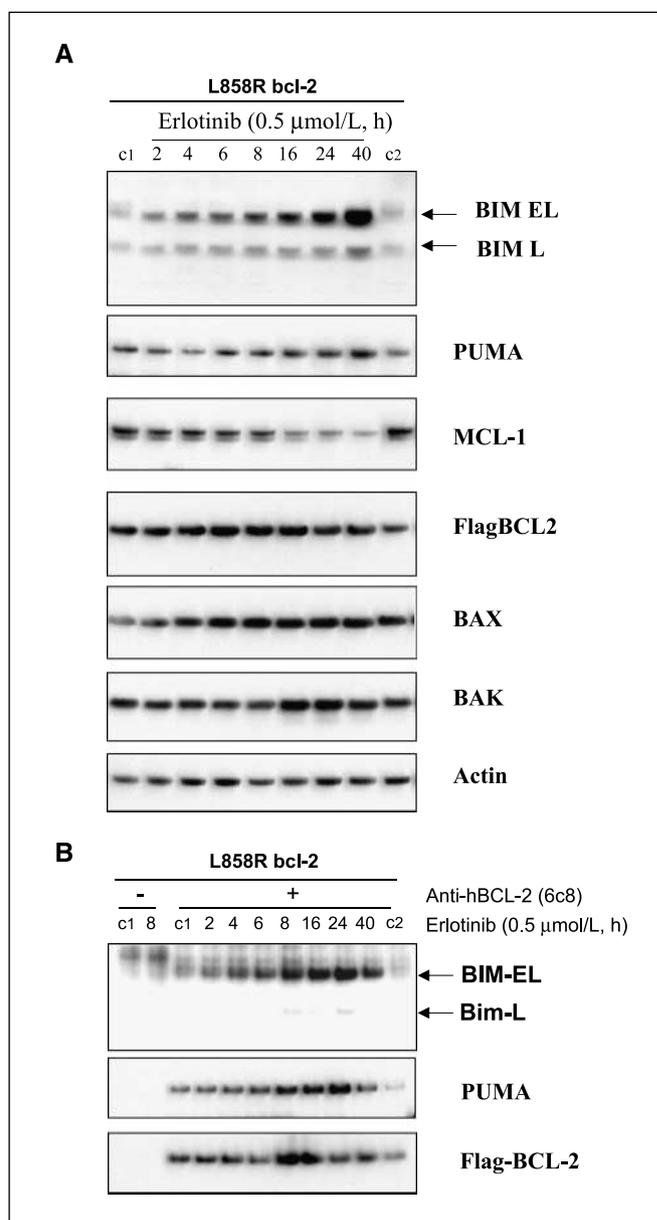

Figure 3. Proapoptotic BH3-only proteins BIM and PUMA are up-regulated by erlotinib and sequestered by BCL2. *A,* protein lysates were prepared from L858R BA/F3 bcl-2 cells treated with 0.5 μmol/L erlotinib over a time course and separated by denaturing electrophoresis. Membranes were blotted with antibodies against BCL-2 family proteins as indicated. *B,* same protein lysates as *A* were subjected to immunoprecipitation with an antibody specific for human BCL-2 (*6C8*). Immunoprecipitants were immunoblotted for BIM, PUMA, and BCL-2 proteins [anti–BCL-2 antibody (/100) was used for immunoblotting after immunoprecipitation].

MCL-1 decrease in HCC827 but not other cell lines. It is not clear that reductions in MCL-1 reflect changes upstream of the initiation of apoptosis, however. BAX and BAK levels do not consistently change with erlotinib treatment (Supplementary Figs. S1–4).

H1650 offers a particularly interesting example of erlotinib resistance because it bears a mutation that would be expected to allow drug interaction with EGFR. To verify that erlotinib indeed can target the EGFR in H1650, we examined phosphorylation of targets downstream of EGFR. These targets became hypophosphorylated after erlotinib treatment, demonstrating that EGFR

activity is indeed inhibited (Fig. 4*D*). Notably, because Akt becomes hypophosphorylated, it rules out resistance due to rescue by MET amplification, a resistance mechanism recently described (9). Therefore, we have identified a new mechanism of resistance to erlotinib downstream of EGFR inhibition that does not involve MET but rather blocks BIM up-regulation.

BIM up-regulation was the most striking difference between sensitive and resistant cell lines. We therefore chose to examine further the role of BIM in conveying the death signal downstream of erlotinib treatment. First, we tested whether NSCLC cells containing naturally selected mutants of EGFR used the intrinsic mitochondrial pathway of cell death after erlotinib inhibition, as in our Ba/F3 models. We found that BCL-2 inhibited cell death, again

implicating the requirement of the mitochondrial apoptotic pathway (Fig. 5*A*). We found that BIM and PUMA could both be found sequestered by BCL-2 after erlotinib treatment (Fig. 5*B*). Again, the up-regulation of BIM was quantitatively more striking than that of PUMA, however. In addition, we found that caspase 9 activation, a hallmark of mitochondrial apoptosis, accompanied erlotinib killing, further supporting the importance of the intrinsic mitochondrial pathway (Fig. 5*C*).

If BIM plays a critical role in the communication of death signals, then its removal should inhibit death. To test the importance of BIM, we used siRNA to knock down BIM levels (Fig. 5*D*). Reduction of BIM levels indeed caused a reduced sensitivity to treatment with erlotinib, consistent with its role as
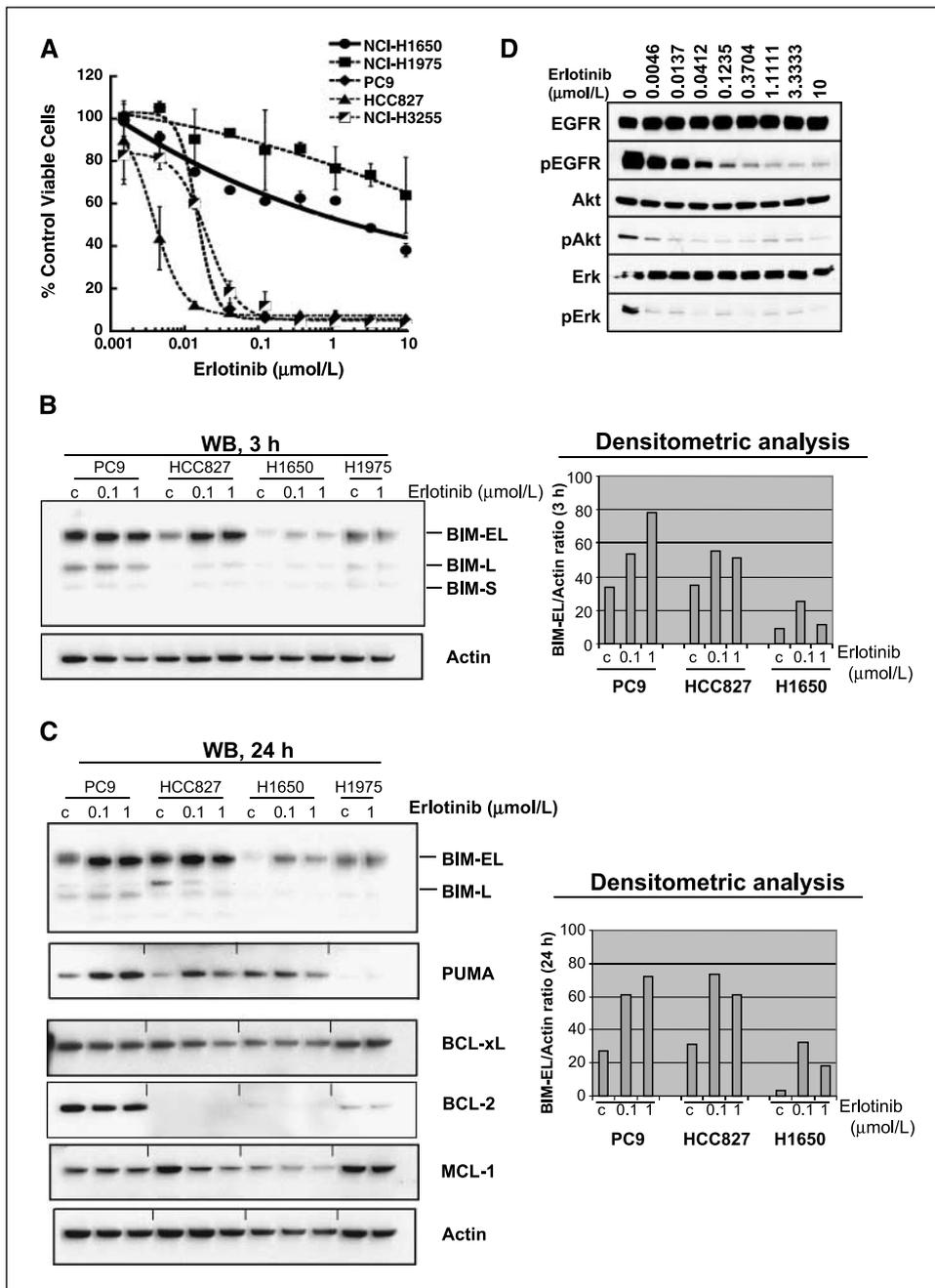

Figure 4. Proapoptotic BH3-only protein BIM is markedly increased by erlotinib in sensitive cell lines (*PC9* and *HCC827*) but not in resistant ones (*H1650* and *H1975*). *A*, NSCLC cells (*PC9*, *HCC827*, *H1650*, *H1975*, and *H3255*) were treated with the indicated doses of erlotinib. Viability was measured by CCK-8 colorimetric assay (Dojindo). *B* and *C*, NSCLC cells (*PC9*, *HCC827*, *H1650*, and *H1975*) were treated with indicated doses of erlotinib for 3 h (*B*) and 24 h (*C*) and harvested for Western blot analysis for BCL-2 family proteins. Actin is a loading control. BIM-EL/Actin ratio for the 3-h blotting was obtained after performing densitometric analysis on the respective blot. BIM-EL/Actin ratio for the 24-h blotting was shown as a mean of two independent blotting (*left*; data not shown). *D*, cell line H1650 was treated with the indicated concentrations of erlotinib. Whole cell lysates were evaluated for protein levels and phosphorylation status of EGFR, AKT, and Erk as indicated by immunoblot. *C*, DMSO-treated control cells.

Figure 5. Sequestering BIM via BCL-2 overexpression or decreasing BIM protein levels protects cells from death triggered by erlotinib. *A*, Western blot analysis of FlagBCL-2 expression level in transfected HCC827 cells and cell viability [determined by Annexin V flow cytometry analysis (*FACS*)] of parental and transfectant HCC827 cells treated with erlotinib for 48 h. Results of cell viability were shown as a percentage of control cells; *error bars*, SD of triplicate experiments. *Vec*, vector. *B*, protein lysates prepared from BCL-2 expressing HCC827 cells (control or treated with erlotinib) were used for immunoprecipitation (*IP*) with an antibody specific for human BCL-2 (6C8). Both protein lysates and immunoprecipitates were separated by electrophoresis and immunoblotted for BIM, PUMA, and BCL-2 [anti–BCL-2 antibody (/100) was used for immunoblotting after immunoprecipitation]. C1 and C2 were duplicates of DMSO treated samples. *, a nonspecific crossreacting band. *C*, caspase 9 activity assay. BCL-2 or vector expressing HCC827 cells were treated with erlotinib (0.1 µmol/L) for 6, 24, and 48 h. At each time point, cells were lysed and analyzed for caspase 9 activity using ApoAlert Profiling Assay plate. The fold changes (erlotinib-treated versus control cells) were shown from two independent experiments. *Columns*, mean; *bars*, SD. *E*, erlotinib. *D*, BIM levels were analyzed by immunoblot in PC9 cells transfected with either scrambled or BIM siRNA and treated with erlotinib. Mock, scramble, or BIM SiRNA–transfected PC9 cells were also subjected to viability assay by Annexin V flow cytometry analysis. Viability was shown as a percentage of control cells; *error bars*, SD of triplicate experiments.
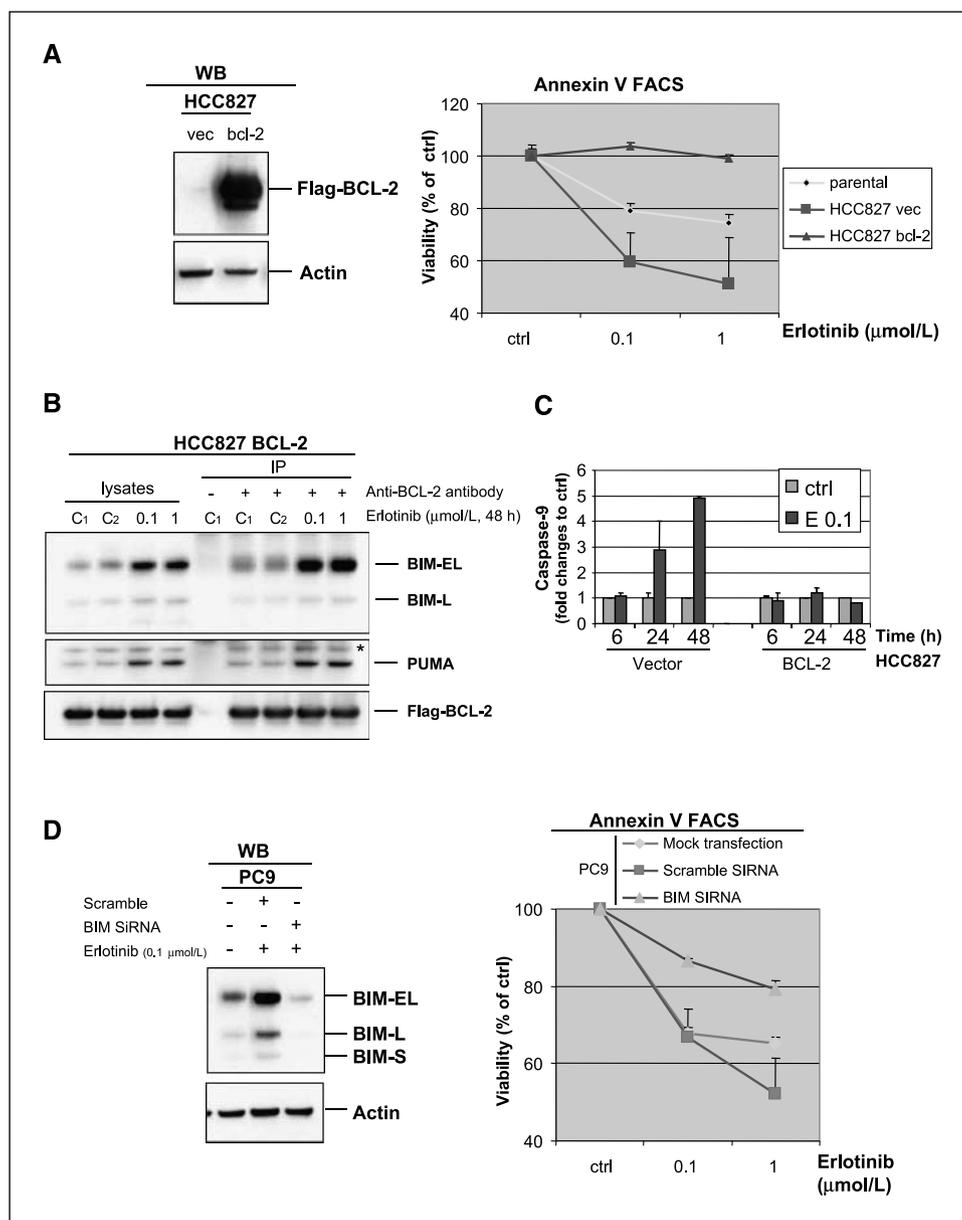
a key signaling molecule downstream of activated EGFR activity in NSCLC (Fig. 5*D*). Similar reduction of PUMA levels had no detectable effect on erlotinib sensitivity, suggesting a more limited role for this protein in erlotinib-induced death signaling (Supplementary Fig. S5).

Discussion

Epithelial cancers are responsible for the vast majority of morbidity and mortality caused by cancer. An exciting new strategy has focused on the targeting of activated EGFR molecules that are often present in carcinomas. This strategy has reached clinical fruition in NSCLC with the approval of erlotinib for the second- or third-line treatment of NSCLC. Erlotinib has been shown to induce responses in cancers that bear activating mutations in EGFR. The molecular pathways connecting EGFR inhibition to cell death have been poorly understood, however.

Furthermore, the alterations in signaling pathways that result in resistance are still incompletely understood.

Here, we definitively connect inhibition of activated EGFR by erlotinib with the intrinsic, or mitochondrial, cell death pathway controlled by the BCL-2 family of proteins. BCL-2, which protects against mitochondrial cell death, inhibits erlotinib toxicity. Moreover, mitochondria isolated from erlotinib-treated, BCL-2–protected cells expressing activated EGFR show evidence of bearing significant quantities of death signals destined for the mitochondrial pathway, as shown by BH3 profiling. We showed the participation of the mitochondrial pathway both in a model hematopoietic cell line as well as in NSCLC lines, suggesting that this is a general feature to be expected of inhibition of activated EGFR in many different cellular contexts.

We identify BIM induction as a key step in signaling EGFR inhibition to the intrinsic apoptotic pathway. We, furthermore, show that reduction of BIM by RNAi affords protection against
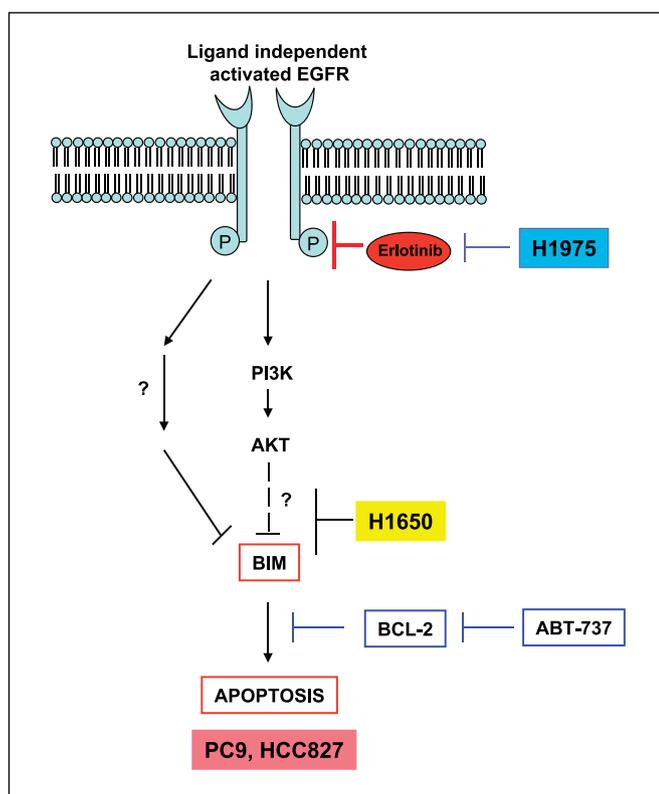
Figure 6. Model of how apoptosis is induced by erlotinib and how it may be blocked. Inhibition of activated EGFR by erlotinib results in BIM up-regulation downstream of loss of kinase activity. H1975 is resistant based on a mutation that inhibits the binding of erlotinib to EGFR. H1650 is resistant based on a novel mechanism that blocks BIM up-regulation downstream of kinase inhibition. BCL-2 can block apoptosis by sequestering BIM and other proapoptotic molecules generated by erlotinib. BCL-2–derived resistance can be reversed by ABT-737. PC9 and HCC827 are killed by the mitochondrial, or intrinsic, apoptotic pathway, downstream of BIM activation.

erlotinib-induced apoptosis, supporting a causal role for BIM in this death signaling. Our comparison of NSCLC cell lines also supports a role for BIM in death signaling. It is striking in Fig. 4B and C that BIM levels are markedly increased in the cell lines sensitive to erlotinib (PC9 and HCC827) but not in those resistant to erlotinib (H1650 and H1975).

Although we have shown that BIM plays an important role in death signaling, this does not rule out the participation of other BCL-2 family proteins. For instance, we note that the proapoptotic BH3-only protein PUMA is up-regulated in cells sensitive to erlotinib. This is interesting, as PUMA was initially identified as a prodeath protein transcriptionally activated by p53. Subsequently, PUMA up-regulation has been observed under conditions that seem to be p53 independent. Because erlotinib is not genotoxic, PUMA up-regulation in this case is likely also to be p53 independent. However, our knockdown results suggest that PUMA plays a more limited role than BIM in death signaling after erlotinib treatment. In addition, we cannot rule out contribution by one of the many other BH3-only proteins that were not examined. However, the BIM siRNA results suggest that a substantial proportion of the BH3-only death signaling is performed by BIM. The balance can be tipped in favor of apoptosis by loss of antiapoptotic proteins, too. MCL-1 is one of the more dynamic antideath proteins. Although we found MCL-1 levels to decrease

after erlotinib treatment in Ba/F3 cells (Fig. 3A), a similar decrease was not consistently found in erlotinib-treated NSCLC cell lines tested (Supplemtary Figs. S1–4). In addition, increases in proapoptotic BAX and BAK were not consistently found in erlotinib-treated NSCLC cells (Supplementary Figs. S1–4).

Although lung cancers bearing activating mutations in EGFR usually respond to erlotinib, the eventual development of resistance is very common. In many of these cases, the cause of resistance is at the level of the target. Many resistant clones survive due to the introduction of secondary mutations in EGFR mutants that are poorly targeted by erlotinib due to steric hindrance. In others, it has recently been shown that activation of MET can substitute for EGFR signaling (9). However, in a significant portion, no change in EGFR sequence or in MET can be identified. This suggests that resistance takes place in the death-signaling pathway somewhere downstream of drug-reaching target. We show here that the proteins in the BCL-2 family are excellent candidates for being altered to allow resistance. For instance, we show that increased BCL-2 overexpression or decreased BIM expression can both afford protection from erlotinib. It will be interesting to test whether either of these alterations are selected for *in vivo*; subsequent studies on clinical samples will include investigation of levels of these proteins. Excitingly, we find that a BCL-2 antagonist, ABT-737, can effectively abrogate resistance to erlotinib caused by BCL-2 overexpression. Should clinical samples of patients resistant to erlotinib show increased BCL-2 expression, addition of a BCL-2 antagonist such as BCL-2 would be an obvious intervention for clinical investigation.

We also identify a novel mechanism of erlotinib resistance in a NSCLC cell. H1650 harbors del E746_A750 EGFR activating mutation, and erlotinib completely suppresses both EGFR phosphorylation and its downstream signaling transduction at low dose (Fig. 4D). However, H1650 is not killed by erlotinib treatment, which suggests the existence of resistance mechanisms downstream of EGFR inhibition. Interestingly, BIM induction upon treatment with erlotinib in H1650 was greatly reduced in magnitude and significantly delayed compared with other erlotinib sensitive NSCLC cell lines. Therefore, we can place the source of resistance in H1650 downstream of EGFR activity inhibition but upstream of BIM activation (Fig. 6).

The signaling pathways that mediate up-regulation of BIM after erlotinib treatment are not yet fully elucidated. BIM levels and function have been shown to be subject to control at the level of transcription, proteosomal degradation, mRNA stability, phosphorylation, caspase cleavage, and subcellular localization (35–43). Some of these mechanisms are in turn controlled by phosphorylation pathways, including MAP/ERK kinase/extracellular signal-regulated kinase, p38MAPK, Ras/Raf, and phosphatidylinositol-3-OH/mammalian target of rapamycin, so that integration with EGFR signaling is certainly plausible (44). Clearly, many testable hypotheses of BIM up-regulation are immediately suggested and are the subject of current investigation. Once one thoroughly understands the pathways at work in BIM up-regulation, one can begin to design rational strategies to augment the up-regulation of BIM in conjunction with erlotinib treatment, or to restore it where lost in clones that acquire resistance to erlotinib. It is noteworthy that it seems that changes in posttranslational modification occur to BIM after EGFR inhibition (Fig. 4B and C; Supplementary Figs. S1 and S2). It has been shown that phosphorylation of BIM decreases its proapoptotic function or half-life in conjunction with growth

factor signaling (35, 36, 38–43). An interesting hypothesis worthy of further study is that loss of EGFR signaling induces loss of BIM phosphorylation, resulting in an augmentation of the proapoptotic signal.

Here we present results that clarify the death-signaling pathways that are used after erlotinib's inhibition of activated EGFR in cancer. Understanding the details of death pathways used by targeted therapies is essential in understanding how to predict what cancers will respond and what rational combination therapies might be most efficacious. Furthermore, understanding the death pathways assists in the understanding of induced resistance and in the design of strategies to counteract resistance. Our knowledge of the molecular operation of cell death pathways has increased tremendously in the past decade. We are now at the stage where this knowledge can begin to be put to use for the improvement of cancer therapy.

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Proapoptotic BH3-Only BCL-2 Family Protein BIM Connects Death Signaling from Epidermal Growth Factor Receptor Inhibition to the Mitochondrion

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