BIM Expression in Treatment-Naïve Cancers Predicts Responsiveness to Kinase Inhibitors

Anthony C. Faber3,4*, Ryan B. Corcoran3,4, Hiromichi Ebii3,4, Lecia V. Sequist3,4, Belinda A. Waltman3,4, Euiheon Chung5, Joao Incio5, Subba R. Digumarthy2,4, Sarah F. Pollack3,4, Youngchul Song3,4, Alona Muzikansky1, Eugene Lifshits1,4, Sylvie Roberge5, Erik J. Coffman3,4, Cyril H. Benes3,4, Henry L. Gómez2, José Baselga3,4, Carlos L. Arteaga6,7, Miguel N. Rivera1,3,4, Dora Dias-Santagata1,3,4, Rakesh K. Jain5, and Jeffrey A. Engelman3,4

Published OnlineFirst July 22, 2011; DOI: 10.1158/2159-8290.CD-11-0106
Cancers with specific genetic mutations are susceptible to selective kinase inhibitors. However, there is a wide spectrum of benefit among cancers harboring the same sensitizing genetic mutations. Herein, we measured apoptotic rates among cell lines sharing the same driver oncogene following treatment with the corresponding kinase inhibitor. There was a wide range of kinase inhibitor-induced apoptosis despite comparable inhibition of the target and associated downstream signaling pathways. Surprisingly, pretreatment RNA levels of the BH3-only pro-apoptotic BIM strongly predicted the capacity of EGFR, HER2, and PI3K inhibitors to induce apoptosis in EGFR-mutant, HER2-amplified, and PIK3CA-mutant cancers, respectively, but BIM levels did not predict responsiveness to standard chemotherapies. Furthermore, BIM RNA levels in EGFR-mutant lung cancer specimens predicted response and duration of clinical benefit from EGFR inhibitors. These findings suggest assessment of BIM levels in treatment-naïve tumor biopsies may indicate the degree of benefit from single-agent kinase inhibitors in multiple oncogene-addiction paradigms.

**SIGNIFICANCE:** In several oncogene-addiction paradigms, assessment of BIM RNA levels identifies those cancers that fail to have substantial apoptotic responses to kinase inhibitors. BIM RNA levels may be assessed in diagnostic cancer specimens to predict which patients will receive less benefit from single-agent kinase inhibitors. Cancer Discovery: 1(4):352–65. © 2011 AACR.

**INTRODUCTION**

Subsets of cancers harboring specific genetic abnormalities are sensitive to specific kinase inhibitors. Two examples of such oncogene-addicted cancers include EGFRTK-mutant lung cancers and HER2-amplified breast cancers. In both cancer types, tyrosine kinase inhibitors (TKIs) or antibodies that disrupt the function of the corresponding receptor tyrosine kinase (RTK) are effective treatments (1–4). Other paradigms of oncogene addiction have more recently emerged, such as EML4-ALK translocated lung cancers treated with the ALK TKI crizotinib (5). Although these therapies are highly successful in these genetically defined populations as a whole, there is a high degree of heterogeneity among patient outcomes within each genetically defined subgroup. For example, although some patients benefit from a targeted therapy for much longer than one year, some only benefit for a few months. In addition, 30% to 40% of patients with EGFR-mutant non–small-cell lung cancers (NSCLCs) and ALK translocated lung cancers fail to achieve responses to targeted therapy based on Response Evaluation Criteria in Solid Tumors (RECIST), for largely unknown reasons (5). The biology underlying this heterogeneity of clinical benefit is not well understood. In addition, biomarkers that identify those who do not benefit as greatly from single-agent targeted therapy would aid in directing them towards novel therapeutic strategies.

The evasion of apoptosis is a hallmark of cancer and is often caused by disruptions in the intrinsic surveillance system regulating the survival of a cancer cell. Critical to this surveillance system is a group of Bcl-2 like proteins that connect growth factor signaling pathways to the mitochondria, the epicenter of apoptosis (6). In oncogene-addicted cancers, the growth and survival signals originating from the oncogene regulate both the expression and the interactions of Bcl-2 family members. When the balance of pro- and anti-apoptotic proteins changes to favor apoptosis, as it often does following effective targeted therapy, two terminal members of the Bcl-2 family, Bak and Bax, homo- and/or hetero-dimerize to form porous channels in the mitochondria, committing the cell to apoptosis (6). Recent data have demonstrated that the pro-apoptotic Bcl-2 family member BIM is a particularly critical mediator of targeted therapy-induced apoptosis in both blood and solid tumor cancers (7–7). This BH3-only Bcl-2 family member directly binds to the antagonistic pro-apoptotic Bcl-2 members, such as Mcl-1 and Bcl-2, by binding into their hydrophobic clefts, countering their pro-survival effects (18). BIM also interacts directly with Bax, activating it to promote cell death (19).

In cancers addicted to receptor tyrosine kinases, the regulation of key intracellular signaling pathways (e.g., PI3K-AKT and MEK-ERK) is under strict control of the corresponding RTK. Pharmacologic inhibition of the RTK with targeted therapies leads to suppression of these signaling pathways and often results in apoptosis (7, 20–21). In EGFR-mutant
NSCLC cells, we and others have demonstrated that apoptosis is triggered by tipping the scale of pro- and anti-apoptotic Bel-2 family members in favor of pro-apoptotic signaling (7–11). BIM levels increase following MEK-ERK pathway suppression, and Mcl-1 levels decrease following PI3K-mTORC1 pathway inhibition downstream of EGFR (7). Normally, the MEK-ERK pathway suppresses BIM expression by direct phosphorylation of BIM, leading to its proteasomal degradation (22, 23). The upregulation of BIM resulting from suppression of MEK-ERK signaling is essential for the induction of apoptosis, but in EGFR-mutant cancers, it is not sufficient. Reducing levels of cellular BIM with small interfering RNA (siRNA) and short hairpin RNA (shRNA) blocks TKI-induced apoptosis (7–11). Other successful targeted therapy paradigms have also demonstrated an integral role for BIM in promoting apoptosis in response to targeted therapies, including BRAF-mutant colorectal cancers (13), BRAF-mutant melanoma cancers (14, 24, 25), Bcr-Abl–translocated chronic myeloid leukemia (CML) cancers (15), and EML4-ALK–translocated lung cancers (26). Importantly, evidence from in vivo studies suggests, but does not prove, that the apoptotic response may be an essential contributor to marked tumor regressions in vivo upon treatment with targeted therapies (7, 27). Thus, we hypothesize that patients with oncogene-addicted cancers who undergo the most dramatic apoptotic responses to kinase inhibitors may enjoy the greatest benefit from kinase inhibitors. However, there has been no biomarker identified to date that accurately predicts which EGFR- and HER2-addicted cancers are most likely to undergo robust apoptosis in response to TKIs. Here, we describe the identification of pre-treatment BIM levels as a functional biomarker that predicts the induction of apoptosis in several oncogene-addiction paradigms. Assessment of this biomarker in clinical samples effectively distinguished the benefit that patients derived from single-agent EGFR TKIs.

**RESULTS**

Pretreatment BIM Levels Predict Apoptotic Responses in EGFR-Mutant Lung Cancers Treated with EGFR Inhibitors

We were interested in studying cancers with disparate apoptotic responses to EGFR TKIs despite harboring the same
activating EGFR predicts drug response. Initially, we compared two EGFR-mutant lung cancer cell lines, PC9 and HCC2279, that both harbor exon 19 deletions (Supplementary Table S1). These two cell lines had markedly different apoptotic responses (PC9 approximately 65% versus HCC2279 <10%) (Fig. 1A; Supplementary Tables S1 and S2), despite similar potent suppression of PI3K-AKT and MEK-ERK signaling following treatment with the EGFR TKI, gefitinib (Fig. 1B). Accordingly, EGFR TKIs led to a similar reduction in S-phase cell cycle distribution in the two cell lines (Fig. 1C), consistent with the observed sensitivity of both cell lines to EGFR TKIs in short-term (72 hour) growth assays (28, 29). In long-term growth assays, the growth of both cell lines was inhibited by gefitinib, but the cell viability of PC9 cells was impacted more than the HCC2279 cells (Fig. 1D), suggesting, but not proving, that the differential induction of apoptosis may even result in differences in growth in the presence of drug over long periods. To understand the differential apoptosis, we examined the regulation of the Bcl-2 family of proteins, and found that the expression of the pro-apoptotic extra long (EL) BIM protein (the most abundant form of BIM) was markedly diminished in HCC2279 cells compared to PC9 cells (Fig. 1E). Gefitinib increased the expression of BIM in both cell lines as expected due to MEK/ERK inhibition (~3-fold increase in PC9 cells and ~4-fold increase in HCC2279 cells; ref. 7), but the level reached in HCC2279 cells remained substantially lower (~3-fold) than the level reached in PC9 cells (Fig. 1E).

These results raised the possibility that the pre-treatment and post-treatment levels of BIM might identify which cancers were most likely to undergo an apoptotic response following treatment with a TKI. Thus, we expanded these analyses to other EGFR-mutant lung cancers. As shown in Figure 2A, the cancers with the most pronounced apoptotic responses following gefitinib treatment tended to possess higher levels of BIM expression both pretreatment (“−TKI”) and posttreatment (“+TKI”). Of note, although gefitinib-treatment led to marked downregulation of PI3K-AKT and MEK-ERK signaling in 7 of 8 cell lines (Fig. 2A), the low BIM-expressing H1650 cell line, which has a PTEN deletion, had retention of PI3K-AKT signaling in the presence of gefitinib (30). However, we found this cell line was also resistant to PI3K and MEK inhibitor combination therapy, suggesting that resistance was due to more than just the loss of PTEN (Supplementary Fig. S1A and S1B). Indeed, PI3K/MEK combination therapy is effective at inducing apoptosis in high BIM-expressing cells but not in low BIM-expressing cells (Supplementary Fig. 1B and ref. 7), further supporting the notion that apoptotic responses to targeted therapies are blunted when cellular BIM levels are diminished.

Since BIM levels were induced by the TKI in all of the cell lines, this suggested that post-translational regulation of BIM was similar in all of the models. Thus, we hypothesized that the RNA levels might differ between the EGFR-mutant cell lines that undergo pronounced versus attenuated apoptotic responses. By performing quantitative real-time (q) reverse transcriptase (RT)-PCR, we found that RNA levels of BIM correlated with the magnitude of apoptosis (Fig. 2B, P = 0.0018), suggesting RNA levels of BIM, like protein levels, predict apoptotic response to gefitinib in these cancers. However, we did not observe any correlation between BIM levels and induction of growth arrests as measured by a reduction in S-phase (Supplementary Fig. S1C), consistent with the downregulation of signaling in both low and high BIM-expressing cell lines (Fig. 2A). Thus, it appears that BIM expression distinguishes apoptotic responses to EGFR inhibitors among these cell lines, but not the induction of growth arrest.

**Figure 2.** Greater induction of apoptosis following EGFR TKI treatment correlates with higher basal BIM expression across a panel of EGFR-mutant lung cancers. A, indicated EGFR-mutant lung cancer cell lines were treated with (+TKI) or without (−TKI) 1 μM gefitinib (2 μM CL-387,785 for H1975 cells) for 24 hours and lysates were probed with the indicated antibodies. Red type indicates high BIM-expressing cells, blue indicates low BIM-expressing cells. B, BIM RNA levels were quantified by qRT-PCR, normalized to β-actin, and plotted against the amount of apoptosis induced by the TKI (over vehicle-control) as determined by annexin FACS. Both RNA values and apoptosis are the mean of at least 3 experiments (see Supplementary Table S1). For linear regression analysis, the r² value was 0.83 and P = 0.0018.
We extended these analyses to a panel of HER2-amplified cancers. BIM was differentially expressed across the cell line panel. As shown in Figure 4A, the cancers with the most pronounced apoptotic responses following lapatinib treatment possessed the highest levels of BIM expression both pre- and post-treatment. Importantly, none of these cell lines harbored PIK3CA hotspot mutations or PTEN loss that might impact sensitivity (Supplementary Table S1). Accordingly, the intracellular signaling was suppressed in all cell lines (Fig. 4A). When BIM RNA expression was assessed in these cell lines by quantitative RT-PCR, BIM RNA levels correlated with the magnitude of apoptosis induced by lapatinib (Fig. 4A, $P < 0.0001$). Of note, HER2 copy number did not correlate with the magnitude of the apoptotic response (Supplementary Table S1).

**BIM Levels Predict Apoptotic Response in PIK3CA-Mutant and BRAF-Mutant Cancers**

In both HER2-amplified and EGFR-mutant cancers, treatment with the corresponding TKI increased BIM expression due to the suppression of MEK-ERK signaling, resulting in increased BIM stability (22, 23). Similarly, we observed that pretreatment BIM levels predicted apoptotic responses in HER2-amplified cancers treated with HER2 Inhibitors.

---

**Figure 3.** Higher basal BIM expression is associated with more robust apoptosis, but not cell cycle arrest, in response to TKI in HER2-amplified cancers. A, HER2-amplified breast cancer cells BT-474 and EFM-192A were treated with (+) or without (−) 1 μM TKI (lapatinib) for 72 hours and stained with propidium iodide (PI) and annexin V and analyzed by FACS to quantify annexin V–positive cells. B, cell lines were treated as above for 6 hours and lysates were probed with the indicated antibodies. C, cell lines were treated as above for 24 hours and cell cycle distribution was determined by propidium iodide staining followed by FACS analyses. Percentage of cells in S-phase is indicated. D, cell lines were treated as above with fresh media and drug replenished every 72 hours until the vehicle control-treated (No Rx) plate grew to confluency. At that time, both vehicle-treated and drug-treated plates were stained with the nuclear acid stain SYTO60. A representative plate is shown (top). The percentage of TKI-treated cells that survived are shown (bottom). Error bars are +/− SD of the mean of 3 experiments. E, cell lines were treated with 1 μM lapatinib for 24 hours and lysates were probed with antibodies against BIM and actin.
found that basal BIM levels predicted apoptotic response in 10 BRAF-mutant colorectal cell lines treated with the MEK inhibitor AZD6244, which effectively suppressed ERK phosphorylation in all of the models (Supplementary Fig. S2; Supplementary Table S1). We also evaluated whether basal levels of BIM predicted apoptosis in other oncogene-addicted cancers that did not rely on the ERK pathway for growth/survival. Thus, we examined the PIK3CA-mutated cancers that are sensitive to PI3K inhibitors and found that basal BIM RNA levels also indicated the apoptotic response to the PI3K-mTOR inhibitor NVP-BEZ235 in cancer cell lines harboring PIK3CA hotspot mutations (E545K and H1047R) (Fig. 4B). This was especially surprising because BIM levels decreased following treatment, possibly as a result of feedback activation of ERK signaling (7, 31). Thus, BIM induction is not caused by PI3K inhibition, but its expression correlated with the magnitude of apoptosis suggesting that its basal expression is necessary in mediating the apoptotic response.

**Apoptotic Responses of Oncogene-Addicted Cancers Are Predicated on BIM Expression**

Although we and others have shown that knockdown of BIM expression abrogates the apoptotic response to EGFR and MEK inhibitors (7–11, 13–14), it is unknown whether BIM mediates the apoptotic response to lapatinib in HER2-amplified cancers and to PI3K inhibitors in PIK3CA-mutated cancers. Because the findings above suggested a potential role in apoptosis in these cancer models as well, we reduced BIM levels using siRNA in HER2-amplified BT-474 and SkBr3 cells (Fig. 5A; Supplementary Fig. S3A). HER2-amplified breast cancer cells are also sensitive to single-agent PI3K inhibitors (7, 27), and BIM knockdown accordingly protected from NVP-BEZ235-induced apoptosis (Fig. 5A; Supplementary Fig. S3A). Similarly, in cell lines with PIK3CA hotspot mutations, BIM knockdown protected cells from NVP–BEZ235–induced apoptosis compared to control cultures (Fig. 5B; Supplementary Fig. S3B).

We next determined if BIM expression also protected from apoptosis induced by a cytotoxic chemotherapeutic agent,
paclitaxel (Taxol; Bristol-Myers Squibb). We chose Taxol because it is a clinically relevant chemotherapy for both lung and breast cancer. Although apoptosis induced by gefitinib correlated with BIM-expression in the EGFR-mutant cancer cell lines (Fig. 2B), we found that Taxol induced similar levels of apoptosis in low BIM and high BIM-expressing cells (Fig. 5C). Accordingly, we observed that BIM knockdown provided a less impressive protective effect from Taxol-induced apoptosis in the HER2-amplified and PIK3CA-mutated cancers, and reached statistical significance in only 1 of the 4 models tested (Fig. 5A and B; Supplementary Fig. S3C). This suggests that the efficacy of the kinase inhibitors seem to be more sensitive to the amount of BIM in the cell than that of Taxol. We also examined whether the amount of apoptosis induced by two other classic chemotherapies, the nucleoside analog gemcitabine and the DNA cross-linker cisplatin, was abrogated following knockdown of BIM. We observed that BIM knockdown had a negligible impact on the ability of these two drugs to induce apoptosis in HER2-amplified SkBr3 cells or PIK3CA-mutant HCC1954 cells (Supplementary Fig. S3D). Similar to the Taxol analyses, BIM RNA levels did not predict apoptotic responses to either gemcitabine or cisplatin among EGFR-mutant lung cancers (Fig. 5C). These data reveal that apoptosis induced by the targeted therapies are markedly more sensitive to BIM levels than apoptosis induced by some of the more classic cytotoxic chemotherapies.

**Induction of BIM Expression Can Restore Robust Apoptotic Responses in Oncogene-Addicted Cancers**

We determined whether induction of BIM expression could sensitize low BIM-expressing cancers to targeted therapies. We used “tetracycline on” expression vectors that express BIM only in the presence of doxycycline, and used concentrations of doxycycline that lead to expression levels of BIM comparable to endogenous levels in high BIM-expressing cells (Supplementary Fig. S4). In H1650 EGFR-mutant NSCLC cells and SKOV3 PIK3CA-mutant ovarian cancer cells, adding doxycycline in combination with lapatinib or BEZ235 (a dual PI3K/mTOR inhibitor) did not sensitize the low BIM-expressing cells to drug-induced apoptosis (Fig. 5A). However, adding doxycycline in combination with Taxol induced similar levels of apoptosis in low BIM and high BIM-expressing cells (Fig. 5C). Accordingly, we observed that BIM knockdown provided a less impressive protective effect from Taxol-induced apoptosis in the HER2-amplified and PIK3CA-mutant cancers, and reached statistical significance in only 1 of the 4 models tested (Fig. 5A and B; Supplementary Fig. S3C). This suggests that the efficacy of the kinase inhibitors seem to be more sensitive to the amount of BIM in the cell than that of Taxol.
with the appropriate targeted therapy resulted in more pronounced apoptosis, as compared to cells that received targeted therapy alone (Supplementary Fig. S4). These data suggest that restoration of BIM expression may re-sensitize some low BIM-expressing oncogene-addicted cancers to targeted therapies.

**Reducing BIM Levels Retards the Apoptotic Response and Tumor Shrinkage Induced by EGFR TKI Therapy**

Because BIM levels in treatment-naïve cells predicted the amount of apoptosis induced by kinase inhibitors, we hypothesized that the level of apoptosis may correlate with clinical benefit. To directly determine if BIM-regulated apoptosis impacts tumor responsiveness in vivo, we used a BIM short hairpin (sh) sequence that is expressed only in the presence of doxycycline. When HCC827 cells were infected with scrambled (SC) or BIM (shBIM) inducible shRNA, we found only the shBIM cells were protected from gefitinib-induced apoptosis in the presence of doxycycline (Supplementary Figs. SSA and SSB), which mitigated the decrease in cell culture number (Supplementary Fig. S5C). The shBIM HCC827 cells were used to develop subcutaneous xenografts. Induction of BIM shRNA with doxycycline led to reduced BIM levels and attenuated tumor regressions and apoptosis following gefitinib treatment (Fig. 6A and B; Supplementary Fig. SSD). Thus, abrogation of apoptosis by BIM knockdown directly impacted the degree of tumor regression in vivo.

Consistent with the results that impairment of apoptosis by BIM knockdown mitigated tumor regressions in vivo, the high BIM-expressing HER2-amplified BT-474 cells were much more sensitive to lapatinib in vivo than the corresponding low BIM-expressing ZR7530 cells (Figs. 6C and D) despite suppression of PRAS40 (i.e., AKT substrate) and ERK phosphorylation in vitro and in vivo in the ZR7530 cells (Supplementary Figs. S6A and S6B). Since BIM levels and knockdown did not greatly impact Taxol-induced apoptosis, we hypothesized that the combination of Taxol and lapatinib in low BIM-expressing cells would yield greater anti-tumor effects than lapatinib alone by promoting both growth arrest and apoptosis, resulting in tumor regressions in vivo. Importantly, we did not observe that the addition of the TKI mitigated Taxol-induced

**Figure 6.** The efficacy of targeted therapies in vivo is dependent on induction of apoptosis. A, HCC827 cells carrying a doxycycline (DOX) inducible sh sequence targeting BIM were injected into 6- to 8-week-old nude mice. When tumors reached approximately 100 mm³, half the mice were switched to DOX chow. When tumors reached approximately 750 mm³ all the mice were treated with gefitinib (35 mg/kg) once daily for 21 days. Average tumor measurements of the 2 groups are shown. Error bars are +/− SEM of the mean tumor measurement of each treatment group. B, HCC827 cells carrying a plasmid with a DOX-inducible sh sequence targeting either a SC or a BIM sequence were injected into 6- to 8-week-old nude mice. Tumors were treated as indicated and were harvested at the indicated times after beginning gefitinib treatment. Lysates were prepared and probed with the indicated antibodies. C and D, 6- to 8-week-old female mice were injected with BT-474 or ZR7530 HER2-amplified breast cancer cells into the mammary fat pad following ovariectomy. When tumors approached 125 mm³ they were treated with vehicle or 100 mg/kg lapatinib and tumor volume was measured for approximately 30 days. Error bars are +/− SEM of the mean tumor measurement of each treatment group.
apoptosis in the low BIM-expressing cells (Supplementary Fig. S7A-B). Accordingly, the combination of lapatinib and Taxol more potently induced ZR7530 tumor regressions in vivo (Supplementary Fig. S7C), and this was associated with induction of apoptosis (Supplementary Fig. S7D).

**Patients with EGFR-Mutant NSCLCs with Low BIM Expression Derive Less Clinical Benefit from EGFR Inhibitors**

The above studies suggest that high BIM levels predict apoptotic response to TKIs and that this translates into more impressive and durable tumor responses in vivo. Thus, we aimed to determine if pre-treatment BIM levels in patient samples would indicate clinical benefit to TKIs. We isolated nucleic acid from pre-treatment tumors in 24 patients with metastatic EGFR-mutant lung cancers who received single-agent EGFR TKIs and assayed for BIM and β-actin RNA levels by qRT-PCR. The patients consisted of 14 men and 10 women, with EGFR mutations including 13 exon 19 deletions, 9 L858R, 1 G719C, and 1 L861Q. All cancers were void of T790M EGFR mutations, KRAS mutations, PIK3CA mutations, or other known confounding genetic abnormality that would be expected to negatively impact response. The EMT status of these cancers was not known. Nineteen (79%) received the TKI in the first-line setting, the rest as the second systemic therapy for their cancer. Fifteen (62%) had high levels of BIM, defined as relative mRNA to β-actin >45, and nine (38%) had low levels of BIM, defined as relative mRNA to β-actin <30. BIM levels did not correlate with any particular type of EGFR mutation. Twenty-two of the twenty-four patients had scans available for quantification of responses. Fourteen patients (64%) achieved a RECIST response to TKI therapy, including 13 with partial responses and 1 with a complete response. Using RECIST measurements, there was a significant correlation between BIM expression and tumor shrinkage. Low BIM patients had only a mean 29% tumor shrinkage, whereas high BIM patients achieved a mean 57% tumor decrease (P = 0.04). Accordingly, the RECIST response rate was 44% among low BIM patients compared to 77% among high BIM patients (nonsignificant trend). The progression-free survival (PFS) was significantly different between the low and high BIM-expressing patients (Fig. 7A) and the median PFS was only 4.7 months for the low BIM group versus 13.7 months for the high BIM group (P = 0.007; Fig. 7A). We also developed BIM immunohistochemistry (IHC) using control cell lines with known low and high BIM levels (Supplementary Fig. S8). In a few cases where tissue was available, we found that the BIM IHC intensity correlated well with qRT-PCR data (Fig. 7B).

**DISCUSSION**

As the scientific and medical communities gain more experience with the effectiveness and limitations of targeted therapies in genetically defined cancers, it has become increasingly clear that there is significant heterogeneity among the responses observed in the clinic even among cancers harboring the same genetic mutations. For example, about 30% of patients with EGFR-mutant lung cancers from a recent clinical trial had modest or no tumor response to treatment with gefitinib (1). In this study, we have found that pre-treatment assessment of BIM levels in several different oncogene-addicted paradigms accurately predicts the apoptotic response to targeted therapies. By comparing models with different levels of BIM and by knocking down expression of BIM in vivo, we observed that differential induction of apoptosis might be a strong contributing factor to tumor responsiveness in vivo. On assessment of BIM mRNA from a series of EGFR-mutant lung cancers, we confirmed that BIM levels do serve as a correlating factor for benefit from EGFR TKIs. Collectively, our data suggest that diminished expression of BIM, which is not only a biomarker for response, but also has critical functional role in the response, may contribute to the heterogeneity of responses observed in the clinic. Lack of BIM expression precludes the cell from undergoing robust apoptosis, which our data suggest is imperative for targeted therapies to impart robust and sustainable therapeutic responses.
BIM Expression Predicts Drug Response

The reasons for the heterogeneity in BIM expression remain poorly understood. In some cases, there may be genetic loss of BIM. For example, the HER2-amplified Calu-3, EGF-R-mutant H1665, and PIK3CA-mutant T47D and SKOV3 cells all have loss of heterozygosity at the BIM locus (32). Each model is a low BIM expressor that fails to undergo robust apoptotic response following targeted therapies (Figs. 2–4; Supplementary Table S1). There is also evidence from other liquid tumors that epigenetics may contribute to suppression of BIM expression. Indeed, aberrant methylation of BIM has been associated with suppression of BIM expression and may contribute to resistance to targeted therapies among pediatric acute lymphoblastic leukemias, CML, and Burkitt lymphoma (17, 33–35). In addition, suppression of BIM RNA levels via micro RNAs (mi-RNAs), including the mir-17-92 family (36), may also contribute to low basal BIM RNA levels. Since we found that re-expression of BIM re-sensitizes the cancers to targeted therapies, a more detailed understanding of the mechanisms of BIM suppression in these tumors may provide opportunities for therapeutic intervention to upregulate BIM in combination with the appropriate targeted therapy. An alternative strategy would be to add an apoptosis-inducing agent to the appropriate targeted therapy in low BIM-expressing cancers. Since BIM expression did not substantially impact responsiveness to cytoxics such as paclitaxel (this study), gemcitabine (this study), and cisplatinum (this study and refs. 37 and 38), it may be advantageous to combine a cyotoxic agent (to achieve apoptosis) and a targeted therapy in low BIM-expressing cancers. Such combinations are commonly used clinically in HER2-amplified breast cancer; perhaps a similar approach could be utilized in low BIM-expressing EGF-R, BRAF, and PIK3CA-mutant cancers that are currently treated with single-agent kinase inhibitors. Theoretically, combining the growth-arresting effect of the targeted therapy with a cyotoxic agent would mimic the growth-arresting and apoptosis-inducing activity achieved by single-agent targeted therapies in the high BIM expressors (Figs. 6C and D; Supplementary Fig. S7). Of note, the benefit of such combinations may be superior in the low BIM expressors in each specific oncogene-addiction paradigm and, in NSCLC, clinical trials have shown that this strategy is not successful when applied indiscriminately (39–41).

The studies in this article also revealed that BIM expression is necessary for a robust apoptotic response following direct PI3K inhibition in PIK3CA-mutant and HER2-amplified cancers, and HER2 inhibition in HER2-amplified cancers. To our knowledge, this had not been reported previously. Indeed, >70% of the BT-474 cells were protected from apoptosis by BIM siRNA following treatment with lapatinib or NVP-BEZ235 (Fig. 5A). To our initial surprise, BIM suppression blocked NVP-BEZ235-induced apoptosis in all cell lines studied, despite the lack of increase in BIM expression following PI3K-mTOR inhibition. Brachmann and colleagues (27) showed NVP-BEZ235 induces apoptosis in HER2-amplified and PIK3CA-mutant cells through a caspase-dependent mechanism. We also have made similar observations in HER2-amplified cancers (7, 42) without detecting any reductions in Bcl-2 anti-apoptotic family members. In these experiments, we failed to detect any consistent decreases in Bcl-2, Bcl-xl, or survivin following PI3K inhibition in the PIK3CA-mutated cancers (Supplementary Fig. S9). Thus, these data suggest that BIM expression is necessary for apoptosis following PI3K inhibition but apoptosis is not triggered by its expression. In the HER2-amplified and PIK3CA-mutant cancers, it seems likely that PI3K inhibition leads to alterations in other Bcl-2 family members (such as phosphorylation of BAD) that require basal BIM expression to promote apoptosis.

We have posited that low BIM expression in patient samples may help identify those with oncogene-addicted cancers that will not benefit as substantially from single-agent kinase inhibition. The in vivo xenograft data suggest apoptosis is a vital component of effective targeted therapy response and that reductions in BIM expression are sufficient to impair tumor response (Fig. 6A). However, because it is more practical to clinically screen for BIM levels prior to treatment, it is important to note that cancers with low BIM expression before treatment were consistently the cancers that had the lowest BIM expression following treatment (Figs. 1–4; Supplementary Fig. S2). Moreover, tumor samples from patients with EGF-R lung cancers that displayed low BIM expression prior to gefitinib treatment predicted poor responses (Fig. 7). Similar results were observed in a small cohort of patients with HER2-overexpressing metastatic breast cancer enrolled in the only published study that used single-agent lapatinib (Supplementary Fig. S10) (43). Although BIM expression is likely suppressed by different mechanisms during cancer progression, the resultant inadequate apoptotic response following targeted therapies translates into less pronounced patient responses. Our findings are supported by a recent study that found that patients with low BIM expression had poorer responses to imatinib in CML compared to those patients with higher BIM expression (35).

Given the complexity of cancer, it would be extremely unlikely that low BIM expression is the sole cause for diminished responsiveness or defective apoptosis in all of the genetically defined cancer paradigms examined in this study, despite the remarkable correlation among cell lines. Other factors, including interindividual variability in drug pharmacokinetics and coexisting genetic changes, are likely contributing factors. It is also worth noting that other BH3 members play key roles in the apoptotic response in oncogene-addicted cancers (44) and cancers with high BIM expression can have other impediments to kinase inhibitor-induced apoptosis. Indeed, our patient data included cancers that had high BIM expression but tempered responses. Despite these possibilities, it is rather remarkable to us that BIM expression predicted for apoptotic response effectively across oncogene-addiction models and predicted patient outcome. We also found it rather notable that BIM expression levels serve as a functional biomarker across a wide range of kinase inhibitors and oncogene-addicted cancer models. Thus, it appears that apoptosis induced by inhibition of RTKs, PI3K-AKT, and MEK-ERK likely involves regulation of the Bcl-2 family members and requires BIM expression to effectively promote apoptosis. These data suggest that analyses of BIM expression in tumor samples may help guide treatment regimens.
involving targeted therapies. A next step would be to better understand why these cancers have diminished BIM expression in order to understand which combination therapy would be most suitable: therapies that may upregulate BIM expression (e.g., HDAC inhibitors or demethylating agents), therapies that may induce apoptosis irrespective of BIM levels (cisplatin, gemcitabine, or Taxol), or therapies that may increase the amount of unbound or free BIM by directly targeting anti-apoptotic Bcl-2 family members, such as BH3 mimetics (46). Indeed, we are currently pursuing these lines of investigation in the laboratory as a potential strategy to improve the efficacy of targeted therapies in cancers with low BIM expression.

METHODS

Cell Lines

BT-474, BT-20, SkBr3, Colo-201, Colo-205, Colo-206F, WiDr, RKO, and SW1417 cells were cultured in DMEM/F12. The H3255 cells were cultured in ALC-4 media. All other cell lines in this study were cultured in RPMI. All the FBS concentrations were 10%, except for HCC827 cells (5%) and EFM-192A cells (20%). BT-474 and SkBr3 cells were from the Engelman laboratory and have been thoroughly characterized (7). MDA-MB-361, H1819, and MDA-MB-453 cells were provided by Dr. Carlos Arteaga (Vanderbilt-Ingram Cancer Center). The remaining HER2-amplified, PIK3CA-mutant, and BRAF-mutant cell lines were provided by the Center for Molecular Therapeutics at Massachusetts General Hospital, which performs routine cell line authentication testing by SNP and STR analysis. These cell lines have been acquired over the past 18 months. The EGFR-mutant cell lines used in this study are from the Engelman laboratory and have been previously tested for mutation status to confirm their authenticity.

Antibodies and Reagents

The following antibodies used for Western blots were from Cell Signaling: BIM (catalog no. 2819), phospho-HER2, phospho-AKT (473), phospho-ERK, phospho-pras40 (246), phospho-S6 (235/236), total HER2, and total ERK. Other antibodies were Actin (Sigma-Aldrich); phospho-EGFR 1068 (Abcam); and total EGFR and total AKT (Santa Cruz Biotechnology). Lapatinib (LC Laboratories) was dissolved in DMSO. Taxol was from the pharmacy at MGH and was diluted with saline. Gemcitabine (Selleck Pharmaceuticals) was dissolved in DMSO. Cisplatin (Sigma-Aldrich) was dissolved in water for immediate use. Antibodies used for immunohistochemistry are listed below.

Western Blotting

For Western blotting, cells were prepared and lysed as previously described (7). Proteins were resolved using the NuPAGE Novex® Midi Gel system on 4% to 12% Bis-Tris Gels (Invitrogen). Representative blots are shown from several experiments.

RNA Extraction and Quantitative RT-PCR

For cell lines, RNA was isolated and purified using the Qiagen RNAeasy Mini kit and further purified by DNAse treatment with Ambion Turbo DNase (47). For tumors, EGRF-mutant lung and HER2-positive breast tumor specimens were extracted prior to TKI treatment and were then paraffin embedded and mounted on glass slides. For EGRF-mutant tumor tissue, total nucleic acid was extracted using a modified FormaPure System (Agencourt Bioscience Corporation) automated on a Beckman Coulter Biomek NX® workstation. For HER2-positive tumors, RNA was extracted and purified from the slides using the Pinpoint Slide RNA Isolation System II (Zymo Research). Following extraction and purification, RNA was reverse transcribed and amplified using superscript First-strand cDNA synthesis (Invitrogen). The amplicon abundance of BIM and β-actin was monitored in real time on a Roche Lightcycler 480 (Roche Diagnostics) by measuring the fluorescence increases of Sybr Green. The primers used for cell lines were: BIM Forward (5'-GATCCCTCCAGTGGATTTTCTTCT-3') and BIM Reverse (5'-ACTGAGATGTTGTTGAGGGCCTG-3'), β-actin Forward (5'-CTGTGCTATCCTCTGTAGGCTC-3') and β-actin Reverse (5'-CATGATGGAGTGGAGTTAGTCTGG-3'). For tumor samples, target amplicons were shortened to 60 to 80 base pairs. To avoid genomic DNA contamination, the BIM Forward primer was designed to overlap exon 2 and exon 3 and specifically amplified BIMcDNA. The primer pairs were BIM Forward (5'-ATCTCAGTGCAATGGCT-3') and BIM Reverse (5'-CAACTCTTGGGGCATTCAATA-3'), and β-actin Forward 5'-GGCATGGGTCAGAAGGATT-3') and β-actin Reverse (5'-AGGATGCGCTTCTTTGCTCTG-3'). Relative BIM RNA levels were calculated using the Delta-Delta threshold cycle (Ct) method as previously described (48). Threshold levels were set for the exponential phase of amplification as previously described (48).

In Vitro and In Vivo shRNA Experiments

BIM sh sequence (5'-ATGTGTTATCTTACGACTGTTA-3') and SC sh sequence (5'-CCTAAGGTTAATCCGCATTGCGGCGAGCGAC-3') were introduced into the tet-on PLKO vector. For the BIM sh studies, high BIM-expressing HCC827 cells were infected with tet-on PLKO BIM sh and tet-on PLKO Shc knockdown vectors and selected for in 2 μg/mL puromycin. For the in vivo experiments, HCC827 cells stably transduced with tet-on shBIM were injected into the left flanks of 6- to 8-week-old male nu/nu nude mice (5 × 10^6 cells per mouse). Tumor size was measured every 3 to 4 days for 21 days. The perpendicular diameters of the tumors were measured using a caliper and the tumor volumes were calculated using the formula: v = 1/2 (w×l)/(μg/L). Mice were randomized to at least 3 per group.

siRNA Experiments

For the siRNA experiments, BIM and negative control oligos (Qiagen) were used at a concentration of 10 nM and transfected with HiperFect following the manufacturer’s protocol (Qiagen) and as previously described (7).

Doxycycline-Inducible pTREX Expression Vectors

BIMcDNA in the pDEST26 vector was generously provided by Dr. Hidesuke Fukazawa (Department of Bioactive Molecules, National Institute of Infectious Diseases) and the cDNA was introduced into the pTREX vector kindly provided by Novartis Pharmaceuticals. Briefly, the BIM sequence was amplified by PCR with the Forward Primer 5'-CACATGGCACAAGAACATCCCTGATG-3' and Reverse Primer 5'-TCAATGCATTCTCCACCCACC-3' and cloned into pENTR using the TOPO cloning method (Invitrogen). The sequence was then cloned into the pTREX vector by the clonase recombination reaction (Invitrogen). pTREX BIM vectors were subsequently verified by DNA sequencing at the MGH DNA Sequencing Core. The low BIM-expressing EGRF-mutant H1650 NSCLC and PIK3CA-mutant SKOV3 ovarian cancer cells were infected with pTREX BIM vectors, and selected in 1 μg/mL puromycin in RPMI supplemented with 10% tetracycline-free FBS. When cells grew to confluence, cells were split and titrated with doxycycline. The purpose was to find a concentration of doxycycline that induced BIM to a comparable level found in high BIM-expressing cells. This concentration was subsequently used to see if this concentration of BIM sensitized the cells to the
Research Article

BIM Expression Predicts Drug Response

appropriate targeted therapy. For these experiments, cells were incubated with doxycycline for 24 hours, followed by treatment with vehicle or with the appropriate targeted therapy. Parallel cultures of cells were treated with vehicle or appropriate targeted therapy without pre-incubation with doxycycline.

Flow Cytometry

Fluorescence-activated cell sorting (FACS) analysis was performed on a BD LSR III (Becton Dickenson). For cell cycle studies and apoptosis measurements, experiments were done as previously described (48). The Annexin V-Cy5 was from Biosoence International. Experiments were carried out in triplicate and standard deviations are shown (Supplementary Tables S1 and S2).

Patient Selection for EGFR-Mutant Lung Cancer Data

The Massachusetts General Hospital (MGH) began screening patient tumors for EGFR mutations in 2004 and expanded the tumor genotyping platform to screen for additional oncogenic mutations in 2009 (50). We retrospectively collected a cohort of EGFR-mutant NSCLC patients seen in our Thoracic Oncology clinic between October 2005 and July 2010 via chart review under an Institutional Review Board-approved protocol. To meet criteria for inclusion, patients had to have 1) documented EGFR mutation (exon 19 del, L858R, G719X or L861Q only, because these have been the most strongly associated with sensitivity to EGFR TKIs); 2) treatment with single agent EGFR TKI, without concurrent chemotherapy, other targeted agent, or radiation; 3) sufficient baseline tissue available for analysis (baseline defined as resected or biopsied prior to initiation of EGFR TKI therapy); and 4) pre-treatment and post-treatment radiographic scans available for tumor measurements. Twenty-four patients ultimately met these criteria, all treated with either erlotinib or gefitinib except one who received a second-generation EGFR TKI, afatinib. Radiographs were centrally reviewed by a single radiologist (S.R. Digumarthy) who was blinded to BIM results. RECIST methods were used to determine standard overall tumor burden quantitative measurements at each time point (the sum of longest diameters of the target lesions) and best response as well as percent decrease of the target lesions) and best response as well as percent decrease from baseline was calculated (51). Time-to-progression (TTP) was calculated as the time from the start of EGFR TKI until documented progression by RECIST. Patients who did not progress were censored at their last known follow-up. If RECIST progression could not be documented because of lack of formal assessment by CT scan or unavailability of films from outside hospitals (n = 7), the date of progression as documented in clinical notes (n = 6), or the start date of next therapy (n = 1) was used. Best response to therapy was compared by BIM high versus low using the Wilcoxon rank sum test and Fisher’s exact test and TTP was calculated with the Kaplan-Meier method and log-rank test. Low BIM levels were defined as relative mRNA to β-actin >45, and low levels of BIM, defined as relative mRNA to β-actin <30. The cut-off for the BIM RNA levels was empirical and chosen because there was a clear separation in values with all values being either below 30 or above 45 (i.e., there were no cancers with values between 30 and 45). This stratification profoundly distinguished patient outcomes, and changing the cut-offs did not improve the distinction in clinical outcomes.

Mammary Fat Pad Xenograft Studies

One week before tumor implantation, 6- to 8-week-old nu/nu female nude mice underwent ovariectomy and were implanted subcutaneously with controlled release pellets containing 0.75 mg of estrogen for 60-day release (Innovative Research). This allows enough time to recover from any estrogen depletion-induced hemodynamic changes and limits any residual effect of endogenous estrogen, which may vary between animals and potentially influence tumor growth rates. High BIM BT-474 (N = 7/treatment group) or low BIM ZR7530 (N = 3/treatment group) HER2-amplified tumor cells (approximately 3 × 10^6 in PBS) were mixed with high-concentration Matrigel (BD Biosciences) at 1:1 ratio and the mixture was injected using a 30-gauge needle under a dissecting microscope subcutaneously into the mammary fat pad just inferior to the third nipple of the anesthetized, ovariectomized female mice. Leakage to subcutaneous space was avoided. Pellets were replenished approximately every 60 days during ongoing experimentation. Tumor size was measured as described above for HCC827 tumors. Once tumors reached an average volume of 100 mm³, mice were treated with either vehicle, 100 mg/kg lapatinib (once a day, oral gavage) and/or 10 mg/kg paclitaxel (injected into mammary fat pad) for the indicated times.

Immunohistochemistry

Immunohistochemistry for tissue sections was performed using standard protocols with the following antibody dilutions, 1:100 BIM (Cell Signaling #2933). 1:100 phosho-ERK (Cell Signaling #4370). 1:200 phosho-Pras40 (Cell Signaling #2997). and 1:200 cleaved caspase 3 (CC3) (Cell Signaling #9661). Briefly, the sections were deparaffinized using Xylene, quenched with hydrogen peroxide, and antigen retrieval was performed using Borg Decloaking Chamber (Biocare Medical). Subsequently, we incubated tissues with primary antibodies overnight followed by incubation with secondary antibodies for 1 hour at room temperature. Signals were detected using the ABC kit for immunoperoxidase staining (Vector Laboratories). Images were taken by a Nikon 90i scope with color camera.

Statistical Analyses

Linear regression analyses, Student’s t tests and log-rank tests were performed when indicated and calculations were performed using GraphPad Software. For linear regression analyses, the slope was considered significantly non-zero when P < 0.05. For Student’s t tests and Log-rank tests, populations were considered significantly different at P < 0.05.

Disclosures of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

The Editor-in-Chief of Cancer Discovery is an author of this article. In keeping with the AACR’s Editorial Policy, the paper was peer reviewed and a member of the AACR’s Publications Committee rendered the decision concerning acceptability.

Acknowledgments

The authors thank Elizabeth Bast for her help in collecting and analyzing the clinical data.

Grant Support

This study is supported by grants from the National Institutes of Health NIH R01CA137008-01 (J.A. Engelman), R01CA140594 (J.A. Engelman), R01CA135257-01 (J.A. Engelman), National Cancer Institute Lung SPORE P50CA090578 (J.A. Engelman), DF/HCC Gastrointestinal Cancer SPORE P50 CA127003 (J.A. Engelman), K08 grant CA120060-01 (J.A. Engelman), the American Lung Cancer Association Lung Cancer Discovery Award (J.A. Engelman), the V Foundation (J.A. Engelman), the Ellison Foundation Scholar (J.A. Engelman), and an Aid for Cancer Research Postdoctoral Fellowship (A.C. Faber). Received May 12, 2011; revised July 14, 2011; accepted July 18, 2011; published OnlineFirst July 22, 2011.
REFERENCES


BIM Expression Predicts Drug Response
