RESEARCH ARTICLE

Mcl-1 and FBW7 Control a Dominant Survival Pathway Underlying HDAC and Bcl-2 Inhibitor Synergy in Squamous Cell Carcinoma

Lei He, Kristine Torres-Lockhart, Nicole Forster, Saranya Ramakrishnan, Patricia Greninger, Mathew J. Garnett, Ultan McDermott, Stephen M. Rothenberg, Cyril H. Benes, and Leif W. Ellisen
INTRODUCTION

Squamous cell carcinoma (SCC) is a relatively common and highly lethal malignancy arising within the stratified epithelium of the skin, lung, esophagus, and upper aerodigestive tract, the last of which is part of the so-called head and neck SCC (HNSCC). Despite intense, multimodality treatment regimens for SCC, including surgery, chemotherapy, and radiation, little progress has been made over the last 30 years in improving overall survival rates. HNSCC in particular is associated with high morbidity and mortality, and accounts for nearly 10,000 deaths annually in the United States (reviewed in ref. 1). Although recent genome-wide analyses of many cancer types have identified activated oncoproteins that may be targeted therapeutically, SCCs do not commonly harbor such somatically mutated oncoproteins (2). Instead, SCCs more commonly harbor inactivating mutations of genes, including TP53 (p53), NOTCH, and CDKN2A (p16), which presents a much greater challenge for the mitochondrial pathway (3, 4). In contrast, other BH3-only proteins such as Noxa do not directly activate Bax and Bak; instead, they act indirectly by neutralizing antiapoptotic proteins (in this case, Mcl-1) and are classified as “sensitizers” or “derepressors” (3, 5). Bim plays a critical role in the apoptotic regulatory machinery engaged by many anticancer therapy agents (6, 7).

It is now apparent that the clinical response to conventional therapies, including cytotoxic chemotherapy, is governed at least in part by the Bcl-2 family (8, 9). Direct therapeutic targeting of the Bcl-2 family in cancer is therefore conceptually appealing but has proved remarkably challenging. This fact is due, in part, to the difficulty in producing effective drugs and in achieving a satisfactory therapeutic index (10). Also important, but less appreciated in this regard, are the potentially complex, tissue-specific interactions among Bcl-2 family members observed in different tumor types (8). The recent development of the highly potent and specific BH3 mimetic small-molecule ABT-737, which displaces Bim from Bcl-2 and Bcl-xL but not Mcl-1, has provided proof-of-concept for targeting the Bcl-2 family in certain hematologic cancers, which express high levels of Bcl-2 (10). However, emerging data suggest that many, if not most, solid tumors may be refractory to this agent and its orally available derivative ABT-263, even though the mechanistic basis for this resistance has yet to be established (11).

ABSTRACT

Effective targeted therapeutics for squamous cell carcinoma (SCC) are lacking. Here, we uncover Mcl-1 as a dominant and tissue-specific survival factor in SCC, providing a roadmap for a new therapeutic approach. Treatment with the histone deacetylase (HDAC) inhibitor vorinostat regulates Bcl-2 family member expression to disable the Mcl-1 axis and thereby induce apoptosis in SCC cells. Although Mcl-1 dominance renders SCC cells resistant to the BH3-mimetic ABT-737, vorinostat primes them for sensitivity to ABT-737 by shuttling Bim from Mcl-1 to Bcl-2/Bcl-xl, resulting in dramatic synergy for this combination and sustained tumor regression in vivo. Moreover, somatic FBW7 mutation in SCC is associated with stabilized Mcl-1 and high Bim levels, resulting in a poor response to standard chemotherapy but a robust response to HDAC inhibitors and enhanced synergy with the combination vorinostat/ABT-737. Collectively, our findings provide a biochemical rationale and predictive markers for the application of this therapeutic combination in SCC.

SIGNIFICANCE: This study reveals the tissue-specific landscape and biochemistry of the Bcl-2 family in SCC, which underlie moderate sensitivity of these tumors to HDAC inhibitor therapy but dramatic synergy in combination with BH3-mimetic therapy. By establishing predictive biomarkers, we provide evidence that tumors most likely to respond to this therapeutic combination, including those harboring somatic FBW7 mutations, are those most resistant to standard chemotherapy. Cancer Discov; 3(3); 324–37. © 2012 AACR.
An additional approach to targeting the Bcl-2 family that has recently emerged involves the use of histone deacetylase (HDAC) inhibitors (12), a treatment that induces expression of multiple proapoptotic Bcl-2 family members, including Bim, Puma, and Noxa (4). Although the direct targets and precise specificity of clinical HDAC inhibitors vary, substantial data support the concept that proapoptotic Bcl-2 family induction involves a direct effect on chromatin (13). For example, we recently showed that the lineage-specific transcription factor p63, which is overexpressed in a large proportion of SCCs, functions as a direct repressor of Puma and Noxa transcription in SCC cells through recruitment of HDAC1/2 (14). Either p63 inhibition or treatment with a clinical HDAC inhibitor results in increased histone acetylation within the regulatory elements of these genes, leading to their upregulation and cell death in a subset of SCC cell lines (14).

Here, we sought to uncover the tissue-specific biochemical landscape of the Bcl-2 family in SCC as a means to rational therapeutic targeting. We reveal Mcl-1 as a dominant survival factor in SCC, which contrasts dramatically with the Bcl-2 dominance of hematologic cancers. Underscoring this observation, we find that disruptive mutations of the E3 ubiquitin ligase complex gene FBW7, which are recurrent in SCC, lead to Mcl-1 stabilization and thereby render mutant tumors refractory to conventional chemotherapy but highly sensitive to HDAC inhibitors. Most importantly, our biochemical studies show how combining HDAC inhibition with a BH3 mimetic agent synergizes in SCC treatment both in vitro and in vivo. This work points to a new treatment approach for SCC, as well as potential genomic and gene expression markers to guide therapeutic decision making.

RESULTS
Role of the Bcl-2 Family in Apoptosis Induced by Vorinostat in SCC Cells

We had previously observed substantial differences in the sensitivity of SCC cells to the clinical class I/II HDAC inhibitor vorinostat (14), and we therefore wished to define key mediators of response and resistance to this agent. We collected a panel of SCC cell lines representing upper aerodigestive tract (HNSCC) and esophageal SCCs. Treatment of multiple lines with vorinostat induced a dose-dependent increase in apoptosis, evidenced by Annexin V/propidium iodide (PI) staining and flow cytometry (Fig. 1A and B and Supplementary Fig. S1A). We then determined the vorinostat IC50 value for each cell line in a dose-response analysis (Supplementary Fig. S1B). Apoptosis induced by vorinostat has been linked to the intrinsic (mitochondrial) pathway (15, 16), and thus was associated in SCC cells with induction of the mRNA and protein for the BH3-only proapoptotic Bcl-2 family members Puma, Noxa, and Bim, and with downregulation of the prosurvival family member Mcl-1 (Fig. 1C and Supplementary Fig. S1C–S1E). Although these 4 family members were regulated in all of the lines following vorinostat treatment, no correlation was found between the sensitivity to vorinostat and either the basal or induced level of mRNA or protein for these factors (Fig. 1D and Supplementary Fig. S1E).

Recent work has defined expression of Bcl-2 itself as a potentially important prognostic factor in squamous cancers, including HNSCC (17–19). We noted that Bcl-2 exhibited the broadest range of expression across the cell lines analyzed (difference between the highest- and lowest-expressing cell line) of all the Bcl-2 family members analyzed (Fig. 1E), although its level was not significantly changed following vorinostat treatment (Supplementary Fig. S1E). Remarkably, however, Bcl-2 levels correlated significantly with sensitivity to vorinostat in SCC cells (Fig. 1E), a finding that has not been observed in other cell types (12). Collectively, these findings suggest that Bcl-2 may have a “gatekeeper” function in supporting survival of SCC cells following vorinostat treatment. Of note, one significant outlier in this analysis was the BICR-78 line, which exhibited sensitivity to vorinostat despite relatively high Bcl-2 levels (Fig. 1E), the molecular explanation for which is discussed below.

Mcl-1 Dominance and Shuttling of Bim Define the Molecular Mechanisms of ABT-737 Resistance and Vorinostat Sensitivity in SCC

On the basis of these findings and the emerging role of Bcl-2 in SCC (17–19), we next tested the ability of the Bcl-2 inhibitor (BH3 mimic) ABT-737 to induce apoptosis as a single agent in SCC cells. As a control for efficacy, we directly compared ABT-737 sensitivity in SCC cells to that in leukemia cells, which are known to be sensitive to this agent (20). Remarkably, all of the SCC cell lines exhibited striking resistance to this agent, with mean IC50 values 20-fold greater in SCC than in leukemia cell lines (Fig. 2A and Supplementary Fig. S2A). Furthermore, in SCC cells, the effect of ABT-737 was almost exclusively cytostatic (not cytotoxic) even at micromolar doses, whereas this agent killed leukemia cells efficiently at nanomolar doses (data not shown).

Resistance to single-agent ABT-737 implies that SCC cells are less dependent on Bcl-2/Bcl-xl for survival than are leukemia cells (21, 22). We hypothesized that this may be related, in part, to an altered balance of prosurvival Bcl-2 family members in SCC versus leukemia cells. Indeed, while Mcl-1 and Bcl-xl levels were comparable in SCC and leukemia cells, Bcl-2 levels were dramatically lower in SCC (Supplementary Fig. S2B), yielding an Mcl-1/Bcl-2 ratio 100- to 1,000-fold higher in SCC than leukemia cell lines (Fig. 2A). This finding was recapitulated upon examination of a large, publicly available gene expression profiling dataset (Fig. 2B; ref. 23). The resulting low combined Bcl-xl and Bcl-2 level suggests that SCC cells may be relatively more dependent on Mcl-1 than on other prosurvival family members (24). Indeed, siRNA-mediated knockdown of Mcl-1 alone dramatically suppressed viability in several SCC cell lines, whereas knockdown of other prosurvival family members (Bcl-2, Bcl-xl, Bcl-w, A1) did not (Fig. 2C and Supplementary Fig. S2C). Thus, the tissue-specific expression pattern of prosurvival Bcl-2 family members observed in SCC is likely to underlie a selective dependence on Mcl-1 for survival.

Recent biochemical studies have suggested that dependence on Mcl-1 versus Bcl-2/Bcl-xl is reflected in the relative distribution of proapoptotic Bim to these factors (22).
Targeting the Bcl-2 Family in Squamous Cell Carcinoma

**Figure 1.** The Bcl-2 family is targeted by vorinostat in SCC. **A,** apoptosis is induced in a dose-dependent manner in SCC cells following vorinostat treatment (24 hours). Percentages indicate sum of Annexin V and/or PI-positive cells, assessed by flow cytometry. **B,** summary of dose–response analysis of apoptosis as in A for the indicated cell lines. Shown is the mean and SD for triplicate samples in a representative experiment. **C,** dose-dependent changes in key Bcl-2 family members following vorinostat treatment (4 hours), assessed by qRT-PCR in JHU-O29 cells normalized to β2-microglobulin. **D,** immunoblots showing basal levels of Bcl-2 family members in SCC cell lines, revealing Bcl-2 as the most variable in expression. β-Tubulin (β-Tub) serves as a loading control. **E,** Bcl-2 protein levels predict vorinostat resistance. Vorinostat IC50 values were calculated from Supplementary Fig. S1B. Protein levels were assessed by densitometry.
Figure 2. Mcl-1 is a dominant survival factor in SCC targeted by vorinostat, which shuttles Bim from Mcl-1 to Bcl-2/Bcl-xl. A, elevated ratio of Mcl-1/Bcl-2 mRNA is associated with resistance to ABT-737 in SCC cell lines (listed in Fig. 1D) compared with leukemia (Hema.) cell lines HL60, SKNO1, and THP. RNA levels were assessed by qRT-PCR; ABT-737 sensitivity was determined at 3 days by sulforhodamine B assay. Note y-axis is log scale. B, markedly low Bcl-2 expression is confirmed across esophageal SCC and HNSCC cell lines (n = 47) versus cancer cell lines of hematopoietic origin (n = 157). Analysis of the Broad Institute Cancer Cell Line Encyclopedia (23). Horizontal bar indicates mean expression. C, unique Mcl-1 dependence in SCC shown by decreased viability following transfection of Mcl-1–directed but not other siRNA duplexes, assayed at 48 hours. Shown are mean and SD of 6 replicates in a representative experiment. D, the majority of Bim is bound by Mcl-1 in SCC cells (JHU-O29, HO1N1), but is bound instead by Bcl-2 and Bcl-xl in leukemia cells (SKNO1), shown by immunoprecipitations (IP) for the indicated proteins followed by immunoblot. Functional Bim isoforms are shown and include short (S), long (L), and extra-long (EL). *, Ig heavy chain. E, vorinostat (3 μmol/L, 12 hours) induces Bim and shuttles it from Mcl-1 to Bcl-xl in SCC cells. IP/immunoblot was conducted as in D; compare the ratio of Bim levels in lanes 2 to 4 versus 6 to 8 for each cell line. Quantitation by densitometry is shown in Supplementary Fig. S2D.
We hypothesized that in cells that are Mcl-1 dependent (and therefore ABT-737 resistant), Bim would be bound at baseline primarily to Mcl-1, whereas in ABT-737–sensitive cells, the majority of Bim would be bound to Bcl-2/Bcl-xl (21, 22). To test this hypothesis we conducted immunoprecipitation for Mcl-1, Bcl-2, and Bcl-xl in SCC and leukemia cells and analyzed associated Bim protein. Indeed, we found that the majority of Bim was associated with Mcl-1 in SCC cells, whereas 20% or less of Bim was associated with Mcl-1 in leukemia cells (Fig. 2D). Strikingly, treatment of SCC cells with vorinostat led to substantial redistribution of Bim from Mcl-1 to Bcl-2 and Bcl-xl, resulting from the concerted effect of Mcl-1 downregulation and Noxa induction (Fig. 2E and Supplementary Fig. S2D–S2F). Together, these observations begin to explain mechanistically the correlation between Bcl-2 expression and resistance to vorinostat treatment in SCC cells. Release of Bim from Mcl-1 can either induce complete redistribution of Bim to Bcl-2/Bcl-xl, resulting in cell survival, or, in the absence of sufficient Bcl-2/Bcl-xl levels, activate the apoptotic cascade, resulting in cell death. As Bcl-xl levels vary little in SCC cells (Fig. 1D), Bcl-2 becomes the key response determinant. Taken together, these observations define SCC as functionally Mcl-1 dependent.

**FBW7 Is Mutated and Controls Mcl-1 Stability and Drug Sensitivity in SCC Cells**

Mcl-1 dependence of SCC cells suggests that factors regulating Mcl-1 might be important contributors to treatment response in this disease. Recent exome sequencing studies of primary HNSCC tumors have uncovered recurrent somatic mutations in *FBW7*, encoding an F-box protein subunit of the SCF (SKP1-CUL1-F-box protein) E3 ubiquitin ligase complex (2, 23–27). *FBW7* is also somatically mutated in several other human cancers and functions as a tumor suppressor in animal models, potentially by controlling degradation of substrates including c-Myc, Notch1, cyclin E, and Mcl-1 (25, 28). Given its potential link to Mcl-1, we sequenced *FBW7* in our SCC cell lines. Remarkably, BICR-78, an esophageal SCC line, harbored a homozygous nonsense mutation, resulting in an arginine to cysteine change at position 505 (Fig. 3A). Arginine 505 is among the 3 most common *FBW7* codons targeted for mutation in human cancers, and tumor-associated mutations at this position have been shown to disrupt substrate binding (29).

We thus hypothesized that *FBW7* mutation would result in stabilization of Mcl-1, rendering mutant cells further “Mcl-1 addicted” and therefore potentially more sensitive to vorinostat treatment (28). Indeed, we noted in our dose-response analysis (Fig. 1E) that BICR-78 was an obvious outlier showing high vorinostat sensitivity despite relatively high Bcl-2 levels. We therefore assayed endogenous Mcl-1 stability in these cells. Because Mcl-1 degradation occurs selectively during mitosis, we tested its stability by synchronizing cells using a double thymidine block, followed by release into nocodazole (29). This experiment showed clearly that Mcl-1 degradation was attenuated in BICR-78, resulting in a longer half-life in this cell line compared with 4 other SCC lines that express wild-type *FBW7* (Fig. 3B and C). Furthermore, BICR-78 showed the highest level of apoptosis of any SCC line following vorinostat treatment (Fig. 3D). To test directly whether loss of *FBW7* function confers sensitivity to vorinostat, we used siRNA to ablate *FBW7* expression in KYSE-150 cells, which are vorinostat resistant at baseline. As predicted, *FBW7* ablation significantly increased apoptosis and decreased viable cell numbers in multiple SCC cell lines following vorinostat treatment (Fig. 3E and Supplementary Fig. S3A). Thus, *FBW7* loss-of-function increases Mcl-1 stability and vorinostat sensitivity in SCC cells.

To pursue these observations in a broader context, we conducted *in silico* analysis of existing genomic and gene expression data on hundreds of human cancer cell lines (23), comparing tumor lines with deleterious *FBW7* mutations with those without such mutations (see Methods). Consistent with an effect on Mcl-1 protein levels, *FBW7*-mutant lines were significantly less likely to exhibit low-level MCL-1 genomic amplification, a common occurrence in human cancers, than were wild-type lines (Supplementary Fig. S3B; ref. 30). As a control, we examined *BCL-XL*, also subject to frequent low-level amplification, which showed no difference between wild-type and *FBW7*-mutant lines (Supplementary Fig. S3B). In keeping with increased Mcl-1 addiction and oncogenic stress, mutant lines also expressed significantly higher levels of Bim than did nonmutant lines (Supplementary Fig. S3C; ref. 28).

Most importantly, we then analyzed extensive dose–response data we had generated as part of a systematic effort to apply a wide variety of therapeutic compounds to hundreds of well-validated cancer cell lines of diverse histologic types (31). This analysis confirmed the specific sensitivity of *FBW7*-mutant cancer cells to 3 distinct HDAC inhibitors, including the class I–specific inhibitor MS-275 (Entinostat; Fig. 3F and Supplementary Fig. S3D). These differences in sensitivity clearly tracked with *FBW7* mutation and were not due to selective enrichment of a particular cell type among the mutant lines, as a comparison exclusively of hematologic cancer lines continued to show a highly significant increase in sensitivity among mutant lines (Supplementary Fig. S3E). As an additional control, we examined the effect of *FBW7* mutation on sensitivity to doxorubicin and epothilone, as *FBW7* disruption is established to confer resistance to such antitubulin agents via Mcl-1 stabilization (29). As predicted, *FBW7*-mutant cells were significantly more resistant to these cytotoxics than were cells without such mutations (Fig. 3F and Supplementary Fig. S3F). Taken together, these findings show that *FBW7* mutation stabilizes Mcl-1 and confers sensitivity to selective HDAC inhibition. Importantly, this mutation also renders tumor cells particularly resistant to traditional cytotoxic therapy.

**Robust Synergy between Vorinostat and ABT-737 In Vitro and In Vivo**

Our data suggest that Mcl-1 is a dominant survival factor in SCC (with or without *FBW7* mutation), that Mcl-1 activity is selectively targeted by vorinostat, and that Bcl-2 and Bcl-xl oppose the effects of this agent. The most important clinical implication of these findings is the predicted enhanced efficacy of vorinostat in the presence of ABT-737 in SCC. In effect, vorinostat treatment would be expected to “prime” cells for sensitivity to ABT-737 by shutting Bim from Mcl-1 to Bcl-2/Bcl-xl, with the latter Bim-containing complexes being susceptible to ABT-737 (Fig. 2; ref. 21). We therefore
Figure 3. FBW7 mutation stabilizes Mcl-1 and induces HDAC inhibitor sensitivity but chemotherapy resistance. A, disruptive FBW7 mutation in BICR-78 cells. Sanger sequencing chromatogram shows wild-type (WT) sequence and homozygous mutation within a common hotspot. B and C, Mcl-1 is stabilized selectively in BICR-78 cells. B, immunoblots from the indicated cell lines following double thymidine block, followed by release into nocodazole (330 nmol/L). The peak of phosphorylated Cdc27 (arrowhead) indicates mitosis (M), during which Mcl-1 is degraded. C, Mcl-1 protein quantitation normalized to β-tubulin (β-tub). D, FBW7 mutation correlates with increased apoptosis following vorinostat treatment in BICR-78 cells compared with SCC cells with wild-type FBW7, assessed by Annexin V/PI staining and flow cytometry at 24 hours. Shown are mean and SD of triplicate samples in a representative experiment. E, FBW7 ablation confers vorinostat sensitivity. SCC cells were transfected with FBW7-directed siRNA or control (Con), and 48 hours later were seeded for treatment with vorinostat (24 hours) at the indicated doses. Viability was determined by sulforhodamine B assay (left), and apoptosis was assessed as in D (right). ***, P < 0.001. F, cancer cells with disruptive FBW7 mutation show increased sensitivity to HDAC class I inhibitor (MS-275) but relative resistance to antitubulin chemotherapy (docetaxel). FBW7 mutation information was derived from the Broad Institute Cancer Cell Line Encyclopedia (23). Normalized IC₅₀ values are shown, obtained from ref 31. Horizontal lines represent mean values.

tested the combination in multiple SCC cell lines. Indeed, the addition of ABT-737 to vorinostat enhanced cell killing, in each case dramatically altering the slope of the dose–response curve and resulting in approximately a 5-fold decrease in the IC₅₀ for vorinostat (Fig. 4A and Supplementary Fig. S4A). Quantitative analysis of this effect using the Chou–Talalay method indicated a synergistic interaction in multiple lines (Supplementary Fig. S4B; ref. 32).

Our model further predicts that the addition of ABT-737 to vorinostat would be sufficient to overcome Bcl-2–mediated vorinostat resistance in SCC. To model the treatment-refractory Bcl-2–expressing subset of human HNSCC, we generated isogenic JHU-O29/GFP and JHU-O29/Bcl-2 cells, the former expressing a control retroviral vector and the latter a Bcl-2 cDNA (14, 17). As anticipated, vorinostat treatment alone was completely ineffective in JHU-O29/Bcl-2 cells (Fig. 4B). Strikingly, however, the addition of ABT-737 completely reversed this resistance and potentiated vorinostat sensitivity, resulting in the same final IC₅₀ for the combination in both lines (Fig. 4B). Thus, ABT-737 can effectively antagonize Bcl-2 and synergize with vorinostat even in cells expressing high ectopic Bcl-2 levels.
Synergism of combination vorinostat/ABT-737 overcomes Bcl-2–mediated resistance and induces tumor regression in vivo. 

A, ABT-737 enhances vorinostat sensitivity in SCC lines. Cell viability was determined by sulforhodamine B assay following treatment with the indicated doses for 24 hours. Shown is the mean and SD of 6 replicates. Calculated combination (synergy) indices are shown in Supplementary Fig. S4B.

B, ABT-737 synergizes with vorinostat and abolishes resistance mediated by Bcl-2. JHU-O29 cells expressing a GFP control (Con) or Bcl-2 cDNA were treated with vorinostat with or without ABT-737 (3 μmol/L) for 24 hours, and viability was determined by sulforhodamine B assay. Shown is the mean and SD of 6 replicates. **, P < 0.001.

C, xenograft study showing in vivo synergy and reversal of Bcl-2–mediated resistance by combination therapy. JHU-O29 cells as in B were implanted subcutaneously in nude mice. Treatment with vorinostat (50 mg/kg/d) and ABT-737 (75 mg/kg/d) was initiated once measurable tumors appeared. Shown is the mean of 7 to 10 tumors per group ± SEM. *, P < 0.05; **, P < 0.001, 2-way ANOVA.

D, apoptosis induced by vorinostat is highly potentiated by combination therapy. Tumors from day 11 and 12, respectively, in C underwent TUNEL staining (brown nuclear stain, arrows). Scale bar, 50 μm.

E, quantitation of apoptosis by TUNEL staining as in D, assessed by counting 5 random fields of more than 300 cells for 3 tumors per arm. Shown is the mean ± SEM.

Figure 4. Synergism of combination vorinostat/ABT-737 overcomes Bcl-2–mediated resistance and induces tumor regression in vivo. A, ABT-737 enhances vorinostat sensitivity in SCC lines. Cell viability was determined by sulforhodamine B assay following treatment with the indicated doses for 24 hours. Shown is the mean and SD of 6 replicates. Calculated combination (synergy) indices are shown in Supplementary Fig. S4B. B, ABT-737 synergizes with vorinostat and abolishes resistance mediated by Bcl-2. JHU-O29 cells expressing a GFP control (Con) or Bcl-2 cDNA were treated with vorinostat with or without ABT-737 (3 μmol/L) for 24 hours, and viability was determined by sulforhodamine B assay. Shown is the mean and SD of 6 replicates. **, P < 0.001. C, xenograft study showing in vivo synergy and reversal of Bcl-2–mediated resistance by combination therapy. JHU-O29 cells as in B were implanted subcutaneously in nude mice. Treatment with vorinostat (50 mg/kg/d) and ABT-737 (75 mg/kg/d) was initiated once measurable tumors appeared. Shown is the mean of 7 to 10 tumors per group ± SEM. *, P < 0.05; **, P < 0.001, 2-way ANOVA. D, apoptosis induced by vorinostat is highly potentiated by combination therapy. Tumors from day 11 and 12, respectively, in C underwent TUNEL staining (brown nuclear stain, arrows). Scale bar, 50 μm. E, quantitation of apoptosis by TUNEL staining as in D, assessed by counting 5 random fields of more than 300 cells for 3 tumors per arm. Shown is the mean ± SEM.
We then validated these observations in vivo, establishing a xenograft model to test the effect of single-agent or combination therapy. When implanted together with 3T3 fibroblasts, JHU-O29 cells form tumors at 100% frequency in immunodeficient mice (14). We implanted JHU-O29/GFP or JHU-O29/Bcl-2 cells and initiated treatment once measurable tumors were evident. Notably, we used well-tolerated doses of these agents and observed no drop in body weight during the study. Consistent with the results in vitro, vorinostat treatment alone was moderately effective in delaying progression of JHU-O29/GFP-derived tumors but was virtually ineffective against JHU-O29/Bcl-2 (Fig. 4C). Interestingly, although ABT-737 alone had little measurable activity in vitro against SCC cells, including JHU-O29, in vivo, this agent produced a measurable antitumor effect in JHU-O29/GFP tumors similar to that of vorinostat alone. Most importantly, combination treatment with vorinostat and ABT-737 did not simply delay progression but instead induced significant regression of established tumors (Fig. 4C and Supplementary Fig. S4C). As was observed in vitro, this dramatic synergy resulted in similar efficacy of the combination regardless of Bcl-2 expression (Fig. 4C). Thus, ABT-737 synergizes with vorinostat and effectively overcomes Bcl-2-mediated vorinostat resistance in vivo.

Histologic evaluation of tumors following combination drug treatment showed a marked increase in apoptotic cells in tumors treated with combination therapy compared with either single agent alone, as evidenced by pyknotic nuclei and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining (Fig. 4D and E). In contrast, assessment of proliferation by Ki67 staining revealed no difference between vehicle and combination therapy–treated tumors (data not shown). As anticipated, we observed increased expression of the same proapoptotic BH3-only family members following either vorinostat or combination therapy in vitro (Supplementary Fig. S4D). Collectively, these observations show that vorinostat promotes apoptotic priming of SCC cells through downregulation of Mcl-1 and induction of Noxa, resulting in shuffling of Bim to Bcl-2/Bcl-xl, which renders cells highly susceptible to treatment with the BH3-mimetic ABT-737.

**Bcl-2 Family Organization and Function Is Recapitulated in Primary HNSCC Tumors**

Finally, we sought to validate directly the key players involved in the response to combination vorinostat/ABT-737 treatment and to determine their relevance in human SCC tumors. Our functional studies predict that McI-1 overexpression would lead to increased Bim sequestration and thereby treatment resistance. Indeed, ectopic McI-1 expression (Supplementary Fig. S5A) induced substantial resistance to single-agent vorinostat and abrogated the ability of ABT-737 to overcome this resistance (Fig. 5A). Similarly, loss of Noxa would be predicted to recapitulate these effects by allowing increased Bim sequestration by McI-1. Noxa ablation by siRNA did in fact closely mimic the effects of McI-1 overexpression on drug sensitivity in multiple SCC cell lines (Supplementary Fig. S5B and S5C). Finally, Bim is predicted to play a critical role in the response to these drugs, functioning as the direct activator of the apoptotic cascade, which is released from McI-1 and Bcl-2/Bcl-xl in response to vorinostat and ABT-737, respectively (3, 8). Consistent with this model, siRNA-mediated ablation of Bim (Supplementary Fig. SSD) induced substantial resistance to the combination (Fig. 5B). Furthermore, we found that endogenous Bim levels were a significant predictor of the response to the vorinostat/ABT-737 combination across our entire panel of cell lines (Fig. S5C and Supplementary Fig. S5E).

To establish whether the Bcl-2 family organization and function are recapitulated in primary human tumors, we collected a cohort of 28 HNSCC cases at our institution, which received treatment with a combination of chemotherapy and radiation. Tumors underwent pathologic review followed by macromisection of viable tumor. We then determined the relative expression levels of Bcl-2 family members by quantitative reverse transcription (qRT-PCR) and compared them with those in the panel of SCC cell lines. This analysis showed a remarkably similar expression pattern in primary tumors and cell lines, both among and between different family members (Fig. 5D). For example, Mcl-1 and Bcl-xl were the most highly expressed members in both cell lines and tumors, and exhibited a relatively narrow range of expression between different cases. Bcl-2, in contrast, exhibited the lowest mean expression and the broadest expression range (Fig. 5D). Of note, we conducted internal validation for the measured gene expression levels in these tumors by showing that expression of the major antiapoptotic factors (Bcl-2, Bcl-xl, Bcl-w, and Mcl-1) was in equilibrium, and therefore linearly correlated, with the key proapoptotic factors (Bim and Noxa; Supplementary Fig. S5F). These findings imply that the expression and organization of the Bcl-2 family is comparable in SCC cell lines and primary tumors.

We next asked whether Bcl-2 family gene expression levels or tumor-specific somatic cancer gene mutations were associated with clinical response. Genomic analysis included a validated assay for “hot-spot” mutation detection for 15 key cancer genes, including KRAS, HRAS, and PIK3CA (33), as well as FBW7 sequencing. For outcome correlation, we included the subset of patients who had all undergone initial treatment using the same standard taxane and platinum chemotherapy–containing regimen, known as “induction” chemotherapy (34) and whose clinical responses were known. Remarkably, even in this relatively small cohort, high Mcl-1 mRNA levels were significantly associated with an inferior response of these patients to standard therapy (Fig. 5E). Furthermore, although no RAS or PIK3CA mutations were observed, one tumor harbored a structure-disrupting missense FBW7 mutation within the substrate-binding domain (Supplementary Fig. S5G), and this tumor showed a poor response to chemotherapy treatment (Fig. 5E). These findings are in accordance with the established correlation between FBW7 mutation/Mcl-1 levels and taxane chemotherapy resistance (29, 35). Of note, high Bcl-2 levels showed a trend toward correlation with poor response in this cohort (Supplementary Fig. S5H), consistent with other recent data (17, 18). Although larger cohorts will be required to confirm these observations, they support our model for Mcl-1 and Bcl-2 as key survival factors in SCC.

Finally, we wished to address the potential promise of the vorinostat/ABT-737 therapeutic combination for treatment of patients with SCC. To date, few or no clinical data are available regarding the use of this combination in SCC. Therefore,
**Figure 5.** Bcl-2 family expression is recapitulated in primary HNSCC tumors and predicts sensitivity to combination vorinostat/ABT-737 treatment. 

A, Mcl-1 is a key effector in the response to vorinostat and ABT-737 in SCC. Dose–response analysis as in A was conducted following infection with a control or Mcl-1–expressing retrovirus. See Supplementary Fig. S5A for immunoblots.

B, Bim mediates the response to combination therapy. Relative ratio of viable cell numbers in ABT-737 (3 μmol/L)–treated cells in the presence or absence of vorinostat (1.5 μmol/L) following transfection of control (siCon) or Bim-directed (siBim) siRNA. **, P < 0.01.

C, Bim levels are associated with overall response to combination therapy. Vorinostat IC50 values in the presence of ABT-737 from Supplementary Fig. S5E are shown. Bim levels were determined by qRT-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase.

D, relative expression and dynamic range of Bcl-2 family expression in cell lines (n = 7) and primary tumors (n = 28), assessed by qRT-PCR. Horizontal lines represent mean values.

E, correlation between Mcl-1 mRNA levels and clinical outcome of HNSCC tumors treated with taxane/platinum chemotherapy and radiation. CR, complete response; PD, progressive disease; SD, stable disease. Horizontal lines, mean values; error bars, SEM. Arrow indicates tumor with disruptive FBW7 mutation. F, application of biomarkers to primary tumors predicts sensitivity to combination therapy. In each case, gray shading denotes biomarker range associated with sensitivity in preclinical studies. Left, Mcl-1/Bcl-2 expression by qRT-PCR from chronic lymphocytic leukemia (CLL; ref. 36) was validated and applied to our HNSCC cohort, predicting resistance to single-agent ABT-737. Right, application of Bim levels from in vivo studies (Fig. 4) predicts sensitivity with combination therapy.
we interrogated our established gene expression biomarkers in these tumors, and we then predicted responses using cutoff values determined from preclinical data. We first tested the Mcl-1/Bcl-2 ratio, which, we showed, correlated with cell line sensitivity to single-agent ABT-737 (Fig. 2), and which was previously shown to do the same in chronic lymphocytic leukemia (CLL; ref. 36). CLL cells have a very low Mcl-1/Bcl-2 ratio and are sensitive to ABT-737, whereas SCC cells have a much higher ratio, are Mcl-1 dominant, and are ABT-737 resistant (Fig. 2). Consistent with these findings, comparing the Mcl-1/Bcl-2 ratio in primary CLL cultures with that in our primary HNSCC tumors predicts resistance of nearly all tumors to single-agent ABT-737 (Fig. 5F). This interpretation is also consistent with early clinical data suggesting very poor single-agent activity of the orally available and more stable analogue of ABT-737, ABT-263, against many solid tumors (10, 11). Most importantly, we then examined Bim levels as a measure of the potential response to combination therapy, as we showed above that Bim correlated with cell killing by this combination in SCC lines (Fig. 5C). Comparing Bim values derived from the xenograft study (Fig. 4) with those observed in the primary HNSCC tumors, we find that the vast majority of cases would be predicted to respond to combination therapy (Fig. 5F). Thus, the molecular profile of the Bcl-2 family in HNSCC tumors predicts that significant treatment responses would be obtained using this drug combination in clinical studies (Fig. 6).

**DISCUSSION**

Here, we uncover the tissue-specific landscape of the Bcl-2 family in SCC, revealing Mcl-1 as a key survival factor and establishing functional biomarkers to guide the application of a novel combination-targeted therapy. The dominant role played by Mcl-1 in this disease compared with hematologic cancers is evidenced by the dramatically elevated Mcl-1/Bcl-2 ratio and by the high fraction of Bim bound to Mcl-1 relative to Bcl-2/Bcl-xL in SCC cells (Fig. 2). Consistent with these observations is the profound resistance of SCC cells to single-agent ABT-737, which is explained by the inability of this agent to displace the large proportion of Bim that is bound to Mcl-1 (3, 4). Of note, the situation in SCC also contrasts with that observed in myeloma cells, which are reported to be ABT-737 sensitive and Bcl-2/Bcl-xL and Mcl-1 “codependent,” based on the distribution of bound Bim among all 3 of these factors (22). Powerful direct evidence of unique Mcl-1 dominance in SCC is our finding that siRNA-mediated ablation of Mcl-1, but not other prosurvival family members, is sufficient to kill SCC cells (Fig. 2 and Supplementary Fig. S2). Finally, the importance of Mcl-1 in this disease is also supported by the presence of recurrent disruptive mutations in FBW7, encoding an SCF component, which is known to target Mcl-1 for degradation. We show that Mcl-1 is stabilized in FBW7-mutant SCC cells, further promoting Mcl-1 addiction and leading to increased HDAC inhibitor sensitivity (Fig. 3).

We show that vorinostat targets Mcl-1 function in SCC, mediating striking redistribution of Bim from Mcl-1 to Bcl-2 and Bcl-xL through the concerted effect of Noxa induction and Mcl-1 downregulation (Fig. 2 and Supplementary Fig. S2). These effects, together with the induction of Bim itself by vorinostat, account for the sensitivity of a subset of SCCs with low Bcl-2 to single-agent vorinostat (Fig. 1). More importantly, we show that vorinostat-induced Bim redistribution dramatically reprograms SCCs from Mcl-1 dependence to Bcl-2/Bcl-xL dependence, an effect that underlies the synergy we have uncovered between vorinostat and ABT-737. In *vivo*, this synergistic effect converts vorinostat from a moderately effective single agent that delays tumor progression to an inducer of apoptosis and tumor regression (Fig. 4 and Supplementary Fig. S4). Perhaps even more exciting is our finding that this combination, *in vivo*, is able to fully reverse resistance in tumors expressing high levels of Bcl-2. This observation is in line with previous data showing that this same combination was able to induce apoptosis in a mouse model of Bcl-2-overexpressing B-cell lymphoma (37). In HNSCC, we and others have shown that Bcl-2 is an
important negative prognostic factor, which is independent of other clinicopathologic factors, including human papillomavirus status (17–19). Taken together, these observations support the view that this therapeutic combination may be particularly effective for the subset of SCCs that are most refractory to standard therapy.

Our study uncovers potential predictive markers, showing that levels of Bim predict the overall response to combination therapy (Fig. 6). This is logical because the net effect of the combination is to disable both submodules for Bim sequestration (Mcl-1 and Bcl-2/Bcl-xl), resulting in free Bim, consequent activation of Bax/Bak, and cell death. Critically, we provide several lines of evidence that the organization of the Bcl-2 family and our model in general are likely to hold true in human HNSCC tumors. We show that the relative expression of Bcl-2 family members is comparable in both SCC cell lines and tumors, and that Mcl-1, which is known to be associated with chemoresistance in vitro (29), predicts a poor response to treatment in patients (Fig. 5). Finally, by analyzing expression of biomarkers, we have established from preclinical data and we provide evidence that a substantial fraction of human HNSCC tumors will be resistant to single-agent ABT-737 but sensitive to the combination. Of note, some of the predictions from this and related studies are already being validated through early-phase clinical trials. Patients with tumors that are Bcl-2 dominant, such as CLL, do indeed respond to single-agent ABT-737 (38), whereas those with carcinomas, including SCC, which may be Mcl-1 dominant, seem largely refractory (11). In addition, patients with HNSCC previously treated with, and refractory to, conventional chemotherapy (presumably through increased Bcl-2 or related mechanisms) are indeed resistant to single-agent vorinostat (39). Our data suggest that such tumors might be successfully treated with the vorinostat/ABT-737 combination.

Of particular importance to predictive cancer molecular diagnostics are mutations and other genomic events that function as drivers of tumorigenesis, impinging on key pathways that can be targeted to therapeutic advantage. Mutations of FBW7 is a challenging example, given the number of potentially relevant cancer effectors that may be activated by its loss-of-function, including c-Myc, Notch, cyclin E, Mcl-1, and others (25). As noted above, we provide evidence that Mcl-1 is a key target of FBW7 in SCC. We also speculate that other FBW7 targets may be activated as well following its mutation and may contribute to increased “oncogenic stress” of FBW7-mutant SCC, reflected by increased Bim levels (Supplementary Fig. S3B). Collectively, our data suggest that FBW7 mutation is likely to serve as an important predictive marker in SCC. These mutations would confer a poor response to chemotherapy and particularly taxane-based therapy, but a robust response to combination HDAC/Bcl-2 inhibitor therapy and potentially other targeted therapies as well (40).

Our detailed analysis of SCC also provides an explanation for one of the paradoxes of this disease: Bcl-2 itself is an important prognostic factor and a key survival factor, yet these tumors are unlikely to respond to single-agent Bcl-2 inhibitors such as ABT-737 (10, 17, 18, 24). Our finding that Mcl-1 plays a central role in sequestering Bim, even in tumors with high Bcl-2 levels, explains why ABT-737 alone is insufficient. This discovery also shows why therapeutic combinations targeting both Mcl-1 and Bcl-2/Bcl-xl survival modules will be required for successful tumor cell killing. Progress in improving clinical outcomes for patients with SCCs including HNSCC has been remarkably slow, owing in part to the lack of readily targeted somatic oncogene mutations and activated pathways observed in these tumors (1). Our study provides fundamental insight into this tumor subtype, revealing the underlying survival mechanisms and how they might be successfully targeted using agents already in clinical use.

**METHODS**

**Cell Lines**

The origin of JHU-O29, JHU-O11, HO1N1, KYSE-30, KYSE-150, and FaDu was described in ref. 14. BICR-78 cells were a generous gift from Dr. S. Michael Rothenberg [Massachusetts General Hospital (MGH), Boston, MA]. SKNO1, HL60, and THP1 cells were generous gifts from Dr. David Sweetser (MGH). Each line was maintained by the MGH Center for Molecular Therapeutics cell line bank and underwent high-density single-nucleotide polymorphism typing during the past 24 months, revealing that each was unique compared with more than 800 other banked lines (31). Cells were maintained at 37°C with 5% CO₂ in RPMI supplemented with 10% FBS, penicillin, and streptomycin.

**Viability Assays**

Cells were plated in 96-well plates at a density consistent with exponential growth, and drugs were added on the next day. Viable cell numbers were determined by sulforhodamine B (SRB) staining for SCC cells, or CellTiter-Glo assay for hematologic cells according to the manufacturer’s instructions (Promega). Comparison between CellTiter-Glo assay and SRB assay were carried out on JHU-O29 cells, resulting in identical IC₅₀ values. Quantitation of apoptosis by Annexin V/PI staining was conducted as described previously (41). Cells were analyzed on a FACScalibur flow cytometer (BD Biosciences). Data were analyzed with FlowJo V5.0 (Tree Star, Inc.).

**Retro viral Production and mRNA Analysis**

Production of virus and mRNA analysis were conducted as described (41). The qRT-PCR for all cell line data using SYBR Green was conducted as described in ref. 41. Primers used for qRT-PCR are shown in Supplementary Table S1. Primary tumor samples were analyzed by TaqMan PCR (LIFE Technologies) according to the manufacturer’s instructions. Probe catalog numbers are listed in Supplementary Table S1. The consistency between these 2 methods was validated directly by applying both platforms to SCC cell lines.

Protein extraction, immunoblot, immunoprecipitation, and Mcl-1 protein degradation were conducted as described in ref. 41 and Supplementary Methods.

**Animal Studies and TUNEL Staining**

All animals were housed and treated in accordance with protocols approved by the Subcommittee on Research Animal Care at the Massachusetts General Hospital. Xenograft tumors were generated and tumor volumes were determined as previously described (14, 42). Mice were injected intraperitoneally with 30 mg/kg vorinostat (Active Biochem) and/or 75 mg/kg ABT-737 (Active Biochem) or the corresponding solvent (50% propylene glycol, 5% Tween-80, 65% of 5% dextrose in water, pH 4) every day starting 13 days after cell implantation. Apoptag Peroxidase In Situ Apoptosis Detection Kit (Millipore) was used to carry out TUNEL staining according to the manufacturer’s instructions. Images were captured using a Nikon Eclipse 80i microscope, Q Imaging Retiga Exi camera, and QCapture version 2.08 software.
Cell-cycle-dependent Mcl-1 protein degradation was conducted as described in ref. 41 and Supplementary Methods.

**Database Analyses**

FBW7 mutation data were obtained from the Sanger Institute COSMIC database and the Broad Institute Cancer Cell Line Encyclopedia (CCLE) database (23). A total of 61 cell lines with deleterious mutations were identified. Analysis of transcript levels, DNA copy number, and drug sensitivity are described in detail in Supplementary Methods.

**Human Samples**

Primary human tumor specimens with the histologic diagnosis of HNSCC were collected before treatment. Use of tissues and clinical data for this study were approved by the local Institutional Review Board. Total RNA preparation and analysis were carried out on mac-rodiced tumor-enriched portions following pathology review (42).

**Statistics**

P values were determined using the Student unpaired t test unless indicated otherwise. For correlation studies, Pearson product-moment correlation coefficient ($r^2$) was calculated, and a 2-tailed $P$ value was generated with GraphPad Prism.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

**Conception and design:** L. He, N. Forster, C.H. Benes, L.W. Ellisen

**Development of methodology:** L. He, N. Forster, L.W. Ellisen

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** L. He, K. Torres-Lockhart, M.J. Garnett, U. McDermott, S.M. Rothenberg, C.H. Benes

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** L. He, K. Torres-Lockhart, N. Forster, S. Ramakrishnan, P. Greninger, L.W. Ellisen

**Writing, review, and/or revision of the manuscript:** L. He, N. Forster, S. Ramakrishnan, C.H. Benes, L.W. Ellisen

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** L. He, S. Ramakrishnan

**Study supervision:** L. He, L.W. Ellisen

**Acknowledgments**

The authors thank Nick Dyson and members of the Ellisen laboratory for comments on the manuscript, and the Dana-Farber/Harvard Cancer Center Specialized Histopathology Core Facility for assisting in the processing of murine tumors.

**Grant Support**

This work was supported by grants National Institute of Dental and Craniofacial Research RO1 DE015945 (to L.W. Ellisen, K. Torres-Lockhart, and S. Ramakrishnan), Congressionally Directed Medical Research Programs/Breast Cancer Research Program BC093523 (to L. He), Deutsche Forschungsgemeinschaft (German Research Foundation) FO 785/1-2 (to N. Forster), and NIH RO8 DE-020139 (to S.M. Rothenberg), and by a grant from the Wellcome Trust (086357; to P. Greninger, U. McDermott, M.J. Garnett, and C.H. Benes).

Received September 10, 2012; revised December 19, 2012; accepted December 19, 2012; published OnlineFirst December 28, 2012.

**REFERENCES**


Targeting the Bcl-2 Family in Squamous Cell Carcinoma


30. Beroukhim R, Mermel CH, Porter D, Wei G, Raychaudhuri S, Dono-


Mcl-1 and FBW7 Control a Dominant Survival Pathway Underlying HDAC and Bcl-2 Inhibitor Synergy in Squamous Cell Carcinoma

Lei He, Kristine Torres-Lockhart, Nicole Forster, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/2159-8290.CD-12-0417

Supplementary Material
Access the most recent supplemental material at:
http://cancerdiscovery.aacrjournals.org/content/suppl/2012/12/31/2159-8290.CD-12-0417.DC1

Cited articles
This article cites 42 articles, 20 of which you can access for free at:
http://cancerdiscovery.aacrjournals.org/content/3/3/324.full#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://cancerdiscovery.aacrjournals.org/content/3/3/324.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.