Mitochondrial Bcl-2 Family Dynamics Define Therapy Response and Resistance in Neuroblastoma

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Abstract

Neuroblastoma is a childhood tumor in which transient therapeutic responses are typically followed by recurrence with lethal chemoresistant disease. In this study, we characterized the apoptotic responses in diverse neuroblastomas using an unbiased mitochondrial functional assay. We defined the apoptotic set point of neuroblastomas using responses to distinct BH3 death domains providing a BH3 response profile and directly confirmed survival dependencies. We found that viable neuroblastoma cells and primary tumors are primed for death with tonic sequestration of Bim, a direct activator of apoptosis, by either Bcl-2 or Mcl-1, providing a survival dependency that predicts the activity of Bcl-2 antagonists. The Bcl-2/Bcl-xL/Bcl-w inhibitor ABT-737 showed single-agent activity against only Bim:Bcl-2 primed tumor xenografts. Durable complete regressions were achieved in combination with noncurative chemotherapy even for highest risk molecular subtypes with MYCN amplification and activating ALK mutations. Furthermore, the use of unique isogenic cell lines from patients at diagnosis and at the time of relapse showed that therapy resistance was not mediated by upregulation of Bcl-2 homologues or loss of Bim priming, but by repressed Bak/Bax activation. Together, our findings provide a classification system that identifies tumors with clinical responses to Bcl-2 antagonists, defines Mcl-1 as the principal mediator of Bcl-2 antagonist resistance at diagnosis, and isolates the therapy resistant phenotype to the mitochondria. Cancer Res; 72(10); 2565–77. ©2012 AACR.

Introduction

Cancer remains a major cause of mortality, and the emergence of therapy resistance is the principal barrier to cure. Nascent cancer cells must bypass numerous apoptotic checkpoints that safeguard against oncogenesis (1, 2), while established cancers encounter therapeutics that interfere with vital functions to activate mitochondrial apoptosis (3, 4). Not surprisingly, alterations that attenuate these pathways are selected for during tumor initiation and progression, and “apoptosis evasion” is considered an essential hallmark of cancer (5). Understanding how specific cancers circumvent the apoptotic program is critical to identifying rational therapeutic approaches. Indeed, numerous agents that target apoptotic mediators are under development and their effective integration into clinical use requires such knowledge.

Neuroblastoma is a highly lethal pediatric solid tumor. Most patients present with high-risk features (6), and despite intensive multimodal therapy the majority die from progression of therapy-resistant disease (7). The Bcl-2 family proteins govern mitochondrial apoptotic responses and are candidate mediators of therapy response (8). They are classified by their highly conserved Bcl-2-homology (BH) domains into the proapoptotic BH3-only proteins (containing only the BH3 death domain), proapoptotic multidomain Bak and Bax (containing BH1, BH2, and BH3 domains), or antiapoptotic multidomain homologues (Bcl-2, Bcl-w, Bcl-xl, Mcl-1, and A1; sharing BH1, BH2, BH3, and BH4 domains). BH3 proteins act as stress sentinels activated by a host of tumor-related insults that include radiation and chemotherapy. 

A subset of BH3 proteins can directly engage Bak or Bax at the mitochondrial membrane, leading to their homooligomerization, mitochondrial outer membrane permeabilization (MOMP), release of apoptogenic factors such as cytochrome c, and death commitment. Such “direct activator” BH3 proteins include Bim, Bid, and Puma (9). Alternatively, antiapoptotic Bcl-2 proteins (e.g., Bcl-2 and Mcl-1) can bind activator BH3’s preventing Bak or Bax activation and providing a buffer against cell death. These tonically neutralized activator BH3 proteins can then be displaced by additional “enabler” BH3 proteins (e.g., Bik, Noxa, and Bad) that have a higher affinity for antiapoptotic docking sites. The state whereby activator BH3...
proteins are sequestered by antiapoptotic Bcl-2 proteins has been termed "primed for death" because such cells are paradoxically sensitized to BH3 proteins (10). Of note, most nontransformed cells lack BH3 priming, providing a conceptual therapeutic index for agents that antagonize the antiapoptotic Bcl-2 proteins (11).

We carried out mitochondrial profiling on neuroblastoma cell lines that were genetically diverse to identify their set point for apoptotic signal transduction following exposure to distinct BH3 death domains, providing a BH3 response profile (12). We identified 3 classes of apoptosis resistance that accurately predicted sensitivity to the Bcl-2 antagonists in vitro. Most neuroblastoma robustly and reproducibly responded to specific enabler peptides, confirming intact Bak/Bax signaling and inferring that endogenous activator BH3 proteins were tonically suppressed but replaceable (cells were primed). One third of high-risk neuroblastomas were most sensitive to BikBH3, suggesting a primary Bcl-2/Bcl-xL/Bcl-w dependence and were equisitely sensitive to the Bcl-2/Bcl-xL/Bcl-w inhibitor, ABT-737, in vitro (IC50 < 50 nmol/L). A second subset had a dominant NoxaBH3 response (indicating Mcl-1 survival dependence) and was relatively ABT-737 resistant. The third subset, consisting solely of cell lines at relapse following therapy, had absent enabler and markedly blunted activator BH3 peptide responses, a profile consistent with the profound therapy resistance seen clinically at relapse.

We hypothesized that relapsed neuroblastomas either lose their primed for death status through repressed Bid, Bim or Puma activation, or repress Bak/Bax-mediated pore formation.

We now directly show that neuroblastomas are primed for death through sequestration of Bim, and that the pattern of Bim binding is predicted by the BH3 response profile. Furthermore, the dominant antiapoptotic site of Bim binding predicts the in vivo response to small-molecule Bcl-2 antagonists. Xenografts from neuroblastoma with Bim sequestered by Bcl-2 are equisitely sensitive to ABT-737 and cures following a single course of therapy were obtained. Importantly, this includes tumors with MYCN amplification and ALK mutations (both R1275Q and F1174L) that are associated with an extremely poor prognosis. Conversely, tumors with Bim sequestered to Mcl-1 are resistant to Bcl-2 antagonists. Using matched tumor cell line pairs obtained at diagnosis and following relapse after therapy, we unequivocally show that relapsed neuroblastomas retain Bim priming and antiapoptotic Bcl-2 dependence patterns indistinguishable from pretherapy cells. That acquired therapy resistance is associated with repression of Bak and/or Bax-mediated apoptotic signal transduction implicates the mitochondria as a major contributor to the postrelapse therapy resistant phenotype.

Materials and Methods

Cell lines

Neuroblastoma cell lines with MYCN amplification [IMR5 (13), NLF, LA-N-5 (30), NGB (14), CHP-134, NB-1643 (15), SMS-SAN, SMS-KCN and SMS-KCNR, SMS-KAN and KANR, SK-N-BE (1) and SK-N-BE (2), BES2, CHLA-15 and CHLA-20, CHLA-122 and CHLA-136 (16) and without [NB69 (17), SK-N-SH and SK-N-AS (18)]] were grown in RPMI-1640 supplemented with 10% FBS, 2 mmol/L l-glutamine, 1% O2I, 100 U/mL of penicillin. Tissue culture was at 37°C in a humidified atmosphere of 5% CO2. All cell lines and isogenic pairs were confirmed with short tandem repeat (STR)-based genotyping (AmpFISTR; Applied Biosciences) and matched to the Children’s Oncology Group (COG) cell line genotype database (www.cogcell.org).

Coimmunoprecipitation

Cells were lysed in CHAPS buffer (10 mmol/L HEPES, 150 mmol/L NaCl, 2% CHAPS (Sigma-Aldrich) and added to antibody–matrix complex [ExactCruz Immunoprecipitation Matrix C plus 1–5 mg immunoprecipitation (IP) antibody] for 4 hours, 24 degrees. Immunoprecipitated proteins were released from the matrix complex using 2× RIPA buffer, run on Nu-PAGE 10% Bis-Tris gels (Invitrogen), transferred to polyvinylidene fluoride membranes and detected for Bcl-2 family proteins as described (10). Primary frozen tumor samples were dissociated through a 0.45 μm sterile filter, washed twice with Red Blood Cell Lysing Buffer (Sigma), lysed, and immunoprecipitated as above.

Antibodies

Anti-Mcl-1 (BD Phamingen), anti-Bcl-2 (DAKO and Santa Cruz Biotechnology; sc-992), and anti-Bcl-xL (clone 7B2.5; gift of L.Boise, Emory University), anti-Bak, anti-Bax (#2772), anti-Puma (#4976), and anti-BID (#2002; Cell Signaling Technology), anti-Bim (Millipore Corporation: AB17003), anti-PARP (Cell Signaling #9664), and anti-Casp3 (Cell Signaling #9664) were used.

Mitochondrial profiling

Heavy membrane fractions enriched for functional mitochondria were obtained from neuroblastoma cells during logarithmic growth, or from tumor xenografts, as described (19). Functional studies were carried out with freshly isolated mitochondria suspended to a final concentration of 1 μg/μL in mitochondrial buffer, as described (20). BimBH3 peptide, recombinant tBid protein (R&D Systems) or 1% dimethyl sulfoxide (DMSO) were incubated with mitochondria for 30 minutes at 30°C and cytochrome c release measured in duplicate by ELISA (R&D Systems). Peptide synthesis and assay details are as in ref. 19.

Whole-cell JC-1 assay

Cells were plated at 2 × 10^4 neuroblastoma cells per well into 384-well plates in 300 mmol/L Trehalose, 10 mmol/L HEPES-KOH, 80 mmol/L KCl, 1 mmol/L ethylene glycol tetraacetic acid, 1 mmol/L EDTA, 0.1% bovine serum albumin, 5 mmol/L succinate; T-EB, and incubated at room temperature with 100 μmol/L BimBH3 peptide, 2 μmol/L JC-1, 20 mg/mL oligomycin, 0.01% digitonin, and 10 mmol/L β-mercaptoethanol, followed by continuous monitoring for JC1 fluorescence (BioTek Synergy Mx plate reader) at 545 ± 20 nm Ex and 590 ± 20 nm Em as described (21).

Bak and Bax oligomerization

Mitochondria from neuroblastoma cells (1 μg/μL per treatment) were treated with BimBH3 peptide (0.0125–50 μmol/L) or DMSO for 30 minutes at 30°C followed by a 30-minute incubation with 0.9 mmol/L of 1,6-bismaleimidohexane (10 mmol/L...
stock in DMSO; Pierce, #22330) at room temperature to cross-link oligomers. Treatments were centrifuged to pellet mitochondria, the pellet was dissolved in 1× NuPage loading buffer (Invitrogen) and immunoblotted for Bak and Bax.

**Cytotoxicity assays**

A total of 2 to 5 × 10⁶ neuroblastoma cells per well were treated with ABT-737, cytotoxic agent, or vehicle controls in RPMI-based media in triplicate. After 48 hours, WST-1 (Roche) was added and incubated at 37°C for 1 hour. Absorbance was recorded at 450 and 620 nm. For log-transformed nonlinear regression curve fit and determination of IC₅₀, Graph Pad Prism software was used (Graph Pad Software). For short interfering RNA (siRNA), 2 × 10⁵ cells/mL were transfected with 50 nmol/L

**Annexin–propidium iodide apoptosis assay**

Neuroblastoma cells were treated with ABT-737, melphalan, both agents, or DMSO vehicle control. Following a 24-hour incubation, cells were harvested, washed twice with PBS, and resuspended in Binding Buffer (8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄ , 0.24 g KH₂PO₄) at a concentration of 1 × 10⁶ cells/mL. Cells were incubated with Annexin V–fluorescein isothiocyanate and propidium iodide (BD Biosciences) for 15 minutes and then analyzed immediately by flow cytometry with the FACSCanto II flow cytometer (BD Biosciences).

**Murine xenograft studies**

Xenografts were established in the flank of nu/nu athymic mice (Jackson Laboratories) as described (19). When tumor volume reached 200 to 400 mm³, mice (n = 10 per arm) were treated by intraperitoneal injections of (i) vehicle control (normal saline, twice weekly for 2 weeks), (ii) cyclophosphamide (CPM; 75 mg/kg, twice weekly for 2 weeks), (iii) ABT-737 (100 mg/kg daily × 2 weeks, as in ref. 22), or (iv) the combination of cyclophosphamide with ABT-737 (according to their monotherapy schedule). Animals were sacrificed when tumor volumes exceeded 2,000 mm³ and all animal work was carried out under a protocol approved by the CHOP and Emory Institutional Animal Care and Use Committee.

**Statistical analyses**

Survival analyses were conducted according to the method of Kaplan–Meier (23) with SEs according to Peto (24). Comparisons of outcome between subgroups were carried out by a 2-sided log-rank test.

**Results**

**Bim is sequestered at the mitochondria of viable neuroblastoma, inducing a primed for death state that defines its antiapoptotic dependence**

We previously used an isolated mitochondrial cytochrome c release assay and inferred that most neuroblastomas were primed for death as shown by robust responses to select enabler BH3 peptides that are incapable of directly activating Bak or Bax (ref. 12 and Supplementary Fig. S1). Using coimmunoprecipitation assays and cell lines from different BH3 response classes, we confirmed that Bim is the principal BH3-only death activator in neuroblastoma (Fig. 1A). All neuroblastomas that had a dominant cytochrome c release to the BīkBH3 peptide had high Bcl-2 expression and endogenous Bim bound to Bcl-2. We predicted these cells would have an activator BH3 protein bound to Bcl-xl or Bcl-w, rather than Bcl-2, as Bik is more avid for these hydrophobic pockets (25–27). However, Bik was discovered in association with Bcl-2 (Bcl-2 interactive killer) and its proapoptotic function can be abrogated by Bcl-2, supporting a functional interaction with Bik (28, 29). In support, BikBKH3 peptide was capable of competitively displacing endogenous Bik from Bcl-2 in neuroblastoma cells with Bim:Bcl-2 priming (Fig. 1B). In contrast, all neuroblastoma cell lines that had a Noxa-dominant BH3 response profile had Bim neutralized almost exclusively by Mcl-1, despite the coexpression of Bcl-2 in many (Fig. 1A). The absence of ABT-737 responses for this subset reflects the inability of this small molecule to antagonize Bim:Mcl-1, as shown for other tumor models (8).

Bim was identified in complex with various antiapoptotic partners in all neuroblastomas assessed, but neither alternative BH3 activators (Bid, Puma) nor multidomain Bak or Bax proteins were sequestered by antiapoptotic homologues (Supplementary Fig. S2). Bim-EL was the only Bim isoform detected in complex with Bcl-2 members and has been proposed as a necessary BH3-only protein downstream of chemotherapy-induced stress (30), and as a tumor suppressor that cooperates with MYC deregulation (30, 31). To test for a functional role for Bim in neuroblastoma, we used siRNA-mediated knockdown and showed Bim loss attenuated etoposide-induced cytotoxicity in line with resistant cell line IC₅₀₈ (Fig. 1C; ref. 32). These data suggest Bim is the major mediator of chemoresponse in neuroblastoma but that its activity is suppressed by antiapoptotic Bcl-2 proteins. Neuroblastoma cells in which Bim is bound to Bcl-2 are extremely sensitive to ABT-737 *in vitro* (12). We therefore reasoned that ABT-737 derived its potency by displacing Bim from Bcl-2 and confirmed this by treating cells in culture with ABT-737 and showing a dose-responsive competitive displacement of Bim (Fig. 1B).

To interrogate whether these Bcl-2 family interactions were inherent to the primary tumor or secondary to cell culture adaptation, we carried out coimmunoprecipitations using fresh-frozen neuroblastoma tumors of different risk groups obtained at diagnosis (Supplementary Table S1). The same heterogeneous Bcl-2 family binding patterns were identified, even within a single clinical risk group. Two tumors showed dominant Bcl-2:Bim priming (NBL-T01 and NBL-T04), one had Bim dominantly sequestered by Mcl-1 (NBL-T02), and 1 tumor that showed no evidence of Bim expression or priming (NBL-T03; Fig. 1D) failed to show other activator BH3’s, Bid, or Puma, bound to Mcl-1 or Bcl-2, suggesting it lacks priming altogether (data not shown).

**Posttherapy relapsed neuroblastomas retain Bim priming, but show repressed apoptotic signaling at the level of Bak/Bax**

The third subset of neuroblastoma cell lines identified by mitochondrial profiling were characterized by repressed enabler BH3 responses and blunted BidBH3 and BimBH3 responses.
(direct activators), suggesting they either had lost Bim priming or acquired apoptotic defects at or downstream of Bak and/or Bax (12). Notably, these neuroblastomas were derived at relapse following therapy. SK-N-AS, CHP-134, and BE2C had high Mcl-1 expression and sequestered Bim in patterns similar to neuroblastomas in the Mcl-1–dependent group, supporting no loss in Bim priming at relapse (Figs. 1A and 2B). Only CHP-134 cells seemed qualitatively to have reduced priming when comparing whole-cell Bim levels with the Bim pulled down with Mcl-1.

We next assayed isogenic matched tumor cell line pairs from the same patients at the time of initial diagnosis and at the time of relapse following therapy (Supplementary Table S2). coinmunoprecipitation again showed that most pre- and postrelapse pairs retained consistent antiapoptotic Bcl-2 family expression as well as indistinguishable Bim priming patterns, suggesting that antiapoptotic Bcl-2 dependence patterns are not substantially altered in response to therapy (Fig. 2A).

Indeed, only KANR showed a gain in expression of an antiapoptotic Bcl-2 homologue (Mcl-1) not expressed at diagnosis (in KAN) yet this acquired Mcl-1 expression did not neutralize Bim appreciably as minimal Bim:Mcl-1 was seen.

Immunoblots of Bim from whole-cell lysates confirmed consistent Bim–EL expression between pre- and postrelapse cells (Fig. 2B). Although the initial cohort of postrelapse cell lines we investigated had Bim:Mcl-1 priming, the additional pre- and postrelapse pair studies identified neuroblastomas with Bim:Bcl-2 priming as well. This suggests that cells in either class could develop therapy resistance associated with maintained Bim priming but a reduction in BH3 responsiveness. We therefore assessed ABT-737 responsiveness before and after relapse in the context of both Bcl-2 and Mcl-1 dependence. SK-N-BE(1) (pretherapy) and SK-N-BE(2) (postrelapse) show Bim: Mcl-1 priming, and as predicted, neither was sensitive to ABT-737 (IC50 > 2 μmol/L; Fig. 2C). In contrast, both CHLA-15

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**Figure 1.** Bim is the primary death activator in neuroblastoma. A, protein from neuroblastoma cell lines of distinct BH3 response classes was immunoprecipitated with antibodies for Mcl-1, Bcl-2, or Bcl-xL and immunoblotted for Bim and the antiapoptotic Bcl-2 family members. The identified dominant Bim interactions with the multidomain antiapoptotic members are consistent with predictions inferred from BH3 profile results (12). B, coimmunoprecipitations of Bcl-2 shows decreased Bim:Bcl-2 interactions following exposure of isolated mitochondrial lysates to a BikBH3 peptide for 30 minutes. Likewise coimmunoprecipitation of Bcl-2 following treatment of intact neuroblastoma cells with ABT-737 shows dose-dependent decreases in Bcl-2 bound Bim, supporting a competitive displacement model. C, Bim or negative control GAPDH were inhibited using siRNA for 24 hours in IMR5 cells, followed by a 48-hour exposure to etoposide (VP-16). Survival was assessed by WST-1. Immunoblots confirmed target protein inhibition by siRNA. D, coimmunoprecipitation of fresh-frozen neuroblastoma tumor at diagnosis shows similar Bcl-2 and Mcl-1 sequestration of Bim. All experiments were replicated at least twice and representative findings shown. WCL, whole cell lysate; IP, immunoprecipitation.
and CHLA-20 (postrelapse, same patient as CHLA-15) had Bim:Bcl-2 priming that predicts ABT-737 sensitivity, yet only the pretherapy CHLA-15 cells were sensitive (IC\textsubscript{50} = 30 nmol/L) while CHLA-20 cells were relatively resistant (IC\textsubscript{50} = 620 nmol/L; Fig. 2C). This suggests that diminished apoptotic responses seen in relapsed neuroblastoma are not primarily due to loss of Bim expression or priming at anti-apoptotic docking sites, but arise downstream of Bim displacement. We reasoned that the defective response to death stimuli in relapsed neuroblastoma may reside at the level of Bak and/or Bax activation or further downstream.

To explore this, we assessed the dose responsiveness of isolated neuroblastoma mitochondria to the direct activator BimBH3. In all cases, the pretherapy cells showed a heightened responsiveness to BimBH3 than did their postrelapse counterparts even at concentrations that saturate antiapoptotic binding sites (100 nmol/L; Fig. 3A). To ensure responses reflected functional signaling, we also assessed cytochrome c release in response to recombinant tBid with similar findings. To confirm this, we used a complementary assay in which mitochondrial membrane potential is measured in intact cells using the JC-1 dye (21). Again, postrelapse cells were less sensitive to direct Bak/Bax activation by BimBH3 as gauged by JC-1 fluorescence as a measure of mitochondrial membrane depolarization. As a positive control, p-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP), a mitochondrial oxidative phosphorylation uncoupler that induces MOMP via a non-Bak/Bax mechanism, was capable of fully inducing JC-1 redistribution (Fig. 3B).

We next investigated the proapoptotic Bak and Bax proteins that are required for Bim-induced mitochondrial apoptosis (33). In cell lines established at relapse postchemotherapy, Bak and/or Bax were reduced in expression (Fig. 3C). SK-N-BE cells had reductions in Bax in the relapsed lines [SK-N-BE(1) and SK-N-BE(2)] with no reduction in overall Bak expression (similar to KAN and KANR). CHLA-122 and CHLA-136 had no difference in total Bax, but Bak levels were reduced in the postrelapse cells (also with KCN and KCNR). CHLA-20 cells, in contrast, showed reductions in both Bak and Bax expression compared with the at-diagnosis isogenic CHLA-15. The functional status of the Bak/Bax axis was assessed by oligomerization assays in which higher order Bak or Bax homooligomers are detected in response to a proapoptotic signal, BimBH3. Indeed, BimBH3-responsive CHLA-15 cells showed higher order oligomerization of Bax in response to Bim that were absent in their matched postrelapse counterpart, CHLA-20 cells (Fig. 3D).

**Bcl-2 antagonists sensitize neuroblastomas with Bim:**

We next investigated whether the Bim:Bcl-2 priming patterns seen in a subset of neuroblastomas could be exploited therapeutically. We tested neuroblastoma cell lines with a Bcl-2–dependent profile (SMS-SAN and LA-N-5), Mcl-1–dependent profile (IMR5), or BH3-resistant profile (SK-N-AS) for the effect of ABT-737 on chemotherapy-induced death in vitro. As predicted, in Bcl-2–dependent neuroblastomas, cell death from doxorubicin and melphalan was enhanced by 5 nmol/L ABT-737 (Fig. 4A). This synergistic loss of viability was due to...
Neuroblastoma cell lines show repressed Bax/Bak activation at the time of relapse. A, isolated mitochondria from isogenic neuroblastoma cell line pairs (\(C3\) posttherapy relapse cells) were challenged with increasing concentrations of BimBH3 peptide or recombinant tBid protein and cytochrome c release was measured as a surrogate for apoptosis commitment. Pretherapy cells (blue) were markedly more responsive to Bim (and tBID, as shown for CHLA-122/136) compared with postrelapse cells (red). Data points represent replicate values and at all points the SE < 5% (error bars omitted). B, differences in mitochondrial responses to Bim in intact whole cells permeabilized with digoxin to incorporate BimBH3. Whole-cell responses mimic isolated mitochondrial responses to Bim and are measured by JC-1 release from mitochondria (decreased fluorescence coincides with MOMP). Top, JC-1 emission over time in response to different BimBH3 concentrations; bottom, JC-1 fluorescence at 90 minutes highlighting decreased depolarization in KCNR compared with KCN. FCCP, positive control for MOMP induction. C, isogenic paired cell lines derived from the same tumor at diagnosis and following relapse were immunoblotted for Bax and Bak. In all cell line pairs, the posttherapy relapse cells had evidence for reduced Bak and/or Bax expression. \(\beta\)-Tubulin serves as a loading control. D, acquired loss of higher order Bax homooligomers in CHLA-20 compared with CHLA-15 following direct Bax/Bak activation with BimBH3. Location of higher order oligomers have been previously defined (20). Nonspecific bands higher than 22 kDa in the Bak immunoblot are not consistent with functional higher order oligomers (20). Data are representative of at least 2 biological replicates.
increased apoptosis, as confirmed by PARP and caspase-3 cleavage, and phosphatidylserine externalization (Fig. 4B and C). In contrast, ABT-737 did not synergize with chemotherapeutics for Mcl-1–dependent (IMR5) or BH3-resistant (SK-N-AS) cells, even at 1 μmol/L concentrations. Similar findings were seen with agents from 2 additional cytotoxic classes with activity against neuroblastoma, cisplatinum (DNA platinator) and etoposide (topoisomerase II inhibitor; data not shown).

**Bcl-2 antagonists have potent activity in vitro against Bcl-2–dependent neuroblastomas**

These data support that BH3 response profiles obtained from neuroblastoma cell lines predict responses to selective Bcl-2 family antagonists (12) as well as synergy with cytotoxics in vitro. However, mitochondrial signaling may be modified by extrinsic survival cues from the microenvironment or additional stress stimuli (e.g., hypoxia, acidosis) that are not recapitulated in vitro. We have shown that neuroblastoma cell line BH3 response profiles are preserved in xenografts from mice supporting that the defining characteristics of these responses are relatively cell autonomous (12). However, to determine the extent to which in vivo responses to Bcl-2 antagonists can be predicted, we treated mice harboring xenografts of neuroblastoma cell lines from different BH3 response classes with ABT-737 alone and with chemotherapy.

ABT-737 monotherapy induced notable tumor regression and extended overall survival by approximately 50% in mice bearing Bcl-2–dependent SMS-SAN xenografts ($P < 0.005$ by
log-rank test), with a median survival of 20.5 ± 10.5 days for ABT-737–treated mice and 14 ± 3.2 days for control mice. For neuroblastomas with Mcl-1 dependence (IMR5) or BH3 resistance profile (BE2C), survival was not significantly extended by ABT-737 (Fig. 5; median survival 17 ± 5 versus 12 ± 3.5 days and 12 ± 5.3 versus 11 ± 1.6 days, respectively). The response of Bcl-2–dependent neuroblastomas to ABT-737 as single agent supports that even in the context of in situ survival signals the tonic repression of activated Bim is required to maintain cancer cell survival and antagonism of this is sufficient for antitumor activity.

Coexposure to a genotoxic agent and a Bcl-2 antagonist was next assessed. We predicted that ABT-737, by saturating anti-apoptotic binding sites, would prevent sequestration of the

Figure 5. Bim priming patterns predict neuroblastoma sensitivity to ABT-737 in vivo. Neuroblastoma cell line xenografts representative of the 3 different BH3 response classes were established in the flank of nu/nu athymic mice. Mice with growing tumors more than 250 mm³ were randomized to receive either intraperitoneal cyclophosphamide (CPM), ABT-737 (A), cyclophosphamide + ABT-737 (CPM + A), or vehicle control (C), as outlined in Materials and Methods. A, Waterfall plot of individual tumor volume as % volume change from tumor volume pretherapy. Negative values define best response tumor shrinkage with −100% representing complete regression. B, Kaplan-Meier curves comparing survival of control (black) versus ABT-737 (red), and CPM (gray) versus CPM + ABT-737 (blue) in representative cell lines with different Bcl-2 dependence patterns. *P* values derived using the log-rank test. For NB-1643, n = 10 mice per arm; for IMR5, BE2C, and SMS-SAN, n = 9 mice per arm.
excess BH3 death stimulus induced by chemotherapy (such as Bim) and increase antitumor activity for tumors functionally dependent on Bcl-2. A noncurative single course of cyclophosphamide was used. For each xenograft model, survival was extended in cyclophosphamide-treated mice compared with control-treated mice ($P < 0.0001$ by log-rank test) consistent with the known antitumor activity of this drug in neuroblastoma, yet all mice had progressive tumor after a transient regression (Fig. 5B, median survival of 22 ± 7 days for SMS-SAN; 25 ± 7.6 days for IMR5; and 18 ± 3.6 days for BE2C in the respective cyclophosphamide-treated arms). Notably, the impact on survival time for SMS-SAN xenografts treated either with cyclophosphamide alone or ABT-737 alone were similar, whereas in IMR5 and BE2C the effect of cyclophosphamide was significantly greater than that of ABT-737.

The addition of ABT-737 to cyclophosphamide induced striking tumor responses in the SMS-SAN (Bcl-2 dependent) model, with complete regression obtained in 5 of 9 mice (Fig. 5A). At the time the last cyclophosphamide treated mouse was sacrificed for tumor progression (day 35), all cyclophosphamide-ABT-737–treated mice had tumors below pretreatment volume, and median survival was extended to 126 days. Indeed, 4 of 9 mice showed no tumor regrowth at the time of sacrifice (>220 days) despite receiving only single cycle of therapy (no tumor was identified at necropsy). The addition of ABT-737 to cyclophosphamide treatment did not significantly increase survival in the IMR5 Mcl-1–dependent model (median survival of 35 ± 10.9 versus 25 ± 7.6 days), but did increase survival in the BE2C BH3–resistant model by log-rank analysis, although the magnitude of this response was modest (median survival 21 ± 6.4 versus 18 ± 3.6 days) and all BE2C tumor–bearing mice succumbed to tumor progression by day 50 (Fig. 5B).

We assessed a second Bcl-2–dependent neuroblastoma cell line, NB-1643, and confirmed robust activity. Tumors regressed and survival was extended for both cyclophosphamide (median survival 64 ± 12.5 versus 42.5 ± 14.4 days) or ABT-737 (median survival 59 ± 10.4 versus 42.5 ± 14.4 days; $P < 0.04$ by log rank) treated mice (Fig. 5). Compared with those treated with cyclophosphamide alone, all mice treated with both cyclophosphamide and ABT-737 had significant tumor regression ($P < 0.001$ by log rank) with 8 of 10 having complete regression and 2 mice sacrificed for tumor progression on days 83 and 95. These findings underscore the utility of this classifier to identify tumors with a marked sensitivity to ABT-737.

To elucidate the mechanisms of Bcl-2 antagonist resistance that might arise in response to ABT-737–selective pressure, we exposed the ABT-737–sensitive SMS-SAN neuroblastoma cell line to ABT-737 at 100 nmol/L in vitro and derived a cell line capable of growth under these conditions. SMS-SAN-derived and ABT-737 resistant (SAN-ABTR) cells expressed most Bcl-2 family proteins at levels similar to parent cells, except Mcl-1, which was overexpressed (Fig. 6A). Coinmunoprecipitation revealed that Mcl-1 also became the dominant site for Bim sequestration (Fig. 6B). SAN-ABTR cells were confirmed to have an $IC_{50}$ of approximately 1 log greater than parental SMS-SAN cells based on this switch to Mcl-1 for its survival bias (Fig. 6C). CHLA-15 cells were similarly exposed in vitro to obtain CHLA15-ABTR that switched from Bim:Bcl-2 priming to Bim:Mcl-1 priming, coincident with acquired ABT-737 resistance (Fig. 6B).

Although standard cytotoxics used to treat neuroblastoma did not appear to select for alterations in the dominant anti-apoptotic protein operative (see, Fig. 2A), exposure to a Bcl-2 antagonist in vitro did. We therefore sought to determine whether SMS-SAN xenografts treated with ABT-737 and cyclophosphamide that regrew had acquired a similar resistance mechanism. The first 2 SMS-SAN xenografts to recur and progress were harvested, and both the xenograft and the rederived cell lines were shown to maintain a Bik-dominant BH3 response profile, nearly identical to the parent cells, and with no change in priming dependency or BH3 profile (Fig. 6D and E). We then retreated the next 2 SMS-SAN xenografts that recurred with a second cycle of ABT-737 and cyclophosphamide. Both xenografts again completely regressed, with one mouse sacrificed at more than 200 days tumor free and the other succumbing to tumor at more than 50 days from the second treatment (Fig. 6D). Thus, over short-term exposures to ABT-737 in combination with an alkylating agent there was no selection for ABT-737 resistance despite our in vitro selective pressure findings with single-agent ABT-737.

**Discussion**

Cancers are extremely heterogeneous, even within a single histiotype. This is apparent in the divergent clinical responses among similarly treated patients, and underscored by deep sequencing results showing few highly recurrent oncogenic lesions but scores of low prevalence mutations. Such heterogeneity provides a practical challenge in an era of targeted therapeutics. Though the hope remains that this process will lead to more personalized and effective therapies, providing such agents for large numbers of distinct tumor genotypes remains daunting. An alternative approach to improve cancer outcomes is to target common downstream pathways that mediate therapy response and resistance, agnostic to the genetic heterogeneity that initiated or drives the tumor.

Targeting apoptotic programs to reengage death signaling downstream of endogenous or therapeutic stressors provides one such opportunity. We hypothesized that identifying the apoptotic signaling set point of neuroblastomas, downstream of their diverse oncogenic drivers and selective adaptations, would inform our understanding of their survival dependencies and provide insight into therapeutic approaches. Importantly, understanding the survival biases (and associated heterogeneity) operative in this often lethal solid tumor ought to have increasing therapeutic relevance as newer agents targeting the apoptotic pathway enter the clinic (Fig. 7).

Mitochondrial profiling identified heterogeneous response patterns in neuroblastoma cell lines that are independent of common driver lesions, as neither MYCN status, 11q LOH, nor ALK aberration segregated within single-response profile. Viable neuroblastomas have activated but tonically neutralized Bim, providing a dependency that can be exploited therapeutically. Although Bim activation could arise as a tissue culture artifact, primary tumor coinmunoprecipitations from fresh-frozen tissue show similar Bim priming states as cell lines for the majority, confirming Bim activation and tonic sequestration exist in situ. Bim was confirmed to be a principal mediator
of chemotherapy response, suggesting its tonic neutralization provides a barrier to chemotherapy-induced apoptosis. Such a role is supported by functional genetic screens in which Bim knockdown induces resistance across multiple chemotherapy drug classes (34) and is consistent with findings from the Letai group correlating mitochondrial priming with chemotherapy response in multiple hematopoietic tumors and ovarian cancer (11). Subsets of neuroblastomas have Bim neutralized by either Bcl-2 or Mcl-1, although we found no tumors in which multiple antiapoptotic homologues bound Bim. Bcl-xL was only modestly expressed and not a site of Bim binding, and Bcl-w was not evaluable as pull down of this family member was not reliably achieved. The determinants of Bim binding when multiple antiapoptotic Bcl-2 homologues are present remain to be elucidated.

Bcl-2–dependent neuroblastomas were exquisitely sensitive to ABT-737, a small-molecule antagonist of Bcl-2/Bcl-xL/Bcl-w (22) and a homologue of ABT-263 (Navitoclax; Abbott

Figure 6. Bcl-2–dependent neuroblastoma cell lines adopt an Mcl-1 dependence in association with ABT-737 resistance in vitro but not in vivo. A, protein lysates from SMS-SAN and its ABT-737–resistant derivative, SAN-ABTR, were immunoblotted for expression of Bcl-2 family members. Increased Mcl-1 protein expression is seen in SAN-ABTR. B, communoprecipitation of Bcl-2, Mcl-1, and Bcl-xL from parent neuroblastoma cell lines and their derived ABT-737–resistant lines show a switch from Bim:Bcl-2 binding to Bim:Mcl-1 binding (for both SAN-ABTR and CHLA-15–ABTR, an ABT-737–resistant variant of CHLA-15). C, dose–response curves (using WST-1 assay) confirm increased resistance of SAN-ABTR to ABT-737 by more than 1 log. D, plot of tumor volume over time for SMS-SAN xenografts treated with ABT-737 and cyclophosphamide (same experiment as in Fig. 5). E, the first 2 tumors to regrow (blue) were harvested and subjected to mitochondrial BH3 profiling directly as a xenograft and after reestablishment as a cell line. Both maintained a Bik-dominant (presumed Bcl-2 dependent) profile. Two subsequent tumors that recurred (red) were retreated with ABT-737/cyclophosphamide combination and showed regression, with durable complete regression obtained for 1 of 2 tumors. Panels A to C and E are representative data of replicate experiments; panel D shows 9 mice in single experiment (as in Fig. 5). Data in panel E histogram represent replicates with an SE < 5%; (error bars not shown). WCL, whole-cell lysate; XG, xenograft; CL, cell line.
Laboratories) that is in clinical trials (35, 36). We correlated a Bik-dominant mitochondrial response profile with ABT-737 sensitivity in vitro, confirmed a mechanism through competitive displacement of endogenous Bim, and showed remarkable in vivo activity in a highly lethal xenograft model. Single-agent activity for ABT-737 was recurrently shown for Bcl-2–dependent tumors and combined with noncurative chemotherapy led to durable complete regressions not previously seen in this model. Neuroblastoma mitochondrial responses to BH3 death domains also identified, for the first time, a novel mechanism of acquired therapy resistance in relapsed neuroblastoma isolated to the mitochondria through Bax/Bak repression. S, sensitive; R, resistant.

Figure 7. Functional mitochondrial assays identify mechanisms of therapy response and relapse in neuroblastoma. Neuroblastoma cell lines derived from the highest risk tumors are primed to die with activated Bim sequestered to prosurvival Bcl-2 or Mcl-1, regardless of concomitant genetic aberrations. Primary human tumors express Bcl-2 and/or Mcl-1 that confer poor prognosis, and diagnostic samples tested thus far show similar Bim sequestration, inferring these findings likely translate to primary disease (41). Bcl-2 sequestration of Bim occurred in a large subset of neuroblastoma cell lines (>50%), suggesting Bcl-2 inhibitors may have utility in treating neuroblastoma in the upfront setting. Neuroblastoma mitochondrial responses to BH3 death domains also identified, for the first time, a novel mechanism of acquired therapy resistance in relapsed neuroblastoma isolated to the mitochondria through Bax/Bak repression. S, sensitive; R, resistant.

ABT-263 across a panel of neuroblastoma cell lines and found 1 of 6 to be sensitive (40). This was NB-1643 that we show to be Bcl-2 dependent with Bim:Bcl-2 binding, predicting this response.

Importantly, Bcl-2 family expression and interactions in cell lines are consistent with those we identified in primary tumors (Fig. 1D; ref. 41). More than 80% of high-risk neuroblastomas express immunohistochemistry-detectable Bcl-2 or Mcl-1, and over a third of primary tumors express Bcl-2 alone, supporting that Bcl-2 antagonists may have clinical utility for a large subset of patients (41). Furthermore, communoprecipitations confirm Bim:Bcl-2 and Bim:Mcl-1 priming in distinct primary tumors, suggesting Bcl-2 sequestration by Bcl-2 or Mcl-1 plays a part in the pathogenesis of untreated tumors and infers a predictive response to BH3 mimetics. For neuroblastoma cell lines showing Bim:Mcl-1 priming, ABT-737 had little impact. However, Mcl-1 is unique among Bcl-2 antiapoptotic
homologues as it has a short half-life and is highly regulated, providing opportunities to re sensitize Mcl-1–dependent tumors to Bcl-2 antagonists through targeting regulators of Mcl-1 expression.

BH3 profiles also defined an apoptosis resistant pattern exclusive to chemoresistant, posttherapy relapsed neuroblastoma (12). Using near isogenic pre— and posttherapy cell line pairs, we show that this reflects attenuation of Bak and/or Bax activation rather than a loss of direct activator BH3 priming or gain of function of anti apoptotic Bcl-2 family members. Though the mechanisms of this repression require further investigation, these findings from mitochondrial assays (and confirmed by a complimentary whole-cell assay) isolate the resistance phenotype to single organelle, the mitochondria, and provide a new conceptual target for reverting chemoresistance. Understanding the broader functional attributes of therapy resistant mitochondria may uncover vulnerabilities that are distinct from both normal cells and pretherapy tumor cells. Importantly, tumors with Bim:Bcl-2 priming (predicted to be ABT-263 responders) are likely to have reduced sensitivity to a Bcl-2 antagonist at the time of relapse based on our findings. This has implications for how ABT-263 and similar agents are studied in the clinic. Our data support integrating such an agent into therapy before relapse, although clinical development standards call for initial testing to be done in patients at relapse, a patient population (based on our data) that are likely to have tumors less sensitive to BH3 mimetics as single agents. However, the data we present here, and preclinical studies combining ABT-737 with fenretinide (42), suggest that recurrent neuroblastomas may be responsive to drug combinations that include BH3-mimetic agents.

The functional mitochondria profiling approach used here was initially applied to the characterization of hematologic neoplasms (10, 43–46) and only more recently to select solid tumors that arise in the context of an organ with dependence on heterotypic cell types, contacts, and signaling (11). Our work shows that despite this complexity, mitochondrial responses from isolated neuroblastoma cells recapitulate in vivo responses to similar stressors, and mitochondrial responses to stressors that promote apoptosis are therefore more cell intrinsic than might have been predicted. A more definitive test of this concept will require assessing the profile of more solid tumor samples harvested directly from their autochthonous primary and metastatic sites, and the subsequent demonstration that these profiles indeed predict response to Bcl-2—directed therapeutics in the clinic.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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