RASSF1A and the BH3-only mimetic ABT-737 promote apoptosis in pediatric medulloblastoma cell lines

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The RASSF1A tumor suppressor is potentially the most important candidate gene identified in medulloblastoma to date, being epigenetically silenced in >79% of primary tumors. However, its functional role has not been previously addressed in this tumor type. Here, we demonstrate that expression of RASSF1A promotes the induction of cell death after activation of both the extrinsic and intrinsic apoptotic pathways in medulloblastoma cells. Treatment of UW228-3 cells stably expressing RASSF1A with an anti-CD95 antibody to induce extrinsic apoptosis and etoposide or cisplatin to activate intrinsic apoptosis augmented tumor cell killing in a caspase-dependent manner. This led to increased activation of the pro-apoptotic BCL-2 family member BAX. On the basis of this knowledge, we demonstrate how the loss of RASSF1A function in medulloblastoma cells might be overcome using the novel BH3-only mimetic ABT-737 in combination with chemotherapeutic agents to target the BCL-2 anti-apoptotic members. We show that ABT-737 increased susceptibility to apoptosis induced by DNA damage regardless of RASSF1A expression status through increased activation of BAX. Our findings identify the RASSF1A tumor suppressor as a promoter of apoptotic signaling pathways. Investigation of its mechanism of action has revealed that these pathways can still be promoted in its absence and how these potentially represent novel therapeutic targets for medulloblastoma.

Keywords: ABT-737, apoptosis, medulloblastoma, RASSF1A, therapy.

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Medulloblastoma arises in the cerebellum and is the most frequently occurring malignant brain tumor of childhood. Medulloblastomas represent a heterogeneous group of tumors that are now divided into at least 3 disease subtypes on the basis of their histology. Although recent developments have improved survival rates, approximately one-third of patients remain refractory to therapy and children overall tend to experience long-term neurological deficits as a result of treatment. To improve outcomes for patients, there is a need to develop less toxic and more effective therapies. Such therapies are likely to be directed against defined molecular targets. The tumor suppressor RASSF1A, an isoform of RASSF1 (Ras association domain family 1), currently represents the most frequently implicated candidate in medulloblastoma. It is epigenetically repressed in >79% of primary tumors. Of importance, this event is independent of factors such as histopathological subtype, age, and sex.

RASSF1A has emerged as a key component of a number of apoptotic signaling pathways. Evasion of apoptosis is a necessary requirement for tumorigenesis and is coordinated through 2 major apoptotic signaling pathways. The extrinsic pathway is activated after binding of death ligands of the tumor necrosis factor receptor (TNF) superfamily to their cognate receptors, whereas the intrinsic pathway is stimulated by a variety of cellular stress signals. The BCL-2 family of pro- and anti-apoptotic proteins plays a major role in determining cell outcome after an apoptotic stimulus or insult. Indeed, these proteins are key regulators of cell death in the central nervous system and are crucially important in its development. BAX is a multidomain pro-apoptotic family member that possesses 3 BCL-2 homology domains (BH1-3). During apoptosis, it undergoes a conformational change allowing it to form homo-oligomers and to induce permeabilization of the outer mitochondrial membrane with the subsequent...
release of apoptogenic molecules, which are involved in bringing about cellular destruction.\textsuperscript{18–21} In cerebellar granule neurons, from which some medulloblastoma subtypes are thought to arise, deletion of BAX can confer increased protection against apoptosis.\textsuperscript{23–25} The mechanism of BAX activation is not yet completely understood, but crucially, regulation of its activity involves both the anti-apoptotic multidomain BCL-2 family members (BCL-2, BCL-xl, BCL-w, MCL-1, and A1/BFL-1) and the single-domain, BH3-only pro-apoptotic members (PUMA, NOXA, BAD, BIM, BID, Bik, BMF, and HKR).\textsuperscript{19–21,26} RASSF1A was shown to promote death receptor-mediated apoptosis and BAX activation via mammalian sterile 20-like kinase 2 (MST2) and subsequent transactivation of PUMA by p73-YAP1.\textsuperscript{14} Another BH3-like protein, modulator of apoptosis-1 (MOAP-1), has also been shown to function as a BAX effector.\textsuperscript{27} MOAP-1 is able to interact with RASSF1A and even depends on it for mediating activation of BAX and cell death in specific contexts.\textsuperscript{11–13} Therefore, to date, BAX has emerged as a target of 2 RASSF1A-dependent extrinsic death pathways involving MST2-p73-PUMA and MOAP-1\textsuperscript{11–14} and is possibly implicated in another through cytochrome C release and upstream signaling through MST1-NDR1/2 kinase.\textsuperscript{16} Inactivation of the prosurvival BCL-2 members by BH3-only proteins is required for BAX activation during apoptosis, and when expressed at high levels in tumor cells, the anti-apoptotic proteins may contribute to chemoresistance. However, it is now possible to target this family therapeutically with small molecule inhibitors that mimic the function of the BH3-only proteins, resulting in BAX activation.

In this study, we were interested in determining the effect of re-introduction of RASSF1A in medulloblastoma cell lines and hypothesized that BAX may be a key effector during RASSF1A-mediated apoptosis. We demonstrate that restoration of the RASSF1A expression status in the UW228-3 medulloblastoma cell line sensitizes them to undergo programmed cell death in response to death receptor ligation and DNA damage, which is characterized by BAX activation and caspase dependence. Furthermore, we present data detailing how the apoptotic machinery can be therapeutically targeted at the level of the mitochondria in the absence of RASSF1A through the use of a novel BH3-only mimetic, ABT-737, in combination with a number of clinical agents.

Materials and Methods

Cell Culture and Transfection Procedure

UW228-3 cells were cultured in DMEM (Gibco) supplemented with 10\% (v/v) fetal bovine serum (PAA Labs). RASSF1A was amplified from normal cerebellar cDNA with use of composite primers that contained a region complementary to the gene sequence bordered by a region with a restriction site. The reverse primer was designed to abrogate the stop codon, allowing read through. The forward and reverse primers were as follows:

\begin{align*}
\text{5'} & \text{GGGAGCAAGTCCATCCGGGACATGTCG}\text{G6'} \\
\text{5'} & \text{GTATGGGTGCCGGCGCCAAGGGCGAGCAGC}\text{G6'}
\end{align*}

The product was digested with HindIII and SrfI and ligated into a pCR3.1 vector that had been engineered to contain an HA tag.\textsuperscript{28} Transfection into the UW228-3 cell line was performed using lipofectamine. The control vector was pCR3.1HA with no insert. Stably transfected cells were selected using 1 mg/mL G418 (Sigma), and individual cells were cloned. Transfected cells were initially identified using an anti-HA antibody (rat monoclonal; Roche) by immunoblotting. One vector-control clone and 3 RASSF1A-expressing clones (A252, A244 and A228) were used in functional studies. Integrity of the cloned sequence was confirmed by isolating RNA from the clones, generating cDNA, and sequencing. Sequence analysis confirmed that no mutation had occurred during the transfection process. Analysis of DNA obtained from the clones confirmed that the endogenous RASSF1A remained hypermethylated. The nMED-1 and nMED-2 cell lines were established and characterized at the Children’s Brain Tumour Research Centre, University of Nottingham, United Kingdom.\textsuperscript{29} The nMED-1 and nMED-2 cell lines were routinely cultured in DMEM supplemented with 15\% (v/v) fetal bovine serum.

Apoptosis Induction

Cell death was induced by 50 \mu M etoposide (Sigma) or cisplatin (TEVA), 10 \mu M ABT-737 (Selleckchem), or 50 ng/mL anti-CD95, CH11 (Upstate), and 10 \mu g/mL cycloheximide (Sigma). Cycloheximide was used to facilitate anti-CD95–induced apoptosis in vitro. The pan-caspase inhibitor z-VAD.FMK (Calbiochem) was added to culture media 1 hour before the addition of apoptotic inducers. Apoptosis was assayed by flow cytometric quantification of hypodiploid cells with degraded DNA (SubG0/G1 cells).

Propidium Iodide Staining and DNA Content Analysis

For DNA content analysis, cells were harvested by trypsinization and were fixed with 70\% (v/v) ice-cold ethanol phosphate-buffered saline (PBS). Pellets were washed with cold PBS containing 0.1\% (w/v) BSA (Sigma) and 0.1\% (v/v) Tween-20 (Sigma). Cells were stained with a stock solution containing 20 \mu g/mL propidium iodide and 200 \mu g/mL Ribonuclease A (Sigma) for 20 minutes in the dark and at room temperature. Cell cycle analysis was conducted using a Coulter FC500 flow cytometer and using WinMDI2.8 (freeware designed by Joe Trotter http://facs.scripps.edu/software.html) and Cylchred software (freeware designed by Terry Hoy http://www.facslab.toxikologie.uni-mainz.de/engl.Websites/Downloads-engl.jsp#Protokolle); 20,000 cells were used for the analysis.

Western Blotting

Proteins from total cell lysates were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis gels
and transferred to polyvinylidene fluoride membranes for immunoblotting. TBS-T-milk (10 mM Tris HCl [pH, 8.0], 150 mM sodium chloride, 0.5% [w/v] Tween 20, and 5% [w/v] nonfat dry milk) was used as a blocking reagent for 1 hour at room temperature. Membranes were incubated overnight at 4°C in primary antibody-blocking reagent solution. Anti-caspase-3, poly(ADP-ribose) polymerase (PARP), and β-tubulin were obtained from Cell Signaling, and anti-caspase-8 was a gift from M. MacFarlane (MRC Toxicology Unit, Leicester University, United Kingdom). Anti-p21 was obtained from Upstate Biotechnology, and anti-RASSF1A (3F3) was purchased from Abcam. All primary antibodies were used at a dilution of 1:1000 with the exception of caspase-8, which was used at 1:30 000. Primary antibodies were detected using antimouse/rabbit horseradish peroxidase-conjugated antibodies (1:2000) with use of an enhanced chemiluminescence system (GE-Healthcare).

Detection of BAX Conformational Change
Immunocytochemistry was used to detect BAX conformational change. Cells were cultured on chamber slides (Labtek) and either treated with CH11 and cycloheximide or with etoposide, as described above. After treatment, the media was removed and cells were fixed with 4% (w/v) paraformaldehyde for 20 minutes at room temperature. Immunodetection of oligomerised BAX was achieved using the anti-BAX 6A7 (Trevigen) antibody. Cells were washed 3 times in PBS before blocking with 20% (v/v) normal goat serum in PBS for 1 hour at room temperature. Cells were incubated with anti-BAX 6A7 (1:200) in 2% (v/v) normal goat serum and 0.1% (v/v) Triton-X100 (Sigma) overnight at 4°C. Anti-BAX was detected using an AlexaFluor 555 anti-mouse antibody (Invitrogen) at a dilution of 1:300 in 5% (v/v) normal goat serum in PBS for 1 hour at room temperature. Cells were washed 3 times before mounting in Vectashield containing DAPI (Vector). Images were acquired using a Leica DMRB fluorescent microscope.

Statistical Analysis
The statistical software SPSS, version 16 (SPSS) was used for analysis of quantitative data. Independent-sample t tests with 95% confidence intervals were used to compare means from 2 groups. Several means were assessed for significance by analysis of variance. P values <.05 are reported as being statistically significant.

Results

RASSF1A Functions to Potentiate Apoptosis Induced by Death Receptor and by DNA Damage Agents
RASSF1A has previously been shown to be fully or partially methylated in medulloblastoma.6–8 Immunoblot analysis of lysates from the medulloblastoma cell lines DAOY, D283, UW228-3, and D458 confirmed that RASSF1A was not expressed at the protein level in comparison with the HeLa cell line, which retains expression of RASSF1A (Fig. 1A). In addition, we did not detect the protein in lysates from the newly derived pediatric medulloblastoma cell lines nMED-1 and nMED-2. To investigate the functional consequences of introduction of RASSF1A in medulloblastoma cells, we created several UW228-3 clones stably expressing the gene (Fig. 1B and C). The potential effects of expression of this tumor-suppressor gene on cell-cycle progression and programmed cell death in UW228-3 cells were determined by measuring DNA content after propidium iodide (PI) uptake. However, we did not observe any notable effects on either of these processes in any of the RASSF1A stably expressing clones (Fig. 1C). There was no significant accumulation of UW228-3 cells expressing RASSF1A in any particular phase of the cell cycle compared with nonexpressing cells (Fig. 1D). Furthermore, the proportion of PI positive cells in the SubG0/G1 phase, representing an apoptotic fraction, remained at an extremely low basal level, within the range of 1.2 + 0.3%–2.6 + 1.6% for RASSF1A-positive cells and 1.4 + 0.16% for nonexpressing cells (Fig. 1D).

To investigate the possibility of a role for RASSF1A in the regulation of the extrinsic apoptotic program in medulloblastoma, the level of apoptosis after activation of this pathway with use of an anti-CD95 monoclonal antibody (CH11) to activate the CD95 receptor was determined in UW228-3 cells stably expressing RASSF1A. Morphological features of apoptosis, such as loss of adherence, cell shrinkage, and rounding, were evident 8 hours after addition of CH11 in cultures stably expressing RASSF1A (data not shown). Apoptosis was measured by assaying DNA fragmentation, leading to the formation of a SubG0/G1 peak 8 hours after addition of CH11 (Fig. 2Ai). This effect was significantly larger in RASSF1A-expressing cells, compared with nonexpressing cells. Relative to vehicle controls, apoptosis in the RASSF1A-expressing subclones increased 14- to 16-fold, compared with only 5-fold in nonexpressing cells in response to activation of the CD95 receptor (Fig. 2Bi). No significant differences were observed between the RASSF1A-expressing subclones.

Next, we sought to determine whether exogenous RASSF1A sensitized UW228-3 to chemically mediated apoptosis with use of DNA damage agents whose killing action is dependent on mitochondrial outer membrane permeabilization and apoptosome formation. RASSF1A expressing UW228-3 cells displayed significantly increased sensitivity to the topoisomerase II poison etoposide (VP-16), evoking a 9- to 11-fold increase in DNA fragmentation compared with only a 2-fold increase in nonexpressing vector controls (Fig. 2Aii and Bii). Similarly, treatment with the DNA cross-linking agent cisplatin also resulted in a significantly greater degree of cell death in the presence of RASSF1A compared with nonexpressing cells (Fig. 2Aii), leading to a 9- to 11-fold increase in cell death, compared with an only 4-fold increase in nonexpressing cells (Fig. 2Bii).
The presence of RASSF1A therefore increased the apoptotic response of UW228-3 cells to CH11 and cisplatin by 2-fold and to etoposide by 3-fold. Therefore, our data clearly demonstrate a role for RASSF1A, both in the regulation of death receptor-induced apoptosis and in the response to DNA damage in UW228-3 medulloblastoma cells.

In summary, restoration of RASSF1A expression in UW228-3 cells resulted in at least a doubling of the apoptotic response to death receptor activation by CH11 and cisplatin by >2-fold and to etoposide by >3-fold. Therefore, our data clearly demonstrate a role for RASSF1A, both in the regulation of death receptor-induced apoptosis and in the response to DNA damage in UW228-3 medulloblastoma cells.

RASSF1A Functions to Promote Caspase-dependent Cell Death

To begin to define how cell death is promoted by RASSF1A in our UW228-3 clones, we first tested whether it was caspase dependent. The A244 clone was selected for downstream analysis because all 3 RASSF1A clones displayed a similar response to each treatment. A244 cells were pretreated with the broad-spectrum caspase inhibitor z-VAD.FMK before the application of an apoptotic stimulus; 10 μM z-VAD.FMK significantly inhibited DNA degradation in response to CH11 treatment, whereas 25 μM z-VAD.FMK effectively blocked apoptosis after exposure to etoposide (Fig. 3A).

To elucidate the mechanism of RASSF1A-mediated apoptosis, western blot analysis was performed on A244 cells after activation of the extrinsic and mitochondrial pathways by CH11 and etoposide, respectively (Fig. 3B and C). The extent of processing and, thus, activation of core components of the apoptotic machinery, namely caspase-8, -3, and the DNA repair protein PARP, was assessed over an 8-hour and 24-hour period.

Stimulation of the CD95 pathway in UW228-3 cells stably expressing RASSF1A resulted in an increase in processed caspase-8, the extrinsic pathway initiator caspase, after 8 hours (41- and 43-KDa fragments) and, possibly, 4 hours (18 KDa) of incubation with the CH11 antibody (Fig. 3B). The addition of a BH3-interacting domain death agonist (tBID) inhibitor,
BI-6C9, did not inhibit CD95-induced apoptosis in RASSF1A-expressing cells (Supplementary material Fig. S1), suggesting that tBID cleavage was not necessary and other key proteins are involved. Caspase-3 was processed mainly to its p19 form in UW228-3 cells lacking RASSF1A after 8 hours compared with expressing cells in which a larger amount of the fully processed p17 fragment of this effector caspase was observed. This caspase-3 activation correlated with the cleavage of its substrate PARP, which was more highly processed in RASSF1A-expressing cells compared with vector control cells (Fig. 3B).

A similar time-dependent processing of caspase-3 and PARP was observed in RASSF1A-expressing UW228-3 cells (visible at 8 hours) after DNA damage by etoposide, whereas in nonexpressing cells, we did not detect...
significant proteolysis of either of these proteins even at 24 hours (Fig. 3C). Upregulation of the cyclin-dependent kinase inhibitor p21 in response to DNA damage was detected in both cell types at 8 hours after challenge with etoposide but was several folds higher at all times in treated RASSF1A-expressing UW228-3 cells (Fig. 3C).

Thus, we see that, for both treatments, RASSF1A results in earlier and more pronounced activation of key apoptotic factors, indicating that RASSF1A is promoting the apoptotic response to these agents. In parallel to RASSF1A promoting a greater increase in response to etoposide (3.5-fold) than CH11 (2.8-fold), the observed effect was greatest in response to etoposide (Fig. 2B).

**RASSF1A Increases Activation of BAX at the Level of the Mitochondria During Apoptosis**

After execution of apoptotic signaling, BAX undergoes a conformational change, exposing its N-terminus before mitochondrial permeabilization. To investigate whether RASSF1A-mediated cell death resulted in activation of BAX, immunocytochemistry was performed on UW228-3 RASSF1A stably expressing cells with use of an antibody specific to conformationally altered BAX after treatment with CH11 and etoposide (Fig. 4A). We saw elevated levels of BAX conformational change after both ligation of the CD95 receptor and DNA damage induced by etoposide in RASSF1A-expressing UW228-3 cells compared with cells stably transfected with the empty vector. Mock treatment of cells with CHX or DMSO did not result in activation of BAX (Supplementary material Fig. S2).

Because the presence of RASSF1A augmented the activation of BAX, we speculated that some of its functions in apoptosis must be partially attributable to an ability to regulate signaling pathways that converge on the BCL-2 family of pro- and anti-apoptotic proteins. Therefore, strategies aimed at targeting the apoptotic machinery and, specifically, BAX through the inhibition of anti-apoptotic BCL-2 members may
be of clinical benefit to patients with medulloblastoma, who lack expression of RASSF1A. This rationale led us to investigate the therapeutic potential of targeting the Bcl-2 family in medulloblastoma cell lines using ABT-737 (Fig. 4B).

**The BAD Mimetic ABT-737 Acts in Synergy with DNA Damage Agents to Induce Apoptosis in Medulloblastoma Cell Lines Regardless of RASSF1A**

ABT-737 acts like a BAD mimic, binding to BCL-2, BCL-xL, and BCL-w with high affinity but not to MCL-1 or A1. As a result, it displays limited single agent efficacy. However, the combination of ABT-737 with chemotherapeutic agents, including etoposide and cisplatin, has been shown to result in MCL-1 inactivation and apoptosis in several tumor types (Fig. 4B). Because exogenous RASSF1A significantly increased the apoptotic response to DNA damage agents, leading to activation of BAX at the mitochondria, we sought to address whether the apoptotic response of medulloblastoma cells (which lack endogenous RASSF1A) could be increased using ABT-737 before treatment with DNA damage agents.

We first examined the expression of MCL-1 and BCL-2 in the UW228-3 clones and in our primary cell lines n-MED1 and n-MED2 (Fig. 5A). High levels of BCL-2 were detected in both newly derived cell lines but not in MCL-1. In contrast, in the UW228-3 clones, we did not detect significant expression of BCL-2, but
saw strong expression of MCL-1. BCL-2 expression was also observed in DAOY, D283, and D458 cells, and preliminary data suggested that MCL-1 was also expressed in the DAOY cell line (data not shown), indicating that members of the BCL-2 family are expressed in medulloblastoma cell lines generally.

Next, we tested whether ABT-737 induced apoptosis in medulloblastoma cell lines when used singly (Fig. 5B). Overall, low levels of apoptosis were observed after 24 hours of treatment with ABT-737 across a range of medulloblastoma cell lines, remaining at <6% even when RASSF1A was present. Furthermore, the nMED-1 and nMED-2 cell lines did not readily undergo apoptosis in response to ABT-737 despite displaying high levels of BCL-2 expression and low MCL-1 levels. Therefore, ABT-263 is unlikely to be useful clinically as a single agent in medulloblastoma.

MCL-1 is a short-lived protein, and its synthesis can be critically reduced by using inhibitors of protein synthesis, such as cycloheximide (CHX), promoting apoptosis in response to ABT-737. In MCL-1-expressing UW228-3 cells, addition of CHX restored sensitivity to ABT-737 as a single agent, regardless of CD95 activation by CH11, suggesting that inhibition of a short-lived anti-apoptotic protein, presumably MCL-1, was occurring, leading to rapid apoptosis (Fig. 5C).

Next, we investigated whether ABT-737 sensitized medulloblastoma cells to apoptosis triggered by a variety of stimuli in the presence or absence of RASSF1A. Stably transfected UW228-3 cells, n-MED1, and n-MED2 cells were incubated either singly with ABT-737, etoposide, or cisplatin or combinations of these. Although exogenous RASSF1A potentiated cell death in response to DNA damage agents in the UW228-3 cell line (Fig. 5D and ii), the presence of ABT-737 significantly increased the cytotoxicity of these agents further in RASSF1A-positive cells. Similarly, we found that apoptosis induced by etoposide and cisplatin was significantly increased by the presence of ABT-737 in RASSF1A-negative medulloblastoma cells (Fig. 5E and F). Thus, ABT-737 increased susceptibility to chemotherapy regardless of RASSF1A expression status. Although the nMED-1 and nMED-2 cells lines appeared to be much more resistant to apoptotic stimuli, significant increases in cell death were observed when ABT-737 was used in combination with cisplatin or etoposide (Fig. 5Fi and ii). Our data suggest that, although RASSF1A can potentiate cell killing in response to DNA damage agents in medulloblastoma cells, a more effective therapeutic strategy may be to target the pathway downstream through inhibition of anti-apoptotic BCL-2 proteins rather than one based on restoring RASSF1A function.

Because we had observed that ABT-737 failed to induce apoptosis as a single agent, even in our newly established cell lines that expressed high levels of BCL-2 but lacked MCL-1, we examined the effect of ABT-737 treatment on BAX conformational change by immunocytochemistry alone and in combination with etoposide in nMED-2 cells (Fig. 6). Addition of ABT-737 was not sufficient to trigger activation of BAX, which is consistent with our earlier observation that cell death was not induced by ABT-737 when used as a single agent (Fig. 6ii). Similarly, low levels of BAX activation were seen 24 hours after addition of etoposide (Fig. 6iii). However, we observed a marked increase in the proportion of cells displaying conformationally altered BAX when ABT-737 was used in combination with etoposide, confirming that ABT-737 acted in synergy with etoposide to promote BAX activation and cell death (Fig. 6iv).

Discussion

This study demonstrated that RASSF1A is not expressed at the protein level in any of medulloblastoma cell lines examined, including 2 newly derived medulloblastoma cell lines. In vitro analyses of function were performed using UW228-3 subclones stably expressing the gene. Therefore, we were able to assign a role to RASSF1A in the regulation of apoptotic signaling in this medulloblastoma cell line. The role of RASSF1A in CD95 apoptosis has been extensively studied in other tumor types, but this is the first demonstration that this tumor suppressor also regulates this pathway in medulloblastoma. We found that RASSF1A-activated apoptosis induced by CD95 was not prevented by the addition of BI-6C9, a tBid inhibitor, suggesting that other key proteins are involved. Similar findings were also reported by Shairaz et al, who found that RASSF1A-dependent death receptor-mediated apoptosis was comparable in wild-type and BID-deficient mouse embryonic fibroblasts. Further studies have revealed that RASSF1A is recruited to death receptors after the time that death-inducing signaling complex components are assembled and, possibly, only after death receptors have been internalized. The pro-apoptotic kinases MST1/2 have been shown to be key effectors of RASSF1A-activated apoptosis after activation of the CD95 receptor. Further studies will be required to determine the involvement of these proteins in this pathway in medulloblastoma cells. During the preparation of this article, Hamilton et al reported that RASSF1A promoted apoptotic signaling in response to cisplatin and etoposide in HeLa, MCF7, and USO2 cells. Here, we have independently confirmed these findings and present a novel role for RASSF1A in the modulation of the response to DNA damage in medulloblastoma cells. Investigations into the mechanism of cell death in both cases identified the pro-apoptotic BCL-2 family member BAX as a protein for which activation is enhanced by the presence of RASSF1A during apoptosis.

Evading apoptosis is a necessary prerequisite for malignant transformation, and restoration of apoptotic signaling could prove to be a major therapeutic strategy to reduce toxicity for patients and improve outcome. Activation of BAX is inhibited by the anti-apoptotic BCL-2 family members. In addition, Sonic Hedgehog, a major developmental signaling pathway in the cerebellum and medulloblastoma, has been shown to promote
Fig. 5. ABT-737 promotes apoptotic signalling in medulloblastoma cell lines. (A) Immunoblot analysis of medulloblastoma cell lines revealed endogenous expression of the anti-apoptotic BCL-2 family members, BCL-2 and MCL-1. Stably transfected UW228-3 clones, expressing RASSF1A (A244) or empty vector, displayed strong expression of anti-apoptotic MCL-1L. The nMED-1 and nMED-2 cell lines lacked endogenous RASSF1A and expressed high levels of BCL-2 but not MCL-1. 15 μg of protein lysates were loaded in each lane and β-tubulin served as a loading control. (B) Medulloblastoma cell lines were incubated with 10 μM ABT-737 for 24 h. ABT-737 induced low levels of apoptosis in medulloblastoma cells, even in the presence of pro-apoptotic RASSF1A. (C) UW228-3 vector control cells or A244 cells stably expressing RASSF1A were incubated with the protein translation inhibitor, CHX (10 μg/mL) in combination with either Anti-CD95, CH11 (50 ng/mL) or ABT-737 (10 μM) for 8 h. RASSF1A expression potentiated killing following CD95 pathway activation by CH11. However, CHX restored sensitivity to ABT-737 (10 μM) as a single agent in stably transfected UW228-3 clones irrespective of CD95 activation (results are reported as the mean ± S.E., t-test, ABT-737 vs CHX and ABT-737 combination: **P < .01). Medulloblastoma cells were cultured in the presence of ABT-737 (10 μM), etoposide or cisplatin (50 μM) or in combinations thereof for 24 h. (D) ABT-737 augmented DNA fragmentation in response to DNA damage induced by etoposide (i) or cisplatin (ii) in UW228-3 vector control cells and in RASSF1A expressing (Clone A244) cells (results are reported as the mean ± S.E., ANOVA, DNA damage agent vs ABT-737 and DNA damage agent combination: *P < .05, **P < .01). (E) ABT-737 acted in synergy with DNA damage agents to promote cell killing in untransfected UW228-3 cells (RASSF1A negative) but did not induce effective cell killing when used as a single agent. (F) ABT-737 increased susceptibility to etoposide (i) and cisplatin (ii) in the newly derived medulloblastoma cell lines nMED-1 and nMED-2. (Error bars represent the standard error of the mean from at least three independent experiments (± S.E., t-test, DNA damage agents vs ABT-737 and DNA damage agent combination: *P < .05, **P < .01)).
medulloblastoma survival via GLI1-mediated transcription of BCL-2. The pharmacological inhibition of this pathway by cyclopamine has been shown to induce cell death, further supporting the hypothesis that the BCL-2 family is a relevant target for therapeutic intervention in medulloblastoma. We demonstrated that the UW228-3 and 2 newly derived medulloblastoma cell lines differentially expressed high levels of BCL-2 and MCL-1. UW228-3 cells are known to express signal transducer and activator of transcription 3 (STAT3). To our knowledge, this is the first study demonstrating that these cells also feature expression of MCL-1, which is one of its known downstream targets.

ABT-737 and the clinical candidate ABT-263 function as BAD mimetics and are able to bind BCL-2, BCL-xL, and BCL-w with high affinity, inhibiting their activity. In addition, they have been shown to potenti-ate the cytotoxicity of a number of chemotherapeutic agents, even when MCL-1 is present, because of upregulation of NOXA, leading to BAX activation and apoptosis. We therefore sought to examine whether apoptosis and BAX activation could be promoted in the absence of RASSF1A through the combination of ABT-737 with chemotherapeutic agents. In a recent study of ABT-737 in high-grade glioma, sensitivity to ABT-737 was shown to depend on the ratio of BCL-2 to MCL-1 expression. We found that sensitivity to ABT-737 as a single agent could not be readily explained by the BCL-2 and MCL-1 ratio and that ABT-737 alone was not sufficient to activate BAX, even in the absence of MCL-1. This finding suggests the involvement of other BCL-2 proteins, such as BCL-xL and/or BCL-w (in UW228-3 cells) or A1 (in n-MED1 and n-MED2 cells). Overall, we conclude that ABT-263 is only likely to be effective in combination with other chemotherapeutic agents in medulloblastoma. This is in accordance with a preclinical study of ABT-737 as a single agent in mice bearing medulloblastoma xenografts. Our results show that, although RASSF1A potentiated apoptotic signaling in
UW228-3 cells in response to etoposide and cisplatin, leading to activation of BAX, the toxicity of these agents was significantly increased by the addition of ABT-737, regardless of RASSF1A expression status. Furthermore, we demonstrated that the combination of ABT-737 and etoposide promoted a striking increase in activation of BAX compared with monotherapy. Therefore, these data suggest that targeting the BCL-2 family of anti-apoptotic proteins in patients with medulloblastoma may be a more effective therapeutic strategy than one based on restoring RASSF1A function. Further studies are, however, required to understand how RASSF1A and ABT-737 regulate the BCL-2 family and promote apoptosis in response to particular chemotherapeutic agents.

To our knowledge, this is the first study of RASSF1A function in medulloblastoma, and these investigations with ABT-737 are the first to experimentally address, in any tumor type, how loss of this important tumor suppressor’s functions might be overcome therapeutically. ABT-737 is reported to be too large to cross the blood-brain barrier, which may limit the clinical applicability of ABT-263 (navitoclax). However, intracranial delivery of these agents might be possible in the future through nanoparticle-mediated delivery, through intra-ventricular administration, or by using implantable wafers. It may also be possible to use other BCL-2 inhibitors, such as obatoclax, which inhibits all BCL-2 family members, including MCL-1, and is presumed to be capable of crossing the blood-brain barrier; however, to date, this has not been tested in vitro in medulloblastoma. Alternatively, it might be possible to target the BCL-2 anti-apoptotic proteins using specific microRNAs. However, understanding precisely how RASSF1A signaling affects the BCL-2 family may ultimately reveal new ways to specifically target apoptotic signaling pathways impaired by its methylation in medulloblastoma, using small molecules mimicking the BH3-only domain, which could be combined with a variety of existing clinical agents as novel therapies. Therefore, more work is needed to define these apoptotic signaling pathways in vitro and, thereafter, in vivo in mouse models of the disease. Overall, the data from this study provide a rationale for further investigation into novel apoptosis-based therapies for the treatment of medulloblastoma that could potentially benefit all patients with medulloblastoma, regardless of RASSF1A expression status.

**Supplementary Material**

Supplementary material is available online at Neuro-Oncology (http://neuro-oncology.oxfordjournals.org/)

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