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Merkel Cell Carcinoma Dependence on Bcl-2 Family Members for Survival

Monique E. Verhaegen[1,*], **Doris Mangelberger**[1], **Jack W. Weick**[1], **Tracy D. Vozheiko**[1], **Paul W. Harms**[1,2,6], **Kevin T. Nash**[1], **Elsa Quintana**[7,†], **Paul Baciu**[1], **Timothy M. Johnson**[1,4,5], **Christopher K. Bichakjian**[1], and **Andrzej A. Dlugosz**[1,3,*]

[1]Department of Dermatology, Comprehensive Cancer Center, University of Michigan School of Medicine, Ann Arbor, MI, 48109, USA.

[2]Department of Pathology, Comprehensive Cancer Center, University of Michigan School of Medicine, Ann Arbor, MI, 48109, USA.

[3]Department of Cell and Developmental Biology, Comprehensive Cancer Center, University of Michigan School of Medicine, Ann Arbor, MI, 48109, USA.

[4]Department of Otolaryngology, Comprehensive Cancer Center, University of Michigan School of Medicine, Ann Arbor, MI, 48109, USA.

[5]Department of Surgery, Comprehensive Cancer Center, University of Michigan School of Medicine, Ann Arbor, MI, 48109, USA.

[6]Michigan Center for Translational Pathology, University of Michigan School of Medicine, Ann Arbor, MI, 48109, USA.

[7]Howard Hughes Medical Institute, Life Sciences Institute, Department of Internal Medicine and Center for Stem Cell Biology, University of Michigan School of Medicine, Ann Arbor, MI, 48109, USA.

Abstract

Merkel cell carcinoma (MCC), a rare but aggressive cutaneous neoplasm with high metastatic potential, has a poor prognosis at late stages of disease with no proven chemotherapeutic regimens. Using an enriched culture medium, we established and characterized 11 MCC cell lines for Bcl-2 family profiling and functional studies. Immunoblot analysis revealed collectively high protein levels of pro-survival Bcl-2 members in cell lines and a panel of MCC tumors. Down-regulation of individual Bcl-2 proteins by RNAi promoted death in a subset of MCC cell lines, whereas simultaneous inhibition of multiple family members using the small molecule antagonist ABT-263 led to dramatic induction of cell death in 10 of 11 lines. ABT-263 induced Bax-dependent apoptosis with rapid cleavage of caspase-3 and PARP, regardless of Bcl-2 family

[*]**Co-corresponding authors:** Andrzej A. Dlugosz 3316 Cancer Center University of Michigan 1500 E. Medical Center Dr. Ann Arbor, MI, 58109-5932 (734) 647-9482 dlugosza@umich.edu *Monique E. Verhaegen 3431 Cancer Center University of Michigan 1500 E. Medical Center Dr. Ann Arbor, MI, 58109-5932 (734) 615-5209 moniquev@umich.edu.

[†]Present address: OncoMed Pharmaceuticals, 800 Chesapeake Drive, Redwood City, CA, 94063 USA

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profile or presence of Merkel cell polyomavirus. Furthermore, ABT-263 treatment led to rapid and sustained growth suppression of MCC xenografts from a representative cell line, accompanied by a striking increase in apoptosis. Our results establish that concurrent inhibition of multiple pro-survival Bcl-2 proteins leads to effective induction of apoptosis, and strongly support the concept that targeting MCC addiction to these molecules may be useful therapeutically by reversing an intrinsic resistance to cell death.

INTRODUCTION

Merkel cell carcinoma (MCC) is a rare but highly aggressive neuroendocrine tumor of the skin, with a propensity for local, regional, and distant metastasis. The primary lesion typically presents in an elderly often immunosuppressed population, on UV-exposed skin as an asymptomatic though rapidly expanding tumor (Bichakjian *et al.*, 2007; Schwartz *et al.*, 2013). Surgical excision is currently the mainstay of therapy for primary lesions, plus lymph node dissection as needed, and often in combination with radiation therapy (reviewed in (Schrama and Becker, 2011)). Although MCC exhibits some sensitivity to chemotherapeutic agents, clinical responses are consistently short-lasting and recurrences are frequent. To date, there is no widely accepted treatment strategy for advanced disease (reviewed in (Desch and Kunstfeld, 2013; Miller *et al.*, 2013)) which invariably carries a poor prognosis. The 5 year survival rate of 0-18% in patients presenting with distant metastases highlights the urgent need for improved therapeutic strategies (Allen *et al.*, 2005).

Mounting evidence suggests that Merkel cell polyomavirus (MCPyV) initially detected in 80% of MCC tumors is a key player in MCC pathogenesis (reviewed in (Arora *et al.*, 2012a). Like other polyomaviruses, MCPyV harbors a T antigen (TAg) oncoprotein locus encoding both large (L) and small (s) TAgs which are predicted to modulate both tumor suppressor and cell cycle regulatory proteins. While the requirement of viral TAgs in MCPyV-positive MCC cells has been demonstrated (Houben *et al.*, 2010), their exact roles in cell survival are not yet clearly defined. However, key mediators of the intrinsic apoptotic pathway, pro-survival Bcl-2 and Mcl-1 have been shown to be highly expressed in up to 85% (Feinmesser *et al.*, 1999; Kennedy *et al.*, 1996; Sahi *et al.*, 2012), and 88% (Brunner *et al.*, 2008) of MCCs, respectively.

Elevation of the pro-survival Bcl-2 family members (Bcl-2, Bcl-x_L, Mcl-1, Bcl-w, and A1) imparts survival advantages to tumor cells by providing an increased apoptotic threshold (reviewed in (Juin *et al.*, 2013)). This offers protection from intrinsic pro-apoptotic tumor-suppressing pathways and oncogenic signaling that would otherwise promote cell death, suggesting tumor cells exist in a “primed for death” state (Certo *et al.*, 2006) and removal of apoptotic blockades may be sufficient to induce tumor cell death. Hence, Bcl-2 was considered an attractive therapeutic target in MCC (Schlagbauer-Wadl *et al.*, 2000) but genetic targeting proved to be ineffective in a phase II trial in patients with advanced MCC (Shah *et al.*, 2009). This may not be surprising since evidence suggests efficient apoptosis of cancer cells may require simultaneous neutralization of multiple Bcl-2 proteins (Chen *et al.*, 2005), driving the development of targeted Bcl-2 family antagonists such as the orally bioavailable ABT-263 (navitoclax) (Park *et al.*, 2008; Tse *et al.*, 2008). This agent binds and

inhibits Bcl-2, Bcl-x_L, and Bcl-w and has provided partial responses in Phase I trials in patients with lymphoid malignancies (Roberts *et al.*, 2012).

Here we demonstrate high expression levels of multiple anti-apoptotic Bcl-2 family members in human MCC tumors. Further analysis of a panel of MCC cell lines that we generated indicate high levels of both anti- as well as pro-apoptotic family members, suggesting that blockade of apoptosis through the collective activity of the anti-apoptotic members contributes to MCC tumor cell survival. In support of this concept, we severely compromise MCC cell viability both *in vitro* and *in vivo* by selectively targeting pro-survival Bcl-2 proteins. These preclinical data uncover the dependence of the majority of human MCC cells on multiple anti-apoptotic Bcl-2 proteins for survival, and provide a strong rationale for evaluating Bcl-2 family antagonists in the treatment of MCC.

RESULTS

Prevalence of Bcl-2 family members in MCC cells

The anti-apoptotic Bcl-2 family members, often elevated in cancer, regulate mitochondrial apoptosis via binding pro-apoptotic proteins (reviewed (Bender and Martinou, 2013)). To gain insight into the role of these proteins in MCC, we focused our analyses on human MCCs as well as 11 UM-MCC cell lines that we have established (Materials and Methods, Table 1, Suppl. Fig. S1-2, Suppl. Table S1). Immunoblotting using 16 human MCC lysates (Fig. 1a) indicates variable but high levels of Bcl-2 protein in 94% of tumors, with Bcl-x_L and Mcl-1 expressed to some degree in all tumors, demonstrating the concurrent expression of multiple pro-survival proteins in MCCs. The UM-MCC cell line panel that we generated reveals expression of Bcl-2, Bcl-x_L, Mcl-1 and Bcl-w to some extent in all lines (Fig. 1b), with some variability and some expressing markedly higher protein. Since cell fate is ultimately regulated by a balance of protein-protein interactions between pro- and anti-apoptotic Bcl-2 members, we also assessed levels of the multi-domain (Bax, Bak) and multiple BH3-only (Bim, Puma, Bad, Noxa, Bmf, and Bik) pro-apoptotic family members (Fig. 1b), and found that several of these proteins are highly expressed in MCC cell lines. These data reveal that in addition to anti- apoptotic proteins, MCC cells also express multiple BH3-only pro-death proteins that may require suppression to maintain cell survival.

Role of anti-apoptotic Bcl-2 family members in MCC cell survival

To assess their roles in MCC cell survival, Bcl-2, Bcl-x_L, and Mcl-1 were down-regulated using lentiviral-driven shRNA constructs in several MCC cell lines. Individual protein knockdown in UM- MCC565 led to dramatic decreases in cell number, with 7%, 47%, and 32% of cells remaining following downregulation of Bcl-2, Bcl-x_L and Mcl-1, respectively. Microphotographs and corresponding immunoblots indicate almost complete downregulation of Bcl-2 and Mcl-1, and to a lesser extent Bcl-x_L (Fig. 2a-c). Interestingly, the effect of individual Bcl-2 protein knockdown was not consistent and downregulation in four additional lines, UM-MCC29 (Suppl. Fig. S4), -MCC40, - MCC32, and -MCC35 (data not shown) had little effect. Exceptionally high levels of multiple BH3- only pro-death proteins in UM-MCC565 (Fig. 1b) may provide one explanation for the sensitivity of these cells to genetic targeting of single Bcl-2 anti-apoptotic family members.

Targeting of multiple Bcl-2 family members induces MCC cell death

To test the concept that Bcl-2 family members collectively contribute to MCC cell survival we utilized ABT-263, a small molecule Bcl-2 family inhibitor that simultaneously binds Bcl-2, Bcl-x_L and Bcl-w, disrupting interactions with pro-death proteins (Tse *et al.*, 2008). Effects of ABT-263 treatment on cell proliferation and viability of MCC cell lines is analyzed in Figure 2d-e. With the exception of UM-MCC31, all lines show decreases in cell number at concentrations as low as 0.5μM, in contrast to control skin cells which were minimally impacted. Furthermore, these lines demonstrate substantial cell death comparable to, or higher than, the sensitive promyelocytic leukemia cell line HL60, with 10 of 11 lines displaying from 46-98% cell death at 24h (Fig. 2e). Note that UM-MCC31 is poorly responsive to ABT-263, more closely resembling the SK-147 melanoma cell line that requires Mcl-1, which is not targeted by ABT-263, for survival (Wolter *et al.*, 2007). Interestingly, the uniquely insensitive UM-MCC31 line displays minimal BH3-only pro-death proteins (Fig. 1b) and remains insensitive even at late time points (not shown).

Apoptotic response of MCC cells to the Bcl-2 family antagonist ABT-263

To begin unraveling the cytotoxic response of ABT-263, a more detailed analysis was performed in 4 select lines (UM-MCC29; -40; -565; -624). Typical MCC aggregates are replaced by what appear to be single cells and debris following ABT-263 treatment (Fig. 3a). Time course studies indicate greater than 85% induction of cell death in all 4 lines (Fig. 3b). Further analysis of downstream signaling reveals cleavage of apoptotic effectors caspase-3 and PARP as early as 12 hours post treatment (Fig. 3c).

To further evaluate MCC cell responsiveness to cytotoxic agents, we compared the effects of ABT-263 and the DNA damaging agent carboplatin (Hospira Inc, IL). We detected ≤ 20% cell death in 3 of 5 MCC lines following carboplatin treatment (100μM), suggesting some level of apoptotic blockade. In contrast, with the exception of UM-MCC31, cells largely unresponsive to carboplatin were highly sensitive to ABT-263 with ≥ 81% cell death (Fig. 4a). Responsive HL60 cells are shown as a positive control for both cytotoxic agents. Immunoblot analysis reveals 5 of 11 cell lines have detectable (stabilized) p53 (Fig. 4b), which is likely mutant or not fully functional, and as expected, these lines are more resistant to carboplatin (Fig. 4a-b), but not ABT-263.

We additionally screened the cell lines for expression levels of apoptotic modulators downstream of the mitochondria, including the inhibitor of apoptosis protein (IAP) family members survivin and XIAP (Fig. 4b), known to block apoptosis at the level of caspase activation. We found high levels of XIAP and variable levels of survivin in all cell lines. Survivin has recently been shown to be deregulated in most MCCs, although regulation differs based on MCPyV status (Xie *et al.*, 2014). While UM-MCC cells with lower levels of survivin (MCC13; MCC565) were more sensitive to ABT- 263 (Fig. 2e), all lines underwent efficient ABT-263 induced death regardless of IAP protein levels.

ABT-263 induces cell death in MCC cells regardless of MCPyV status

Since some MCCs do not express MCPyV, we examined whether MCPyV status altered responsiveness to ABT-triggered cell death. We initially established 5 MCPyV-positive cell

lines from 11 patients (45%), however 2 lines were lost due to contamination and hence our current panel includes only 3 MCPyV-positive lines (Table 1, Suppl. Table S1, Suppl. Figs. S1-2, Fig. 4b). Our initial prevalence rate, while substantially lower than the worldwide average of 74% (reviewed in (Arora *et al.*, 2012a), is in keeping with the 46% prevalence of MCPyV we previously reported in the University of Michigan MCC cohort (Harms *et al.*, 2013). As previously described (Fischer *et al.*, 2010), we also found no correlation between phenotypic characteristics of our MCC cell lines and MCPyV status (Suppl. Fig. S1-S2; Table S1), and MCPyV-positive UM-MCC lines expressed TAg transcripts at levels similar to those in MKL-1 (Rosen *et al.*, 1987) and MKL-2 (Van Gele *et al.*, 2002) confirmed MCPyV-positive MCC cell lines (Houben *et al.*, 2010; Shuda *et al.*, 2008) (Suppl. Fig. S2d). Loss of cell viability in nearly all UM-MCC cell lines examined (Fig. 2e), and a more detailed comparison of two positive and two negative cell lines following ABT-263 treatment (Fig. 3,4) demonstrates a dramatic induction of apoptosis irrespective of MCPyV status.

ABT-263 induces cell death in MCC cells regardless of non-targeted Mcl-1 levels

One of the potential caveats of ABT antagonists are their inability to bind Mcl-1, which leads to a reduced apoptotic response in lymphomas and small cell lung cancers (Konopleva *et al.*, 2006; van Delft *et al.*, 2006). Surprisingly, MCC cell lines with elevated Mcl-1 levels (Fig. 1b) were responsive to ABT-263. Assessment of Bcl-2 family members during the course of treatment reveals that Mcl-1 levels decrease dramatically in 3 of the 4 lines studied, while levels of anti-apoptotic Bcl-2, Bcl-x_L, and Bcl-w as well as pro-apoptotic effectors Bax and Bak, remain relatively stable (Fig. 4c). In contrast to other family members, Mcl-1 turns over rapidly via ubiquitination and proteasomal degradation (Zhong *et al.*, 2005), and can undergo caspase cleavage (Herrant *et al.*, 2004), which may explain the Mcl-1 loss. Although the responsive UM-MCC624 maintains stable Mcl-1 levels during treatment (Fig. 4c), it may be that the presence of high Bak levels confer sensitivity to ABT by sequestering Mcl-1 as has previously been shown (Konopleva *et al.*, 2006).

Requirement for pro-apoptotic Bax in ABT-263 induced MCC cell death

Based on what is known of its mechanism of action, ABT-263 should activate the pro-apoptotic executioners Bax and Bak at the mitochondria, leading to outer membrane permeabilization, release of apoptogenic factors and activation of downstream caspases and proteases (reviewed in (Bender and Martinou, 2013)). To define their role in ABT-263 driven apoptosis in MCC cells, Bax or Bak were down-regulated using lentiviral driven shRNA. Bax downregulation reduced cell death by approximately 80% (24h) and 98% (48h) in UM-MCC29, whereas Bak downregulation had little effect (Fig. 5a-c). Furthermore, effects of Bax shRNA reduced the killing activity of ABT-263 by 79%, 82%, and 94% in UM-MCC32, -35, and -565 cells, respectively (Fig. 5d).

ABT-263 induces apoptosis and blocks growth of MCC xenografts

Of the 9 UM-MCC cell lines tested, all generate tumors in xenografts (Table 1) with histology closely resembling the human MCCs from which they were derived (Suppl. Fig. S3; data not shown). UM-MCC29 was used to test the response to ABT-263 *in vivo*. Growth

kinetics of tumors in control versus ABT-263 treated mice shows a dramatic and sustained halt in growth (Fig. 6a). A separate experiment confirmed these results. H&E histology revealed the typical small blue-cell morphology characteristic of MCC in both control and treated tumors (Fig. 6b), but the latter were distinguished by the frequent presence of apoptotic bodies (arrowheads). This was corroborated by a markedly increased number of cells immunostaining for cCasp3 and exhibiting a punctuate distribution of Bax indicative of mitochondrial translocation (Fig. 6c). Based on cCasp3 quantification, the frequency of apoptotic cells was increased and maintained by approximately 10-fold (Fig. 6d bottom). In contrast, staining for Ki67 and pHisH3 (Fig. 6c) was quantitatively similar in tumors from control versus treated mice at early and late time points (Fig. 6d top).

DISCUSSION

In this study we identified and targeted anti-apoptotic Bcl-2 proteins in MCC tumor cells in an effort to better understand survival mechanisms that may be exploited for improved treatment of this deadly malignancy. Classical DNA damaging agents are largely ineffective in MCC with short-lasting responses, and are associated with high morbidity in elderly patients (Voog *et al.*, 1999). This has encouraged the development of mechanism-based targeted therapy. However, the disco very of novel strategies to improve prognosis has remained challenging, with progress in the field impaired partly by the relative paucity of validated tumorigenic MCC cell lines. We have established and characterized a panel of 11 MCC cell lines for functional studies using an enriched growth medium.

While overexpression of anti-apoptotic Bcl-2 proteins has been implicated in tumor pathogenesis, it is not necessarily a predictor of tumor cell addiction (reviewed in (Juin *et al.*, 2013)). We therefore utilized genetic as well as pharmacological targeting of the anti-apoptotic proteins to functionally define their role in MCC cell survival and ascertain the feasibility of Bcl-2 family targeting as a treatment rationale. Our data indicates cell death in nearly all MCC lines could be triggered by simultaneously targeting multiple Bcl-2 survival proteins with the antagonist ABT-263, whereas genetic inactivation of individual anti-apoptotic Bcl-2 proteins results in decreased cell viability in a subset of MCC cell lines. Our findings may help explain why individual genetic targeting of Bcl-2 was ineffective in MCC patients (Shah *et al.*, 2009). The frequent expression of multiple pro- survival proteins in MCC implies that their collective activity, rather than any one particular Bcl-2 family member, provides an increased apoptotic threshold which therefore requires broader targeting, as can be achieved with ABT-263.

While the exact binding hierarchy of anti- and pro-apoptotic proteins remains somewhat controversial, interactions resulting in Bax/Bak activation at the mitochondria leads to activation of the apoptotic signaling cascade (reviewed in (Bender and Martinou, 2013)). In keeping with this concept, we find that genetic inactivation of Bax almost completely abrogates ABT-induced MCC apoptosis in both MCPyV-positive and negative lines (Fig. 5c-d). Additionally, the widespread sensitivity of MCC cells to ABT-263 compared to carboplatin indicates targeting strategies at the level of the mitochondria may be more effective than those acting upstream which likely rely on functional p53 signaling. Moreover, ABT-induced cell death in MCC cells with high levels of IAP family members

indicates this agent can also efficiently bypass defects downstream of the mitochondria as well.

With the discovery of MCPyV, the notion of targeting viral proteins or associated pathways has become an attractive MCC treatment option. For instance, the survivin-targeted imidazole small- molecule inhibitor YM-155 was shown to promote irreversible, non-apoptotic MCPyV-positive MCC cell death (Arora *et al.*, 2012b). However, here we demonstrate that the Bcl-2 antagonist, ABT-263, induces widespread apoptotic cell death as a single agent in MCC cells regardless of MCPyV status. Furthermore, this apoptotic response was accompanied by suppression of tumor growth *in vivo*. Complete regression was not observed most likely due to unimpaired proliferation of tumors in treated mice (Fig. 6), suggesting that combined therapy with ABT-263 and a cytostatic agent may be a more effective approach to treatment.

Our results, using a recently-developed and characterized panel of cell lines, establish that nearly all MCCs require the collective activity of the anti-apoptotic Bcl-2 family members to restrain intrinsic pro-death signals. Use of the Bcl-2 antagonist ABT-263 induces Bax-dependent apoptosis both *in vitro* and *in vivo*, regardless of additional apoptotic signaling defects, or MCPyV status. These data identify the increased apoptotic threshold in MCCs as a potential point of vulnerability, warranting further investigation into the use of Bcl-2 family antagonists for the treatment of these aggressive malignancies.

MATERIALS AND METHODS

See Supplementary Materials and Methods for further experimental details and antibodies.

Acquisition of human tumors

Human tissue samples were collected from patients that provided written informed consent for use of surplus tissue according to a protocol approved by the University of Michigan Institutional Review Board (IRB Study ID: HUM00050085) in adherence to Helsinki Guidelines. Tissue was collected according to protocols outlined and approved under the same IRB. MCC diagnosis was established by a board-certified dermatopathologist. Patient demographics and other relevant information are listed in Table 1. Fresh tumor or normal skin tissue was collected in cold Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, MD) supplemented with 500U/mL penicillin/streptomycin until further processing.

Establishment of MCC cell lines and tissue culture

MCC cell lines were established from tumor tissue procured as described above. Cell lines UM-MCC9, 13, 29, 31, 32, 34, 35, and 40 were derived directly from solid patient tumors; UM- MCC565, 623, and 624 were established from tumors arising in NOD /SCID/IL2Rγ^{null} (NSG) mice (Jackson Laboratories), following injection of human MCC cells in a xenotransplantation model as previously described (Quintana *et al.*, 2008) (Suppl. Fig. S1; Table 1). All cell lines were established in a modified neural crest stem cell self-renewal medium (Molofsky *et al.*, 2005) supplemented with 15% chick embryo extract (Stemple and Anderson, 1992). Melanocytes, keratinocytes and fibroblasts, as well as melanoma cell

lines, were isolated and cultured as previously described (Verhaegen *et al.*, 2006). HL60, MKL-1, and MKL-2 cell lines were maintained in RPMI supplemented with 10% fetal bovine serum (Atlanta Biologicals, GA).

Reagents

ABT-263 (Accela Chem Bio Inc, CA) was diluted as needed in culture medium or resuspended daily by sonication in 60% Phosal 50 PG (Phospholipid GmbH, Germany), 30% polyethylene glycol (PEG) 400 (Sigma-Aldrich, MO) and 10% ethanol for *in vivo* studies.

Analysis of cell proliferation and cell viability

Assessment of cell proliferation was performed using an XTT Cell Proliferation Assay kit (ATCC, VA) and cell death was determined by standard Trypan blue exclusion assays. All experiments were performed in triplicate and data are presented as the mean + SEM.

Protein immunoblots

Total cell line lysates were obtained by Laemmli extraction and human tumor lysates were prepared by mechanical homogenization in RIPA buffer supplemented with HALT phosphatase inhibitor cocktail (Thermo Scientific, IL) and Complete mini protease inhibitor cocktail (Roche, IN). Protein was quantified by standard Bradford method using Bio-Rad Protein Assay dye reagent (Bio- Rad Laboratories, CA) and separated on 4-20% gradient SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, MA). Detection was carried out with SuperSignal West Pico or Femto chemiluminescent substrates (Thermo Scientific, IL).

Genetic inactivation: RNA interference

Down-regulation of apoptotic mediators Bcl-2, Bcl-x_L, Mcl-1, Bax and Bak was accomplished using lentiviral driven shRNA constructs as previously reported (Verhaegen *et al.*, 2006). Lentiviruses were generated in 293FT cells and viral titers were collected for infection of MCC cell lines.

Treatment of UM-MCC cell lines as tumor xenografts

Athymic nude-*Foxn1*^{nu} mice (Harlan) were kept in specific pathogen-free conditions and used at 5-6 weeks of age in procedures approved by the University Committee on the Use and Care of Animals (animal protocol #PRO00004440). ABT-263 effectiveness *in vivo* was analyzed by oral daily dosing at 100mg/kg for 18 days (d18) when UM-MCC29 xenografts reached a volume of 250-300 mm^3. Control mice were dosed with resuspension diluent (n=12 tumors per condition). Mice were sacrificed 8 days post-treatment (d8) or upon treatment termination (d18).

Immunohistochemical staining

Human or mouse xenograft tumors were fixed in 10% neutral buffered formalin followed by routine paraffin embedding and processing for immunohistochemical staining (including deparaffinization, rehydration, and antigen retrieval in boiling 0.01M citric acid solution, pH

6.8). Vector M.O.M. fluorescein or peroxidase Immunodetection Kits (Vector Laboratories, CA) were used for detection with SigmaFast diaminobenzidene (DAB) as a peroxidase substrate. Nuclei were counterstained with hematoxylin.

Statistics

Statistical analysis of tumor growth *in vivo* following ABT-263 treatments was done using an unpaired two-tailed Student's *t* test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MCC	Merkel cell carcinoma
MCPyV	Merkel cell polyomavirus
LTAg	large T antigen
sTAg	small T antigen
cCasp3	cleaved caspase-3
pHisH3	phosphorylated histone H3
H&E	hematoxylin and eosin
h	hours

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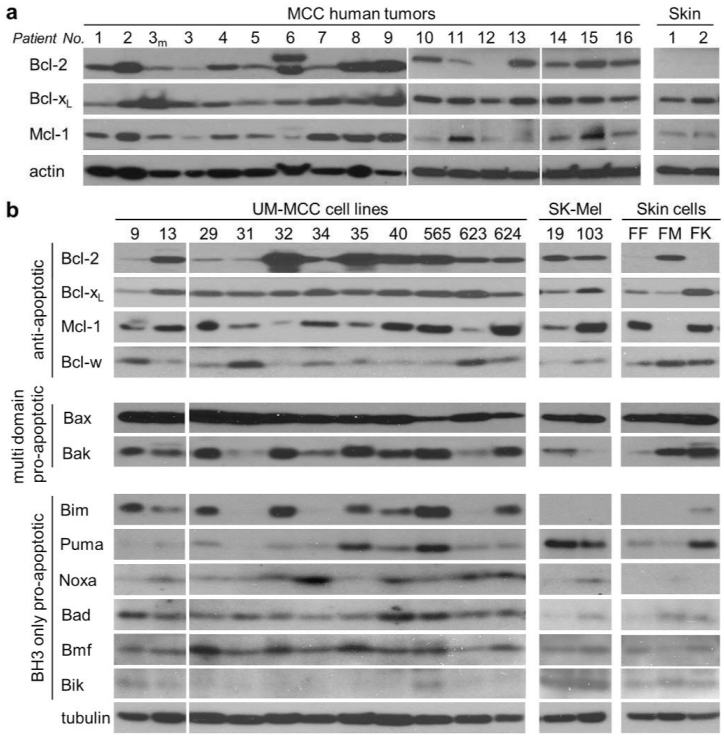

FIGURE 1.

Expression patterns of Bcl-2 family members in MCC tumors and cell lines. a) Immunoblotting depicts levels of Bcl-2, Bcl-x_L, and Mcl-1 in a panel of MCC tumors and normal skin. b) Basal levels of anti-apoptotic (Bcl-2, Bcl-x_L, Mcl-1, Bcl-w), multi-domain (Bax, Bak) and BH3-only (Bim, Puma, Noxa, Bad, Bmf, Bik) Bcl-2 family members in UM-MCC cell lines. See Table 1 for patient tumor numbers and corresponding cell lines. Skin and skin cells: foreskin (F) fibroblasts (F), keratinocytes (FK), and melanocytes (FM) are shown to indicate normal tissue also expresses select anti-apoptotic members presumably needed for tight regulation of apoptosis. Melanoma cell lines (SK-Mel-19, -103) known to express high levels of pro-survival Bcl-2 members are shown as positive controls (Verhaegen *et al.*, 2006).

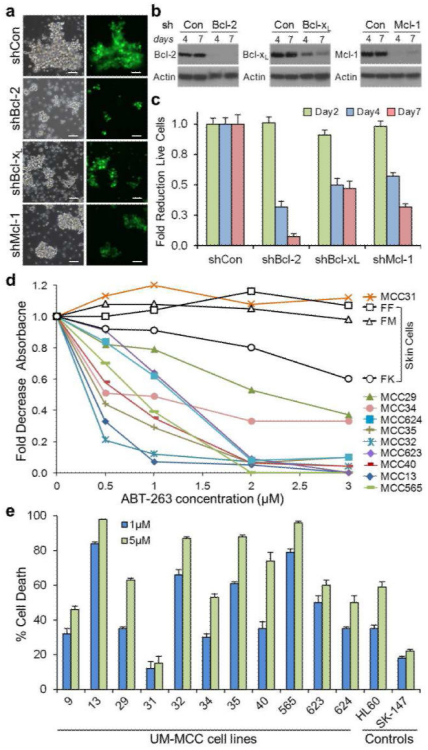

FIGURE 2.

Targeting anti-apoptotic Bcl-2 proteins decreases MCC cell viability. a) Effects of individual Bcl-2 family knockdown in UM-MCC565 via GFP-expressing lentiviral driven shRNA shown by brightfield and fluorescent microphotographs (day 4). Scale bars = 50μm. b-c) Downregulation of targeted proteins is shown by immunoblotting and quantitative analysis of viable cells is determined by Trypan blue exclusion assay. A scrambled shRNA construct was used as a control (shCon). d-e) Effects of ABT-263 on proliferation as determined by XTT assay (48h) (d) or cell viability as determined by Trypan Blue exclusion assay (24h) (e) in UM-MCC cell lines and control skin cells (fibroblasts FF; melanocytes FM; keratinocytes FK) or tumor cell lines (SK-Mel-147; HL60). Cells were treated with ABT-263 or DMSO vehicle in complete medium.

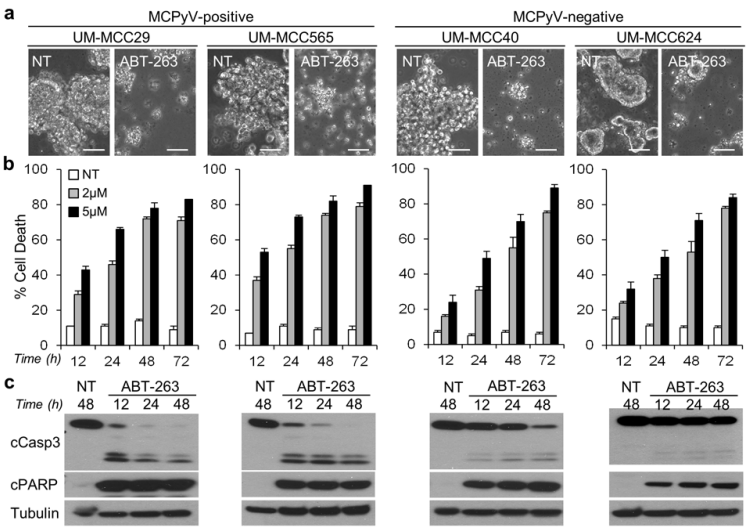

FIGURE 3.

ABT-263 is a potent inducer of apoptosis in MCC cells. a) Microphotographs of representative MCPyV-positive or negative UM-MCC cell lines following treatment with ABT-263 (5μM) for 24h. Scale bars = 50μm. b) Quantitative analysis of cell death by Trypan blue exclusion assay over indicated time course at 2μM (gray) and 5μM (black) concentrations. Similar responses at 2μM and 5μM are most likely due to Bcl-2 binding saturation (Vogler *et al.*, 2009). Cells were seeded, treated and analyzed in triplicate. Vehicle control treated cells are indicated as 'non-treated' (NT). Error bars indicate SEM. c) ABT-263 (5μM) induces apoptosis as depicted by immunoblotting for cleaved (c) Caspase3 (cCasp3) and cPARP as early as 12h post-treatment.

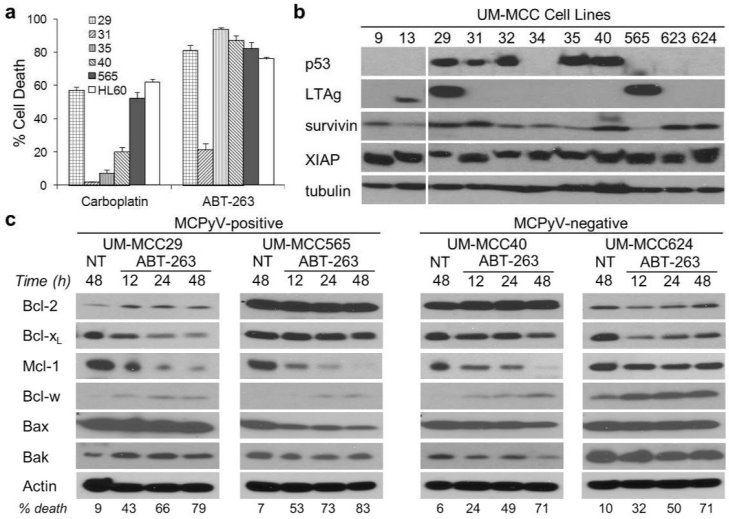

FIGURE 4.

ABT-263 induces widespread apoptosis in MCC cells regardless of additional defects in apoptotic pathways, MCPyV status, or Bcl-2 profile. a) UM-MCC cell lines respond variably to the classical DNA damaging agent carboplatin (100μM) compared to ABT-263 (5μM), with UM-MCC31 uniquely resistant. Cell death was assessed by Trypan blue exclusion assay at 48h. b) Immunoblotting for basal levels of p53, MCPyV LTAg, survivin, and XIAP in UM-MCC cell lines. c) Effects of ABT-263 on key Bcl-2 apoptotic modulators anti-(Bcl-2/Bcl-x_L/Mcl-1/Bcl-w) and pro-apoptotic (Bax/Bak) family members in 4 select UM-MCC lines by protein immunoblotting. Corresponding % cell death as assessed by Trypan blue exclusion assay is shown at each time point. Vehicle control treated cells are indicated as ‘non-treated’ (NT).

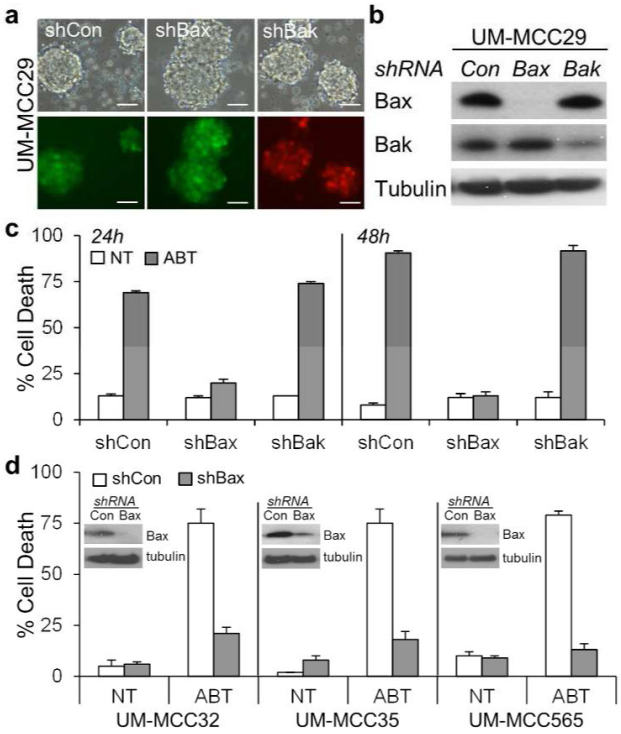

FIGURE 5.

Induction of MCC cell death by ABT-263 is Bax dependent. a-c) Effects of Bax or Bak knockdown on apoptotic response of ABT-263 treatment in UM-MCC29. Brightfield or fluorescent microphotographs (a) are shown with GFP (shCon, shBax) or RFP (shBak) under control of the human ubiquitin-C promoter to monitor transduction efficiency. Scale bars = 50μm. Efficacy and selectivity of downregulation are validated by immunoblotting (b). Quantitative analysis by Trypan blue exclusion assay following ABT-263 (5μM) treatment (c). d) Quantitative analysis of cell death by Trypan blue exclusion assay 24h post treatment with ABT-263 (2μM) following Bax downregulation (insets show Bax immunoblots) in cell lines UM-MCC32, -35, and -565. Error bars indicate SEM. NT indicates vehicle control treated cells.

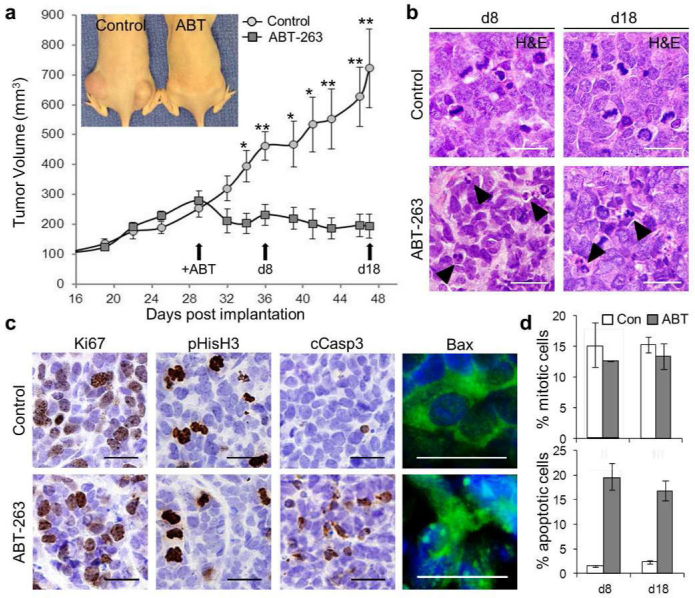

FIGURE 6.

ABT-263 inhibits MCC tumor growth and induces apoptosis *in vivo*. a) Tumor volume ($V=L\times W^2/2$; L = length, W = width) of UM-MCC29 xenografts in control or ABT-263 treated mice. Photo depicts mice 18 days post ABT-263 (d18). Error bars indicate SEM (n=12). * and ** indicate *P*-values <0.05 or 0.005, respectively. b) H&E staining at d8 and d18. Arrowheads indicate apoptotic cells. c) Immunostaining for proliferation markers Ki67 and pHisH3 as well as apoptotic markers cCasp3 and Bax translocation (d18). d) Quantitative analysis of pHisH3 and cCasp3 cell counts at d8 (bars indicate range for n=2) and d18 (bars indicate SEM for n=4). 1,500 cells from randomly-selected fields were counted for each tumor analyzed. All scale bars = 25μm.

TABLE 1

UM-MCC cell lines and patient demographics. Listed are MCC tumor type, site, age, gender and disease stage at the time of tissue acquisition. Corresponding cell line designation is shown for each patient tumor. Source of each UM-MCC cell line indicates direct establishment from patient tumors (human) or from human tumor xenografts in NOD/SCID/IL2Rγ^{null} (NSG) mice. Yes (Y) indicates tumorigenicity of MCC lines in athymic nude mice.

Patient No.	MCC Tumor					Cell Line		
	Tumor type	Site	Age	Gender	Disease Stage	Designation	Source	Growth in vivo
1	primary	scalp	92	M	1B	UM-MCC9	human	Y
2	metastasis	leg	54	F	3B	UM-MCC13	human	ND
3	primary	arm	54	M	3B	UM-MCC623	NSG mice	Y
3	metastasis	axillary LN	54	M	3B	UM-MCC624	NSG mice	Y
4	metastasis	inguinal LN	56	M	3B	UM-MCC29	human	Y
5	metastasis	axillary LN	85	M	3B	UM-MCC31	human	Y
6	primary	scalp	72	F	3B	UM-MCC32	human	ND
6	metastasis	post-auricular LN	73	F	3B	UM-MCC35	human	Y
7	metastasis	axillary LN	88	M	3B	UM-MCC34	human	Y
8	metastasis	inguinal LN	79	F	3B	UM-MCC40	human	Y
9	primary	eyelid	71	F	1B	UM-MCC565	NSG mice	Y
10	metastasis	axillary LN	79	F	3B	UM-MCC19*	human	ND
11	metastasis	leg	62	M	3B	UM-MCC49*	human	ND

LN = lymph node; ND = not determined.

* indicates cell lines lost to contamination.