Azacitidine and MCL1 inhibitors in combination may provide novel treatment for patients with melanoma

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Abstract

Treatment options for melanoma have progressed in recent years, with the rise of targeted therapy and immunotherapy. However, options are still limited for BRAF wildtype patients, which account for 50-60% of all patients, and for those who do not respond to immunotherapy. The BCL2 family of proteins regulates apoptosis, and the anti-apoptotic members are implicated in the development of treatment resistance in a variety of cancers. Drugs mimicking the pro-apoptotic BH3 only proteins, termed BH3 mimetics, are a promising therapeutic option for patients who are resistant to or ineligible for other therapies. The BH3 mimetic ABT-199 (venetoclax) is approved for use as a single agent in chronic lymphocytic leukemia (CLL), and was recently approved for use in combination with the hypomethylating agent azacitidine in selected patients with acute myelogenous leukemia (AML). This combination was found to selectively target leukemia stem cells (LSCs), and greatly improved the treatment options for leukemia patients not eligible for more toxic chemotherapy. A hallmark of melanoma is resistance to chemotherapy and increased expression of anti-apoptotic proteins. We therefore examined similar combinations on fourteen patient-derived and commercially available melanoma cell lines. The BH3 mimetics tested include MCL1 inhibitors (S63845, S64315), an inhibitor of BCL2, BCLXL, and BCLW (navitoclax/ABT-263), a BCL2 inhibitor (venetoclax/ABT-199), and a BCLXL inhibitor (A1331852). Our data showed that treatments with MCL1 inhibitors plus azacitidine were effective in reducing melanoma cell viability in vitro. The MCL1 inhibitor, S63845, was the most potent compound when combined with azacitidine, with ~85% of melanoma cell lines achieving less than 50% viability at the highest dose. Treatment with azacitidine in combination with ABT-263 or A1331852, had appreciably less effect with only 64% and 50% of cell lines responding at the highest dose, respectively. Response to ABT-199 with azacitidine was considerably lower with

only 21% of cell lines demonstrating a decrease in viability at the highest concentration. The S63845 plus azacitidine combination increased the level of MCL1 protein, a result that has been shown by other groups to be due to increased protein stability. Additionally, treatment with S63845 plus azacitidine disrupted sphere formation in melanoma initiating cell (MICs), a subset of melanoma cells thought to be responsible for relapse. Finally, knockdown of BIM and MCL1 was shown to sensitize cells to treatment with S63845 plus azacitidine, an effect that may be due to the metabolic roles of these proteins. Our data demonstrate that treatment with the MCL1 inhibitor S63845 in combination with azacitidine was highly effective in suppressing growth of melanoma cells in vitro including rare uveal, acral and mucosal subtypes. Mechanistic experiments exploring the potential role of disruption in energy metabolism to explain these results are planned. A better understanding of how this novel combination works may provide an alternative therapeutic approach for patients with melanoma.

Introduction

Introduction to melanoma, cutaneous and rare types

The incidence of melanoma has been steadily rising over the past 30 years (American Cancer Society, 2019). Melanomas arise from melanocytes, which are neural crest derived cells that populate the skin, eyes and certain mucosal surfaces (Shain & Bastian, 2016) and provide protection against UV damage. Melanomas can be divided into a variety of subtypes based on their anatomic location. Although melanoma is a relatively rare form of skin cancer, making up only 3-4% of all cases, it accounts for a majority of skin cancer related deaths (Cummins et al., 2006). Melanomas identified before metastasis generally have a favorable prognosis. However, metastatic melanoma is a devastating disease, with a 10 year survival of 10-15% (O'Neill & Scoggins, 2019).

Cutaneous melanoma is the most common form, making up approximately 90% of all cases (Chang et al., 1998). One of the most common molecular alterations in cutaneous melanoma are mutations in the BRAF gene. BRAF mutations are present in approximately half of cutaneous melanomas, frequently through a valine to glutamic acid substitution at codon 600 (BRAFV600E). This leads to constitutive activation of the mitogen-activated protein kinase (MAPK) pathway, and subsequently an increase in cell proliferation (Ascierto et al., 2012).

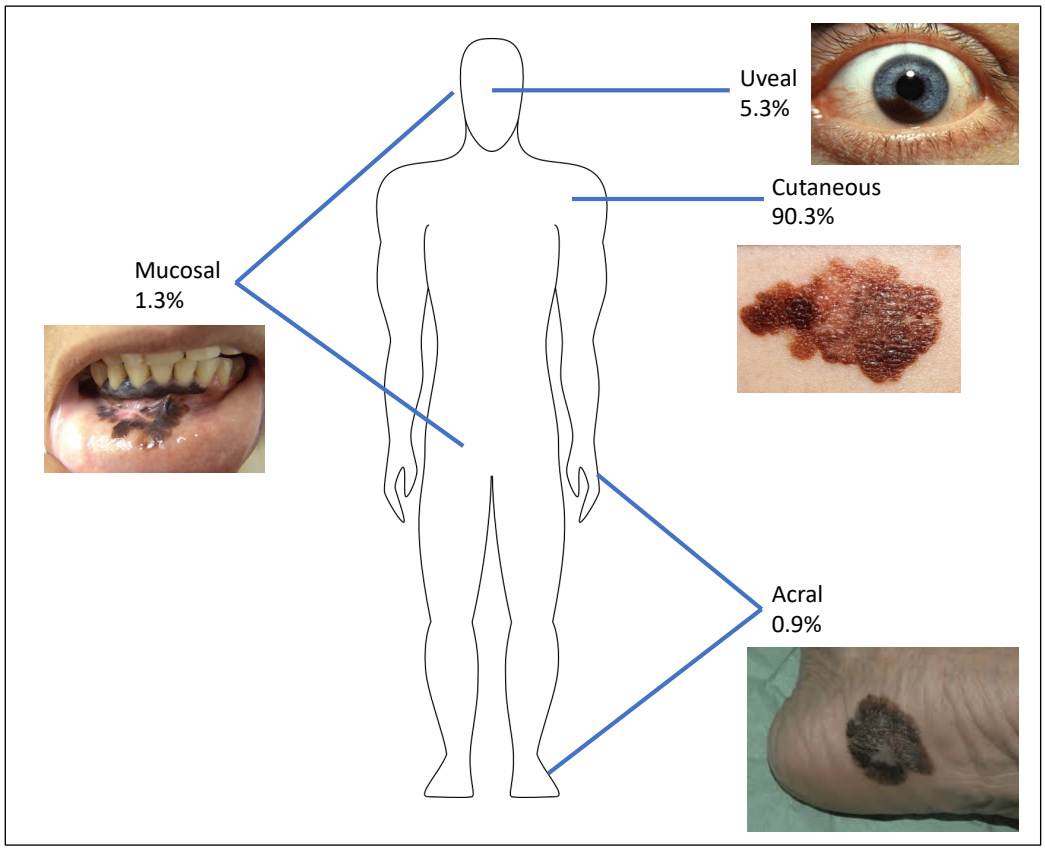

Figure 1: Cutaneous and rare melanoma subtype locations and percent of total incidences. Data adapted from Chang et al., 1998.
Image sources: http://www.pcds.org.uk/, https://en.wikipedia.org/wiki/Melanoma, https://www.curemelanoma.org, http://atlasgeneticsoncology.org

Melanomas, other than cutaneous, are divided between a range of rare subtypes, including uveal, mucosal, and acral. As opposed to cutaneous melanoma, these subtypes infrequently have BRAFV600E mutations. Approximately 20% of acral melanomas have been reported to have a

BRAF alteration, while in mucosal melanomas the incidence of BRAF mutations is only 10% (Yde et al., 2018; Yeh et al., 2019). In a series of three studies assessing the mutation status BRAF in 150 uveal melanomas, none were found (Cruz et al., 2003; Edmunds et al., 2003; Rimoldi et al., 2003). The most common mutations in uveal melanoma include those in GNAQ, GNA11, SF3B1 and BAP1 (Harbour et al., 2010, 2013; Van Raamsdonk et al., 2009, 2010). Mucosal melanomas more commonly harbor mutations in SF3B1 and KIT (Nassar & Tan, 2020). Mutations in KIT, NRAS, NF1, and GNAQ, are reported to be relatively common in acral melanoma (Moon et al., 2018). Although the incidence of BRAFV600E mutations in these subtypes is relatively low in comparison to cutaneous melanoma, many of the predominant alterations in these subtypes involve proteins directly involved in or which modulate the MAPK pathway. These proteins include NRAS, KIT, and NF1. The MAPK pathway is able to modulate the activity of several pro- and anti- apoptotic members of the BCL2 family (Inamdar et al., 2010). Alterations in GNAQ and GNA11 have also been shown to confer resistance to apoptosis, with knockdown of these mutant proteins inferring apoptotic sensitivity to therapeutics (Y. Li et al., 2019; Liu et al., 2015). Due to the dysregulation of apoptosis induction in these rare subtypes as well as in cutaneous melanoma, targeting the apoptotic pathway in these cancers may be an effective mode of therapy.

Treatment options and drawbacks

In the past decade, the treatment options for advanced melanoma patients have rapidly progressed, with the approval of targeted therapies and immunotherapies. Due to the high mutation rate and role of the MAPK pathway in melanoma growth and survival, targeting mutant BRAFV600E is an attractive option. In 2011, the first BRAF inhibitor, vemurafenib, was approved for the treatment of metastatic melanoma with a BRAFV600E mutation. Since this approval,

several other MAPK inhibitors have reached the clinic, such as dabrafenib (BRAF inhibitor), trametenib (MEK inhibitor), and cobimetenib (MEK inhibitor). Additionally, several combinations of BRAF and MEK inhibitors have been approved in order to overcome issues with relapse and resistance to single agent BRAF inhibition. Immune checkpoint blockade has also yielded substantial improvements over historical treatments. Currently, three immunotherapeutics targeting PD-1 or CTLA-4 are approved for the treatment of melanoma (Luke et al., 2017).

Although the approval of MAPK inhibitors and immunotherapeutics has revolutionized treatment of advanced melanoma, issues with these regimens remain formidable. Most patients treated with BRAF inhibitors relapse, and at least 50% of patients do not respond to immunotherapy, or have significant toxicities associated with treatment (Luke et al., 2017; Wagle et al., 2011). Rare melanomas in particular have extremely limited treatment options. Due to the lack of BRAF mutations in codon 600, targeted BRAF inhibition is not a viable therapy for these patients. In addition, these rare melanomas generally have a lower response to immune checkpoint blockade than cutaneous melanoma (Yang et al., 2018; Yde et al., 2018). As a result new alternative treatment options are particularly needed for these patients.

Apoptosis and the BCL2 family

Apoptotic cell death can be triggered by one of two major pathways. The extrinsic pathway requires a ligand/receptor interaction to activate downstream signaling that ultimately leads to caspase activation and death (Derakhshan et al., 2017). Apoptosis can also be initiated via the intrinsic pathway, which relies on intracellular signaling induced by events such as DNA damage, the unfolded protein response, or oxidative stress. The intrinsic pathway is mediated in part by interactions between members of the BCL2 family of proteins.

The BCL2 family is made up of three categories: 1) anti-apoptotic, 2) pro-apoptotic multi-domain effectors and 3) pro-apoptotic BH3 only sensitizers and activators. The anti-apoptotic members consist of BCL2, BCLXL, MCL1, BCLW and BFL1/A1. These proteins contain four BCL2 homology domains, and are able to bind to the BH3 domain of the pro-apoptotic BCL2 family members using a shallow hydrophobic groove (Montero & Letai, 2018). The pro-apoptotic effectors, BAX and BAK, are sequestered in the inactive form through this interaction with the anti-apoptotic BCL2 members.

The BH3 only proteins can be categorized as sensitizers or activators by their ability to directly interact with and activate the pro-apoptotic effectors. All BH3 only proteins, both the sensitizers and the activators, are able to displace BAX and BAK from their interactions with the anti-apoptotic members. In addition to this function, activators are also able to bind BAX and BAK

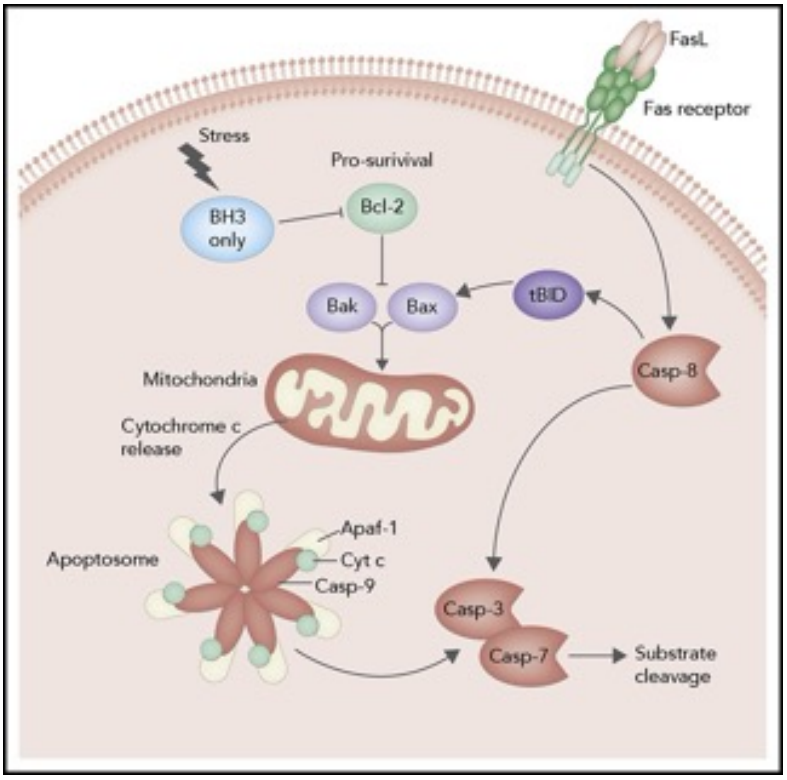

Figure 2: The intrinsic and extrinsic pathways of apoptosis: The intrinsic pathway is initiated by intracellular stress, which induces expression of BH3 only proteins, ultimately leading to the release of pro-apoptotic effectors and triggering the apoptotic cascade (McArthur et al., 2018).

using a BH3-BH3 interaction and activate them. Active BAX and BAK oligomerize and initiate the formation of pores in the outer mitochondrial membrane, facilitating the release of cytochrome c (Kale et al., 2018). The presence of cytochrome c in the cytosol triggers the activation of initiator and executioner caspases, ultimately leading to death of the cell.

(McArthur et al., 2018)

Inducing the intrinsic pathway: BH3 mimetics in cancer treatment

In recent years, the intrinsic apoptotic pathway has been evaluated as a potential therapeutic target for the treatment of cancer through the use of small molecules termed BH3 mimetics. These compounds act by initiating apoptosis through binding and inhibiting pro-apoptotic BCL2 proteins. In their landmark paper *Hallmarks of Cancer*, Hanahan & Weinberg describe a variety of mechanisms cancer cells use to avoid apoptosis, including the alteration of expression of BCL2 members (Hanahan & Weinberg, 2011). One of the rationales for the use of BH3 mimetics is the observation that cancer cells are at times more "primed" for apoptosis than their somatic neighbors, due to stressors in oncogenic transformation that lead to a shift in the ratio of pro- and anti-apoptotic BCL2 proteins. Specifically, the presence of pro-apoptotic BH3 only proteins or BAX and BAK in cancer cells leads to a primed state in which addition of synthetic BH3 peptides or BH3 mimetics is sufficient to release the pro-apoptotic effectors and initiate apoptosis (Chonghaile et al., 2011).

The development of high affinity drugs inhibiting the main BCL2 anti-apoptotic members has been an area of rapid progression over the past several years, culminating in the production of compounds selectively targeting BCL2, MCL1, and BCLXL. Currently, only one BH3 mimetic has reached clinical approval, although several show promising results in preclinical research. This

drug, venetoclax (ABT-199), a selective BCL2 inhibitor, is approved for the treatment of CLL and AML, and is in clinical trials in a variety of other hematologic malignancies.

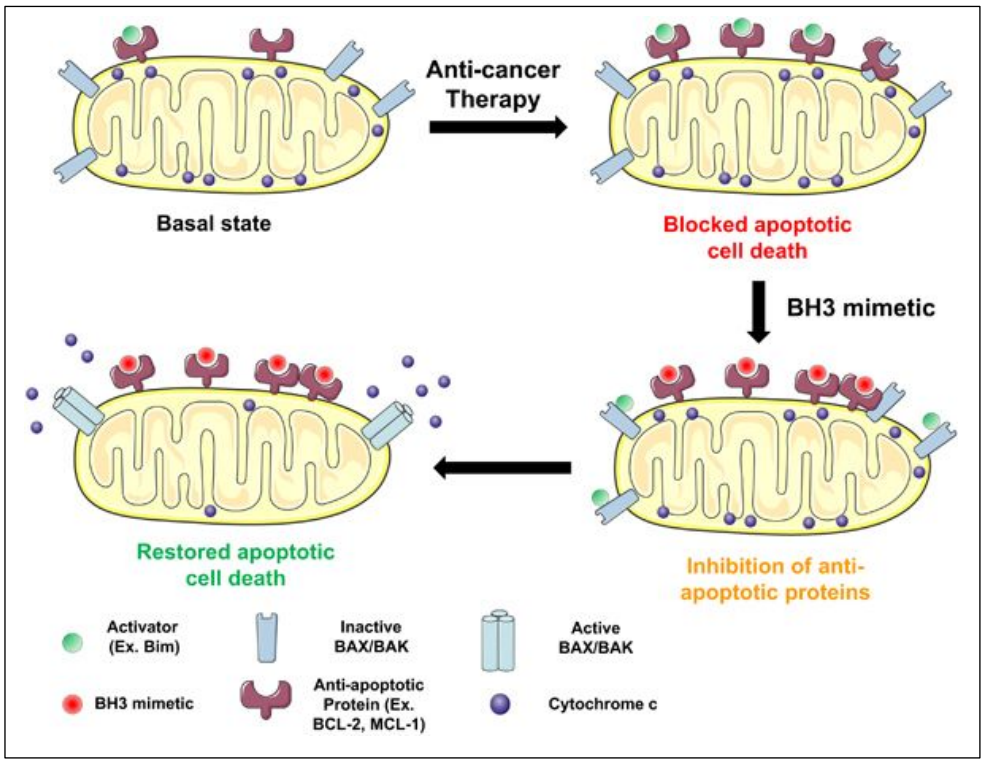

Figure 3: Method of action of BH3 mimetics: BH3 mimetics competitively bind to anti-apoptotic BCL2 family proteins and release proapoptotic activator and effector (BAX and BAK) proteins, allowing for mitochondrial outer membrane permeabilization (MOMP) and cell death (Montero & Letai, 2018).

The BCL2 family of proteins have been demonstrated to play a role in the development of both normal melanocytes and melanoma. BCL2 has been shown to be highly expressed in melanocytes, and BCL2 null mice lose the ability to regenerate melanocytes after hair stripping (van den Oord et al., 1994; Yamamura et al., 1996). These proteins also play a key role in the oncogenic transformation to malignant melanoma. In melanoma, overexpression of MCL1 or BCLXL has been shown to confer resistance to both classical chemotherapeutics and the more modern targeted therapies such as MAPK inhibitors, suggesting that BH3 mimetics may help to overcome resistance to these therapies (Fofaria et al., 2015; Heere-Ress et al., 2002). The efficacy of BH3 mimetics targeting BCL2, MCL1, BCLXL, and the pan-BCL2 inhibitor navitoclax have

been assessed in melanoma in the preclinical setting. Although these drugs yielded only modest effects as single agents, combinations targeting both MCL1 and BCLXL were shown to be highly effective (Lee et al., 2019; Mukherjee, Skees, et al., 2020a). MCL1 inhibition alone has also been shown to sensitize melanoma cells to targeted therapy with trametinib, vemurafenib or selumetinib (Sale et al., 2019). Taken together, these studies suggest that combinations targeting these proteins may be a promising treatment for melanoma.

Azacitidine, ABT-199 and AML

Treatment with single agent hypomethylating agents (HMA), such as azacitidine, is frequently used in AML patients who are not eligible for more rigorous cytotoxic chemotherapeutics (Pettit & Odenike, 2015). Azacitidine is a cytidine analog that influences methylation with the goal of inducing the expression of genes that were previously transcriptionally silent due to hypermethylation at the promoter site. Azacitidine is able to incorporate into DNA and RNA, and promotes the degradation of DNA methyltransferase (Stresemann & Lyko, 2008).

In November 2018, the BCL2 inhibitor venetoclax was approved for combination treatment with one of two hypomethylating agents, azacitidine or decitabine, for the treatment of AML. The combination of HMA and venetoclax was a significant improvement over single agent HMA treatment, with an overall response rate of 68% for venetoclax plus HMA treatment, while single agent HMA therapy had an overall response rate of 10-50%. Additionally, overall survival was lengthened from under 1 year with HMA monotherapy, to 17.5 months with the combination regimen (DiNardo et al., 2019). This combination was found to selectively kill LSCs through

targeting their reliance on oxidative phosphorylation (OXPHOS) for energy production (Jones et al., 2018; Pollyea et al., 2018).

Our hypothesis and aims

Overall Aim and Hypothesis:

The impressive success of azacitidine in combination with venetoclax in treating AML led us to examine similar combinations in melanoma cells. Our goal was to assess the efficacy of azacitidine plus various BH3 mimetics in melanoma cell lines from a variety of subtypes and mutations to determine the potential efficacy of this combination in melanoma. We theorized that these combinations of BH3 mimetics plus azacitidine would be potent to kill melanoma cell lines, similar to what has been seen in other cancers such as AML.

Specific aims:

1) To determine the impact of each treatment with BH3 mimetics in combination with azacitidine on cell viability. In order to assess this, we utilized a CellTiter-Glo ATP assay. Additionally, we monitored apoptosis induction and cellular proliferation using IncuCyte live cell analysis in the most promising treatment combination, S63845 plus azacitidine, determined based off of ATP assay results.
2) To assess the effect of each treatment on BCL2 protein expression. Using an immunoblot, we evaluated protein level between treatment with DMSO control and treatment with single agent azacitidine, BH3 mimetics, or their combination.

3) To gauge the impact of treatment on Melanoma Initiating Cells (MICs). We used a primary sphere assay, which measures the ability of MICs to form spheres in nonadherent conditions as a proxy for their functionality.

4) To evaluate whether the expression of select pro- and anti- apoptotic BCL2 family members is required for efficacy of single agent and combination treatment of S63845 plus azacitidine. We created sh mediated knockdown lines for NOXA, BIM, and MCL1, and utilize a CellTiter-Glo assay to determine impact of treatment on viability.

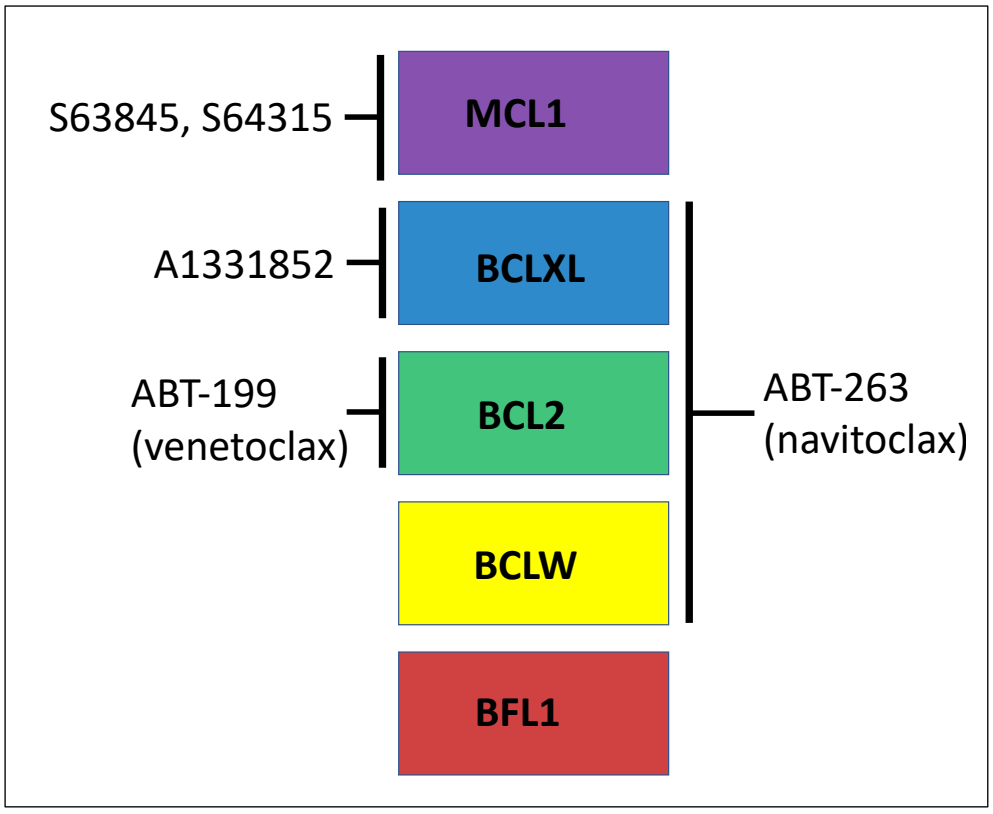

Figure 4: Selectivity of BH3 mimetics tested: Each BH3 mimetic can bind and inhibit a subset of the anti-apoptotic BCL2 family proteins.

Materials and methods

Cell lines and patient sample derived lines

Patient derived cell lines were established from tumor samples of patients at the University of Colorado Hospital (study use agreement COMIRB 05-0309). Patient lines were short tandem

repeat (STR) profiled, with a >80% match cutoff to peripheral blood or tumor specimen from the same patient. The cell lines MP41 and MP46 were purchased from the American Type Culture Collection (ATCC, Manassas, VA), 92-1 and Mel202 were purchased from Sigma Aldrich (St. Louis, MO). Cells were maintained in RPMI media with 10% FBS and 5% penicillin-streptomycin.

Drugs and Dosages

The drugs S63845, S64315, ABT-199, ABT-263, A1331852, and azacitidine were purchased from MedChem Express (Monmouth Junction, NJ). All drugs were used at a dose between 0.156 uM and 2.5 uM. For all in vitro assays, cells were treated for 48h and kept at 36.5°C and 5% CO_2.

ATP cell viability assay

Cells were plated at a density of 3000-5000 cells per well in tissue culture treated 96 well plates. After 24 hours, drugs were added at a concentration of 0.156- 2.5uM for BH3 mimetics, and 0.625-2.5uM for azacitidine. All treatments were done in triplicate wells. After 48h, cell viability was assessed using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI). 25 uL of CellTiter-Glo reagent was added to each well, and the contents of each well was moved to an opaque 96 well plate (Greiner Bio- One, #655075, Monroe, LA) and fluorescence was read using a BioTek Synergy 2 plate reader (Biotek, Winooski, VT). The CellTiter-Glo assay measures the amount of ATP in a sample as a readout for the number of live cells.

Lysate collection

Cells were plated on 10 cm dish at a density of 30,000 cells per plate. After 24h, cells were treated at a concentration of 0.156- 2.5uM for BH3 mimetics, and 0.625-2.5uM for azacitidine. Lysates were collected 48h after treatment. Floating and adherent cells were collected, and centrifuged at 270G. The supernatant was aspirated, and the pellet was resuspended using PBS, transferred to a 1.5 mL eppendorf tube and centrifuged again at 270G. The pellet was then lysed using 2x Lamelli buffer (Bio-Rad, Hercules, CA) with 5% 2-mercaptoethanol (MP Biomedicals, Santa Ana, CA). Lysates were then boiled at 95°C for 6 minutes and centrifuged at full speed for 10 minutes at 4°C. The lysates were stored at -80°C.

Immunoblot

Lysates were loaded into SDS-PAGE 4–15% acrylamide gradient gels (Bio-Rad, Hercules, CA) and run at 200V for 35-40 minutes. Blots were then transferred to polyvinyl difluoride (PVDF) filter membranes (Bio-Rad, Hercules, CA) using a Semi- Dry transfer cell (Bio-Rad, Hercules, CA) for 30 minutes at 15V. The membranes were washed 3X for 5 minutes in TBST. The membranes were blocked in BSA solution for 1h at room temperature, and probed with primary antibodies diluted 1:1000 in BSA overnight. The antibodies used were MCL1, PARP (#819,#9532, Santa Cruz Biotechnology, Dallas, TX), NOXA (#114C307, Millipore Sigma, Burlington, MA) BIM, and α/β tubulin (#2933, #2148, Cell Signaling Technologies, Danvers, MA). Anti-rabbit or anti- mouse IgG secondary antibodies (#7074S, #7076, Cell Signaling Technologies, Danvers, MA), were diluted 1:10,000 in 5% nonfat milk solution. Blots were washed thrice in TBS for 5 minutes each, and incubated for 2 hours in anti-rabbit or anti- mouse secondary antibody solution. Blots were then rewashed 3X with TBS for 5 minutes each, and developed with Super Signal chemiluminescent substrate (Thermo Scientific, Rockford, IL).

IncuCyte live cell analysis

Cells were seeded in 96 well tissue culture plate 24h prior to drug addition at a density of 3000-5000 cells per well. Cells were treated with vehicle, single drug or combination of S63845 and azacitidine, or S64315 and azacitidine. All treatments were done in triplicate wells. At the time of treatment, IncuCyte caspase 3/7 Green or Red Apoptosis Assay Reagent (Essen Bioscience Cat. No. 4440, No. 4704, Ann-Arbor, MI) was added to treatment media per manufacturer's instructions. Over 48h, cell confluence and caspase 3/7 activity was measured using IncuCyte S3 Live-Cell Analysis System (Essen Bioscience, Ann Arbor, MI). Images were acquired using the phase, and green or red fluorescent channels every 4h over a total of 48h.

Creation of short hairpin RNA transduced cell lines

Short hairpin RNA (shRNA) expressing lines against various BCL2 family members, or scrambled control, were constructed using shRNA Lentiviral Particles from Santa Cruz Biotechnology (Santa Cruz, CA) according to the manufacturer's instructions with slight modification. Cells were seeded in 12-well plates for 24h at concentrations sufficient to reach 50% confluency. The media was removed from each well and replaced with 1 ml of chilled polybrene working solution (5 μg/ml in RPMI1640 medium) and incubated at RT for 5 min. The solution was removed and replaced with 1 ml of chilled polybrene working solution with up to 20 μl of viral particle and incubated at 37 °C. The solution was removed 24 h later, and cells were rinsed once with fresh media, and then 1 ml of fresh media was added. Cells were grown until sufficient numbers were available for selection. Transduced cells were selected by supplementing the media with puromycin (1-4 μg/ml) for at least 7 days, replacing the medium with fresh puromycin-containing medium every 3-4 days until resistant colonies could be identified. Knockdown of genes of interest was measured by immunoblot.

Primary sphere forming assay

The assay was conducted in commercially available ultra-low attachment plates or polyhema-coated 24-well plates. Cells were seeded at a density of 5k-10k cells/mL with stem cell media. Stem cell media is a serum free media made with DMEM as the base media with added growth factors. After 120h, cells were treated with DMSO, azacitidine, S63845, or a combination. All drugs were dosed at 2.5 uM. After 48h of treatment, spheres were counted and representative images were taken. Spheres were defined as clumps of cells with a minimum diameter of 50 um.

Statistical analysis

Graphs for ATP assay, IncuCyte analysis, CI values, and sphere assay were created using GraphPad Prism 8 software. All graphs show mean +/- standard error of mean. Statistical significance was evaluated using two way ANOVA with a follow up Tukey post-hoc test. Combination index (CI) values were calculated using CompuSyn software (version 1). CI values indicate the synergistic, additive, or antagonistic effect of a drug combination. Values <0.9 indicate synergy, values 0.9-1 indicate an additive effect, and values >1 indicate antagonism (Chou, 2006).

Results

A combination of an MCL1 inhibitor and azacitidine is sufficient to kill melanoma cells in vitro.

We first examined the potency of single agent BCL2 family inhibitors, S63845 (MCL1 inhibitor), A1331852 (BCLXL inhibitor), ABT-263 (BCL2, BCLXL and BCLW inhibitor), and ABT-199 (BCL2 inhibitor), as well as the effect of single agent azacitidine treatment. We found that doses up to 2.5 uM were not effective in suppressing melanoma cell growth in this diverse array of melanoma subtypes and molecular profiles (Figure 5a, Table 1). Single agent treatment with S63845 was most effective, although only ~35% of cell lines had viability of less than 50%

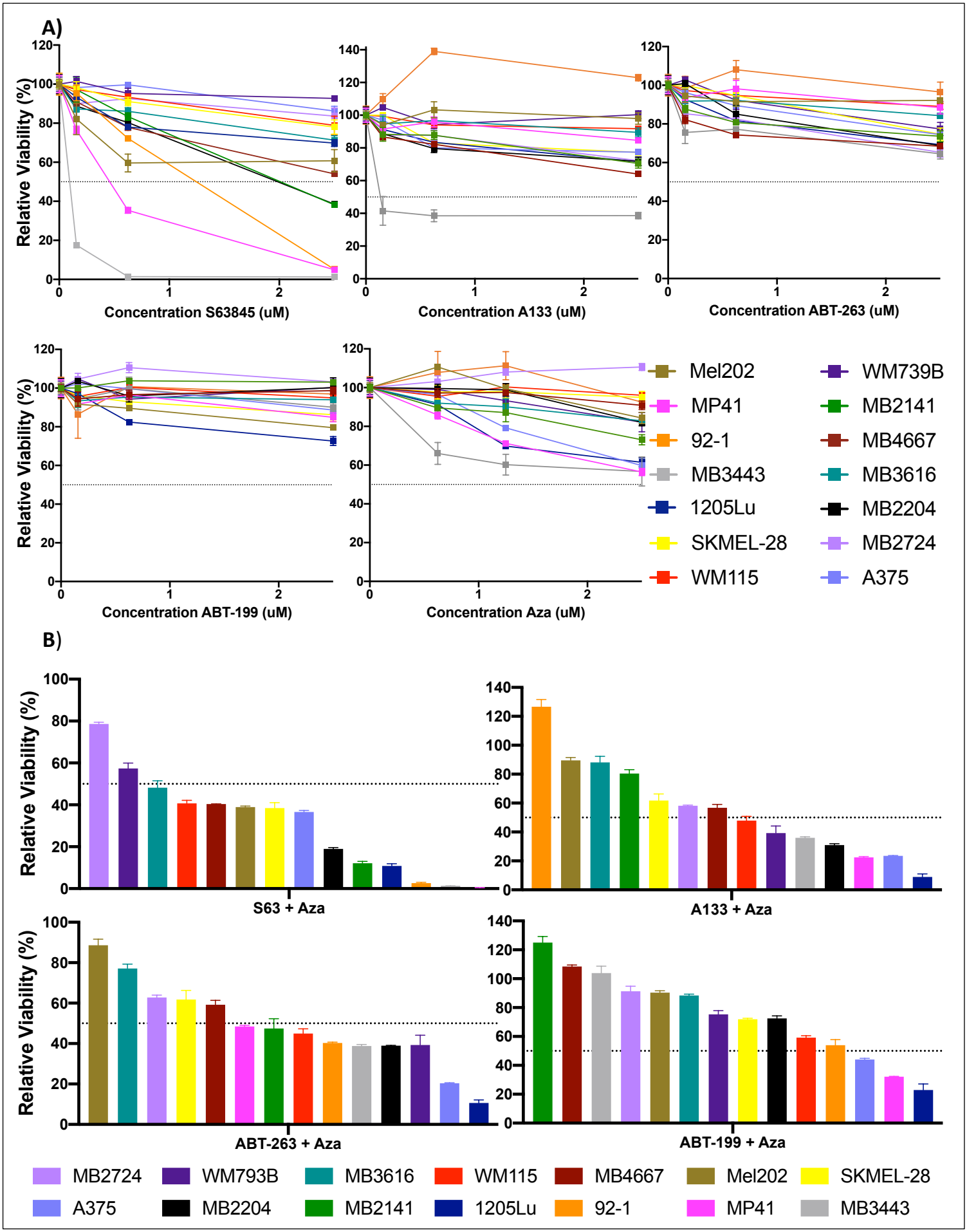

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Figure 5: Single agent BH3 mimetics or azacitidine have little impact on melanoma cell line viability; S63845 + azacitidine significantly reduce viability in the majority of melanoma cell lines. A) ATP assay of single agent BH3 mimetics or azacitidine dosed at a range of 0.156 - 2.5 uM for BH3 mimetics, and 0.625 - 2.5 uM for azacitidine. Error bars indicate +/- SEM. Y axis indicates cell viability relative to DMSO treated control, set to 100%. X axis indicates dosage in uM. B) ATP assay of combination BH3 mimetics plus azacitidine as indicated. All drugs were treated at a dose of 2.5 uM. For clarity, significance measurements are shown in Table 2. Dashed line indicates 50% viability. Error bars represent +/- SEM. Y axis indicates viability relative to DMSO control, set to 100%. X axis indicates treatment.

at the highest dose. Interestingly, of those cell lines sensitive to single agent MCL1 inhibition, all were from rare melanoma subtypes, two each from mucosal and uveal subtypes, and one from the acral subtype.

We next treated cell lines with azacitidine in combination with BH3 Mimetics S63845, ABT-199, A1331852, or ABT-263 (Figure 5b). In all combinations, we observed overall increased responses relative to that of single agent treatment. Combination treatment with the MCL1 inhibitor S63845 plus azacitidine was the most effective, with ~86% of cell lines reaching a viability below 50% relative to controls with DMSO only. This was significant ($p<0.001$) when compared to DMSO controls or azacitidine as a single agent in all cell lines, and in comparison to S63845 single agent treatment ($p <0.01$) in 10/14 cell lines (Table 2). The combinations of ABT-263 plus azacitidine and A1331852 plus azacitidine were less potent, with 64% and 50% of cell lines responding at the highest dose, respectively. The comparison of ABT-263 plus azacitidine and A1331852 plus azacitidine was significant ($p<0.01$) in 10/14 and 9/14 cell lines, respectively. ABT-199 plus azacitidine was the least effective combination, with only 21.4% of cell lines responding to combination treatment at the highest dose (Figure 5b). The difference between combination treatment and single agent or control was significant ($p<0.05$) in 12/14 cell lines (Table 2).

In order to determine the effect of treatment with single agent or combination azacitidine plus BH3 inhibitors on expression of select anti-apoptotic BCL2 family members, we performed

an immunoblot using cells treated with single agent BH3 mimetics or azacitidine, or their combination. We did not observe any consistent changes in expression of BCLXL, BCL2, or BCLW after any treatment. However, treatment with S63845 or ABT-263, alone or in combination with azacitidine increased the expression of MCL1 (Figure 6 a-b). S63845 has previously been demonstrated to increase the level of MCL1 protein without increasing the levels of MCL1 mRNA (Hird & Tron, 2019; Kotschy et al., 2016; Z. Li et al., 2019). This is due to a stabilizing effect of S63845 interacting with the BH3 binding site, which increases the half-life of the MCL1 protein. Upregulation of MCL1 after treatment with ABT-263 has been reported by several groups as a possible resistance mechanism to ABT-263, it has also been noted that reduction of MCL1 is able to sensitize cells to treatment with ABT-263 (Faber et al., 2015; Mukherjee, Skees, et al., 2020b; Yecies et al., 2010). ABT-263 may also directly impact MCL1 protein levels by stabilizing MCL1

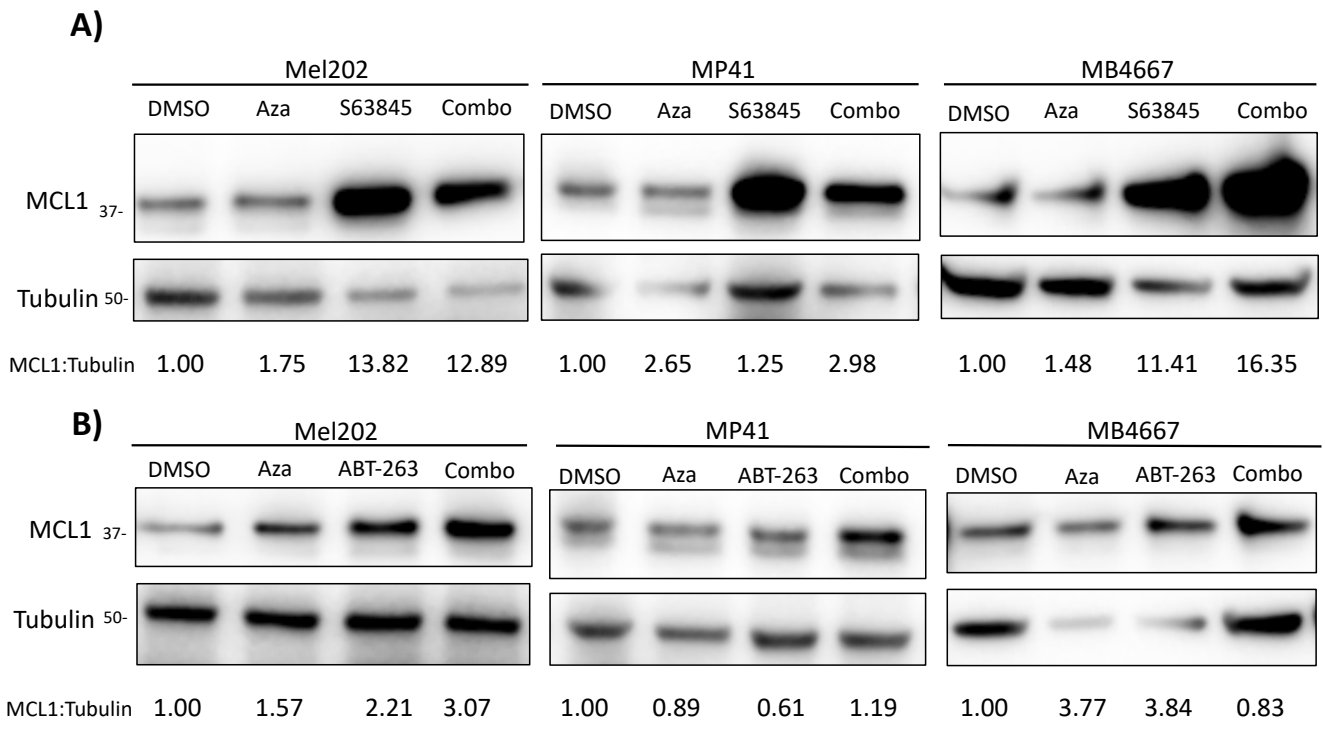

Figure 6: Single agent treatment with S63845 or ABT-263, or their combination with azacitidine increases MCL1 expression. A,B) Immunoblot using lysates from 48h after addition drugs as indicated. Quantification normalized to DMSO treatment for each cell line shown at base.

mRNA (Wang et al., 2014). These findings of increased MCL1 protein level after treatment with S63845 or ABT-263 are consistent with previous findings from other groups, and did not warrant further investigation.

S63845 or clinical grade version S64315/MIK665 in combination with azacitidine are able to induce apoptosis and reduce proliferation in melanoma cell lines

Due to the higher efficacy of the S63845 plus azacitidine combination, we narrowed our focus to this pairing for the remainder of our studies. We also evaluated the efficacy of S64315 (MIK665), the clinical grade counterpart of S63845. Preliminarily, we examined the activity of the S64315 plus azacitidine combination in two cell lines with mid-range sensitivity to S63845 plus azacitidine (Figure 7 a-b). We found that the combination of S64315 plus azacitidine was overall comparable to that of S63845, the difference between the combination treatments was not significant at the 2.5 uM dose. Additionally, combination index values calculated for these two treatments indicated that S63845 plus azacitidine treatment was synergistic in eleven of fourteen cell lines, while S64315 plus azacitidine was synergistic in both cell lines evaluated (Figure 7c).

In order to determine the mechanism of cell reduction induced by the combination of S63845/S64315 plus azacitidine, we utilized IncuCyte live cell imaging with a fluorescent caspase 3/7 reagent in order to monitor apoptosis induction, as well as cellular proliferation after treatment. The caspase reagent is made of a DNA dye bound to DEVD, a substrate of caspase 3 and 7. Once DEVD is cleaved off by active caspase 3 or 7, the DNA dye is free to move to the nucleus and bind DNA, where it fluoresces ("NucView® Caspase-3 Substrates," n.d.). Therefore, fluorescent staining is exclusive to apoptotic cells. Our data shows that combination treatment with S63845/S64315 plus azacitidine was able to significantly decrease proliferation and increase active caspase in all cell lines tested (Figure 8 a-b).

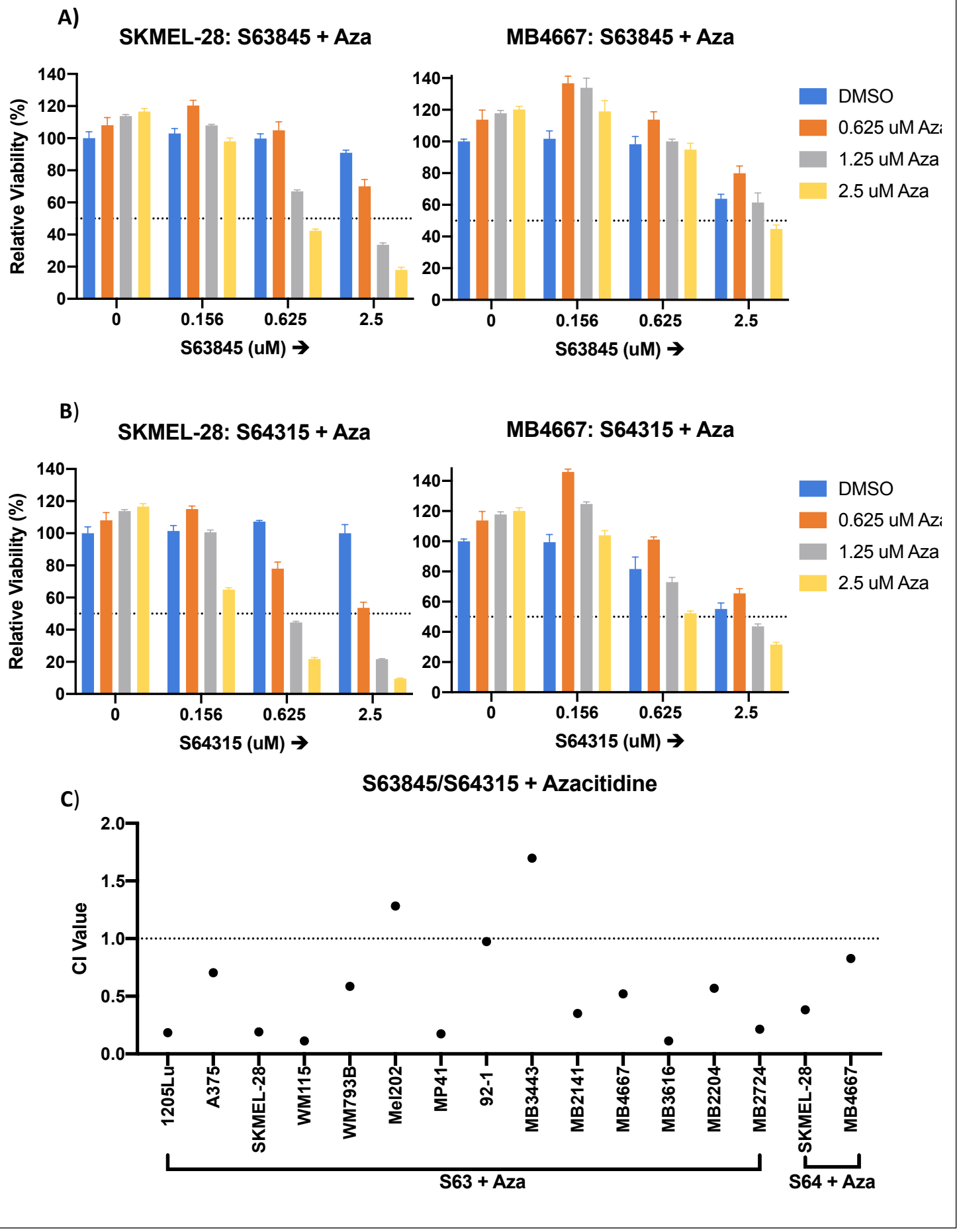

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Figure 7: S63845 and clinical grade version S64315 (MIK665) have similar efficacy in melanoma cell lines: A) ATP assay of S63845 plus azacitidine in two lines. Dashed line indicates 50% viability. Y axis indicates viability relative to DMSO control, set to 100%. X axis indicates S63 dosage. Error bars represent +/- SEM. B) ATP assay of S64315 (MIK665) plus azacitidine. Dashed line indicates 50% viability. Y axis indicates viability relative to DMSO control, set to 100%. X axis indicates S63 dosage. Error bars represent +/- SEM. C) Combination index (CI) values for S63845 + azacitidine and S64315 + azacitidine treatment. CI <0.9 indicates synergy, 0.9-1 indicates additivity, >1 indicates antagonism. CI values are calculated at the 2.5 uM dose using CompuSyn (version 1) software.

S63845 plus azacitidine decreases sphere formation of melanoma cell lines

Within melanoma, there is thought to be a subset of cells termed melanoma initiating cells (MICs), which share some features with cancer stem cells (CSCs) such as slow cycling time and the ability give rise to new tumors when transplanted into mice (Ahmed & Haass, 2018; Villani et al., 2015). This subset of cells has been implicated in relapse to targeted therapies, and so it is imperative to find treatments that target these cells as well as bulk tumor cells. The primary sphere

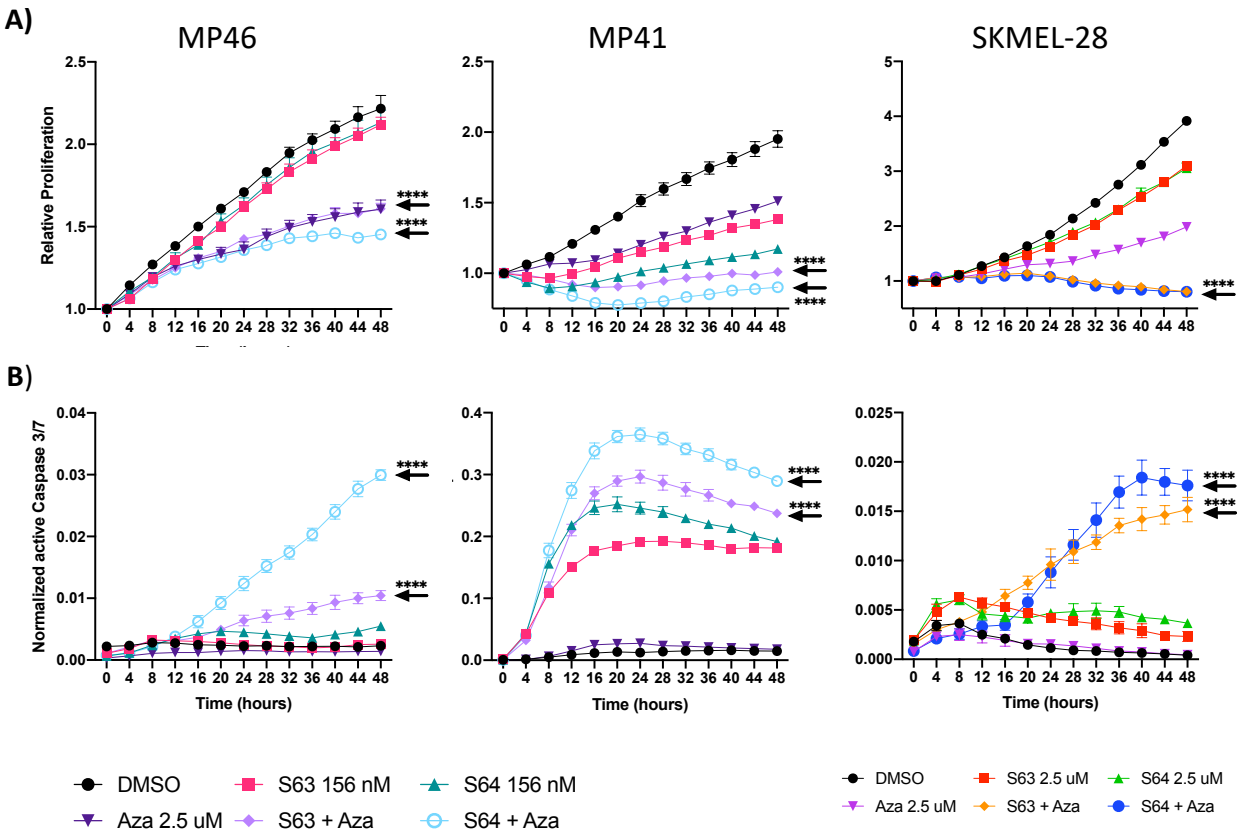

Figure 8: Treatment with S63845/S64315 plus azacitidine decreases proliferation and induces apoptosis. A) IncuCyte live cell analysis of cell proliferation. Y axis represents confluence relative to 0h. X axis indicates time in hours. B) IncuCyte live cell analysis of active caspase 3/7. Y axis indicates ratio of area expressing fluorescent signal. X axis indicates time in hours. For all graphs, error bars represent +/- SEM, ****indicates $p < 0.001$.

assay measures the impact of a drug treatment on viability of CSCs, including MICs. This technique hinges upon the ability of CSCs to survive and form spheres in nonadherent conditions (Johnson et al., 2013; Mukherjee, Amato, et al., 2020; Mukherjee, Skees, et al., 2020b; Stecca et al., 2013). After 48 hours, combination treatment with S63845 plus azacitidine significantly decreased sphere formation compared to DMSO controls ($p < 0.001$), in the melanoma cell line SKMEL-28, the difference between single agents and combination treatment was also significant ($p < 0.05$) (Figure 9a). Additionally, brightfield imaging showed substantially disrupted spheres in wells treated with combination versus single agent or control (Figure 9b).

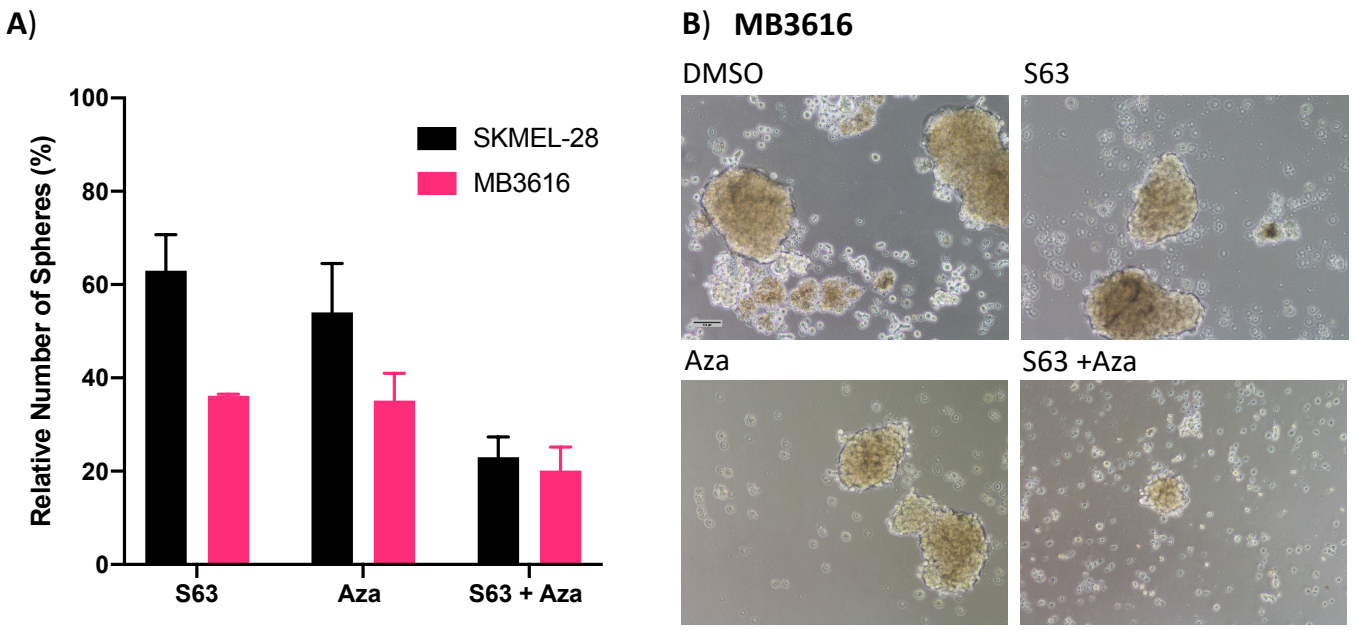

Figure 9: S63845 plus azacitidine treatment disrupts sphere formation. A) Quantification of number of primary spheres after 48h of indicated treatments. Y axis represents sphere number relative to DMSO control, set to 100%. X axis indicates treatment. For visual clarity, significance measurement is shown in Table 3. Error bars represent +/- SEM. B) Brightfield images of representative wells of MB3616 spheres after 48h of indicated treatment. Scalebar represents 100 um.

MCL1 or BIM knockdown sensitizes cells to S63845 plus azacitidine treatment; NOXA knockdown has no effect on combination potency

In order to determine the role of BCL2 family protein expression on single agent and combination treatment efficacy, we created knockdown lines for MCL1, BIM, and NOXA in the A375 cell line. We found that knockdown of BIM or MCL1 was able to significantly ($p < 0.01$) protect against cell death induced by single agent S63845. Both shBIM and shMCL1 significantly ($p < 0.05$) sensitized cells to single agent azacitidine, as well as the combination of S63845 plus azacitidine (Figure 10 a-b). Knockdown of NOXA appeared to have a slightly protective effect against S63845 or azacitidine single agent treatment, although this trend did not reach statistical significance.

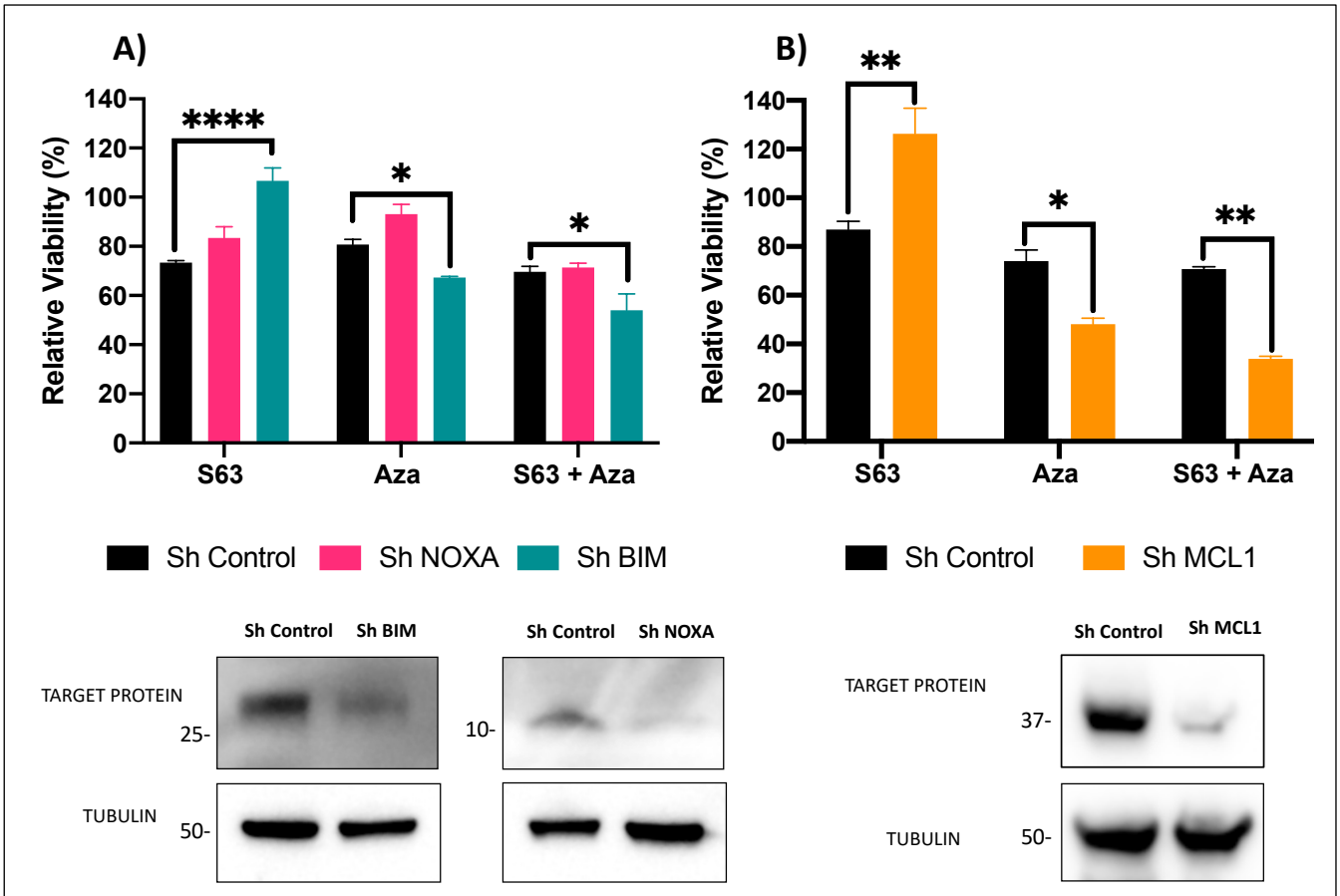

Figure 10: Knockdown of BIM or MCL1 sensitizes cells to combination treatment with S63845 plus azacitidine. A, B) Knockdown NOXA, BIM and MCL1 lines were created in A375 cells using shRNA technology. Western blots confirm knockdown in each line vs. sh Control. Error bars represent +/- SEM. Y axis indicates viability relative to DMSO control, set to 100%. X axis indicates treatment. *Indicates $p < 0.05$; **indicates $p < 0.01$;

Discussion

Treatment options for patients with advanced melanoma have expanded greatly since the introduction of targeted therapies and immunotherapies. However, the number of patients ineligible for these treatments due to a lack of targetable mutations or non-response to current immunotherapy has led to the search for novel treatment options. The studies reported here indicate that a combination of MCL1 inhibition plus azacitidine may provide such an option for melanoma patients that is not dependent on mutation status or melanoma subtype. The cell lines assayed in this study included lines from all of the main melanoma subtypes (cutaneous, uveal, mucosal, and acral) as well as both BRAF mutant and wildtype lines (Table 1). Our findings demonstrate that this combination is able to synergistically induce apoptosis in the majority of melanoma cell lines, and without a clear trend in response to treatment based of mutation status or subtype. Most importantly, we have shown that this combination is able to target MICs, a subpopulation of cells that are thought to be responsible for relapse after treatment, thus indicating that this combination may be able to prevent recurrence.

Surprisingly, we found that the least effective combination was ABT-199 plus azacitidine, which is the only combination that is currently clinically approved. This treatment has been shown to selectively target LSCs in AML, and has revolutionized the treatment of AML patients not eligible for more cytotoxic chemotherapeutics (DiNardo et al., 2019; Pollyea et al., 2018). The weakness of this combination in melanoma may be due to the reliance of melanoma on MCL1 and BCLXL for survival rather than BCL2, which has been previously described by our group as well as others (Lee et al., 2019; Mukherjee, Skees, et al., 2020b).

While these findings suggest that MCL1 inhibition plus azacitidine may be a promising combination in melanoma treatment, it will be necessary to determine the toxicity of this

combination in vivo before moving into human trials. Due to the general anti-apoptotic role of MCL1, there have been concerns regarding potential on-target toxicity in somatic cells (Hird & Tron, 2019). However, multiple in vivo studies using MCL1 inhibitors have been carried out in mice models with manageable toxicities (Brennan et al., 2018; Kotschy et al., 2016). Additionally, past studies in our group have achieved tolerable doses of S63845 in combination with ABT-199, A1338152, or ABT-263 (Mukherjee, Amato, et al., 2020; Mukherjee, Skees, et al., 2020). S64315 is currently involved in three phase 1 clinical trials to evaluate efficacy, all of which are in hematopoietic malignancies (clinicaltrials.gov, NCT02992483, NCT02979366, NCT03672695). So far, no data for the safety of these treatments have been reported. Although the combination of S63845/S64315 plus azacitidine has not been fully tested in animal studies or in clinical trials, there is a phase 1 clinical trial currently evaluating the safety of the MCL1 inhibitor AMG176 in combination with azacitidine (clinicaltrials.gov, NCT02675452). This trial consists of once weekly IV infusion of AMG176 paired with azacitidine treatment (doses not specified). Although no data have been published for this trial, it has been updated recently and is on track to reach primary completion by 2022. Azacitidine is generally considered a safe and tolerable treatment, with lower toxicity than the standard treatment in AML of cytarabine paired with daunorubicin, hence its use as the standard of care in elderly AML patients with extensive comorbidities (Schuh et al., 2017). Azacitidine treatment is associated with adverse events related to hematologic cell types, such as neutropenia, anemia, and thrombocytopenia (Xie et al., 2015). Although in vivo data to determine potential toxicities are needed, the clinical approval of azacitidine, paired with the results of in vivo work previously done with S63845 suggests that it may be possible to achieve a tolerable dosing schedule of the combination.

S63845 has been demonstrated to induce cell death selectively through its ability to bind and inhibit MCL1, and therefore should not be effective in cells lacking MCL1, which rely entirely on other anti-apoptotic proteins for their survival (Villalobos-Ortiz et al., 2020). The findings of our knockdown study are consistent with this mechanism, where the shMCL1 line had significantly increased viability relative to the shControl line after treatment with single agent S63845. BIM is a pro-apoptotic BH3 only protein that is able to bind and inhibit all of the pro-apoptotic BCL2 members (BCL2, MCL1, BCLXL, BCLW, BMF1/A1), as well as activate BAX and BAK (Shukla et al., 2017). Therefore, it follows that BIM expression would be requisite for killing via a BH3 mimetic. In our shBIM line, knockdown of BIM completely protected from cell death by S63845.

Knockdown of either BIM or MCL1 sensitizes cells to combination treatment with S63845 plus azacitidine, a result that is unexpected based on their roles in intrinsic pathway of apoptosis. However, both BIM and MCL1 have roles outside their involvement in apoptosis. Recent studies have shown that both MCL1 and BIM are involved in mitochondrial dynamics and OXPHOS (Andersen & Kornbluth, 2012, Perciavalle et al., 2012; Wali et al., 2018). Notably, the mechanism of cell reduction induced by the azacitidine plus ABT-199 combination is thought to operate through inhibition of OXPHOS (Jones et al., 2018). Both LSCs and potentially also MICs are reliant on OXPHOS, rather than glycolysis for energy production (Lagadinou et al., 2013; Roesch et al., 2013). It is possible that the higher sensitivity of MCL1 and BIM knockdown lines is due to a role of these proteins other than their canonical apoptotic function. In order to further explore this possibility, it will be necessary to carry out a series of metabolic studies to determine the impact of treatment on mitochondrial energy production.

Overall, our data strongly suggest that the combination of MCL1 inhibitor with azacitidine can be an effective means of killing melanoma cells irrespective of their mutational status. Our limited mechanistic studies indicate that this is through effects on the OXPHOS metabolic pathway, and we anticipate that further studies will verify this. Further in vivo studies of efficacy and toxicity are warranted to determine if this combination can be brought to clinic.

Future Directions

Moving forward, we would like to start by confirming the results of our knockdown studies in KD lines derived from a cell line with higher sensitivity to combination treatment with azacitidine plus S63845. We will also investigate the potential metabolic impact of this treatment similar to that described by Jones et al., 2018. This group examined the impact of treatment on OXPHOS in LSCs using a combination of metabolomics and assessment of the role of amino acids in energy production through their incorporation into metabolic intermediates. Initially, we will determine if the combination treatment of S63845 plus azacitidine has an impact on overall mitochondrial respiration. This can be done using a Seahorse analyzer to determine oxygen consumption rate. If the combination treatment of S63845 plus azacitidine impacts mitochondrial energy dynamics, it will be necessary to determine the basis for this alteration. In order to examine this, we will begin by looking at the potential role of amino acid catabolism in energy production, similar to Jones et al, 2018.

Additionally, we hope to evaluate the efficacy of this combination in vivo in a mouse xenograft model, which is considered to be a better indicator of efficacy in humans than cell culture. This would also allow us to determine the impact on somatic cells, any dose limiting

toxicities as well as a possible range of maximum tolerated dose. Finally, this laboratory is already planning to begin clinical trials of this combination in the near future.

Acknowledgements

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Supplemental Materials

Table 1: Genotype and subtype of commercially available or patient derived cell lines used

CELL/MB LINE	GENOTYPE	SUBTYPE
A375	BRAF V600E	Cutaneous
1205LU	BRAF V600E	Cutaneous-Metastatic
SKMEL-28	BRAF V600E	Cutaneous
WM793B	BRAFV600E	Cutaneous
WM115	BRAFV600D	Superficial spreading
MB2141	EML4-ALK	Mucosal
MB3443	NRAS Q61H	Mucosal
MB3616	NRAS Q61K	Superficial spreading
MB4667	NRAS Q61R	Acral
MB2724	Triple WT (WT for BRAF, NRAS and NF-1)	Acral
MB2204	Triple WT (WT for BRAF, NRAS and NF-1)	Acral
MEL202	SF3B1 R625G, GNAQ Q209L, R210K	Uveal
92-1	GNAQ Q209L	Uveal
MP46	GNAQ Q209P	Uveal
MP41	GNA11 Q209L	Uveal

Table 2: *p* values for figure 5b
S63845 + azacitidine

	DMSO Vs. Combo	Aza Vs. Combo	S63 Vs. Combo
MB2141	<0.0001	<0.0001	<0.0001
MB4667	<0.0001	<0.0001	0.0011
Mel202	<0.0001	<0.0001	<0.0001
MP41	<0.0001	<0.0001	ns
MB3616	<0.0001	<0.0001	<0.0001
MB3443	<0.0001	<0.0001	ns
MB2204	<0.0001	<0.0001	<0.0001
MB2724	<0.0001	<0.0001	ns
WM793B	<0.0001	<0.0001	<0.0001
WM115	<0.0001	<0.0001	<0.0001
SKMEL-28	<0.0001	<0.0001	<0.0001
1205Lu	<0.0001	<0.0001	<0.0001
A375	<0.0001	<0.0001	<0.0001
92-1	<0.0001	<0.0001	ns

A1331852 + azacitidine

	DMSO Vs. Combo	Aza Vs. Combo	A133 Vs. Combo
MB2141	<0.0001	ns	0.0346
MB4667	<0.0001	<0.0001	ns
Mel202	0.0237	ns	ns
MP41	<0.0001	<0.0001	<0.0001
MB3616	0.0091	ns	ns
MB3443	<0.0001	<0.0001	ns
MB2204	<0.0001	<0.0001	<0.0001
MB2724	<0.0001	<0.0001	0.0014
WM793B	<0.0001	<0.0001	<0.0001
WM115	<0.0001	<0.0001	<0.0001
SKMEL-28	<0.0001	<0.0001	0.0005
1205Lu	<0.0001	<0.0001	<0.0001
A375	<0.0001	<0.0001	<0.0001
92-1	<0.0001	<0.0001	ns

ABT-263 + azacitidine

	DMSO Vs. Combo	Aza Vs. Combo	ABT-263 Vs. Combo
MB2141	<0.0001	<0.0001	<0.0001
MB4667	<0.0001	<0.0001	ns
Mel202	0.0133	ns	ns
MP41	<0.0001	ns	<0.0001
MB3616	<0.0001	ns	ns
MB3443	<0.0001	<0.0001	<0.0001
MB2204	<0.0001	<0.0001	<0.0001
MB2724	<0.0001	<0.0001	ns
WM793B	<0.0001	<0.0001	<0.0001
WM115	<0.0001	<0.0001	<0.0001
SKMEL-28	<0.0001	<0.0001	0.0047
1205Lu	<0.0001	<0.0001	<0.0001
A375	<0.0001	<0.0001	<0.0001
92-1	<0.0001	<0.0001	<0.0001

ABT-199 + azacitidine

	DMSO Vs. Combo	Aza Vs. Combo	ABT-199 Vs. Combo
MB2141	<0.0001	<0.0001	<0.0001
MB4667	ns	0.411	<0.0001
Mel202	0.0410	0.0198	ns
MP41	<0.0001	<0.0001	<0.0001
MB3616	0.0112	ns	ns
MB3443	ns	0.0019	<0.0001
MB2204	<0.0001	<0.0001	0.0468
MB2724	ns	0.0089	<0.0001
WM793B	<0.0001	ns	<0.0001
WM115	<0.0001	<0.0001	<0.0001
SKMEL-28	<0.0001	<0.0001	0.0013
1205Lu	<0.0001	<0.0001	<0.0001
A375	<0.0001	0.0004	<0.0001
92-1	<0.0001	<0.0001	<0.0001

Table 3: *p* values for figure 8c

	DMSO Vs. Combo	Aza Vs. Combo	S63 Vs. Combo
SKMEL-28	<0.0001	0.0370	0.0065
MB3616	<0.0001	ns	ns

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