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Co-targeting BET proteins and MCL1 induces synergistic cell death in melanoma

Short Title: Combined BET and MCL1 inhibition in melanoma

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Abbreviations

ΔΨm: change in mitochondrial membrane potential

BCL2: B cell lymphoma 2

BET: bromodomain and extra-terminal

DMEM: Dulbecco's Modified Eagle Medium

CI: combination index

HDF1314: Human dermal fibroblast 1314

IHC: Immunohistochemistry

RPMI: Roswell Park Memorial Institute

RPPA: Reverse phase protein array

Novelty and Impact

Inducing apoptosis in melanoma has proven difficult due to high expression of multiple anti-apoptotic proteins. We show combining BET and novel MCL1 inhibitors is highly effective at killing many melanoma cell lines by inducing a pro-apoptotic state while also targeting anti-apoptotic MCL1. This novel treatment strategy may benefit the large proportion of melanoma patients who fail current treatment with targeted therapies and/or immunotherapies.

Abstract

The treatment of melanoma has been markedly improved by the introduction of targeted therapies and checkpoint blockade immunotherapy. Unfortunately, resistance to these therapies remains a limitation. Novel anti-cancer therapeutics targeting the MCL1 anti-apoptotic protein have shown impressive responses in haematological cancers but are yet to be evaluated in melanoma. To assess the sensitivity of melanoma to new MCL1 inhibitors, we measured the response of 51 melanoma cell lines to the novel MCL1 inhibitor, S63845. Additionally, we assessed combination of this drug with inhibitors of the bromodomain and extra-terminal (BET) protein family of epigenetic readers, which we postulated would assist MCL1 inhibition by downregulating anti-apoptotic targets regulated by NF-kB such as BCLXL, BCL2A1 and XIAP, and by upregulating pro-apoptotic proteins including BIM and NOXA. Only 14% of melanoma cell lines showed sensitivity to S63845, however, combination of S63845 and I-BET151 induced highly synergistic apoptotic cell death in all melanoma lines tested and in an *in vivo* xenograft model. Cell death was dependent on caspases and BAX/BAK. Although the combination of drugs increased the BH3-only protein, BIM, and downregulated anti-apoptotic proteins such as BCL2A1, the importance of these proteins in inducing cell death varied between cell-lines. ABT-199 or ABT-263 inhibitors against BCL2 or BCL2 and BCLXL respectively, induced further cell death when combined with S63845 and I-BET151. The combination of MCL1 and BET inhibition appears to be a promising therapeutic approach for metastatic melanoma, and presents opportunities to add further BCL2 family inhibitors to overcome treatment resistance.

Introduction

The past few years have seen major advances in treatment of metastatic melanoma based on immunotherapy with immune checkpoint blockade[1, 2] and drugs that target mutated BRAF pathways[3, 4]. Both approaches, however, have limitations in that over a third of patients do not respond to immunotherapy and the majority of patients develop resistance to the targeted therapies via a diverse range of mechanisms including MAPK pathway reactivation and epigenetic reprogramming[5-8]. Therapies aimed at killing melanoma failing these first-line treatments remain an unmet need. In past studies, therapies aimed at inducing cell death in melanoma have been frustrated by its high expression of multiple anti-apoptotic proteins and the heterogeneity of this expression both between and within melanoma tumours.

The apoptotic pathway controlling death in melanoma have been well described[9]. In brief, the intrinsic apoptotic pathway is regulated by members of the B-cell lymphoma (BCL)2 protein family that can be subdivided into the pro-apoptotic "activator" BCL2 homology domain 3 (BH3)-only proteins (BIM, BIK, BAD, BID, HRK, BMF, NOXA and PUMA), which interact with and inhibit the anti-apoptotic family members (BCL2, BCLXL, BCLW, BCL2A1 and MCL1) that in turn functionally repress the pro-apoptotic effectors BAK and BAX. The latter initiate apoptosis by mediating cytochrome C release from the mitochondria[10]. Activation of pathways such as MEK/ERK, NF-kB and PI3K/AKT, hold apoptosis in check by shifting the balance towards anti-apoptotic proteins by transcriptional and post-translational mechanisms[11, 12]. Although there is some redundancy in the function of the anti-apoptotic proteins, a number of studies have pointed to the importance of MCL1 in inhibiting apoptosis in melanoma.

Previous immunohistochemical (IHC) studies showed that melanoma progression was associated with an increase in levels of MCL1 and BCLXL proteins and a reduction in levels of BCL2[13]. MCL1 is also critical for survival of melanoma cells undergoing endoplasmic reticulum stress[14] and confers drug resistance[15, 16].

MCL1 is the largest BCL2 family member due to a large N-terminal which contains a number of regulatory regions, including sites ubiquitinated by a number of E3 ligases such as MULE, which promotes its degradation by proteasomes[17]. It is bound by practically all BH3-only proteins but preferentially binds to NOXA[9, 18]. The transcription of MCL1 is altered by a range of factors and its promotor contains binding sites for NF-kB, STAT3 and cAMP responsive element binding protein (CREB)[18]. MCL1 can be regulated by the MEK/ERK pathway through ETS transcription factors[19], hence, inhibition of MEK can downregulate MCL1[20]. The MCL1 promoter is directly repressed by the E2F transcription factor[21].

Given the importance of MCL1 in melanoma survival, it has been the target of many attempts to develop inhibitors. The initial BH3-only mimetics like ABT-737 and ABT-263 (Navitoclax) were unable to inhibit binding of MCL1 to BH3-only proteins. As reviewed elsewhere[9], there are now 4 new agents (Servier: S63845, Amgen: AMG-176, AstraZeneca: AZD-5991 and Boehringer Ingelheim: VU661013) that show potential as inhibitors of MCL1 in pre-clinical and early clinical studies[22-26]. One of these, referred to as S63845, was found to have high specificity for MCL1 and to have cytotoxic activity against a number of haematological malignancies at dose levels that did not induce toxicity against normal tissues in mice[22].

In the present studies we have examined the potential effectiveness of S63845 against a large panel of melanoma lines. As melanomas are heterogeneous and may express a range of other proteins to protect them against MCL1 inhibition, we tested whether combination with an inhibitor of bromodomain and extra terminal domain (BET) proteins may increase its effectiveness. BET proteins are small family of epigenetic reader proteins that bind acetylated histone residues, as well as transcriptional regulators such as pTEFb – thereby connecting the histone landscape of a cell to transcription. BET proteins, especially BRD4, are increased in melanoma and alter the expression of many genes, including those involved in survival[27]. Inhibitors of this epigenetic reader protein family were selected for two reasons – the first being their ability to upregulate pro-apoptotic BIM, which pushes cells towards apoptosis[28]. Secondly, BET inhibitors are known to inhibit NF-kB[29], which is one of the known transcription factors regulating MCL1, XIAP[30], and BCL2A1[31, 32]. Importantly, BET inhibitors were shown to downregulate BCL2A1 proteins, which are not inhibited by BCL2, BCLXL and MCL1 inhibitors[33, 34].

Materials and Methods

Cell lines

The panel of human melanoma cell lines used in the drug screen (Supplementary Table 1) were generated by Professor Nicholas Hayward, QIMR Berghofer, Queensland. Cells were cultured in Roswell Park Memorial Institute Medium (RPMI) 1640 (Gibco, Thermofisher, North Ryde, NSW), supplemented with 10% fetal bovine serum (FBS) (Hyclone, GE Life Sciences, NSW, Australia). Human epithelial melanocytes (HEMn-MP) were from Thermofisher (North Ryde, NSW) and cultured in medium 254, supplemented with HGMS (Gibco, Thermofisher, North Ryde, NSW), while human dermal fibroblast (HDF1314) were from Prof Helen Rizos (Macquarie University, NSW, Australia) and cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, Thermofisher, North Ryde, NSW) supplemented with 10% FBS (AusGeneX, Molendinar, QLD). The use of these cell lines was approved by the Sydney Local Health District Human Research Ethics Committee and QIMR Berghofer Medical Research Institute Human Research Ethics Committee. All human cell lines have been authenticated using STR profiling within the last three years and all experiments were performed with mycoplasma-free cells.

Antibodies and Reagents

I-BET151 was purchased from Selleckchem (Houston, TX). S63845, AMG-176 and AZD-5991 were purchased from MedChemExpress (Monmouth Junction, NJ). The pan-caspase inhibitor, Q-VD-OPh (SM Biochemicals, Anaheim, CA), or the proteasome inhibitor, MG132 (Sigma-Aldrich, Castle Hill, NSW), was added 1 h before other additional treatment.

Antibodies used were purchased from Cell Signaling Technology [BIM (2933); caspase-3 (9668); caspase-8 (9746); caspase-9 (9502); PUMA (4976); BAX (2772)]; Santa Cruz Biotechnology [BCL2 (C-2); BAK (G-23); BCLXL (H-5); PARP (F-2); GAPDH (6C5)]; Sigma-Aldrich [β-Actin (AC-74)]; Imgenex [NOXA (114C307.1)] and BD [MCL1 (22/MCL1); XIAP (20/hILP/X)].

Cell drug sensitivity and viability assay

Cells were seeded into white 96-well plates at 4,000 cells per well and treated the next day with 7 different concentration of each drug ranging from 20 nM-20 mM, or carrier control. Biological duplicates were performed. After 72 h, cell viability was measured using CellTitre-Glo (Promega, Alexandria, NSW) reagent. Luminescence was detected using POLARstar Omega microplate reader (BMG Labtech, Mornington, VIC). Luminescence values were normalised to a percentage of controls cells. IC50 was calculated using djvMixedIC50 in R Studio.

For other cell viability assays, cells were seeding in 96-well plates and treated with DMSO, S63845, I-BET151 or combination of S63845 and I-BET151 at the indicated concentrations for 72 h and measured with CellTitre-Glo as above. Synergy of drug interactions was calculated using the fixed ratio/combination index (Chou-Talalay) method [35]. Combination index (CI) was calculated using CalcuSyn software Version 2.1 (Biosoft, Cambridge, UK).

Analysis of cell death and mitochondrial membrane potential

Apoptotic cells were quantified using Annexin-V/propidium iodide staining as described by the manufacturer (Becton Dickinson, Macquarie Park, NSW). Changes in mitochondrial membrane potential ($\Delta\Psi m$) were measured using the cationic dye, JC-1, according to the manufacturer's instructions (Molecular Probes, Eugene, OR). Flow cytometry was performed on a BD FACSCantoII (BD Biosciences, North Ryde, NSW) and analysed using FlowJo software (BD Biosciences, North Ryde, NSW).

Clonogenic Assay

Cells were seeded at 2,000 cells per well onto 6-well plates and allowed to attach and grow for 24 h followed by treatment. Cells were kept in culture for another 12 days, replenishing with fresh media every three days. On day of harvest, cells were fixed with methanol and stained with 0.5% crystal violet. The images were captured with ChemidocMP image system (Bio-Rad, Gladesville, NSW) and processed using Fiji software.

Western blotting

Western blot analysis was carried out as described previously[28]. Labelled bands were detected by Clarity horseradish peroxidase chemiluminescence kit (Bio-Rad, Gladesville, NSW), and images were captured with the ChemidocMP image system (Bio-Rad, Gladesville, NSW).

Reverse phase protein array (RPPA)

Protein lysates were prepared in CLB1 (cell lysis buffer 1) and RPPA was performed by the Victorian Centre for Functional Genomics (Australia). Lysates were arrayed on glass slides

using a GeSim Nanoplotter, probed with 124 antibodies and quantified using Zeptosens ZeptoReader. The results were global rank invariant normalised and log_2 transformed.

Lentiviral production and transduction

Lentiviral vectors pSIH-H1-coGFP, pSIH-H1-coGFP-shMCL1 #1, and pSIH-H1-coGFP-shMCL1 #2 were produced as described previously[36]. Lentiviral vectors pSIH-H1-copGFP-shBIM #1, pSIH-H1-copGFP-shBIM #3, pSIH-H1-copGFP-shNOXA #1, pSIH-H1-copGFP-shNOXA #3, pSIH-H1-copGFP-shBAX #2, pSIH-H1-copGFP-shBAX #3, pSIH-H1-copGFP-shBAK #2, pSIH-H1-copGFP-shBAK #3, pSIH-H1-copGFP-shBCL2A1 #1, pSIH-H1-copGFP-shBCL2A1 #3, pSIH-H1-copGFP-shXIAP #1, and pSIH-H1-copGFP-shXIAP #3 were constructed in-house (Supplementary Table 2). Vectors were transfected into HEK293T packaging cells using the calcium phosphate precipitation method as described previously[37]. Cells were transduced with virus in the presence of polybrene (8μg/ml) (Sigma-Aldrich, Castle Hill, NSW) by spinoculation at 800 g, 32°C for 90 min.

Quantitative reverse transcription and real-time PCR (qPCR)

Total RNA was isolated using RNeasy mini kit (Qiagen, Doncaster, VIC) and reverse transcribed into complementary DNA using qScript cDNA Supermix (Quantabio, Beverly, MA) following the manufacturer's instructions. qPCR was performed using the Stratagene Mx3005P system (San Diego, CA) with specific gene primers (BCL2A1 forward, 5'-GGC AGA AGA TGA CAG ACT GTG AAT TTG GAT ATA TT-3'; BCL2A1 reverse, 5'-CCT TTC TGG TCA ACA GTA TTG CTT CAG GAG AGA TAG-3'; 18S forward, 5'-GTA ACC

CGT TGA ACC CCA TT-3'; 18S reverse, 5'-CCA TCC AAT CGG TAG TAG CG-3'). The following PCR conditions were used: 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min using Power SYBR Green PCR mastermix (Thermo Fisher Scientific, North Ryde, NSW).

3D Spheroid cultures

The 3D melanoma spheroid model mimics *in vivo* tumour architecture and microenvironment and is used for investigating growth, invasion and viability of melanoma cells[7]. Spheroids were prepared by seeding 5,000 cells per well into ultra-low attachment plate and embedded into collagen 3 days later. After embedding, media was added on top of the collagen layer containing the indicated drugs (final concentration of drugs was 1 µM S63845 and 2 µM I-BET151). Following 3 days of treatment, spheroids were stained with Calcein AM/Ethidium Homodimer-1 and imaged under a Nikon eclipse Ti Microscope (Nikon, Rhodes, NSW) or dissociated by collagenase (Sigma-Aldrich, Castle Hill, NSW) digestion and stained with PI (Sigma-Aldrich, Castle Hill, NSW) and Annexin V (BD, Macquarie Park, NSW) to measure cell death by flow cytometry (BD, Macquarie Park, NSW). Images were processed by Fiji software.

In vivo experiments

All animal experiments were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and with approval from the NSW Sydney Local Health District Animal Ethics Committee (2019/014). $2x10^6$ of C002M were

subcutaneously injected into the right flank of male nod-scid gamma (NSG) mice. Once tumours reached to approximately 100 mm^3, mice were randomized into four groups of 8-10 mice per group and each group administered S63845 (30mg/kg, i.v., on days 2 and 5), I-BET151 (15mg/kg, i.p., qdx5), both drugs or the control solvent carrier (Figure 5f). S63845 was dissolved in 20% hydroxypropyl β-cyclodextrin in 20mM HCl and I-BET151 in 1% methyl cellulose. The palpable size of the tumour was measured by vernier callipers. Treatments were continued for 5 days and mice were monitored until ethical endpoint.

Statistical analysis

Graphs are presented as mean ± SEM and are representative of 2-4 replicates unless otherwise stated. Statistical significance between groups was determined in Graphpad Prism 8 using ANOVA with Sidak's multiple comparisons test, unless otherwise indicated. The values of p-values are indicated as ns = >0.05 (not significant); * <0.05; ** <0.01; *** <0.001.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Results

Combined S63845 and I-BET151 treatment synergistically kills human melanoma cells

We measured the sensitivity of a panel of 51 human melanoma cell lines to single-agent treatment with the MCL1 inhibitor, S63845. We also assessed sensitivity to the BET inhibitor I-BET151, as we hypothesised that BET inhibitors would increase the efficacy of MCL1 inhibition by targeting a number of other apoptotic proteins. The cell line panel exhibited a broad range of sensitivity to the drugs, and using previously published suggested cut-offs for drug sensitivity[22, 29, 38], only 14% of the cell lines were sensitive to S63845 (IC50<1 μM), and 39% were sensitive to I-BET151 (IC50<10 μM) (Figure 1a and Supplementary Table 1). The IC50 of either inhibitor was not correlated with mutation status (Supplementary Figure 1a) and the sensitivity to S63845 did not correlate to sensitivity to I-BET151 (Supplementary Figure 1b). There was poor correlation between MCL1 mRNA expression from RNA-seq analysis and sensitivity to S63845, indicating that MCL1 mRNA expression could not be used as biomarker to predict responses (Supplementary Figure 1c). Similarly, there was no correlation between BCL2, BCLW, BCLXL and BCL2A1 mRNA expression and sensitivity to S63845 (Supplementary Figures 1d-g). Nevertheless, our RPPA data showed that there was a positive correlation of sensitivity with BCLXL, β-catenin and phosphorylated c-Jun proteins, and negative correlation with phosphorylated SHP2 (Supplementary Figures 1h-n).

We investigated the effects of combining these two drugs in 11 melanoma cell lines which had a range of sensitivity to I-BET151, from very sensitive to very resistant as shown by the asterisks in Figure 1a. Combination index (CI) was calculated using the Chou and Talalay

method, where a value of less than 1 indicate synergy[35]. Despite the heterogeneity in response to the single drugs, combining S63845 and I-BET151 was synergistic in all melanoma lines tested (CI=0.2-0.8) (Table 1), but not in human fibroblasts. Strong synergy was observed in C002M and C065M, with a combination index of 0.2 and 0.3, respectively (Table 1 and Supplementary Figures 2a-b).

Three melanoma cell lines were selected from this screen (Table 1) to assess the kinetics of the single and combination drug treatment. In agreement with IC50 data Figure 1a, I-BET151 alone did not induce significant death in C065M at 2 μM but induced moderate cell death in C002M and C057M, only after 48 h or 72 h of treatment (Figure 1b and 1c). S63845 did not induce apoptosis in C002M or C065M and caused cell death of C057M with relatively rapid kinetics (24 h) (Figure 1b and 1c). Treatment with both drugs combined caused high levels of cell death (Figure 1b). The synergistic killing induced by the combination treatment peaked at 48 h or 72 h, suggesting the kinetics of the combination followed that of I-BET151, rather than the quicker action of S63845. Synergy was not specific to the inhibitors used as we obtained similar results when we combined S63845 with two other BET protein inhibitors, JQ-1 and OTX-015 (Figure 1d and Supplementary Figure 2c), or combined I-BET151 with two other MCL1 inhibitors, AMG-176 and AZD-5991 (Figure 1e and Supplementary Figure 2d).

Combined S63845 and I-BET151 induces caspase dependent death

We explored the mechanism of cell death induced by the combination of S63845 and I-BET151. As expected, activation of caspases appeared to be involved as inhibition of caspases with the

pan-caspase inhibitor, Q-VD-OPh, abrogated cell death in both C002M and C065M treated for 72 h with the combination, but to a lesser extent in C057M (Figure 1f). Further investigation of the C057M cell line showed caspase inhibition strongly reduced cell death at a shorter timepoint of 24 h, suggesting that the cell death mechanism transitioned from caspase-dependent to caspase-independent at 72 h as described in another study[39].

Apoptosis was associated with depolarisation of mitochondrial potential ($\Delta\Psi m$), which was not prevented by caspase inhibition (Supplementary Figure 2e). These results argued against mitochondrial depolarisation being driven by activation of caspases upstream of the mitochondrial apoptotic pathway, such as caspase-8, and was consistent with activation of BAX/BAK by members of the BCL2 family.

Changes in expression of caspases and BCL2 family proteins upon treatment

We performed western blotting to measure caspases in cell lines treated with S63845 and I-BET151 for 6, 24 or 48 h. The combination of S63845 and I-BET151 induced cleavage of caspase-8, caspase-3, caspase-9 and PARP (Figure 2a). The drugs also induced a number of changes in expression of BCL2 family proteins (Figure 2b). MCL1 was strongly upregulated in all cell lines treated with S63845 or the combination of S63845 and I-BET151. This was considered to be due to increased MCL1 protein stability resulting from binding of S63845 to MCL1, thereby inhibiting the binding of E3 ligase that targets MCL1 for degradation[17, 22, 40].

As reported previously, I-BET151 upregulated BIM and NOXA in all cell lines[28]. NOXA was also strongly reduced in all cell lines by S63845 treatment. This may be due to loss of stability provided by MCL1 and reflect increased proteasome activity[41]. BCLXL was very slightly decreased by I-BET151 treatment and BCL2 was not markedly changed by the drug treatments.

Involvement of BIM, NOXA, BAX, BAK and XIAP in apoptosis induced by the combination

To assess whether inhibition of MCL1 with S63845 was equivalent to reducing MCL1 protein levels, we knocked down MCL1 in C002M and C065M cells with shRNA and treated these cells with I-BET151. Cell death induced by the combination of S63845 and I-BET151 was comparable to MCL1 knockdown and I-BET151 treatment in C002M, however, this was not observed in C065M (Supplementary Figures 3a-b), suggesting S63845 may have additional effects other than just inhibiting MCL1.

To define which BH3-only protein may have been involved in induction of apoptosis, we carried out a number of knock down studies. We have reported previously that I-BET151 upregulates BIM and NOXA in melanoma cells[28]. Knockdown of these proteins by shRNA revealed that induction of cell death by the combination in C002M appeared partially dependent on BIM but not NOXA, whereas either knockdown of BIM or NOXA had no effects on apoptosis in C065M, suggesting other mechanisms were involved (Figure 3a-c).

It is known that BAX and BAK play important roles in intrinsic apoptotic pathways by formation of oligomeric pores in the mitochondrial outer membrane, resulting in the release of

cytochrome c and other initiators of apoptosis. Knockdown of BAX or BAK with shRNA partially inhibited apoptosis induced by the combination in the cell lines tested, consistent with their role as effectors (Figures 3a, d-e).

XIAP levels decreased with many of the treatments (Figure 2b), and these changes correlated with the level of apoptosis induced at that timepoint, suggesting that the changes in XIAP were the result of its cleavage by caspases[42]. However, we also investigated if changes in XIAP were also contributing to the induction of apoptosis by the combination. XIAP knockdown did not increase apoptosis in the control or drug treated C002M and C065M cells (Supplementary Figures 4a-b), suggesting that changes in XIAP we observed were more likely a consequence of cell death and were not a driver of cell death.

BCL2A1 suppression by BET inhibition contributes to death induced by the drug combination

BCL2A1 is a transcriptional target of NF-kB, which we previously reported could be inhibited by BET protein inhibitors[29]. To investigate the role of BCL2A1 in the observed cell death induced by the drugs, we first confirmed that I-BET151 inhibited mRNA expression of BCL2A1 (Figure 4a). Studies were carried out on mRNA as antibodies against human BCL2A1 were not satisfactory[43]. BCL2A1 knockdown only moderately increased apoptosis induced by S63845, indicating downregulation of BCL2A1 by I-BET151 contributes slightly to the mechanism of death, but is not the main driver (Figures 4b-c). Additionally, we observed an increased apoptosis induced by I-BET151 following BCL2A1 knockdown of C002M, but not in C065M.

Combined BCL2 family and MCL1 inhibition

To determine whether other BCL2 proteins were responsible for the resistance of C065M to S63845 or I-BET151, we tested them in combination with ABT-199 (Venetoclax), which inhibits BCL2, or with ABT-263 (Navitoclax), which inhibits both BCL2 and BCLXL[44]. When S63845 was added in combination with ABT-199, in C065M induction of cell death was comparable to the level when combining S63845 with I-BET151 (Figure 4d). However, when combining all three inhibitors (S63845, I-BET151 and ABT-199), cell death was almost complete. Similar results were observed using ABT-263 in place of ABT-199 (Figure 4d). These results suggest that BCL2, BCLXL and MCL1 play important roles in the survival of C065M. The triple combination was also highly effective against C002M and C057M lines (Supplementary Figure 4c).

Effectiveness of combinations of S63845 and I-BET151 against melanoma grown in 3D spheroids

Clonogeneic assays showed that the combination of S63845 and I-BET151 was superior to either single treatment alone (Figures 5a-b). The efficacy of S63845 and I-BET151 was then tested in 3D spheroids of C002M. Cells were grown into spheroids, embedded in collagen matrix and treated with S63845 and/or I-BET151 for 72 h. A halo of live cells can be seen in the control spheroids that invade the surrounding collagen. This is reduced in the spheroids treated with the combination of drugs or S63845 alone. Interestingly, spheroids that are treated with I-BET151 alone showed a greater invasion than the control (Figure 5c), suggesting that

BET proteins may play an anti-invasive role. We next stained these spheroids with Calcein-AM and Ethidium homodimer-1 to visualise the live and dead cells under a fluorescent microscope. However, due to poor dye penetration in collagen embedded spheroids, staining was done in floating spheroids. As expected, there was more dead (red) cells dissociated from the spheroids in the combination treated group compared to controls (Figure 5d). Analysing the floating spheroids by flow cytometry showed that spheroids treated with the combination of S63845 and I-BET151 had more dead cells compared to control or single drug treatment groups (Figure 5e).

To assess whether the drugs would limit melanoma growth *in vivo*, we established xenograft tumours of the C002M cell line in NSG mice and treated them with vehicle controls, S63845, I-BET151 or combination of S63845 and I-BET151. Previous experiments have shown as few as 1-3 doses of S63845 reduced the growth of some tumour models[22], so we used a minimal dosing schedule where I-BET151 was given for 5 days with S63845 given on days 2 and 5 (Figure 5f), as the kinetics of I-BET151 effect are slower than S63845 (Figure 1b, c). Following 5 days of treatment, tumour growth was monitored until ethical endpoints were reached (Figure 5a and Supplementary Figure 5). Mice treated with the combination had smaller tumours, with tumour volume ratios significantly lower than those treated with the vehicle controls or either drug alone (Figure 5g). S63845 alone did not reduce tumour growth, while I-BET151 treatment showed some reduction in growth, although this did not reach statistical significance. Individual drugs were well tolerated with little weight reduction in the mice, however some

initial weight reduction was observed in mice receiving combination treatment (Supplementary Figure 5c).

Discussion

Progression of melanoma and other cancers is known to be associated with development of pro-survival pathways that include resistance to apoptosis mediated by the BCL2 family[45, 46]. Newly developed inhibitors of the BCL2 family are proving effective against haematological malignancies but are yet to define a role against solid cancers[47, 48]. In the case of melanoma, practically all anti-apoptotic members appear to be involved but with varying importance in individual tumours. MCL1 has proven particularly important as it is upregulated by most of the known signalling pathways involved in progression and has not been inhibited by currently available inhibitors against BCL2 and BCLXL.

The present study was initiated to test whether newly available potent inhibitors of MCL1 may prove effective against melanoma. We first tested a large panel of 51 melanoma lines against the inhibitor as a single agent in the belief that MCL1 alone may be critical for the survival of a subset of lines. This revealed only 13% of the lines were sensitive to S63845 at concentrations less than 1 μM, which was the concentration cut-off for sensitivity in studies on human chronic myeloid leukemia (CML) lines[22] and breast carcinoma lines[23]. Similarly, poor responses against melanoma were reported in the initial studies on S63845[22].

In the CML study, sensitivity to S63845 was not associated with levels of MCL1 but was related to levels of BCLXL proteins[22]. In our study, although analysis from RNA-seq data revealed that the sensitivity to S63845 was not associated with levels of MCL1 or BCLXL, analysis from RPPA data showed that sensitivity was positively correlated with BCLXL

proteins expression, suggesting the importance of post-transcriptional modification and that protein expression may be a better predictive marker (Supplementary Figures 1k). However, high levels of heterogeneity between melanomas and redundancy in the function of anti-apoptotic BCL2 family members indicate targeting MCL1 alone is insufficient. This provides a strong basis for combinations of agents that target a range of apoptotic-related proteins in melanoma.

Given the evidence from these studies of the heterogeneity of anti-apoptotic mechanisms in melanoma we reasoned that inhibitors of BET proteins may target a number of anti-apoptotic proteins. In particular, BET inhibitors were reported to inhibit BCL2A1[31, 32], an anti-apoptotic protein that is not impeded by inhibitors of MCL1 or BCL2 inhibitors[34]. We have previously shown that the BET inhibitor, I-BET151, is a potent inhibitor of NF-kB, which is the main regulator of BCL2A1 and XIAP[29]. BET inhibitors also induce pro-apoptotic BIM in melanoma, which further pushes cells towards death, but does not always translate to high levels of cell death[28]. The initial screening studies confirmed that I-BET151 targeted a wider range of melanoma lines (Figure 1a) and there was very little cross sensitivity to the individual drugs (Supplementary Figure 1b). However, when combined, they induced synergistic death in all eleven melanoma lines tested.

More detailed studies on three melanoma cell lines (C002M, C057M and C065M) confirmed the marked heterogeneity in the apoptotic mechanism involved with the combination of both drugs. The BH3-only proteins involved in the death caused by the drug combination varied by

cell line, and targeting single proteins such as BIM or NOXA rarely completely inhibited the death induced by the drugs. While mitochondrial depolarisation, caspase activation and BAX/BAK were involved, other components of the apoptotic machinery had variable contributions.

As reported by others, we have also shown that MCL1 protein was stabilised by S63845 in melanoma cells (Figure 3b)[22]. Similarly, this was also observed in the combination of S63845 and I-BET151, though this increment was not sustained. It is postulated that MCL1 may be cleaved by the strong activation of caspases[49].

We initially hypothesised that I-BET151 might be acting via downregulation of XIAP that can inhibit caspase-3/7 activation, but I-BET151 treatment alone only slightly decreased XIAP levels and the marked decrease seen with the combination may reflect cleavage by activated caspases[42]. XIAP ablation by shRNA also did not increase cell death induced by S63845 alone, which suggested XIAP was not a major inhibitor of cell death in this setting.

Previous studies have suggested that growth of melanoma in spheroids mimics results obtained with mice xenografts[50]. It was found that the effects of S63845 and I-BET151 on the spheroids established from C002M was similar to the results from the 2D cultures (Figure 5e). In view of these results, we examined the effects of these drugs on xenografts of C002M in NSG mice. As expected, S63845 was ineffective as a single agent but the strong synergistic effects seen in 2D and 3D models were reproduced *in vivo*. Studies on xenografts of breast carcinoma lines

also found that S63845 was ineffective as a single agent and its effects on tumour growth was only apparent when given with chemotherapy[23]. Although I-BET151 alone showed a trend in the reduction of tumour growth (Supplementary Figures 5a-b and 5d), this did not reach statistical significance. We postulated that this may be due to regrowth of tumour once drugging has stopped. Given the excellent *in vitro* and *in vivo* results against melanoma, this combination could potentially be an alternative treatment strategy for melanoma patients.

We were also very interested into assessing possible toxicity of the drugs as specific knockout of MCL1 in animal models have shown that MCL1 is critical for survival of hematopoietic stem cells, lymphocytes and a variety of normal tissues (reviewed in[23]). Nevertheless, S63845 was well-tolerated as a single agent, as previously reported in other preclinical studies in mice[22]. The combination of S63845 and I-BET151 was tolerated in NSG mice, however, we did note an initial weight reduction. Further work may be needed to optimise dosing schedules and drug formulations to reduce toxicity. S63845 has lower affinity to mouse MCL1 compared to human MCL1[51], which should be considered when these drugs are translated to the human setting.

Another question is whether other combinations may extend the effectiveness of the MCL1 inhibitor. This was tested on the C065M line that did not show sensitivity to S63845 or I-BET151 as single agent. When S63845 was tested in combination with Venetoclax or Navitoclax, the results were similar to the S63845 and I-BET151 combination. However, when the latter were used in combination with Venetoclax or Navitoclax, the triple combination proved to be potent inducers of cell death. This is consistent with previous studies showing that

most cell lines depend on at least one combination of BCL2 protein inhibitors[34]. That study used CRISPR/Cas9 screens to show that BCL2A1 and BCLW were not inhibited by combinations of BCL2, BCLXL and MCL1 inhibitors and provides further support for our inclusion of I-BET151 in combinations tested in the present studies.

Taken together, this study on large numbers of melanoma lines have emphasised the heterogeneity of pro- and anti-apoptotic mechanisms in melanoma. Only a relatively small proportion of the lines were dependent on MCL1 for survival and we were unable to identify correlates of single MCL1 inhibitor responses. A much larger proportion of lines were sensitive to inhibitors of BET proteins which is not surprising considering that these drugs inhibit multiple anti-apoptotic proteins and upregulate pro-apoptotic BIM and NOXA proteins. The mechanisms engaged by I-BET151 varied again between the cell lines but the broad spectrum of changes induced by the drug particularly in combination with the MCL1 inhibitor, S63845 suggests this combination may be effective against a wide range of melanoma despite their heterogeneity. Responses to the MCL1 inhibitor and I-BET151 combination were further increased when Venetoclax against BCL2 or Navitoclax against both BCL2 and BCLXL were added. Combined inhibition of BET and MCL1 is a therapeutic strategy that could be applied to melanoma patients failing targeted and immunotherapy therapy, and has the potential to be extended by the addition of further BCL2 family inhibitors.

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Conflict of Interest

Professor Grant McArthur is an employee of the Peter MacCallum Cancer Centre, which receives reimbursement for the cost of clinical trials from Array Biopharma and Roche Genentech.

References

1. Ribas A, Hamid O, Daud A, Hodi FS, Wolchok JD, Kefford R, Joshua AM, Patnaik A, Hwu WJ, Weber JS, Gangadhar TC, Hersey P, et al. Association of Pembrolizumab With Tumor Response and Survival Among Patients With Advanced Melanoma. JAMA 2016;315:1600-9.

2. Hodi FS, Chiarion-Sileni V, Gonzalez R, Grob JJ, Rutkowski P, Cowey CL, Lao CD, Schadendorf D, Wagstaff J, Dummer R, Ferrucci PF, Smylie M, et al. Nivolumab plus ipilimumab or nivolumab alone versus ipilimumab alone in advanced melanoma (CheckMate 067): 4-year outcomes of a multicentre, randomised, phase 3 trial. Lancet Oncol 2018;19:1480-92.

3. Robert C, Grob JJ, Stroyakovskiy D, Karaszewska B, Hauschild A, Levchenko E, Chiarion Sileni V, Schachter J, Garbe C, Bondarenko I, Gogas H, Mandala M, et al. Five-Year Outcomes with Dabrafenib plus Trametinib in Metastatic Melanoma. N Engl J Med 2019.

4. Dummer R, Ascierto PA, Gogas HJ, Arance A, Mandala M, Liszkay G, Garbe C, Schadendorf D, Krajsova I, Gutzmer R, Chiarion-Sileni V, Dutriaux C, et al. Encorafenib plus binimetinib versus vemurafenib or encorafenib in patients with BRAF-mutant melanoma (COLUMBUS): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol 2018;19:603-15.

5. Hugo W, Shi H, Sun L, Piva M, Song C, Kong X, Moriceau G, Hong A, Dahlman KB, Johnson DB, Sosman JA, Ribas A, et al. Non-genomic and Immune Evolution of Melanoma Acquiring MAPKi Resistance. Cell 2015;162:1271-85.

6. Shaffer SM, Dunagin MC, Torborg SR, Torre EA, Emert B, Krepler C, Beqiri M, Sproesser K, Brafford PA, Xiao M, Eggan E, Anastopoulos IN, et al. Rare cell variability and drug-induced reprogramming as a mode of cancer drug resistance. Nature 2017;546:431-35.

7. Gallagher SJ, Gunatilake D, Beaumont KA, Sharp DM, Tiffen JC, Heinemann A, Weninger W, Haass NK, Wilmott JS, Madore J, Ferguson PM, Rizos H, et al. HDAC inhibitors restore BRAF-inhibitor sensitivity by altering PI3K and survival signalling in a subset of melanoma. Int J Cancer 2018;142:1926-37.

8. Emran AA, Chatterjee A, Rodger EJ, Tiffen JC, Gallagher SJ, Eccles MR, Hersey P. Targeting DNA Methylation and EZH2 Activity to Overcome Melanoma Resistance to Immunotherapy. Trends Immunol 2019;40:328-44.

9. Adams JM, Cory S. The BCL-2 arbiters of apoptosis and their growing role as cancer targets. Cell Death Differ 2018;25:27-36.

10. Hata AN, Engelman JA, Faber AC. The BCL2 Family: Key Mediators of the Apoptotic Response to Targeted Anticancer Therapeutics. Cancer Discov 2015;5:475-87.

11. Balmanno K, Cook SJ. Tumour cell survival signalling by the ERK1/2 pathway. Cell Death Differ 2009;16:368-77.

12. Hersey P, Zhang XD. Treatment combinations targeting apoptosis to improve immunotherapy of melanoma. Cancer Immunol Immunother 2009;58:1749-59.

13. Zhuang L, Lee CS, Scolyer RA, McCarthy SW, Zhang XD, Thompson JF, Hersey P. Mcl-1, Bcl-XL and Stat3 expression are associated with progression of melanoma whereas Bcl-2, AP-2 and MITF levels decrease during progression of melanoma. Mod Pathol 2007;20:416-26.

14. Jiang CC, Lucas K, Avery-Kiejda KA, Wade M, deBock CE, Thorne RF, Allen J, Hersey P, Zhang XD. Up-regulation of Mcl-1 is critical for survival of human melanoma cells upon endoplasmic reticulum stress. Cancer Res 2008;68:6708-17.

15. Tseng HY, Jiang CC, Croft A, Tay KH, Thorne RF, Yang F, Liu H, Hersey P, Zhang XD. Contrasting effects of nutlin-3 on TRAIL- and docetaxel-induced apoptosis due to upregulation of TRAIL-R2 and Mcl-1 in human melanoma cells. Mol Cancer Ther 2010;9:3363-74.

16. Lucas KM, Mohana-Kumaran N, Lau D, Zhang XD, Hersey P, Huang DC, Weninger W, Haass NK, Allen JD. Modulation of NOXA and MCL-1 as a strategy for sensitizing melanoma cells to the BH3-mimetic ABT-737. Clin Cancer Res 2012;18:783-95.

17. Zhong Q, Gao W, Du F, Wang X. Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. Cell 2005;121:1085-95.

18. Thomas LW, Lam C, Edwards SW. Mcl-1; the molecular regulation of protein function. FEBS Lett 2010;584:2981-9.

19. Dong L, Jiang CC, Thorne RF, Croft A, Yang F, Liu H, de Bock CE, Hersey P, Zhang XD. Ets-1 mediates upregulation of Mcl-1 downstream of XBP-1 in human melanoma cells upon ER stress. Oncogene 2011;30:3716-26.

20. Wang YF, Jiang CC, Kiejda KA, Gillespie S, Zhang XD, Hersey P. Apoptosis induction in human melanoma cells by inhibition of MEK is caspase-independent and mediated by the Bcl-2 family members PUMA, Bim, and Mcl-1. Clin Cancer Res 2007;13:4934-42.

21. Croxton R, Ma Y, Song L, Haura EB, Cress WD. Direct repression of the Mcl-1 promoter by E2F1. Oncogene 2002;21:1359-69.

22. Kotschy A, Szlavik Z, Murray J, Davidson J, Maragno AL, Le Toumelin-Braizat G, Chanrion M, Kelly GL, Gong JN, Moujalled DM, Bruno A, Csekei M, et al. The MCL1 inhibitor S63845 is tolerable and effective in diverse cancer models. Nature 2016;538:477-82.

23. Merino D, Whittle JR, Vaillant F, Serrano A, Gong JN, Giner G, Maragno AL, Chanrion M, Schneider E, Pal B, Li X, Dewson G, et al. Synergistic action of the MCL-1 inhibitor S63845 with current therapies in preclinical models of triple-negative and HER2-amplified breast cancer. Sci Transl Med 2017;9.

24. Caenepeel S, Brown SP, Belmontes B, Moody G, Keegan KS, Chui D, Whittington DA, Huang X, Poppe L, Cheng AC, Cardozo M, Houze J, et al. AMG 176, a Selective MCL1 Inhibitor, Is Effective in Hematologic Cancer Models Alone and in Combination with Established Therapies. Cancer Discov 2018;8:1582-97.

25. Tron AE, Belmonte MA, Adam A, Aquila BM, Boise LH, Chiarparin E, Cidado J, Embrey KJ, Gangl E, Gibbons FD, Gregory GP, Hargreaves D, et al. Discovery of Mcl-1-specific inhibitor AZD5991 and preclinical activity in multiple myeloma and acute myeloid leukemia. Nat Commun 2018;9:5341.

26. Ramsey HE, Fischer MA, Lee T, Gorska AE, Arrate MP, Fuller L, Boyd KL, Strickland SA, Sensintaffar J, Hogdal LJ, Ayers GD, Olejniczak ET, et al. A Novel MCL1 Inhibitor Combined with Venetoclax Rescues Venetoclax-Resistant Acute Myelogenous Leukemia. Cancer Discov 2018;8:1566-81.

27. Segura MF, Fontanals-Cirera B, Gaziel-Sovran A, Guijarro MV, Hanniford D, Zhang G, Gonzalez-Gomez P, Morante M, Jubierre L, Zhang W, Darvishian F, Ohlmeyer M, et al. BRD4 sustains melanoma proliferation and represents a new target for epigenetic therapy. Cancer Res 2013;73:6264-76.

28. Gallagher SJ, Mijatov B, Gunatilake D, Tiffen JC, Gowrishankar K, Jin L, Pupo GM, Cullinane C, Prinjha RK, Smithers N, McArthur GA, Rizos H, et al. The epigenetic regulator I-BET151 induces BIM-dependent apoptosis and cell cycle arrest of human melanoma cells. J Invest Dermatol 2014;134:2795-805.

29. Gallagher SJ, Mijatov B, Gunatilake D, Gowrishankar K, Tiffen J, James W, Jin L, Pupo G, Cullinane C, McArthur GA, Tummino PJ, Rizos H, et al. Control of NF-kB activity in human melanoma by bromodomain and extra-terminal protein inhibitor I-BET151. Pigment Cell Melanoma Res 2014;27:1126-37.

30. Oeckinghaus A, Ghosh S. The NF-kappaB family of transcription factors and its regulation. Cold Spring Harb Perspect Biol 2009;1:a000034.

31. Wang CY, Guttridge DC, Mayo MW, Baldwin AS, Jr. NF-kappaB induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. Mol Cell Biol 1999;19:5923-9.

32. Zong WX, Edelstein LC, Chen C, Bash J, Gelinas C. The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-kappaB that blocks TNFalpha-induced apoptosis. Genes Dev 1999;13:382-7.

33. Esteve-Arenys A, Valero JG, Chamorro-Jorganes A, Gonzalez D, Rodriguez V, Dlouhy I, Salaverria I, Campo E, Colomer D, Martinez A, Rymkiewicz G, Perez-Galan P, et

al. The BET bromodomain inhibitor CPI203 overcomes resistance to ABT-199 (venetoclax) by downregulation of BFL-1/A1 in in vitro and in vivo models of MYC+/BCL2+ double hit lymphoma. Oncogene 2018;37:1830-44.

34. Soderquist RS, Crawford L, Liu E, Lu M, Agarwal A, Anderson GR, Lin KH, Winter PS, Cakir M, Wood KC. Systematic mapping of BCL-2 gene dependencies in cancer reveals molecular determinants of BH3 mimetic sensitivity. Nat Commun 2018;9:3513.

35. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 1984;22:27-55.

36. Becker TM, Boyd SC, Mijatov B, Gowrishankar K, Snoyman S, Pupo GM, Scolyer RA, Mann GJ, Kefford RF, Zhang XD, Rizos H. Mutant B-RAF-Mcl-1 survival signaling depends on the STAT3 transcription factor. Oncogene 2014;33:1158-66.

37. Tiffen JC, Gunatilake D, Gallagher SJ, Gowrishankar K, Heinemann A, Cullinane C, Dutton-Regester K, Pupo GM, Strbenac D, Yang JY, Madore J, Mann GJ, et al. Targeting activating mutations of EZH2 leads to potent cell growth inhibition in human melanoma by derepression of tumor suppressor genes. Oncotarget 2015;6:27023-36.

38. Dawson MA, Prinjha RK, Dittmann A, Giotopoulos G, Bantscheff M, Chan WI, Robson SC, Chung CW, Hopf C, Savitski MM, Huthmacher C, Gudgin E, et al. Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. Nature 2011;478:529-33.

39. Samejima K, Tone S, Kottke TJ, Enari M, Sakahira H, Cooke CA, Durrieu F, Martins LM, Nagata S, Kaufmann SH, Earnshaw WC. Transition from caspase-dependent to

caspase-independent mechanisms at the onset of apoptotic execution. J Cell Biol 1998;143:225-39.

40. Warr MR, Acoca S, Liu Z, Germain M, Watson M, Blanchette M, Wing SS, Shore GC. BH3-ligand regulates access of MCL-1 to its E3 ligase. FEBS Lett 2005;579:5603-8.

41. Craxton A, Butterworth M, Harper N, Fairall L, Schwabe J, Ciechanover A, Cohen GM. NOXA, a sensor of proteasome integrity, is degraded by 26S proteasomes by an ubiquitin-independent pathway that is blocked by MCL-1. Cell Death Differ 2012;19:1424-34.

42. Hornle M, Peters N, Thayaparasingham B, Vorsmann H, Kashkar H, Kulms D. Caspase-3 cleaves XIAP in a positive feedback loop to sensitize melanoma cells to TRAIL-induced apoptosis. Oncogene 2011;30:575-87.

43. Hind CK, Carter MJ, Harris CL, Chan HT, James S, Cragg MS. Role of the pro-survival molecule Bfl-1 in melanoma. Int J Biochem Cell Biol 2015;59:94-102.

44. Timucin AC, Basaga H, Kutuk O. Selective targeting of antiapoptotic BCL-2 proteins in cancer. Med Res Rev 2019;39:146-75.

45. Chen DS, Mellman I. Elements of cancer immunity and the cancer-immune set point. Nature 2017;541:321-30.

46. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646-74.

47. Yamaguchi R, Lartigue L, Perkins G. Targeting Mcl-1 and other Bcl-2 family member proteins in cancer therapy. Pharmacol Ther 2019;195:13-20.

48. Korycka-Wolowiec A, Wolowiec D, Kubiak-Mlonka A, Robak T. Venetoclax in the treatment of chronic lymphocytic leukemia. Expert Opin Drug Metab Toxicol 2019;15:353-66.

49. Herrant M, Jacquel A, Marchetti S, Belhacene N, Colosetti P, Luciano F, Auberger P. Cleavage of Mcl-1 by caspases impaired its ability to counteract Bim-induced apoptosis. Oncogene 2004;23:7863-73.

50. Beaumont KA, Mohana-Kumaran N, Haass NK. Modeling Melanoma In Vitro and In Vivo. Healthcare (Basel) 2013;2:27-46.

51. Brennan MS, Chang C, Tai L, Lessene G, Strasser A, Dewson G, Kelly GL, Herold MJ. Humanized Mcl-1 mice enable accurate preclinical evaluation of MCL-1 inhibitors destined for clinical use. Blood 2018;132:1573-83.

Figure Legends

Figure 1. Combination of S63845 and I-BET151 synergistically kills a subset of human melanoma cells. (a) Heatmap of IC50 $\log_{10}$ I-BET151 or S63845 drug concentrations in a panel of melanoma cell lines. *n* = 4. (b) C002M (top), C057M (middle) and C065M (bottom) were treated with the MCL1 inhibitor (S63845, 1 μM) and/or BET inhibitor (I-BET151, 2 μM) for the indicated period of time and cell death was measured by Annexin V staining; early apoptosis: Annexin V positive, late apoptosis: Annexin V and PI positive. *n* = 2-4. Total cell death (early apoptosis + late apoptosis) from all treatment groups were compared to their respective DMSO control group within the same timepoint, **p < 0.01; ***p < 0.001. (c) C002M (top), C057M (middle) and C065M (bottom) were treated with the MCL1 inhibitor (S63845, 1 μM) and/or BET inhibitor (I-BET151, 2 μM) for the indicated period of time and cell viability measured by CellTiter-Glo. *n* = 2-6. All treatment groups were compared to their respective DMSO control group within the same timepoint, *p < 0.05; ***p < 0.001. (d) C002M and C057M were treated with the MCL1 inhibitor (S63845, 1 μM) and/or BET inhibitor (JQ-1, 1 μM) for 72 h and cell death was measured by Annexin V staining. n = 4. All treatment groups were compared to their respective DMSO control group, ***p < 0.001. (e) C002M and C057M were treated with the MCL1 inhibitor (AMG-176, 5 μM) and/or BET inhibitor (I-BET151, 2 μM) for 72h and cell death was measured by Annexin V staining. n = 4. ***p < 0.001. (f) C002M (left), C057M (middle) and C065M (right) were pre-treated with Q-VD-OPh (10 μM) for 1 h followed by S63845 (1 μM) and/or I-BET151 (2 μM) for 72 h. Additionally, C057M was also pre-treated with Q-VD-OPh (10 μM) for 1 h followed by

S63845 (1 μM) for 24 h. Cell death was measured by Annexin V staining. *n* = 2-4. ***p < 0.001.

Figure 2. Changes in the expression of caspases and BCL2 family proteins. (a) Representative western blot images from three independent experiments of PARP and caspases in C002M, C057M and C065M. (b) Representative western blot images from three independent experiments of BCL2 family proteins in C002M, C057M and C065M.

Figure 3. Apoptosis induced by the combination is independent of BIM or NOXA but dependent on BAX, BAK. (a) C002M (top) and C065M (bottom) were transduced with the indicated lentiviral particles followed by treating with S63845 (1 μM) and/or I-BET151 (2 μM) for 72 h. Cell death was measured by Annexin V staining in the GFP positive population. *n* = 4. All treatment groups in the knockdown cells were compared to the respective treatment in cells transduced with shcon, *p < 0.05; **p < 0.01; ***p < 0.001. (b) Representative western blot images from three independent experiments of BIM knockdown in C002M and C065M. (c) Representative western blot images from three independent experiments of NOXA knockdown in C002M and C065M. (d) Representative western blot images from three independent experiments of BAX knockdown in C002M and C065M. (e) Representative western blot images from three independent experiments of BAK knockdown in C002M and C065M.

Figure 4. BCL2A1 suppression by BET inhibition moderately contributes to death induced by the drug combination. (a) BCL2A1 mRNA expression normalised to 18S in C002M and C065M after treating with S63845 (1 μM) and/or I-BET151 (2 μM) for 24 h. *n* = 4-6. (b) C002M (left) and C065M (right) were transduced with shBCL2A1 lentiviral particles followed by treating with S63845 (1 μM) and/or I-BET151 (2 μM) for 72 h. Cell death was measured by Annexin V staining in the GFP positive population. *n* = 4. (c) Validation of BCL2A1 knockdown in C002M and C065M by BCL2A1 mRNA expression measurement. *n* = 2-3. (d) C065M was treated with the MCL1 inhibitor (S63845, 1 μM), the BET inhibitor (I-BET151, 1 μM), and/or the BCL2 inhibitors (ABT-199 or ABT-263, 1 μM) for 72 h and cell viability measured by Annexin V staining. *n* = 4. All treatment groups were compared to the DMSO control group, *p < 0.05; **p < 0.01; ***p < 0.001.

Figure 5. Effectiveness of combined S63845 and I-BET151 against melanoma grown in spheroids and *in vivo*. (a) Representative images of clonogenic assays of C002M, C057M and C065M. Cells were treated with the MCL1 inhibitor (S63845, 1 μM) and/or BET inhibitor (I-BET151, 2 μM) for 72 h with media changed every three days thereafter. Images of crystal violet stained colonies taken at 12-15 days. *n* = 2. (b) Colony counts of (a) using Fiji software. All treatment groups were compared to their respective DMSO control group *p < 0.05; **p < 0.01; ***p < 0.001. (c) Representative brightfield images of collagen embedded spheroids of C002M after treatment for 72h with S63845 (1 μM) and/or I-BET151 (2 μM). All images were acquired at 4x magnification with scale bars representing 500 μm. *n* = 4-6 spheroids per treatment. (d) Representative images of floating spheroids of C002M stained with Calcein-AM

(2 μM) and Ethidium Homodimer-1 (4 μM) after treatment for 72h with S63845 (1 μM) and/or I-BET151 (2 μM). All images were acquired at 4x magnification with scale bars representing 500 μm. *n* = 4-6 spheroids per treatment. (e) Following treatment, C002M spheroids within each treatment were pooled and cell death was measured by flow cytometry. Monolayer cultured cells were performed in parallel for comparison. *n* = 2. All treatment groups were compared to the DMSO control group, **p < 0.01; ***p < 0.001. (f) Dosing schedule for *in vivo* model. (g) Mean tumour volume ratio of C002M xenografted in NSG mice treated with vehicle control, S63845 and/or I-BET151. Tumour volume ratio was calculated by normalising tumour volume to the first day of treatment. All treatment groups were compared to the vehicle control group, *p < 0.05.