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Selective BCL-X_L inhibition promotes apoptosis in combination with MLN8237 in medulloblastoma and pediatric glioblastoma cells

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Running title: BH3-mimetics/MLN8237 in pediatric brain tumors

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Abstract

Background: Central nervous system tumors (CNS), including medulloblastoma and pediatric GBM (pGBM) account for the majority of solid pediatric malignancies. There remains an unmet need to identify novel treatment approaches in poor prognosis and relapsed pediatric brain tumors, where therapeutic options are limited. Small-molecule BCL-2/BCL-X_L inhibitors may enhance tumour cell killing when combined with conventional and targeted chemotherapeutic agents. We investigated the effect of disrupting BCL-2/BCL-X_L protein function using ABT-263, ABT-199 and WEHI-539 in medulloblastoma and pGBM cells following treatment with MLN8237, an Aurora kinase inhibitor under investigation as a novel agent for the treatment of malignant brain tumors

Methods: Tumor cell growth and viability was determined by MTT/WST-1 assays and flow cytometry. Effects on cell phenotype, cell cycle progression and ploidy were determined by live cell imaging and DNA content analysis. Apoptosis was determined by Annexin V/PI staining and time-lapse microscopy, and confirmed by measuring caspase-3/7 activity, western blotting, and by siRNA-knockdown.

Results: ABT-263, in combination with MLN8237, reduced mitotic slippage and polyploidy, and promoted the elimination of mitotically defective cells via a BAX/BAK-dependent, caspase-mediated apoptotic pathway. The BCL-X_L antagonist, WEHI-539, significantly augmented tumor cell killing when used in combination with MLN8237, and also sensitized resistant brain tumor cells to a novel BAX activator, SMBA1. In addition, siRNA mediated knockdown of BCL-X_L sensitized pGBM and medulloblastoma cells to MLN8237, and mimicked the effect of combination drug treatment.

Conclusions: Selective small-molecule inhibitors of BCL-X_L may enhance the efficacy of MLN8237, and other targeted chemotherapeutic agents.

Importance of the study

Medulloblastoma and pGBM account for the majority of brain tumor related deaths in children and present a significant challenge for conventional multimodal therapy. Although targeted therapies represent an important approach to improving outcomes for children suffering from these aggressive brain tumors, drug resistance remains a significant problem, and contributes to tumor recurrence after initial treatment. Using ABT-263, ABT-199 and WEHI-539, we identify BCL-X_L as a candidate drug target. Ours is the first study in pediatric brain tumors to demonstrate that the BCL-X_L antagonist, WEHI-539, can promote apoptosis when used in combination with other chemotherapeutics, including MLN8237, an Aurora kinase inhibitor currently being tested in clinical studies for the treatment of CNS tumors, and a novel BAX agonist, previously untested in brain tumor models. Such therapeutic combinations represent novel, targeted treatments that could improve the clinical management of these aggressive, and therapeutically challenging, brain tumors.

Key words: Brain tumors, apoptosis, MLN8237, ABT-263, WEHI-539.

Introduction

Medulloblastoma is the most prevalent (type of) brain tumor in the pediatric population,[1] and can be classified into at least four subtypes (WNT, SHH, group 3 and group 4) based on their distinct molecular biology.[2] In children over 3 years old,

effective treatment consists of surgical resection, irradiation and chemotherapy.[3] Survival rates for patients classified as having average-risk disease (total surgical resection, non-metastatic, non-*MYC/MYCN* amplified tumours; *TP53*-wild type SHH, Group 3, and non-metastatic Group 4; without chromosome 11 loss) are greater than 75%.[4] However, effective treatment options for high-risk patients with poor prognosis tumors are limited, and current therapies lack target specificity and are associated with significant neurocognitive sequelae and psychosocial deficits.[5,6] Tumor recurrence following conventional treatment remains a significant problem and the majority of patients with relapsed medulloblastoma succumb to the disease.[7] Other high grade tumors including glioblastoma, are rarer in children and adolescents (<3.0% of CNS tumors).[8] However, the outlook for these patients remains poor (5 year survival: ~18%)[9], and is partly due to the intrinsic resistance of these tumors to radiation and chemotherapy. The clinical management of aggressive brain tumors in children remains a significant challenge and there is a need for novel, efficacious therapies directed against defined molecular targets.

BCL-2 family proteins containing only the BH3 (BCL-2 homology 3) domain are inducers of apoptosis, and include BAD, which promotes apoptosis via selective binding of anti-apoptotic BCL-2, BCL-X_L and BCL-w.[10] Navitoclax (ABT-263), is a BAD-like BH3-mimetic and inhibits BCL-2, BCL-X_L and BCL-w, but not MCL-1.[11] Other selective BCL-2 and BCL-X_L inhibitors have been developed and, when used in combination with chemotherapy, may help to elucidate the roles of BCL-2 and BCL-X_L in drug response. ABT-199 (Venetoclax/GCD-0199) selectively inhibits BCL-2,[12] and WEHI-539, a small-molecule inhibitor of BCL-X_L has been described.[13] This agent has also been shown to sensitize tumor-initiating, colon cancer stem-cell like cells to a variety of chemotherapeutic agents.[14] The combination of BCL-2/BCL- X_L

inhibitors with other targeted therapeutics may represent an efficient way to selectively kill brain tumor cells and could lead to a more durable response than single agent treatment. Aurora kinase A (AURKA) and B (AURKB) regulate cell cycle events from G_2 phase to cytokinesis, and have emerged as attractive drug targets in medulloblastoma and pGBM.[15-17] MLN8237 (Alisertib) an Aurora kinase inhibitor[18] has shown *in vitro* and *in vivo* activity in pre-clinical pediatric cancer models.[19] Orally administered MLN8237 was tolerated by children with relapsed/refractory solid tumors in a phase I pediatric study,[20] and has been evaluated as a single agent in pediatric patients with recurrent atypical teratoid/rhabdoid tumors (AT/RT).[21] However, the factors contributing to drug response and the mechanisms involved in tumor cell elimination following disruption of mitosis are not fully understood.

In medulloblastoma and pGBM cells, we demonstrate that ABT-263 significantly decreased cell survival, increased caspase-mediated apoptosis, and decreased mitotic exit following mitotic disruption by MLN8237. Combined WEHI-539/MLN8237 treatment revealed that ABT-263 increases sensitivity to MLN8237 via a mechanism of BCL-X_L inhibition. In addition, we show that WEHI-539 augments tumor cell killing in resistant pGBM cells when combined with a newly developed BAX agonist.[22] Our findings confirm BCL- X_L as a relevant anti-apoptotic target in pediatric brain tumor cells which may have implications in the context of targeted combination therapy for these aggressive tumors.

Materials and methods

Cell Culture

pGBM cell lines SF188 (H3F3A wild-type) and KNS42 (H3F3A G34V-mutant) were donated by C. Jones (Institute of Cancer Research, London, United

Kingdom), and cultured in DMEM (Sigma-Aldrich) containing 10% (v/v) fetal bovine serum (FBS). DAOY (SHH MB)[23] and D283 (Group 3 MB)[24], were purchased from American Type Culture Collection (ATCC) and cultured in Improved MEM Zinc Option (Gibco, Life Technologies) supplemented with 10% (v/v) FBS, 1% (v/v) NEAA (Sigma-Aldrich) and 0.002% Phenol Red (Sigma-Aldrich). The identity of cell lines was confirmed by short tandem repeat profiling and all were cultured at 37ºC in 5% CO_2.

Chemicals

All chemicals were purchased from Sigma Aldrich unless otherwise stated. Stock solutions of MLN8237, ABT-263, ABT-199 (Selleckchem), WEHI-539 (Bioquote) and z-VAD-FMK (BD) were prepared in DMSO. SMBA1[22] was donated by J. Zhou (University of Texas Medical Branch, Galveston, Texas, United States).

Cell Viability Assays

Cells were seeded in triplicate prior to treatment. Viability was determined by MTT assay for adherent cell lines (SF188, DAOY, KNS42) and by WST-1 for the semi-adherent D283 cell line, as per the manufacturers' instructions.

Analysis of Cell Death

Membrane integrity was determined by flow cytometric quantification of propidium iodide uptake. Cells were seeded in triplicate and after treatment were harvested and washed with ice-cold PBS containing sodium azide (0.1% v/v). Cell pellets were re-suspended in PBS/sodium azide solution containing propidium iodide (0.83 mg/ml). Apoptosis was quantified using the FITC Annexin V apoptosis detection kit (BD Pharmingen) according to the manufacturers' instructions. Cell

death was analyzed using an Attune® Acoustic focusing cytometer (Applied Biosystems) and Attune® Cytometric Software.

Cell cycle analysis

Treated cells and supernatants were harvested and pelleted before fixation with ice-cold 70% (v/v) ethanol/PBS. Cells were stained in FACS buffer (PBS + 0.1% (w/v) BSA and 0.1% (v/v) TWEEN-20) containing 20µg/ml PI and 200µg RNaseA. Cells were analyzed using an Attune® Acoustic focusing cytometer (Applied Biosystems) and ModFit LT™ software (Verity Software House).

Live cell imaging

Cells were seeded at sub-confluent densities in imaging dishes (Ibidi, Germany), and placed in a Nikon BioStation IM CELL-S1 microscopy system (Nikon UK Ltd, UK). Phase contrast images (x20) were captured every 3 minutes for 72h. At least 50 mitotic cells were analyzed/condition and data was processed for the following parameters: total time in mitosis, completed/uncompleted mitosis, mitotic slippage and cell death during mitosis.

Apoptosis was monitored in real-time by time-lapse microscopy, using a reversible, polarity sensitive Annexin-V probe (pSIVA-IANBD) according to the manufacturers' instructions (Abcam). Live cell imaging was performed using the IncuCyte™ zoom live cell imaging system (Essen Bioscience). Whole well scans (x4), and higher resolution scans (x20) over multiple fields of view were performed every 30 minutes (for 72h) and pSIVA-IANBD fluorescence was measured using conventional green filter sets (excitation maximum 488 nm and emission maximum 530 nm).

Quantification of Caspase 3/7 Activity

Caspase 3/7 activity was assayed using the Caspase-Glo® assay (Promega) according to the manufacturers' instructions. Luminescence was measured using a Mithras LB 940 multimode plate reader.

Western Blotting

Cell lysates were prepared and separated by SDS-PAGE, and probed with antibodies against PARP, Lamin A/C, BCL-X_L, BCL-2, BCL-w, BAX, BAK, Bid, Puma, Bad, p-Bad (Ser112), Bik, Bim, phospho-Aurora A (Thr288)/Phospho-Aurora B (Thr232)/Phospho-Aurora C (Thr198), and total Aurora A and B (Cell Signalling Technologies). Antibody binding was detected using the Odyssey imaging system (LI-COR Biosciences) or using the Supersignal West Pico Mouse and Rabbit IgG detections kits (Life Technologies). Equal loading was confirmed using a β-actin antibody (Sigma-Aldrich).

RNA interference (RNAi) studies

On Target plus Smartpool^{TM} siRNAs were purchased from Thermo Scientific. Cells were transfected with 75nM of BCL-X_L, BCL-2, and BAX or BAK siRNA alone or in combination. A control siRNA was included. Transfections were carried out in Optimem (Gibco, Life Technologies) using Lipofectamine RNAiMax (Invitrogen) for 6–7h. Media were replenished with serum and supplements and cells cultured for 48h. Cells were treated with either MLN8237/ABT-263/ABT-199/WEHI-539 alone or in combination. Lysates were prepared in parallel and subjected to SDS-PAGE and membranes probed to confirm successful target knockdown.

Statistical Analysis

SPSS (IBM) was used to analyze quantitative data from independent experiments. Statistical significance between multiple groups was determined by Kruskal-Wallis ANOVA tests which were followed by Bonferroni-corrected Mann-Whitney U tests for paired comparisons.

Results

ABT-263 sensitizes paediatric brain tumor cells to MLN8237

We observed detectable levels of Aurora A and B in two medulloblastoma (DAOY, D283) and pGBM (SF188, KN42) cell lines under normal culture conditions, and in the presence of the proteasome inhibitor MG132, and confirmed the inhibitory effect of MLN8237 on auto-phosphorylation of Aurora A (Thr288) and Aurora B (Thr232)[18] (Fig.1A-B). DAOY has been shown to be representative of SHH MB[23] and the mitotic kinases have been identified as drug targets in this disease subtype.[16] KNS42 cells feature mutant *H3F3A* (G34V) which encodes histone H3.3,[17,25, 27] and occurs in ~15% of cerebral hemispheric high grade gliomas in adolescents and young adults,[27] and has been associated with transcriptional upregulation of multiple genes, including *MYCN.*[17] We used these cell lines as *in vitro* models of medulloblastoma and pGBM to assess the effect of MLN8237 on cell cycle progression (Fig.1C). Exposure of DAOY cells to 0.1μM MLN8237 for 24h induced a significant G2/M accumulation (*$P \leq 0.05$, vehicle versus 0.1μM MLN8237). We also observed a significant increase in the proportion of cells exhibiting 8N DNA content after treatment with 0.5-2.5μM MLN8237 (*$P \leq 0.05$, 0.1μM versus 0.5μM MLN8237). By 48h, the proportion of polyploid (>4N) cells had significantly increased to over 60% suggesting that higher concentrations of MLN8237 induced mitotic slippage

(**$P \leq 0.01$, 24 versus 48h 0.5µM MLN8237). Similar results were observed in the KNS42 cell line. However, whilst treatment with 0.5µM MLN8237 induced a pronounced G2/M delay and a significant increase in cells with 4N DNA content (13.0% ± 1.3% to 54.0% ± 3.8%, compared with vehicle, **$P \leq 0.01$) only 21% ± 3.2% were polyploid after 72h, compared with DAOY cells.

Sustained mitotic arrest and induction of polyploidy can increase the susceptibility of various cancer cell lines to the BCL-2/BCL-X_L targeting drug, ABT-263.[28-31] Based on our cell cycle data, we reasoned that the efficacy of MLN8237 may be improved by combination with ABT-263. Cell lines exhibited differential expression of BCL-2 family members and when cultured with ABT-263, and the selective BCL-2 and BCL-X_L antagonists, ABT-199 and WEHI-539, we found that ABT-263 was the most effective in reducing cell survival, with D283 cells (MCL-1 deficient), being most sensitive (Supplementary Fig.S1). We found that high concentrations of MLN8237 (>0.1µM) were required to attenuate tumor cell growth in vitro (Fig.2A). We then examined whether the efficacy of MLN8237 could be enhanced by lowering the apoptotic threshold with ABT-263. We used a sub-lethal concentration of ABT-263 (1µM) in combination with MLN8237, which reduced the IC_{50} for MLN8237 after 72h of treatment (KNS42; >2.5µM versus 138nM, DAOY; 1.1µM versus 75.8nM, Fig.2A), with maximal inhibition of tumor cell growth observed with 0.5µM MLN8237 and 1µM ABT-263 (KNS42; 21.2% ± 2.1% viability, DAOY; 5.7% ± 1.5% viability, **$P \leq 0.01$ combination compared with MLN8237 alone). To determine whether the drug combination was cytotoxic, we quantified apoptosis by Annexin V/PI staining (Fig.2B and Supplementary Fig.S2B) and monitored the effect of drug treatment on Annexin V exposure by time-lapse microscopy (videos S1-S2). Compared with MLN8237 treatment alone, the

combination of ABT-263 with MLN8237 significantly increased apoptosis in KNS42 and DAOY cells (Fig.2B). We obtained similar results in SF188 and D283 cells (Supplementary Fig.S2A). Next, we investigated the mechanism of ABT-263 and MLN8237 cytotoxicity in KNS42 and DAOY cells using the general caspase inhibitor, zVAD-fmk, and by measuring caspase-3/7 activity and PARP and Lamin A/C cleavage (Fig.2B-C). Inhibition of caspase activity with zVAD-fmk decreased apoptosis to levels observed in vehicle controls (Fig.2B and Supplementary Fig.S2B) and partially restored cell viability in KNS42 and DAOY cells (Supplementary Fig.6C). The combination of ABT-263 and MLN8237 led to a 2.5-fold increase in caspase-3/7 activity in KNS42 and DAOY cells, compared with MLN8237 or ABT-263 alone, and was accompanied by increased cleavage of PARP and Lamin A/C (Fig.2C). Taken together, these results confirm that ABT-263 promotes caspase-dependent apoptosis following MLN8237 treatment.

ABT-263 promotes caspase-dependent cell death following disruption of cell division by MLN8237

In order to determine whether ABT-263 promoted caspase-dependent cell death during MLN8237-induced mitotic disruption, we examined the effect of drug treatment on cell cycle progression, and on cell phenotype via time-lapse microscopy (Fig.3A-C and Supplementary Fig.S3). In KNS42 cells, DNA content analysis revealed that in comparison to MLN8237 treatment alone, combination of ABT-263 and 0.5μM MLN8237 led to a small but significant increase in the proportion of cells with 4N DNA content (53.5% ± 3.8% to 71.0% ± 2.9%, $*P \leq 0.05$), and a decrease in the proportion of cells with 8N DNA content (21.1% ± 3.2% to 8.2% ± 1.3%, $*P \leq 0.05$). Similar effects were observed in the DAOY cell line which was more

prone to polyploidization. The ABT-263/MLN8237 combination significantly decreased the proportion of cells with 8N DNA content (63.1% ± 1.3% to 42.7% ± 5.7%), and increased the proportion of cells with 4N DNA content (7.3% ± 0.7% to 29.7% ± 4.9%) compared with MLN8237 alone (*P≤0.05, 0.5µM MLN8237 versus 0.5µM MLN8237/1µM ABT-263). Time-lapse microscopy confirmed that vehicle or ABT-263 (1µM) treated cultures underwent normal mitosis (Supplementary Fig.3 and videos S3-S4). KNS42 and DAOY cells, treated with 0.5µM MLN8237 alone, showed clear evidence of protracted and defective mitoses, with the majority of cells exiting mitosis without division. However, ABT-263 addition decreased mitotic exit following cell division failure during MLN8237 treatment, increased cell elimination and reduced polyploidization (Fig.3B-C).

Selective inhibition of BCL-X_L increases cell death in combination with targeted chemotherapeutic agents

To assess the specific roles of BCL-2 and BCL-X_L in promoting cell survival, we used ABT-199 and WEHI-539 in combination with MLN8237 (Fig.4A and Supplementary Fig.4). In KNS42 and DAOY cells, WEHI-539 (BCL-X_L selective) induced apoptosis as effectively as ABT-263 when used in combination with MLN8237. These results were reproducible in the SF188 and D283 cell lines, where combination of MLN8237 with WEHI-539, but not ABT-199, significantly increased cell death and mimicked the effect observed with ABT-263 and MLN8237 (Supplementary Fig.5). In order to address the functional role of BCL-X_L in the response to MLN8237 treatment, we used siRNA to silence BCL-X_L expression in KNS42 and DAOY cells (Fig.4B). Knockdown of BCL-X_L significantly increased cell death following treatment with MLN8237 and levels of cell death were

comparable to those observed with MLN8237/ABT-263 or WEHI-539 (Fig.4B). These data indicate that MLN8237 renders tumor cells increasingly dependent on BCL-X_L for survival.

To further investigate whether BCL-X_L contributes to chemotherapy resistance, we examined whether WEHI-539 could sensitize tumor cells to other chemotherapeutic agents and to a novel small molecule BAX activator, SMBA1[22] (Fig.5). All cell lines expressed comparable levels of BAX protein (Supplementary Fig.S1A), but exhibited differential sensitivity to the BAX agonist (Fig.5A). In DAOY cells, we observed a significant increase in cell death following treatment with SMBA1 compared with vehicle controls (34.1% ± 2.7% - 52.1% ± 2.6%, 10-20µM of SMBA1, $^{**}P \leq 0.01$). By contrast, KNS42 cells showed no significant increase in cell death indicating that the observed decrease in viability was due to cytostatic rather than cytotoxic effects. Next, we examined the effect of combining ABT-263, ABT-199 and WEHI-539 with SMBA1 in KNS42 cells (Fig.5C). Addition of 1µM ABT-263 or WEHI-539, but not ABT-199, sensitized KNS42 cells to SMBA1 and significantly increased cell death, indicating that resistance to the BAX agonist was mediated by BCL-X_L rather than BCL-2. We also investigated the use of these compounds in combination with the DNA damaging agent, cisplatin (Fig.5D). Our data show that WEHI-539 and ABT-263 potentiated cell death when used in combination with cisplatin in the DAOY cell line. Together, these data indicate that selective targeting of BCL-X_L could increase the sensitivity of brain tumor cells to chemotherapeutic agents.

MLN8237 and WEHI-539 cytotoxicity is attenuated by caspase inhibition and requires BAX/BAK

Our data indicated that ABT-263 promoted caspase-dependent apoptosis following MLN8237 treatment. Similarly, we found that caspase inhibition significantly reduced cell death and increased viability following WEHI-539/MLN8237 treatment (Fig. 6A and Supplementary Fig.S6A-C). Next, we examined the role of the mitochondrial death effectors, BAX and BAK (Fig.6B-C). Immunoblotting confirmed knockdown of BAX and BAK (Fig.6B and Supplementary Fig.6D). In DAOY cells, cytotoxicity induced by a combination of MLN8237/ABT-263 or WEHI-539 was only significantly reduced in cells with a double BAX/BAK knockdown (Fig.6C). Cell death induced by MLN8237/ABT-263 was reduced by 30% following BAX/BAK knockdown (56.2% ± 3.4% - 26.7% ± 2.4%), and by 18% in cells treated with MLN8237/WEHI-539 (48.0% ± 1.8% - 29.6% ± 3.2%) compared with controls. Similarly, BAX/BAK knockdown reduced cell killing in KNS42 cells by 30% and 21% following treatment with MLN8237/ABT-263 or MLN8237/WEHI-539. In addition, specific knockdown of BAK significantly attenuated cell death following treatment with ABT-263 or WEHI-539 alone, and in combination with MLN8237 (**$P \leq 0.01$, BH3-mimetic/control siRNA versus BH3-mimetic/BAK siRNA). Overall, these data indicate that ABT-263 and WEHI-539 promote BAX/BAK dependent, caspase-mediated apoptosis in pediatric brain tumor cells following MLN8237-induced mitotic disruption.

Discussion

Pharmacological targeting of the apoptotic mitochondrial pathway represents a novel strategy for cancer therapy, and small-molecules targeting BCL-2 family proteins, including ABT-263, can augment tumour cell killing when combined with other chemotherapeutic agents.[28-32] There is considerable interest in Aurora kinase

inhibitors as novel, targeted therapies for aggressive pediatric CNS tumors including, AT/RT, GBM and medulloblastoma[15-17, 21, 33,34] and MLN8237 is a promising drug candidate since it readily crosses the blood-brain barrier.[35] MLN8237 significantly prolonged survival in models of relapsed medulloblastoma, via disruption of Aurora-A/MYCN complexes and MYCN degradation.[34] Here, we demonstrate that selective inhibition of BCL-X_L significantly enhances the sensitivity of pediatric brain tumor cells to MLN8237 and augments tumor cell killing via activation of the mitochondrial apoptotic pathway. BAX is a key pro-apoptotic mitochondrial effector, and when deleted in ND2:SmoA1 transgenic mice, accelerates SHH-driven medulloblastoma formation.[36] The discovery of new compounds which target BAX provides an additional and important route for apoptosis-based therapies.[22] In this study, we demonstrated that a novel BAX agonist, SMBA1, reduced the growth of pediatric brain tumor cell lines in vitro and, when combined with WEHI-539, overcame apoptotic resistance in the pGBM cell line, KNS42. Our study particularly identifies BCL- X_L as a potential therapeutic target in pediatric brain tumour cells, and is the first to demonstrate that selective inhibition of BCL-X_L using WEHI-539 can sensitise medulloblastoma and pGBM cells to other targeted chemotherapeutic agents, including MLN8237Live cell imaging and DNA content analysis revealed MLN8237/ABT-263 decreased mitotic exit, reduced polyploidy and increased cell death., and a novel BAX activator.

In a range of solid tumors, ABT-263 has been shown to potentiate tumor cell killing when used in combination with chemotherapeutics, predominantly via a mechanism of BCL-X_L inhibition.[13,28, 29, 31, 37] Ham et al., (2016) recently reported that MYCN-amplified neuroblastomas, characterized by low BCL-X_L and high BCL-2 expression, retain sensitivity to ABT-199 partly due to the MYCN-driven

upregulation of the MCL-1 antagonist, NOXA.[38] In addition, combination of MLN8237 with ABT-199 was shown to increase apoptosis in vitro, and improve tumor control in vivo. Differing expression levels and protein-protein interactions amongst individual BCL-2 family members are likely to contribute to differences observed in other tumor models where drug induced mitotic arrest and polyploidization has been shown to increase BCL- X_L dependency.[28,29,31] Similarly, in our investigations in pediatric brain tumour cells, BCL- X_L was revealed as the key target, since ABT-263/WEHI-539 treatment or siRNA mediated knockdown of BCL-X_L sensitized tumor cells to MLN8237 and potentiated cell death, whereas ABT-199 treatment did not. Our new findings have potentially important implications for brain tumor therapy, since addition of sub-lethal concentrations of ABT-263 significantly reduced cell viability, lowered the IC50 of MLN8237, and augmented tumor cell killing. Post-chemotherapy survival of giant polyploid cells may contribute to tumor progression, relapse and increased drug resistance via the generation of diploid escape cells.[39] Thus, therapeutic combinations incorporating MLN8237 with BCL-X_L targeting drugs may reduce the risk of tumor progression and relapse by decreasing mitotic slippage, suppressing polyploidization,[28,31] or by eliminating polyploid cells. However, in this context, tumor regression appeared to be the consequence of cytostasis rather than apoptotic induction.[29] In a pediatric phase 1 trial of MLN8237, when patients were treated with the recommended maximum tolerated dose of 80mg/m^2/day, the peak plasma drug concentration was 7.5µM (2h post administration), and declined below 1µM after 24h.[20] Our in vitro studies indicated that ABT-263 and WEHI-539 could enhance sensitivity to MLN8237 below this physiological range, indicating that the efficacy of MLN8237, at clinically relevant doses, could be enhanced via combination with BCL-X_L inhibitors, such as A-

1155463.[37] However, on-target thrombocytopenia associated with BCL-X_L targeting drugs,[12] and adequate CNS penetration are significant challenges affecting their use in the clinical setting.

In this study, we identify BCL- X_L as a candidate drug target for sensitization to chemotherapy, and present new evidence demonstrating how specific targeting of BCL-X_L can promote apoptosis in pediatric brain tumour cell lines, in response to either MLN8237, or a small-molecule therapeutic designed to target pro-apoptotic BAX. Overall, our data indicates that combinatorial therapy with selective BCL- X_L inhibitors and other targeted agents may be more effective against pediatric brain tumor cells than single agent treatment. Here, we utilised KNS42 (H3F3A G34V-mutant) pGBM cells and DAOY, an established medulloblastoma cell line (previously subtyped as SHH-MB[23], TP53-mutant[40]), as in vitro models. TP53-mutant, SHH medulloblastomas are associated with a poor prognosis (<50% survival),[4] and the H3F3A G34V-mutation occurs in up to 15% of cerebral hemispheric high grade gliomas in children and adolescents.[27] Follow-up pre-clinical studies focusing on pediatric high grade gliomas and specific medulloblastoma subtypes should be undertaken and include patient-derived cell lines and xenograft models to further determine treatment efficacy and durability of response. Our findings suggest that such studies are warranted in order to develop new, targeted treatment approaches for these aggressive pediatric brain tumors.

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Figure legends

Figure 1. MLN8237 induces G2/M accumulation and polyploidy in medulloblastoma and pGBM cell lines. (A) Expression of Aurora A and B was determined by immunoblotting in cells cultured +/- MG132 (20µM) for 2h (N≥3). (B) DAOY and KNS42 cells were synchronized with 100ng/ml Nocodazole (16h) and treated with MLN8237 + MG132 for 2h. Equal loading was confirmed using a β-actin antibody. (C) Cells were treated with MLN8237 for 24-72h and DNA content was quantified by PI uptake. Representative cell cycle profiles are shown; quantitative data is reported as mean ± S.E.M (N≥3).

Figure 2. ABT-263 enhances sensitivity of pediatric brain tumor cells to MLN8237 promoting caspase-mediated apoptosis. (A) Cells were treated with MLN8237 +/-ABT-263 (1µM). Cell viability was assessed after 72h by MTT assay. Data represent mean ± S.E.M (N≥9): $^{**}P \leq 0.01$ MLN8237/ABT-263 versus

MLN8237 alone. (B) Cells were treated with 0.5µM MLN8237 (+/-1µM ABT-263) and apoptosis was quantified by Annexin V/PI staining 48h (DAOY) or 72h (KNS42) after treatment. Data (percentage Annexin V positive cells) represent the mean ± S.E.M (N≥9): ***P*≤0.01 MLN8237/ABT-263 combination versus MLN8237 alone or combination + 50µM zVAD-FMK. (C) Cells were treated with 0.5µM MLN8237 (+/-1µM ABT-263) and caspase 3/7 activity was quantified. Data represent the mean ± S.E.M (N≥9): ***P*≤0.01 MLN8237/ABT-263 versus MLN8237 or ABT-263 alone. Cleavage of PARP and Lamin A/C were detected by immunoblotting (N≥3). Equal loading was confirmed using a β-actin antibody. Annexin-V time-lapse microscopy was performed following addition of 0.5µM MLN8237 and 1 µM ABT-263. Representative images are shown (72h). Green fluorescence indicates Annexin-V positive cells.

Figure 3. ABT-263 reduces MLN8237-induced polyploidy and increases death in mitosis. (A) Cells were treated with MLN8237 +/- ABT-263 (1µM), and DNA content was quantified by PI uptake. Representative cell cycle profiles are displayed and quantitative data represent the mean ± S.E.M (N≥3). (B) Alterations in cellular ploidy were quantified following cell cycle experiments, KNS42 (72h) and DAOY (48h) (N≥3): **P*≤0.05 MLN8237/ABT-263 versus MLN8237 alone. (C) Cells were imaged for 72h by time-lapse microscopy. A minimum of 50 cells per condition were analysed.

Figure 4. Selective inhibition of BCL-X_L sensitizes pediatric brain tumor cells to MLN8237. (A) Cells were treated with 0.5µM MLN8237 +/- BH3-mimetics (1µM) and apoptosis was quantified by Annexin V/PI staining 48h (DAOY) or 72h (KNS42). Data (percentage Annexin V positive cells) represent the mean ± S.E.M (N≥9): ***P*≤0.01 MLN8237/ABT-263 or WEHI-539 combination versus MLN8237

alone. (B) Cells were treated with BCL-X_L or control siRNA and lysates were immunoblotted using antibodies against BCL- X_L and BCL-2 (N≥3). Equal loading was confirmed using a β-actin antibody. Cell death was determined by PI uptake 48 – 72h after drug treatment. Data represent the mean ± S.E.M (N≥9): ***P*≤0.01 MLN8237/control siRNA versus MLN8237/BCL- X_L siRNA.

Figure 5. WEHI-539 increases cell death in combination with a BAX activator or cisplatin. (A) Cells were treated with SMBA1 for 72h and cell viability determined by MTT (SF188, KNS42, DAOY) or WST-1 (D283) assay. Data represent the mean ± S.E.M (N≥9). (B) DAOY and KNS42 were treated with SMBA1 and cell death was determined by PI uptake after 72h. The data represent the mean ± S.E.M (N≥9): ***P*≤0.01 10-20µM SMBA1 versus controls. (C) KNS42 cells were treated with SMBA1 +/- BH3-mimetics (1µM) for 72h. Cell death was determined by PI uptake. Data represent the mean ± S.E.M (N≥9):***P*≤0.01 15-20µM SMBA1+ABT-263/WEHI-539 versus SMBA1 alone. (D) DAOY cells were treated with cisplatin +/- ABT-263/WEHI-539 (1µM) for 72h. Cell death was determined by PI uptake. Data represent the mean ± S.E.M (N≥9): **P*≤0.05, cisplatin/WEHI-539 versus cisplatin alone; ***P*≤0.01 cisplatin/ABT-263 versus cisplatin alone.

Figure 6. Combination of WEHI-539 with MLN8237 promotes caspase mediated apoptosis and is BAX/BAK-dependent. (A) Cells were pre-treated with zVAD-fmk (50µM) for 1h before addition of WEHI-539 (1µM), MLN8237 (0.5µM)or both agents. Cells were treated with ABT-263 (1µM) and MLN8237 (0.5µM), alone or in combination as a control. Cell death was determined by PI uptake. Data represent the mean ± S.E.M (N≥9): ***P*≤0.01 MLN8237/ABT-263 versus MLN8237/ABT-263 + zVAD-fmk; MLN8237/WEHI-539 versus MLN8237/WEHI-539 + zVAD-fmk (B) DAOY and KNS42 cells were treated with BAX and BAK, or control siRNAs prior to

treatment with MLN8237 (0.5µM) and ABT-263 and WEHI-539 (1µM) for 48– 72h. Expression levels of BAX and BAK are shown (N≥3). Equal loading was confirmed using a β-actin antibody. (C) Cell death was determined by PI uptake. The data represent the mean ± S.E.M (N≥9): *$P \leq 0.05$, **$P \leq 0.01$ MLN8237/BH3-mimetic + control siRNA versus MLN8237/BH3-mimetic + BAX/BAK siRNA.