Neuroblastoma

Crossing borders in targeted therapy

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NEUROBLASTOMA:

CROSSING BORDERS IN TARGETED THERAPY

organoids

BCL2

EZH2

Laurel Tabe Bate-Eya

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TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

INTRODUCTION

Cancer research involves the study of deregulated pathways and the use of a wide range of molecular biology techniques and tumor material in order to discover new therapeutic strategies. In this thesis, we describe the isolation and generation of new neuroblastoma cell lines from patient-derived tumor tissues and their future potential in neuroblastoma research. Additionally, we exploited the functional role of EZH2 in neuroblastoma and the therapeutic potential of the BCL2-specific inhibitor venetoclax (ABT199) including strategies to overcome resistance. The current introduction gives a synopsis of the pathology and genomic landscape of neuroblastoma, the currently used treatment protocol, fundamental model systems used in neuroblastoma research and deregulated pathways that can be utilized for the development of novel treatment strategies.

1. NEUROBLASTOMA PATHOLOGY

Neuroblastoma is a solid tumor that arises from the sympathoadrenal system of the adrenal gland during early neural crest formation (1) (**Figure 1**). Histologically, these tumors are composed of undifferentiated neuroectodermal cells or neuroblasts that appear as small round blue cells (2, 3). Advances in clinical and translational research reveal that neuroblastic tumors can be classified according to the differentiated state of the cells, with the ganglioneuroblastomas composed of more differentiated neuroblasts surrounded by Schwannian stroma and ganglioneuromas composed of Schwann cells with differentiated cells of ganglional lineage (4). Neuroblastomas are the most aggressive neuroblastic tumors and can be classified into four stages: stage 1, 2 and 3 neuroblastomas presenting loco-regional tumors with or without lymph node involvement and stage 4 neuroblastomas present tumors with distant metastases. There is one additional subgroup of neuroblastoma tumors (4S), characterized by minimal dissemination to the skin and bone marrow and spontaneous regression (5, 6). Primary anatomical locations of neuroblastoma are in the adrenal medulla, organ of zuckerkandl, and the sympathetic nervous system of the neck and mediastinum and retroperitoneum. The metastatic pattern of neuroblastoma usually involves the bone, bone marrow, lymph nodes and skin (7-10).
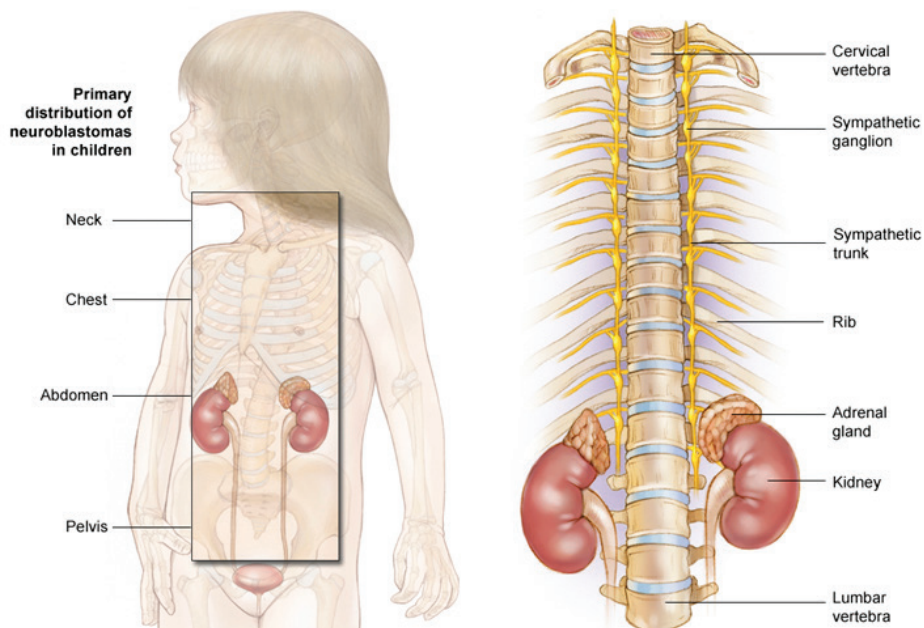
Figure 1: Anatomical representation of the sympatho-adrenal system and neuroblastoma metastatic sites by the American Society of Clinical Oncology.

1.1. The genomic landscape of neuroblastoma.

Several genomic aberrations have been identified in neuroblastoma, such as aberrations in the ploidy status (11). Near-triploidy presented with whole chromosome gains and losses is usually associated with a better prognosis and lower stage of the disease. Ploidy status in neuroblastoma is of prognostic significance in infants of less than 1 year while its prognostic relevance is lost in patients older than 1 year of age.

Partial chromosome gains and losses are frequently observed in neuroblastoma, of which 17q gain and 1p and 11q losses are the most notable. Gain of chromosome 17q occurs in more than 50% of all neuroblastoma tumors and is most frequently observed in high stage tumors. Unbalanced gain of 17q frequently occurs as a translocation between chromosome 1 and 17. Different breakpoint regions of chromosome 17q are observed but a preferential gain in the 17q22 arm results in a dosage effect of genes in this region such as *BIRC5* (survivin) and *PPM1D* (protein phosphatase 1D) (12).

Deletions in chromosomes 1p and 11q are also associated with a worse prognosis. Loss of 1p occurs in 30-35% of all neuroblastoma tumors and is thought to be a tumor-

promoting event because known tumor suppressor genes *CAMTA1*, *CHD5*, *KIF1B*, *CASZ1* and *miR-34a* are located on this region (13-16). In addition, 1p loss is often associated with amplification of the oncoprotein *MYCN*. Loss of 11q was predominantly observed in non-MYCN amplified neuroblastoma patients and was correlated to decreased survival in these group of patients (17).

Amplification of MYCN (located on chromosome 2) is one of the most notable genomic events in neuroblastoma. It is observed in 22% of all neuroblastoma patients and strongly correlates with a poor prognosis (18, 19). MYC oncoproteins (c-MYC, N-MYC, L-MYC, S and B-MYC) are basic helix-loop-helix leucine zipper (b-HLH-Zip) transcription factors (20). The DNA binding and transcription factor roles MYCN requires its dimerization with Max, a small b-HLH-Zip protein leading to the activation of genes involved in cellular processes such as cell cycle arrest, migration and apoptosis (21, 22).

Amplification of the *MDM2* proto-oncogene located on chromosome 12 has been described in 2% of neuroblastomas (23-25). MDM2 is an E3 ubiquitin ligase responsible for the degradation of the master transcriptional regulator *TP53* (p53) by catalyzing its ubiquitylation and subsequent proteasomal degradation upon binding. p53 is a well-known tumor suppressor involved in cell cycle arrest, DNA repair and apoptosis (26-29).

Recently, chromothripsis has been identified in about 18% of the neuroblastoma tumors (30). Chromothripsis is a genomic event involving the local shredding and subsequent random reassembly of chromosomes, resulting in the loss of tumor suppressor genes and amplification of oncoproteins leading to tumor-promoting events (31, 32). The mechanisms causing chromothripsis are unknown, but advances in gene sequencing have demonstrated that these mechanisms might involve the segregation and reassembly of a single chromatid from a micronucleus (33, 34).

Somatic and single nucleotide mutations have been identified in a number of genes in neuroblastoma by whole genome and exome sequencing. The anaplastic leukemia kinase gene (*ALK*) has been identified as one of the driving oncogenes involved in familial neuroblastoma (35, 36). ALK is a protein tyrosine kinase receptor which through the phosphorylation activities of its kinase domain activates a variety of downstream signaling pathways involved in oncogenesis. In addition to activating somatic and germline mutations (F1174L and R1275Q), copy number gains of this gene have been observed in neuroblastoma (37, 38). Targeted inhibition of ALK with small molecule inhibitors crizotinib and alectinib has shown promise in a variety of

tumor types, including neuroblastoma (39-41). Somatic mutations have also been described for *PTPN11* (activating mutations in 2.9% of the neuroblastoma patients) and *ATRX* (inactivating mutations in 2.5% of the neuroblastoma patients) while germline mutations have been described in the PHOX2B gene (missense mutations and whole allele deletions) (30, 42).

1.2. Current treatment regimen and prognosis of neuroblastoma.

Treatment of neuroblastoma is based on the Dutch Childhood Oncology Group (DCOG) risk stratification scheme, using the INSS stage, MYCN status, tumor differentiation grade, chromosome 11q status and DNA ploidy to determine which treatment neuroblastoma patients will receive (**Figure 2**).
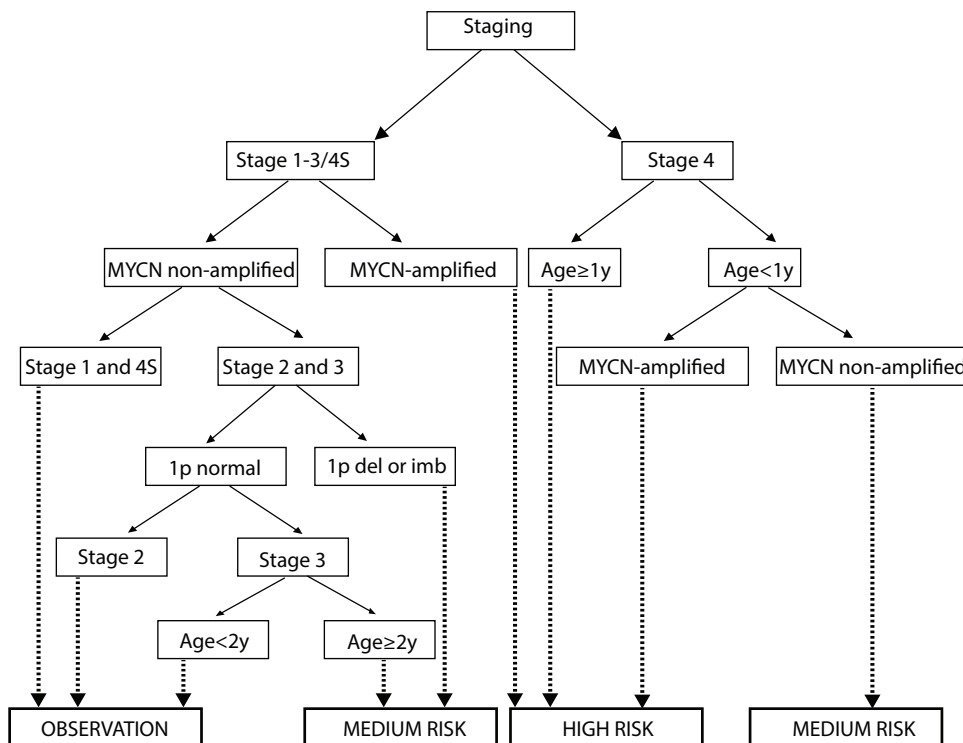

Figure 2. Risk stratification of neuroblastoma tumors according to the DCOG NBL 2009 trial.

1

Figure 3 shows the treatment regimen for high risk neuroblastoma patients. In the DCOG protocol, treatment of high risk neuroblastoma patients is started with 2 upfront courses of radiolabeled ^{131}I-MIBG. Radiolabeled MIBG is a neurotransmitter-like substance that can be used for treatment (^{131}I) or for diagnostic (^{123}I) purposes (43-45). Following MIBG treatment, high risk patients receive 6 cycles of N5 chemotherapy (cisplatin, vindesine and etoposide) and N6 chemotherapy (ifosfamide, vincristine, dacarbazine and doxorubicin). When necessary, tumor debulking is performed to minimize the amount of tumor left after treatment with N5/6 chemotherapy cycles. Next, myelosuppressive high dose chemotherapy (etoposide and melphalan) is given, followed by autologous stem cell transplantation and retinoic acid treatment is then carried out in 6 cycles with a short break and then 3 additional cycles. Medium risk patients receive similar treatment (i.e. without upfront ^{131}I-MIBG courses), while for the low risk patients a wait and see approach is applied.
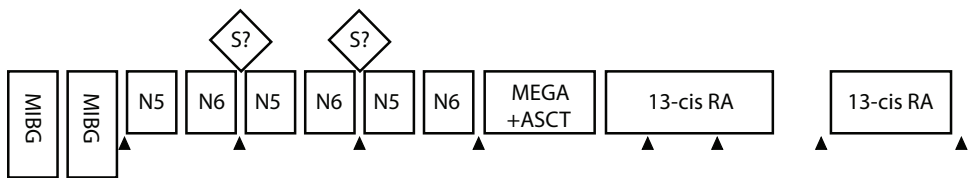
Figure 3: Overview of the DCOG NBL2009 treatment protocol of high risk neuroblastoma patients (S= surgery, N5/6= chemotherapy cycles, MIBG= MIBG treatment, MEGA+ASCT= myeloablative high dose chemotherapy with autologous stem cell transplantation, 13-cis-RA=13-cis-retinoic acid).

Despite of the intensive multimodality therapy, high risk neuroblastoma patients have an overall survival chance of only <50% (46). It is thus imperative to test new targeted therapies that might improve the survival rate patients diagnosed with high risk neuroblastoma. Pre-clinical evaluation of new therapies in adequate *in vitro* and *in vivo* model systems closely mimicking the clinical situation is essential to facilitate the eventually necessary pre-clinical to clinical translation.

2. *IN VITRO* AND *IN VIVO* SYSTEMS FOR NEUROBLASTOMA BIOLOGY.

Most tumor cells exist within a 3D microenvironment comprised of surrounding stromal and immune cell infiltrates. However, upon excision of tumor material, tumor-derived cells are frequently cultured and maintained in a 2D culture system. It has

been shown in neuroblastoma and other solid tumors that cell lines cultured under 2D conditions fail to adequately recapitulate the expression profiles of the corresponding tumors from which they were derived (47-50). Tissue-specific and cell-cell interactions are lost upon culturing of cells under 2D conditions. Key cellular events such as cell proliferation, migration and apoptosis are frequently based on cellular context and the common consensus is that mimicking cell-cell and cell-extracellular matrix interactions in 3D culture can better portray tumor-specific conditions (51). Additionally, cell lines maintained under 2D conditions are cultured with medium not containing growth factors present within the tumor microenvironment. Hence, there is a need to develop specially formulated medium enriched with growth factors found in the tumor microenvironment (52). For neuroblastoma as well as other types of solid tumors, long-term culturing of cells in 2D culture conditions leads to the acquisition of non-tumor-specific mutations, rendering these cell lines unsuitable for cell based assays (53, 54). This has led to the generation of so-called "tumor-initiating cells (TICs)" or "organoids", i.e. short-term tumor-derived cells maintained in culture under 3D conditions.

Organoids can be defined as an *in vitro* cluster of cells derived exclusively from primary tissues, embryonic stem cells or immature pluripotent stem cells and capable of self-renewal properties with the ability to exhibit similar functional roles as its organ of origin (55, 56). Most organoid culture systems lack basement membrane and extracellular matrix components that constitute the normal tissue architecture. In order to facilitate the self-renewal and self-organizing properties of these structures, organoids are most often cultured in rigid matrices that closely resemble the components of the extracellular matrix (57) . In order to maintain the 3D architecture of neuroblastoma, TIC lines or organoids are cultured on reconstituted rat extracellular matrix (matrigel) or 1% methylcellulose solution. Because of the limited knowledge of the growth factors and lineage commitment factors required by cells of the neuronal lineage, establishment of the optimal conditions for 3D culturing of neuroblastoma cells remains challenging. Currently, neuroblastoma TIC lines and organoids are cultured using neuronal stem cell medium supplemented with EGF and FGF which are important components necessary for neuronal cell maintenance and survival (58). Under these conditions, neuroblastoma cells have been shown to recapitulate the genotype and phenotype of the primary tumors from which they are derived (49, 52, 59, 60).

The ability to culture self-renewing organoids provides model systems that can be used for multiple research applications as well as translational research. Organoids

provide relevant *in vitro* platforms to study tissue homeostasis and the onset of disease in tissues. Introducing well-known driver mutations in *APC*, *KRAS*, *TP53* and *SMAD4* by CRISPR/Cas9 DNA editing in organoids derived from healthy intestinal tissues resulted in their transformation into organoids mimicking the genomic and signaling profile of colorectal tumors. After implantation in mice, mutated organoids formed tumors with genomic properties near identical to human colorectal cancers (61). For solid tumors, high-throughput drug screens in 3D culture models yielded the discovery of new therapeutic targets that were not found in 2D culture models (62-66).

As already shortly addressed above, organoids are also used for the development of more representative *in vivo* models. Organoids are injected in immune-compromised mice at two main locations: 1) subcutaneous (i.e. directly under the dermis), or 2) orthotopic (i.e. at their site of origin). Alternative models are patient-derived tumor xenograft (PDTX) models whereby the patient-derived tumor material is directly engrafted into immune-compromised mice (67, 68). Organoid- and patient-derived xenograft models have served as robust model systems for efficacy testing of novel targeted inhibitors. The shift from *in vitro* 2D culture systems to 3D organoids and organoid- and patient-derived xenograft models has ushered in a new era of better predicting and understanding the effects of drug responses in patients.

In this thesis, we describe the generation and propagation of patient-derived neuroblastoma TIC lines. We show that neuroblastoma TIC lines retain the phenotypic and genotypic characteristics of the primary tumors from which they are derived, with excellent sphere forming potential. The above topic will be more extensively discussed in **chapter 2.**

3. DEREGULATED PATHWAYS IN NEUROBLASTOMA

3.1. Targeting the apoptotic pathway

Apoptosis or programmed cell death is a cellular process whereby activation leads to DNA shredding, cytoplasmic fragmentation, membrane reorganization and cell death without lysis of surrounding cells (69). The balance between cell viability and apoptosis plays an important role in maintaining normal cell homeostasis. Alterations in the apoptotic pathway has been shown to promote neoplastic transformation as well as resistance to therapy (70, 71). The apoptotic pathway consists of the extrinsic and intrinsic apoptotic pathway **Figure 4**. The main players of the extrinsic apoptotic pathway are the tumor

necrosis factor (TNF) superfamily of receptors and TNF-related apoptosis inducing ligand (TRAIL) (72). TRAIL binds to two types of receptors: death receptors that can activate apoptosis and decoy receptors that can prevent apoptosis from occurring by functioning as gatekeepers. Activation of the extrinsic apoptotic pathway occurs upon TRAIL binding to TNF receptors leading to their oligomerization and subsequent recruitment of FAS-associated protein with death domain (FADD) to their death domains. FADD in turn recruits initiator caspase-8 or caspase-10 via its death effector domain (DED), which is then cleaved and activated within the death-inducing signaling complex (DISC). Activated initiator caspases cleave and activate effector caspase-3, resulting in DNA fragmentation by DNAse endonucleases, nuclear disorganization, cytoplasmic condensation, cellular shrinkage and, eventually, cell death (73, 74).
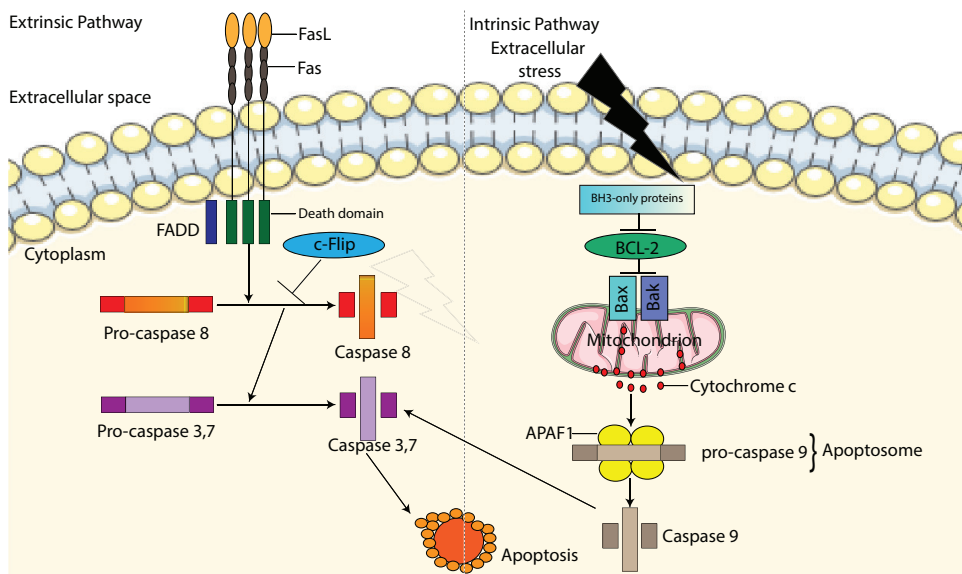
Figure 4: Schematic representation of the extrinsic and intrinsic apoptotic pathway.

In numerous tumors, resistance to TRAIL-mediated apoptosis has been shown to be attributed to downregulation of death receptors, CASP8 inactivation and overexpression of anti-apoptotic proteins such as the caspase 8 and FADD-like apoptosis regulator (CFLAR) or c-FLIP (75-77). The observation that in most high stage neuroblastomas *CASP8* is inactivated due to hypermethylation or deletion of the gene provides rational for targeting the extrinsic apoptotic pathway in these tumors (78, 79). Non-small cell

1

lung carcinoma (NSCLC) patients showed partial or complete responses in phase I and II clinical trials upon treatment with recombinant human Apo2 ligand/TRAILZ (dulanermin). Combination studies of TRAIL with chemotherapeutics are currently being carried out to assess the feasibility of improving the response rate of these patients to TRAIL (NCT00092924). In neuroblastoma cell lines, restoration of caspase-8 levels by combination treatment with soluble TRAIL ligands and demethylating agents triggered apoptosis. Sensitization of neuroblastoma cell lines to TRAIL was shown to be effective upon additional activation of the intrinsic apoptotic pathway (80, 81). These finding suggest that combined activation of both the extrinsic and intrinsic apoptotic pathway might be beneficial in neuroblastoma patients with inactivating events in both pathways.

 The B-cell lymphoma (BCL2) family of proteins are key players in the intrinsic apoptotic cascade. It consists of the anti-apoptotic proteins BCL2, BCL-extra-large (BCL-X_L), myeloid cell leukaema sequence 1 (MCL1), BCL-2-like protein 2 (BCL-W) and BCL2 related protein A1 (A1) (82), the multi-domain pro-apoptotic proteins BCL2-associated X (BAX), BCL2 homologous antagonist/killer (BAK) and BCL2 related ovarian killer (BOK) and the BH3-only pro-apoptotic proteins BCL2-like protein 11 (BIM), BH3 interacting domain death agonist (BID) and BCL2-antagonist of cell death (BAD)(83). Activation of the intrinsic apoptotic pathway occurs primarily at the outer mitochondrial membrane. Apoptosis is induced by external stimuli such as DNA damage caused by chemotherapy or radiation or by growth factor redrawal, resulting in the displacement of pro-apoptotic proteins (primarily BIM, Bid or Bad) from anti-apoptotic members. Displaced pro-apoptotic proteins causes oligomerization of BAX/BAK, leading to the formation of pores on the surface of the mitochondria. Cytochrome c, Second mitochondria-derived activator of caspases/ direct IAP binding protein with low PI (SMAC/DIABLO), HtrA Serine peptidase 2 (HtrA2/Omi), apoptosis–inducing factor (AIF) and endonuclease G are then released from the mitochondria into the cytosol. Cytochrome c together with the apoptotic protease activating factor 1 (Apaf1) forms the apoptosome. The apoptosome is responsible for the recruitment and cleavage of effector caspases such as caspase-3 and -9, leading to cell-mediated apoptosis (84). Regulators of apoptosis Smac/DIABLO and HtrA2/Omi play various roles in the inactivation of inhibitors of apoptosis (IAPs), while AIF and endonuclease G are involved in chromatin condensation and DNA fragmentation (85, 86). IAPs bind to effector caspases preventing their activation and subsequent induction of apoptosis (87). In humans, eight family members of these proteins have been identified: neuronal apoptosis inhibitory protein (BIRC1), cellular IAP1 (c-IAP$_1$ or

BIRC2), c-IAP$_2$ (or BIRC3), X-chromosome-linked IAP (XIAP or BIRC4), survivin (TIAP or BIRC5), Bruce (BIRC6), Livin (BIRC7) and IAP-like protein 2 (BIRC8) (88).

In numerous malignancies, inactivating genomic events in the intrinsic apoptotic pathway have been observed, including loss of *BAX* or *BAK* and overexpression of *BIRC5* or the anti-apoptotic BCL2 family member proteins (89, 90). *BIRC5* is located on chromosome 17q, which is frequently gained in neuroblastoma tumors. Inhibition of BIRC5 with small molecule inhibitor YM155 lead to a strong apoptotic response and complete tumor regression in neuroblastoma xenograft mouse models (90), underscoring the potential of BIRC5 as a therapeutic target for neuroblastoma treatment. The anti-apoptotic protein BCL2 is also highly expressed in the majority of all neuroblastoma patients (91-93). Previous studies have shown that BCL2 knockdown in BCL2 high-expressing neuroblastoma cell lines causes a strong apoptotic response. Thus, targeting this pathway with small molecule inhibitors might prove useful in the treatment of neuroblastoma patients with high BCL2 expression (91).

Earlier attempts to target the intrinsic apoptotic pathway were primarily through the use of antisense oligonucleotides specifically targeting BCL2 protein expression. The first agent which was specifically developed to target BCL2 protein expression was the antisense oligonucleotide oblimersen (94, 95). Oblimersen showed great promise during pre-clinical development, but its clinical development was halted due to off-target effects observed in phase III trials. Off-target effects were mainly due to the induction of apoptosis via other mechanisms than BCL2 (96-98).

Several compounds such as histone deacetylase (HDAC) inhibitors, sodium butyrate and depsipeptide were discovered to directly regulate the expression of the anti-apoptotic BCL2 family proteins BCL2, BCL-X$_L$ and MCL1 in lymphoid malignancy cell lines (99, 100). Other agents such as the cyclin-dependent kinase (CDK) inhibitor flavopiridol inhibited apoptosis specifically by MCL1 downregulation (101). However, the specificity of these compounds is limited compared to small molecules which directly interact with the anti-apoptotic BCL2 family proteins, hence the need for such compounds.

Under normal physiological conditions, a group of BH3-only proteins can directly bind to and activate BAX and BAK, leading to activation of the intrinsic apoptotic pathway (82). The term "primed for cell death" was coined to describe the state whereby activator proteins in this group such as BIM, Bid and Puma can bind to anti-apoptotic proteins BCL2, MCL1 etc., preventing BAX and BAK1 oligomerization and hence apoptosis (93).

Based on this hypothesis, a group of small molecule inhibitors (BH3 mimetics) were developed. These small molecule inhibitors directly bind to the hydrophobic groove of the BH3 domain of the anti-apoptotic family proteins, displacing the BH3-only proteins triggering apoptosis. Gossypol (AT-101, Ascenta), a phenolic pigment, was one of the first compounds that showed significant inhibitory effects on BCL2, BCL-X$_L$ and MCL1 (102-104). AT-101 binds to BCL2, BCL-X$_L$ and MCL1 at low micromolar concentrations and is currently in phase II clinical trial for chronic lymphocytic leukemia (CLL; in combination with rituximab) and hormone refractory prostate cancer (in combination with docetaxel) (105-107). Because in a phase I/II clinical trial for prostate cancer dose-limiting gastrointestinal toxicity was observed, the gossypol analog apogossypol was developed. Apogossypol exhibited a stronger binding affinity to BCL2, BCL-X$_L$ and MCL1 (0.32, 0.48 and 0.18μM) and the systemic toxicities observed with gossypol were notably decreased (108).

Obatoclax (GX15-070) is a small molecule inhibitor that was developed as a hydrophobic pan-BCL2 family inhibitor (109). Studies performed in acute myeloid leukemia (AML) cell lines showed that the compound displays low binding affinities to BCL2, BCL-X$_L$, BCL-W and MCL1 (3,5 3, 2.9 and 5μmol/L, respectively). *In vitro* pre-clinical evaluation of the compound in AML cell lines showed moderate responses, which could be attributed to partial BIM displacement and activation of BAX and BAK. Additionally, G$_2$-arrest was observed in the cell lines which might be due to possible off-target effects of the compound (110). Phase I clinical trials in hematological malignancies showed only 4% partial responses and one complete remission after obatoclax treatment. Low response rates in clinical trials led to the discontinuation of further development of the compound that has now an orphan status (111).

ABT-737 (Abbott Laboratories) is a small molecule inhibitor that binds to the anti-apoptotic proteins BCL2, BCL-X$_L$, MCL1, A1 and BCLW ((K$_i$=0.001 , 0.078, 0.03, 0.46 and 0.197 nmol/L) (100) displacing the pro-apoptotic BH3-only proteins, triggering BAX and BAK oligomerization ultimately inducing apoptosis in cancer cells (112). Thus, cell lines with high BCL2, MCL1, BCL-X$_L$ and BCL-W expression were most sensitive to the compound than cell lines with low expression. Various studies have shown that ABT-737 in combination with radiation and chemotherapeutic agents increased the apoptotic response of CLL cell lines and xenografts (113, 114). Pre-clinical evaluation of ABT-737 in combination with chemotherapeutics also showed promising results for AML, multiple myeloma (MM), small cell lung cancer (SCLC) and acute lymphoblastic

leukemia (ALL) (115-117). Because ABT-737 was not suitable as an oral agent, the orally bioavailable analog ABT-263 (navitoclax) was developed (118). Similar to ABT-737, navitoclax exhibits high binding affinities to BCL2, BCL-X$_L$ and BCL-W (K$_i$=0.044, 0.055 and 7nmol/L, respectively) (119). *In vitro* pre-clinical evaluation showed that navitoclax selectively caused a strong apoptotic response in neuroblastoma cell lines with high BCL2 expression levels. At the *in vivo* level, navitoclax delayed the onset of tumor formation and induced almost complete regression of established tumors in a neuroblastoma BCL2 high-expressing xenograft mouse model (91). In a phase I/II clinical trial for patients with relapsed and refractory CLL and Hodgkin's lymphoma, overall response rates (ORRs) of, respectively, 22% and 35% were observed with a progression free survival of about 25 months (120, 121). However, the clinical use of navitoclax was associated with dose-dependent thrombocytopenia, caused by the on-target inhibition of BCL-X$_L$ in the platelets (122).

Due to the toxic side effects of navitoclax in early clinical trials, it was crucial to develop a BCL2-specific inhibitor. ABT-199 (venetoclax) binds over 50 times more potent to BCL2 (K$_i$< 1 nmol/L) than to anti-apoptotic BCL2 family members BCL-X$_L$, BCL-W and MCL1 (K$_i$~50, 245 and >444 nmol/L, respectively) (123). Like navitoclax, *in vitro* pre-clinical studies showed that neuroblastoma cell lines with high BCL2 and BIM/BCL2 complex levels responded more potently to venetoclax than low-expressing lines. In mice, venetoclax strongly inhibited the growth of BCL2 high-expressing neuroblastoma xenografts, but complete tumor remission was not observed (124, 125). Resistance to venetoclax could be attributed to upregulation of MCL1, which then prevents the occurrence of apoptosis by sequestration of BIM displaced from BCL2. MCL1-mediated resistance could be effectively abrogated upon treatment of BCL2 high-expressing cell lines with venetoclax in combination with MCL1specific inhibitors as well as Aurora kinase inhibitors that act mainly by downregulating MCL1 protein levels (124). Additional studies in ALL and CML have shown that phosphatidyl-inositol -3 kinase (PI3K), serine/threonine kinase 1 (AKT), mammalian target of rapamycin (mTOR) and cyclin-dependent kinase (CDK) inhibitors also successfully abrogated venetoclax resistance by directly regulating MCL1 expression (126). In hematological malignancies, combining venetoclax with the Bruton's tyrosine kinase (BTK) inhibitor ibrutinib or the anti-CD20 monoclonal antibody rituximab gave excellent responses both *in vitro* and *in vivo* (123, 127). Clinical trials combining venetoclax with the MEK inhibitor cobimetinib are currently ongoing in hematological malignancies (NCT02670044). In hematological malignancies, it has been observed that resistance to venetoclax can also occur because missense mutations

in the BH3 domain of BCL2 prevent binding of the inhibitor. Strategies to circumvent this type of resistance mechanism are still subject to further investigation (128). A dose-escalation phase I study showed that 79% of chronic lymphocytic leukemia (CLL) or small lymphocytic lymphoma (SLL) patients demonstrated a favorable response to the compound with 22% of the patients exhibiting a complete remission (127). The current thesis describes the preclinical evaluation of the BCL2-specific inhibitor venetoclax in neuroblastoma, including mechanisms causing neuroblastoma resistance to venetoclax and combination strategies to prevent this. Based on these results, a clinical trial will be conducted in neuroblastoma patients to determine the response rates to venetoclax treatment.

3.2. The clinical relevance of targeting the EZH2 pathway.

Epigenetics can be described as the study of factors that exert gene control without modification of DNA sequences (129). Epigenetics processes play an important role in normal cellular functions in development as well as in disease initiation and progression. Epigenetic modulation of a gene often results in the differential expression of the modulated gene. Several epigenetics processes have been identified: methylation, acetylation, sumolyation, phosphorylation and ubiquitylation (130). The most studied epigenetic modifications are DNA methylation and chromatin or histone modifications, of which the latter will be discussed in-depth in this thesis. DNA methylation is a process whereby methyl groups are added to the DNA (131) in regions whereby a cytosine nucleotide is located adjacent to a guanine nucleotide (known as CpG sites) (132). The methylation of CpG sites is carried out by enzymes known as DNA methyl transferases (DNMT) (133, 134). Addition of a methyl group leads to DNA modification and interaction of the transcription machinery of the gene with the DNA. As stated above, DNA methylation often occurs at CpG sites in normal cells. However, there are stretches of DNA within CpG-rich regions that are normally non-methylated. These are known as CpG islands and are located near the promoter regions of genes (135, 136). In cancer cells, CpG island methylation can be modified and this phenomenon has been identified as an early tumor promoting event (137). Several DNA demethylating agents have been developed and pre-clinically tested for the treatment of DNA methylation driven tumors. These inhibitors are capable of reactivating genes silenced by DNA methylation. 5-azacytidine and 5-aza-2'-deoxycytidine are the two pre-clinically most extensively tested and validated DNA demethylating agents and have shown promising results in numerous tumor types (138).

Histones are a family of proteins which together with DNA condense to form chromatin (139). Modifications of histones mainly occur via methylation, acetylation or phosphorylation and might result in either gene activation or silencing (130). Acetylation is a process whereby acetyl groups are added to lysine amino acids present in histones. Lysine acetylation usually results in gene activation, while methylation of lysine residues causes gene silencing (140, 141). Methylation of histones occurs primarily on lysine and arginine side chains. Methylation and acetylation of histone tails at lysine is mediated by histone methyl and acetyltransferases (142, 143). Histone methyltransferases are a SET (SU[VAR]3-9, Enhancer of Zeste and Trithorax) containing group of enzymes that together with a group of genes form the polycomb repressor complex (PRC).

Polycomb repressor genes play a key role in cell fate determination, cell cycle regulation and hematopoiesis. These genes encode a group of proteins that form two distinct complexes: polycomb repressor complex 1 and 2 (PRC1 and PRC2) (144). Polycomb repressor group proteins are transcriptional repressors that act via methylation of the histone tails of genes leading to gene silencing. The enhancer of zeste homologue 2 (EZH2) together with suppressor of zeste 12 (SUZ12) and embryonic ectoderm development (EED) form part of the PRC2 complex (141, 145, 146). SUZ12 functions by binding to and stabilizing EZH2 within the PRC2 complex. Additionally, SUZ12 enhances PRC2 activity via recruitment of RbApb48, which in turn enhances EZH2 histone methyltransferase activity (147-150). EED via its carboxy-terminal domain binds to the histone tails of histone 3 leading to the activation of the histone methyltransferase activity of the PRC2 complex (151). EZH2 consists of a catalytic SET domain, through which methyl groups are transferred to histone 3 di-methylated at lysine 27, resulting in tri-methylated histone 3 (H3K27me3) and subsequently gene silencing (152-154) **(Figure 5)**.

EZH2 is overexpressed in numerous cancer types and plays a crucial role in cancer cell proliferation and survival (155-158). Several oncoproteins such as MYC mediate EZH2 overexpression by downregulating miR-26a and miR26b, which are negative regulators of EZH2 (159, 160). Cell cycle regulators such as E2F can also regulate EZH2 expression via the pRB-E2F pathway (160, 161). In diffuse B-cell lymphomas, it has been observed that Y641 and A677G somatic point mutations in the SET transactivation domain of EZH2 result in enhanced stability and histone methyltransferase activity of the gene (162). EZH2 overexpression in neuroblastoma was correlated to increased hypermethylation and silencing of tumor suppressor genes *CASZ1*, *RUNX3*, *NGFR3* and *CLU* (163, 164).
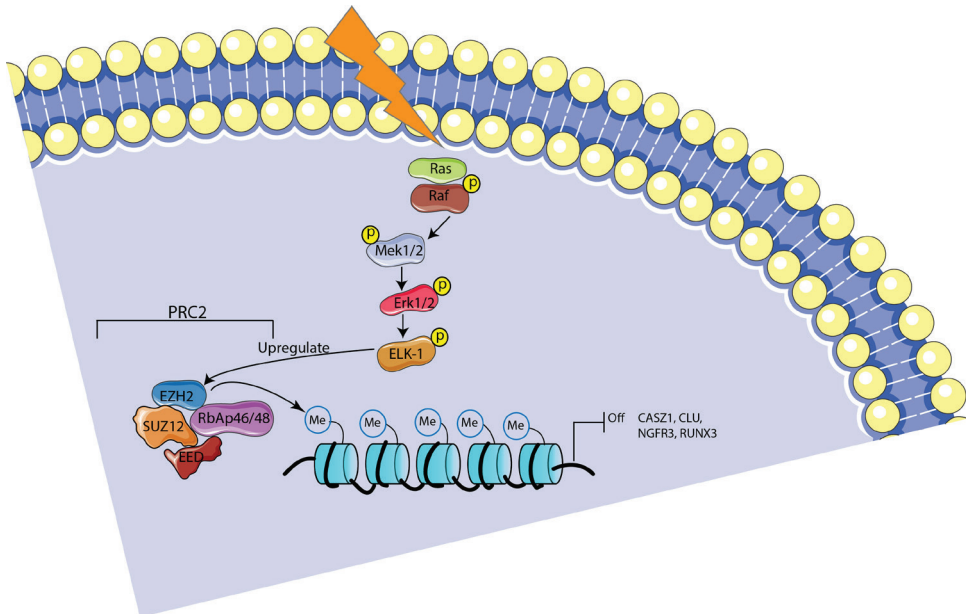
Figure 5: Schematic representation of the histone methyltransferase activity of EZH2 and the transcriptional repression of known target genes.

Because tumor promoting events are often associated with increased histone methyltransferase activity of EZH2, several small molecule histone methyltransferase inhibitors were developed. 3-Deazaneplanocin (DZNep), a first line histone methyltransferase inhibitor, was reported to cause inhibition of H3K27me3 as well as H4K20me3 (165-167). Treatment of neuroblastoma cell lines expressing high EZH2 levels with DZNep resulted in a strong apoptotic response. At the *in vivo* level, almost complete tumor regression was observed upon treatment with the inhibitor. Molecular analysis on neuroblastoma cell lines and xenografts treated with DZNep revealed upregulation of genes that are normally silenced by EZH2 (163). DZNep was shown to be an s-adenosyl homocysteine hydrolase inhibitor, indirectly also inhibiting other histone methyltransferases. Because this resulted in possible side-effects, further development of the compound was discontinued (168). This led to the development of the two new EZH2-specific methyltransferase inhibitors GSK126 and EPZ6438. *In vitro* pre-clinical evaluation of both histone methyltransferase inhibitors showed potent responses in diffuse B-cell lymphoma (DBCL) cell lines harbouring the Y641 activating mutation. A strong cell cycle arrest and apoptotic phenotype was observed upon treatment of these cells with both compounds. At the *in vivo* level, complete tumor regression was

observed upon treatment with GSK126 and EPZ6438 (169-171). The *in vitro* and *in vivo* responses observed in DBCL suggests that patients harbouring the Y641 mutation might benefit from treatment with these inhibitors.

In this thesis, we described the local gain and overexpression of *EZH2* in neuroblastoma. We attempted to study and elucidate the histone methyltransferase functions of EZH2 in neuroblastoma using small molecule inhibitors. We report on a histone methyltransferase independent function of EZH2 in neuroblastoma and the relevance of targeted inhibition of the EZH2 protein as a whole in neuroblastoma.

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CHAPTER 2

NEWLY-DERIVED NEUROBLASTOMA CELL LINES PROPAGATED IN SERUM-FREE MEDIUM RECAPITULATE THE GENOTYPE AND PHENOTYPE OF PRIMARY NEUROBLASTOMA TUMOURS.

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ABSTRACT.

Recently protocols have been devised for the culturing of cell lines from fresh tumors under serum-free conditions in defined neural stem cell medium. These cells, frequently called Tumor Initiating Cells (TICs) closely retained characteristics of the tumors of origin. We report the isolation of 8 newly-derived neuroblastoma TICs from 6 primary neuroblastoma tumors and 2 bone-marrow metastases. The primary tumors from which these TICs were generated have previously been fully typed by Whole Genome Sequencing (WGS). Array CGH analysis showed that TIC cell lines retained essential characteristics of the primary tumors and exhibited typical neuroblastoma chromosomal aberrations such as MYCN amplification, gain of chromosome 17q and deletion of 1p36. Protein analysis showed expression for neuroblastoma markers MYCN, NCAM, CHGA, DBH and TH while haematopoietic markers CD19 and CD11b were absent. We analysed the growth characteristics and confirmed tumor-forming potential using sphere-forming assays, subcutaneous and orthotopic injection of these cells into immune-compromised mice. Affymetrix mRNA expression profiling of TIC line xenografts showed an expression pattern more closely mimicking primary tumors compared to xenografts from classical cell lines. This establishes that these neuroblastoma TICs cultured under serum-free conditions are relevant and useful neuroblastoma tumor models.

2

1. INTRODUCTION

Neuroblastoma is a neuroendocrine tumor that arises from the peripheral sympathetic nervous system[1]. INSS stage 1 and 2 tumors display excellent prognosis while stage 3 and 4 tumors have poor clinical outcome with a survival rate of 30% [2-5]. Neuroblastoma can genomically be characterised by aberrations such as gain of chromosome 17q, partial loss of chromosome 1p or 11q and MYCN amplification [6]. MYCN amplification occurs in about 20% of tumors and strongly correlates with poor prognosis [7-8]. Gain of 17q is the most frequent genomic abnormality which is present in over 90% of high grade neuroblastomas [9].

Cancer cell lines have been regarded as valid systems to study cancer biology. Nevertheless, these cell systems are cultured in non-physiological conditions and maintained for many years allowing considerable artificial adaptation. The genetic makeup and phenotypic characteristics of these cells can thus differ substantially from their original tumors [10-11]. In recent years, protocols have been devised to culture cells from fresh tumors in serum-free conditions in neural stem cell medium [12-13]. Such cells retained much of the characteristics of the original tumors and were able to initiate tumors in immuno-compromise mice. They were therefore frequently called Tumor Initiating Cells (TICs), a terminology that we use in this paper to refer to cells cultured by these protocols. TICs isolated from neuroblastoma have recently been reported, but the origin of these cells has been under debate [14-17].

Here we report the unambiguous establishment of TIC cell lines from neuroblastoma. These TICs reflect the primary tumors based on the genotype and phenotype and are not contaminated with cells from the haematopoietic lineage. We also show xenografts of neuroblastoma TICs recapitulate the genotype of primary neuroblastoma and showed similar mRNA expression compared to primary tumors as opposed to xenografts from classical neuroblastoma cell lines.

2. MATERIALS AND METHODS.

2.1. Patients, isolation and culture of neuroblastoma TICs.

Freshly resected human neuroblastoma tissue was obtained directly at surgery. Primary neuroblastoma cells were derived by mechanical disaggregation followed by enzymatic digestion of sheared tumor tissues with Liberase DH (500μl/25ml) (Roche)

for 45mins. Digests were passed through a 40μM cell strainer (BD Biosciences) and cells were cultured in TIC total medium containing DMEM/ F12 (GIBCO) supplemented with 40ng/ml bFGF, 20ng/ml EGF, 1X B27supplement (GIBCO) and 500U/ml of penicillin/ streptomycin, 1000mg/ml glucose and 110mg/ml pyruvate. Neuroblastoma TICs were designated by "AMC" (Academic Medical Centre), the name of the research institute, followed by the patient number and in case of TIC lines the letter "T" or "B" was added (T indicating that the TIC cell line was derived from the primary tumors and B from bone marrow metastases).

2.2 Array CGH analysis

aCGH was performed by hybridizing 100ng genomic DNA to a 180K platform (Agilent Technologies). DNA was labelled by random priming with CY5-dCTP and CY3-dCTP respectively and hybridized at 65°C for about 17h. The chips were scanned on an Agilent G2565BA DNA microarray Agilent scanner. Digital analysis was performed with the R2 bioinformatics platform (http://r2.amc.nl).

2.3 mRNA expression profiling and Principal component analysis

RNA was extracted from tumors and cell lines with TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer's protocols. RNA concentration and quality were determined using the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies). Fragmentation of cRNA, hybridization to hg-u133 plus 2.0, microarrays and scanning were carried out according to the manufacturers protocol (Affymetrix inc. Santa Barbara, CA). The mRNA gene expression data were normalized with the MAS5.0 algorithm within the GCOS program of Affymetrix Inc. Target intensity was set to 100. All data were analyzed using the R2 genomic analysis and visualization platform (http://r2.amc.nl).

2.4 Animal experimentation

Athymic NMRI-*nu/nu* and NOD-CB17-Prkdcscid/NCrHsd were subcutaneously injected with 1x10^6, 2,5x10^6 and 5x10^6 cells of the TIC lines. The size of the tumors was recorded for a period of about 3 months after which tumors were formalin fixed and paraffin sections routinely analysed by haematoxylin-eosin staining. Tumor pieces were serially xeno-transplanted into mice recipients for up to 14 passages. Orthotopic injection of luciferase 2 expressing AMC711T and AMC691B cells was also performed. Briefly, 1x10^6, 2,5x10^6 and 5x10^6 cells were injected into the fat tissue surrounding the adrenal glands of NMRI nu/ nu. Tumor take was evaluated after a period of 3 months by exponential increase of the intensity of luciferase signal within the mice using a highly-cooled CCD camera.

Newly-derived neuroblastoma cell lines propagated in serum-free medium
recapitulate the genotype and phenotype of primary neuroblastoma tumours

2

3. RESULTS

3.1 Clinical and genomic characteristics of patients tumors

Tumor material was obtained from six stages 4 neuroblastoma patients aged 15-115 months. Primary tumors were typed by H&E and by immunohistochemistry staining for NCAM, CHGA and Syp (Figure 1A and B and Supplementary Figure 1). All primary tumors were positive for nuclear imaging using MIBG I127 and all patients were positive for urine catecholamines. aCGH analysis showed *MYCN* amplification in four out of six tumors and multiple other aberrations at chromosomes 1p36, 11q and 17q. *ALK* mutation status of the primary tumors and neuroblastoma TIC cell lines was also reported (Table 1). These primary tumors were previously characterized using whole genome sequencing as described by Molenaar *et. al* [18] and the data is available at the European Genome-phenome Archive under the accession code EGAS00001000222.

Patient	INSS Stage	Age at diagnostics (months)	Gender	Site	MYCN amplification	ALK mutation status	Chromosomal aberrations	Cell line
N691	4	73	F	Adrenal	Yes	No	1p36 Loss 11q Normal 17q Gain	AMC691T AMC691B
N700	4	115	F	Adrenal	Yes	No	1p36 Loss 11q Normal 17q Normal	AMC700T AMC700B
N711	4	15	M	Abdominal side chain	Yes	No	1p36 Loss 11q Normal 17q Gain	AMC711T
N717	4	18	M	Adrenal	Yes	No	1p36 Loss 11q Normal 17q Gain	AMC717T
N753	4	25	M	Abdominal side chain	No	No	1p36 Normal 11q Loss 17q Gain	AMC753T
N772	4	36	M	Adrenal	No	No	1p36 Normal 11q Loss 17q Gain	AMC772T

Table 1. Clinical and genomic characteristics of patient tumours.

A
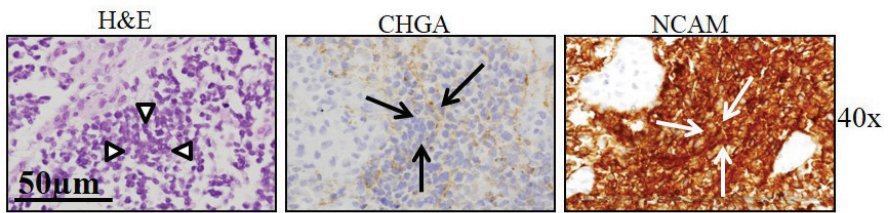
B

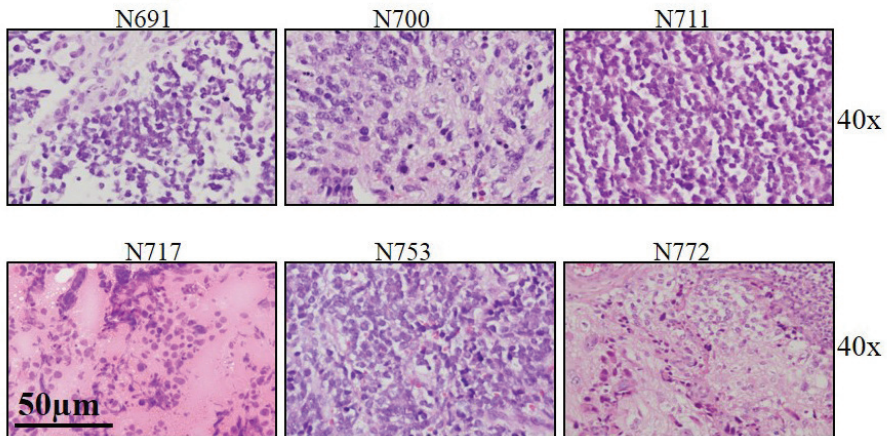**Figure 1:** H&E, CHGA and NCAM staining in human neuroblastoma tissue specimens. (A) H&E staining and immunohistochemistry for CHGA and NCAM was performed for patient N691. Neuroblastoma cells in the H&E staining were indicated with white arrowheads. Cells staining positive for CHGA are indicated with black arrows and NCAM with white arrows. (B) H&E staining in N691, N700, N711, N717, N753 and N772.

3.2 Neuroblastoma TICs express neuroblastoma markers and harbour neuroblastoma specific chromosomal aberrations.

Chemically dissociated cells obtained from primary neuroblastoma tumours could be isolated and cultured in serum free media containing bFGF and EGF under conditions used for TIC culture [12-13]. To verify that the newly-derived TIC lines are indeed neuroblastoma cells, Western blot analysis with specific neuroblastoma markers was performed on TICs and compared to 4 established classical neuroblastoma cell lines (Figure 2A). All TICs expressed MYCN including the two MYCN single copy cell lines. Also, NCAM was expressed albeit at varying levels. AMC691T did not express the neuroblastoma markers CHGA, DBH and TH, whereas AMC691B did express these 3 proteins. This indicates the heterogeneity in neuroblastoma tumours. To exclude that neuroblastoma TICs were contaminated with haematopoietic cells, protein expression of two haematopoietic markers CD19 and CD11b was assessed in all TIC lines. Lymphocytes served as positive controls.

In addition we performed FACS analysis with the same two haematopoietic markers CD19 and CD11b. First AMC691B was spiked with CD19 positive lymphocytes to determine the sensitivity of this method. Flow cytometry analysis revealed that the limit of detection of contamination of TIC cell lines with hematopoietic cells was observed at 1:100 (Supplementary Figure 2C). Subsequent FACS analysis of all neuroblastoma TICs showed that these were negative for CD19 and CD11b (Supplementary Figure 2D and 2E) as previously shown by Western blot in Figure 2A. We therefore concluded that the neuroblastoma TICs are not contaminated with haematopoietic cells.

The origin of neuroblastoma TICs was verified by comparing the aCGH profile of the primary tumour with the aCGH profiles of neuroblastoma TICs (Figure 2B). Chromosome 17q aberrations were most frequently seen. Copy number increases were observed in MYCN amplified TIC cell lines as well as their respective parent tumours (Supplementary Figure 2A). The identical position of the chromosomal breakpoints of the TICs cell lines and original tumours showed that the TICs were derived from the neuroblastoma tumours. In 5 out of 8 TICs a limited number of chromosomal aberrations were not identical with those in the primary tumours. A loss of chromosome 11q and gain of 1q appeared only in AMC691T and loss of 19p only in AMC691B. Gains of parts of 1p31 and 17q were observed in AMC700T and AMC700B. AMC717T had gains in parts of chromosome 1 and 8q and did not have a 1p deletion, whereas the primary tumour did show 1p deletion. AMC772T had unique gains in chromosome 12 and 14.
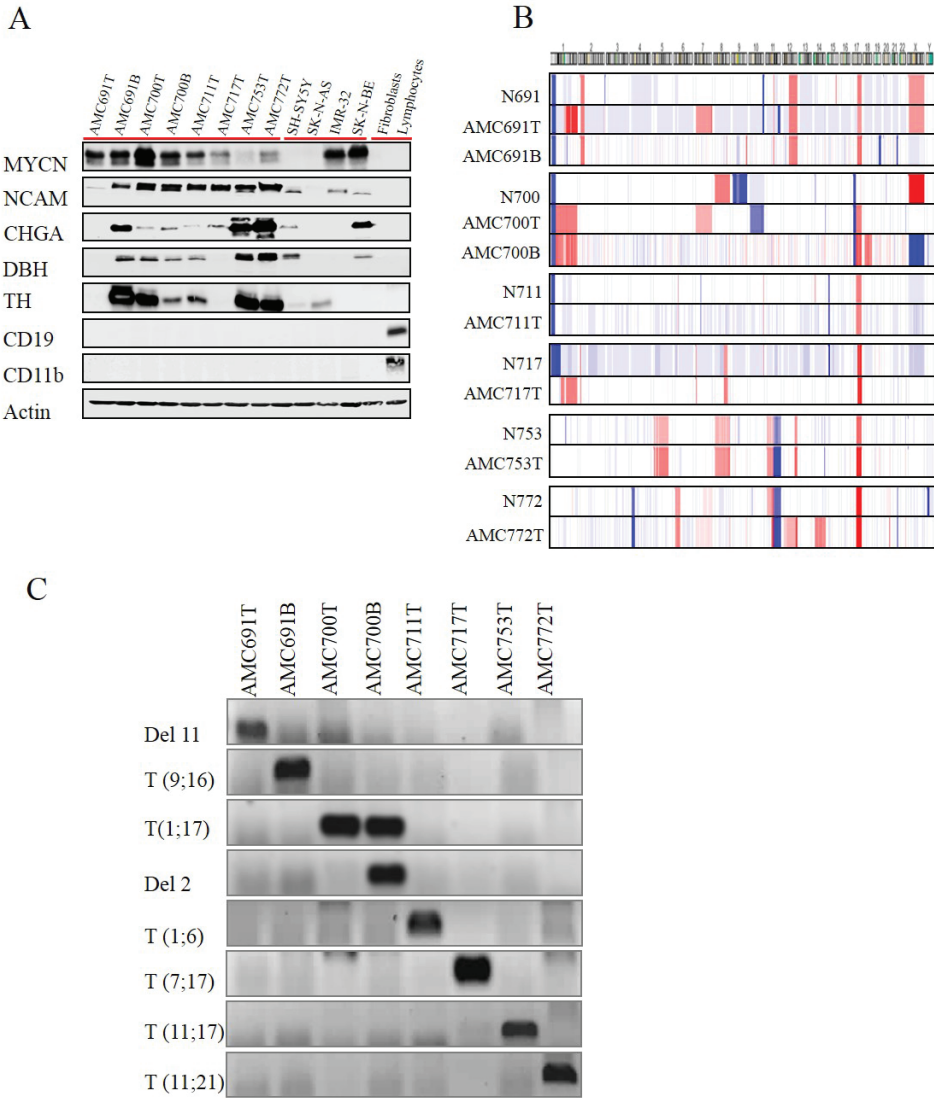
Figure 2: Genomic, protein characterization and DNA typing of neuroblastoma TIC cell lines. (A) Western blot analysis of various neuroblastoma markers MYCN, NCAM, CHGA, DBH and TH and haematopoietic markers CD19 and CD11b in neuroblastoma TIC lines (n=8), classical neuroblastoma cells (n=4), fibroblasts (n=1) and lymphocytes (n=1). Lymphocytes isolated from whole blood served as positive controls for CD19 and CD11b. B-actin was used as a loading control. (B) aCGH profiles comparing primary tumors and neuroblastoma TIC lines. Chromosome gains and losses were detected using the R2 software with blue indicating loss and red indicating gain. (C) Identification of unique breakpoints in neuroblastoma TIC cell lines by breakpoint PCR. Deletions in chromosome 11 were validated for AMC691T, t(9;16) for AMC691B, t(1;17)(AMC700T and AMC700B), deletion chromosome 2 (AMC700B), t(1;6)(AMC711T), t(7;17)(AMC717T), t(11;17)(AMC753T) and t(11;21)(AMC772T).

There were no major differences between the genomic profiles of N711 and N753 and their corresponding TIC lines AMC711T and AMC753T. The discrepancies in the genomic profiles between the tumours and TIC lines most likely indicate the presence of different sub-clones within primary tumours.

3.3 Typing of TIC lines by unique chromosomal breakpoints and short tandem repeat analysis

Correct identification of cell lines is essential and therefore, we performed two typing procedures for the TIC lines. The structural defects identified by aCGH (Figure 2B) allow identification of unique breakpoints in each sample. Based on WGS data (Supplementary Figure 2B), unique breakpoints were identified by JunctionDiff using the Complete Genomics Analysis suite software and were validated by breakpoint PCR with primers specifically designed for each of these breakpoints (Figure 2C and Supplementary Table 1). For AMC700T no unique breakpoint could be identified compared to AMC700B. To allow typing of these two TIC lines a breakpoint for AMC700T and B and a breakpoint unique for AMC700B were included.

Short tandem repeat profiling of cells has previously been described as a method permitting the unambiguous identification of cell lines [19-21]. This method was used to obtain the DNA fingerprints of the 8 neuroblastoma TICs using probes for 16 short tandem repeat markers (Supplementary Table 2). All TIC cell lines showed a distinct DNA fingerprint with the exception of the pair AMC691T and AMC691B, and the pair AMC700T and AMC700B which were derived from the primary tumour and bone marrow metastasis of the same patients.

3.4 Neuroblastoma TIC lines have distinct phenotypic characteristics and exhibit tumour sphere-forming capacity.

Upon isolation from the primary tumour mass and bone marrow aspirates, neuroblastoma TICs were cultured in serum-free medium supplemented with bFGF and EGF. Significant phenotypic differences between the neuroblastoma TICs were observed ranging from the adherent phenotype (AMC691T, AMC691B and AMC700T), to semi-attached (AMC700B and AMC711T) and spheroids (AMC717T, AMC753T and AMC772T) (Figure 3A). All neuroblastoma TICs exhibited different proliferative capacities with doubling times ranging from 76 hrs (AMC700T) to 204 hrs (AMC772T) (Figure 3B). In order to explore the *in vitro* tumourigenic potential of neuroblastoma TICs cells, we determined their ability to form tumour spheres from single cells under ultra-low attachment

conditions in 1% methylcellulose. All neuroblastoma TICs formed spheres within 30 days (Figure 3C). Sphere-forming potential varied among the TIC lines with AMC691B and AMC711T having the highest sphere-forming capacity as shown in Figure 3D.
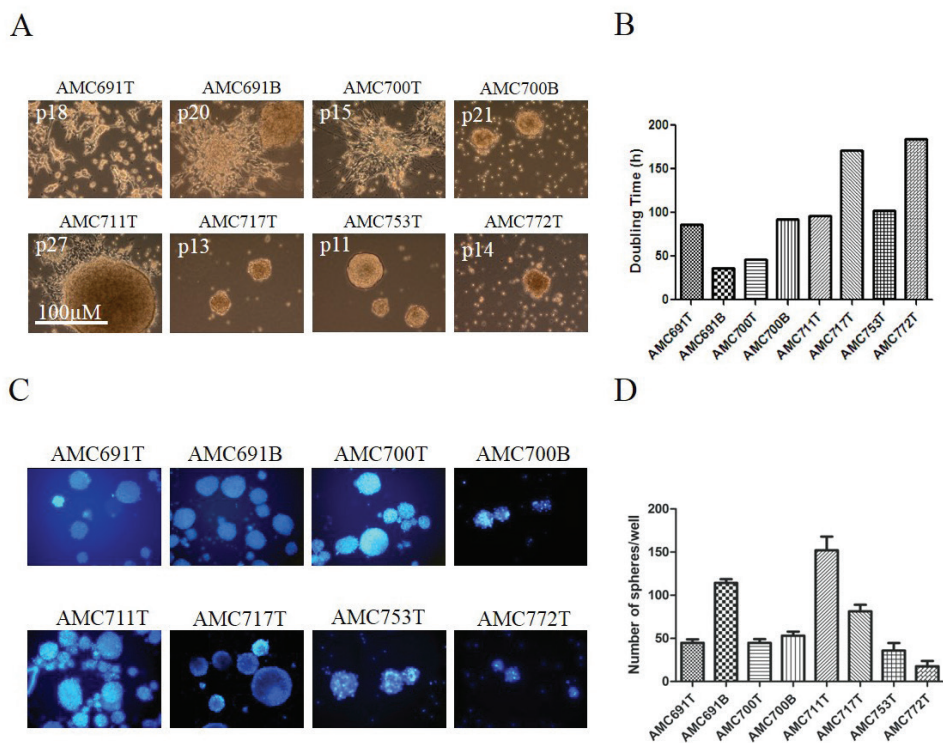
Figure 3: *In vitro* characterization of neuroblastoma TICs cell lines. (A) Light microscopy images of TICs cell lines AMC691T, AMC691B, AMC700T, AMC700B, AMC711T, AMC717T, AMC753T and AMC772T. (B) Doubling times of TICs cell lines as determined by MTT assays. The y-axis values represent the doubling time in hours of each cell line. (C) Fluorescent microscopy images of TIC cell line spheres stained with DAPI in 1% methylcellulose. (D) Sphere forming frequency of each TIC cell lines. An average of 2500 cells was seeded in each well and the number of spheres was estimated for on average 6 wells per cell line. The y-axis represents the number of spheres for on average n=6 wells.

3.5 Neuroblastoma TICs form tumours in mice and recapitulate the genotype of primary neuroblastoma tumours.

We used subcutaneous and orthotopic adrenal mouse models for evaluating tumour formation of all TICs cell lines. Subcutaneous engraftment was primarily performed in NMRI *nu/nu* mice. For TIC lines that showed low tumour take rates, additional xenografting was performed in NOD-SCID mice. All cell lines were tumourigenic in NMRI *nu/nu* and/or NOD-SCID mice, except AMC 691T and AMC753T (Supplementary Table 3). H&E staining and immunohistochemistry showed that these tumours consisted of small round blue immature neuroblasts and were positive for neuroblastoma markers NCAM, CHGA and Syp (Fig. 4A and supplementary Figure 3A). Serial passaging of small tumour pieces in secondary and tertiary mice recipients was performed to improve the tumour take rate of the TICs. This markedly increased the rate of engraftment and early onset of tumour formation (Figure 4B) (Supplementary Figure 3B).

In addition, we performed orthotopic xenografting to validate that TIC lines can form tumours in the microenvironment where neuroblastoma tumour initiation normally occurs. Injection of luc2 expressing AMC711T in the adrenals of immune compromised mice resulted in an increase of luciferase activity and corresponding tumour growth (Supplementary Figure 3C).

Xenografts from cancer cells have been reported to retain the genomic aberrations of parent tumours [22-29]. In order to confirm the origin of the xenografts from the neuroblastoma TICs, AMC691BX, AMC700TX and AMC711TX aCGH profiles of xenografts were compared with the primary tumour and neuroblastoma TIC lines. Genomic aberrations of the TIC xenografts clearly resembled the aberrations of the primary tumours and TIC lines from which they were derived (Figure 4C). A limited number of genomic aberrations were unique and most likely reflect clonal variation between the bulk of the TIC line and the sub-population from which the xenografts formed.

A
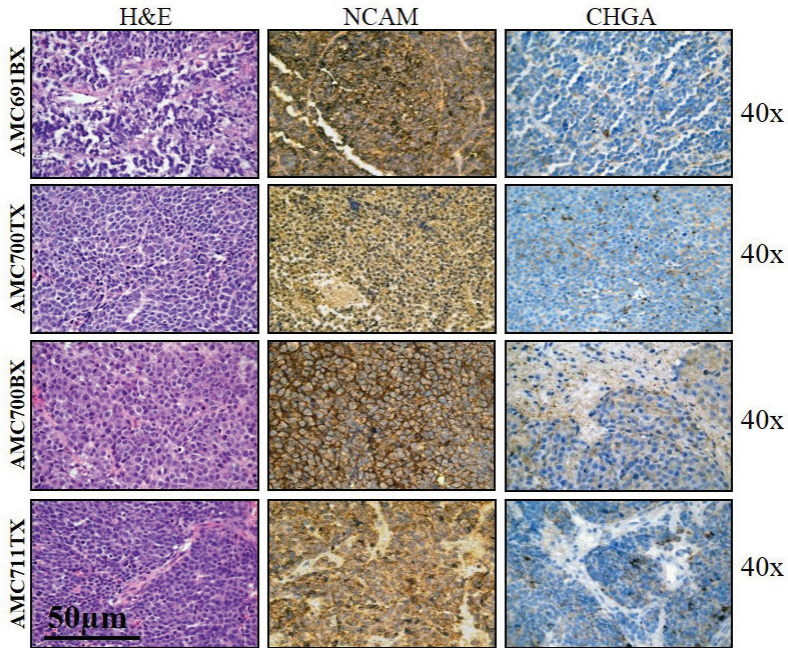
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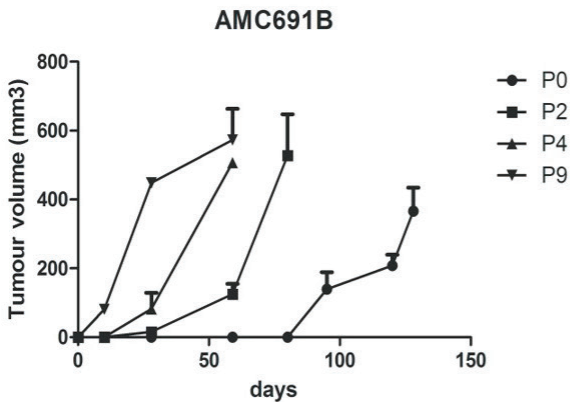
Figure 4: *In vivo* characterization of neuroblastoma TIC cell lines. (A) H&E, NCAM and CHGA staining of neuroblastoma tumors in mice was carried out on p6-p10 of serially passaged mice tumors. (B) Serial xenotransplantation of mice tumor pieces of AMC691B into secondary and tertiary recipients was performed to improve tumor engraftment. The y-axis represents tumor volume in mm^3

C
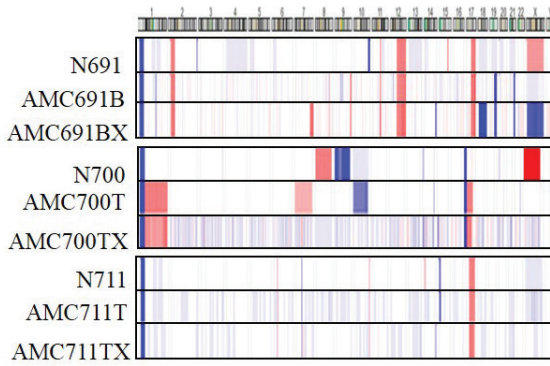
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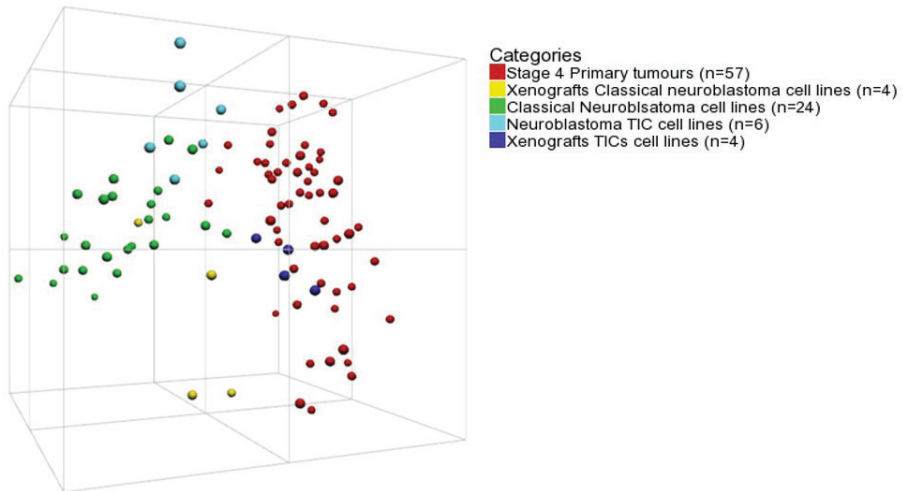
Figure 4: *In vivo* characterization of neuroblastoma TIC cell lines. (C) aCGH profiles of primary tumors, neuroblastoma TIC cell lines and mice xenografts were compared for similarities and differences in genomic aberrations. The in-house R2 software was used to generate the aCGH profile heat map. Blue and red in the heat map represents chromosomes losses and gains respectively. (D) Principal component analysis of 57 stage 4 primary tumors, 6 neuroblastoma TIC cell lines, 24 classical neuroblastoma cells, xenografts from 4 neuroblastoma TIC cell lines AMC691B, AMC700T, AMC700B and AMC711T and xenografts from 4 classical neuroblastoma cells SH-SY5Y, SK-N-AS, IMR-32 and SK-N-BE. Colours of the balls indicate the origin of the samples. PCA was performed in 3 major components.

3.6 Transcriptional analyses reveal a close relationship in the gene expression profiles of primary neuroblastoma tumours and neuroblastoma TIC xenografts

We subsequently investigated whether the phenotypes of xenografts derived from TIC lines better reflected primary tumours as compared to xenografts derived from classical neuroblastoma cell lines. Gene expression profiles of the TIC cell lines (n=6), of neuroblastoma xenografts derived from TIC lines (n=4) and xenografts derived from classical cell lines (n=4) were compared to previously generated profiles of classical cell lines (n=24) and stage 4 primary tumours (n=57). Unsupervised principal component analysis of all samples showed that xenografts from classical neuroblastoma cells clustered further away from primary tumours than neuroblastoma TIC xenografts. The only exception was a xenograft from the classical neuroblastoma cell line SH-SY5Y (Figure 4D and Supplementary Figure 4D). In addition, an unsupervised Euclidian distance-based hierarchical clustering using 500 highest standard deviation containing genes in all samples revealed 2 clusters with distinct gene expression patterns. Neuroblastoma TIC xenografts clustered within the group of primary tumours (Supplementary Figure 3E) while almost all classical cell line xenografts clustered together with the cell line population. All cell lines had the tendency to cluster away from the primary tumours irrespective of whether they were TICs or classical neuroblastoma cells. These results suggest that xenografts from neuroblastoma TICs retained the gene expression profiles of the primary neuroblastoma tumours.

4. DISCUSSION

In this study, we isolated and cultured 8 neuroblastoma TICs from primary neuroblastoma tumors and bone marrow metastases in serum-free medium supplemented with bFGF and EGF. We use the terminology of tumor initiating cells (TICs) to refer to our cell lines although we have not studied stem cell properties of these cell lines in depth. Nevertheless, we will continue to call these cell lines TICs as they were established with the protocols and techniques previously used to establish TIC lines for glioblastoma [30-31]. In this paper we aim to report the full phenotyping and genotyping of these cell cultures. We could confirm the origin of neuroblastoma TICs isolated in this study by western blot analysis of neuroblastoma markers and aCGH profiling. Neuroblastoma TICs were positive for known neuroblastoma markers and negative for the haematopoietic markers CD19 and CD11b, excluding contamination with cells of the haematopoietic lineage. There was no clear pattern of expression of neuroblastoma markers between the TICs derived from the primary tumors and bone marrow derived TICs. For example, AMC691T lacks expression of many neuroendocrine markers while AMC691B does express these markers. In AMC700B the TH and DBH markers show marked lower expression compared to the corresponding tumor derived TIC line. Thus the existence of different phenotypes of neuroblastoma cells is not restricted to certain niches.

aCGH profiling confirmed that the TICs were indeed derived from their parent tumors. The neuroblastoma TICs showed key genomic aberrations found in the primary tumors such as 17q gain and MYCN amplification. However, the TICs also contained unique chromosomal aberrations that were not detected in their tumors of origin. Loss of chromosome 11q was found in AMC691T and not in AMC691B and the corresponding primary tumor. Gain of chromosome 17q was present both in AMC700T and AMC700B and not in the primary tumor. This suggests that highly recurrent genomic aberrations as 17q gain and 11q loss can also appear as late events in sub-clones of neuroblastoma. This could have implications for targeted drug development strategies that aim at genes located at these chromosomal locations. *In vitro* tumorigenicity of TICs is defined by their ability to form spheres in suspension culture [32-35]. AMC711T and AMC691B showed high sphere forming capacity and xenografted with high efficacy while AMC691T and AMC753T did not form tumors in nude mice and had low sphere-forming capacity. The absence of tumor-forming potential *in vivo* of two of our eight TIC lines is in contrast with the previously described potential of TIC cells to form tumors *in vivo* [36-37]. This could relate to specific characteristics of these sub-clones which is a subject of further studies. PCA

and Euclidian distance clustering showed that the xenografts from neuroblastoma TICs clustered more with the primary neuroblastoma tumors as opposed to xenografts from classical cell lines. This suggested that the TIC cells more closely resemble the primary tumors compared to classical cell lines. In conclusion, we think that neuroblastoma TICs isolated in this study closely resemble the human neuroblastoma biology and are therefore useful additions to model systems for the study of neuroblastoma tumor biology and targeted drug development.

Newly-derived neuroblastoma cell lines propagated in serum-free medium
recapitulate the genotype and phenotype of primary neuroblastoma tumours

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SUPPLEMENTARY INFORMATION
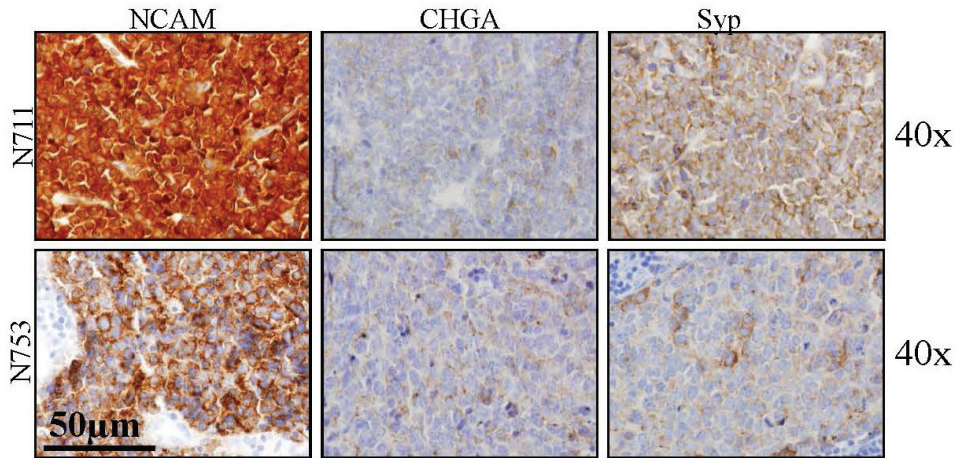
Supplementary Figure 1: IHC staining of human neuroblastoma tumor specimens. Immunohistochemistry was performed for NCAM, CHGA and Syp for patient N711 and N753.

A
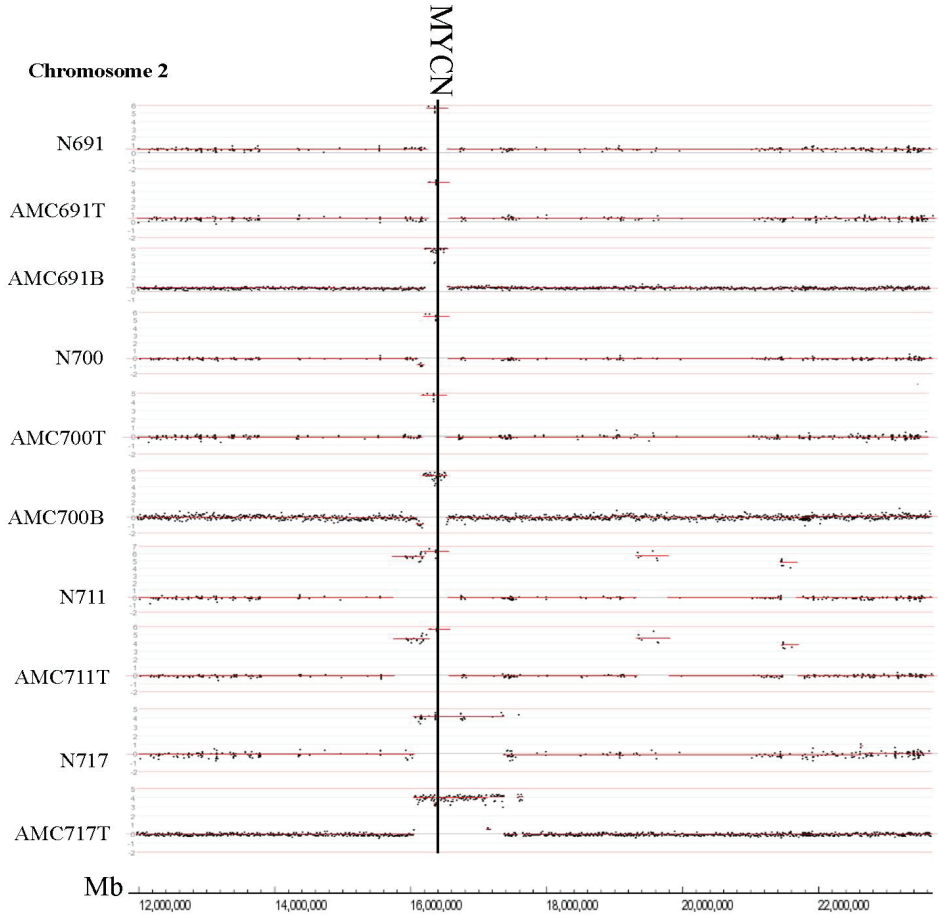
Supplementary Figure 2: MYCN amplification, identification of unique chromosomal breakpoints of neuroblastoma TIC cell lines and flow cytometry analysis of hematopoietic cell surface markers on neuroblastoma TIC cell lines. (A) Zoom in on chromosome 2 bearing the MYCN amplified region in MYCN amplified primary tumors and TIC cell lines. Black line indicates regions of MYCN copy number gains.

B
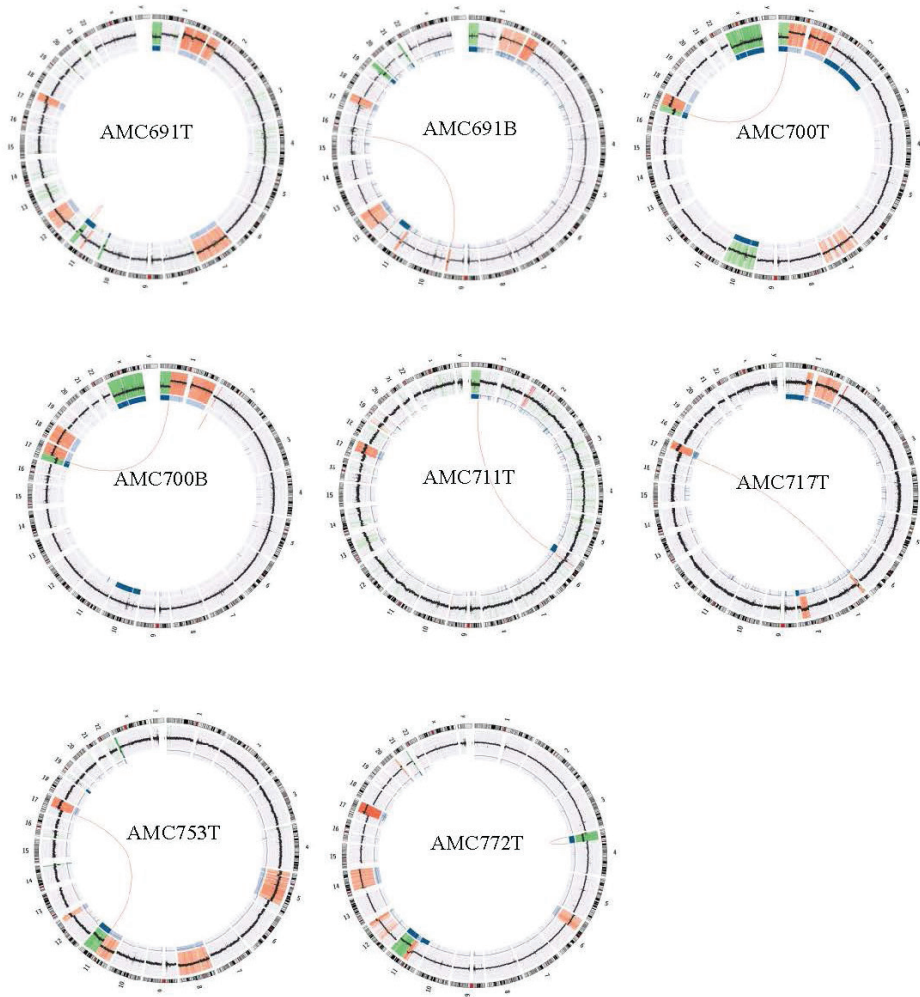
Supplementary Figure 2: MYCN amplification, identification of unique chromosomal breakpoints of neuroblastoma TIC cell lines and flow cytometry analysis of hematopoietic cell surface markers on neuroblastoma TIC cell lines. (B) Circos plots of neuroblastoma TICs cell lines with breakpoint regions validated highlighted in red.

Supplementary Figure 2: MYCN amplification, identification of unique chromosomal breakpoints of neuroblastoma TIC cell lines and flow cytometry analysis of hematopoietic cell surface markers on neuroblastoma TIC cell lines. (C) Flow cytometry analysis of AMC691B spiked with CD19 positive lymphocytes with spiking concentration ranges 1:1, 1:10, 1:100, 1:1000 and 1:10000.

D

TIC cell lines   CD19 Positive cells

Supplementary Figure 2: MYCN amplification, identification of unique chromosomal breakpoints of neuroblastoma TIC cell lines and flow cytometry analysis of hematopoietic cell surface markers on neuroblastoma TIC cell lines. (D) Flow cytometry analysis of the level of expression of CD19 cell surface markers on TIC cell lines. CD19 lymphocytes served as positive controls.

E
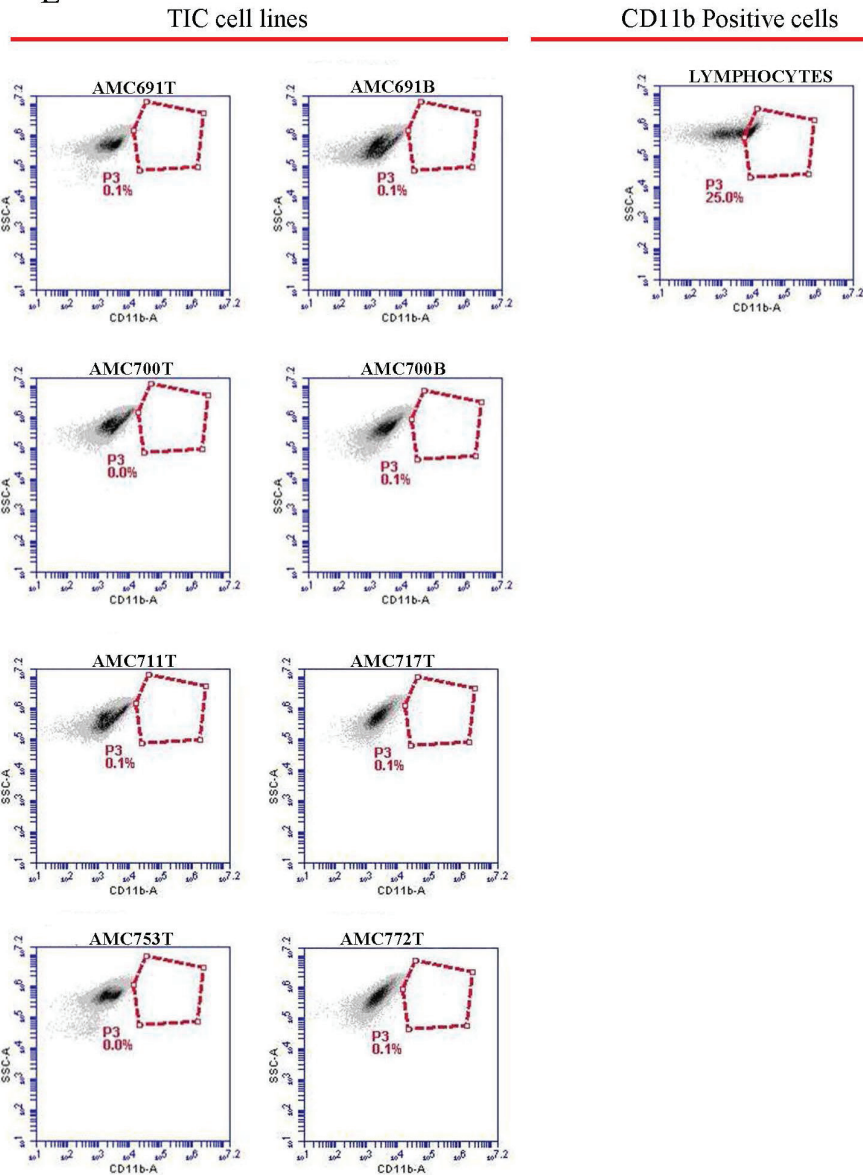
TIC cell lines
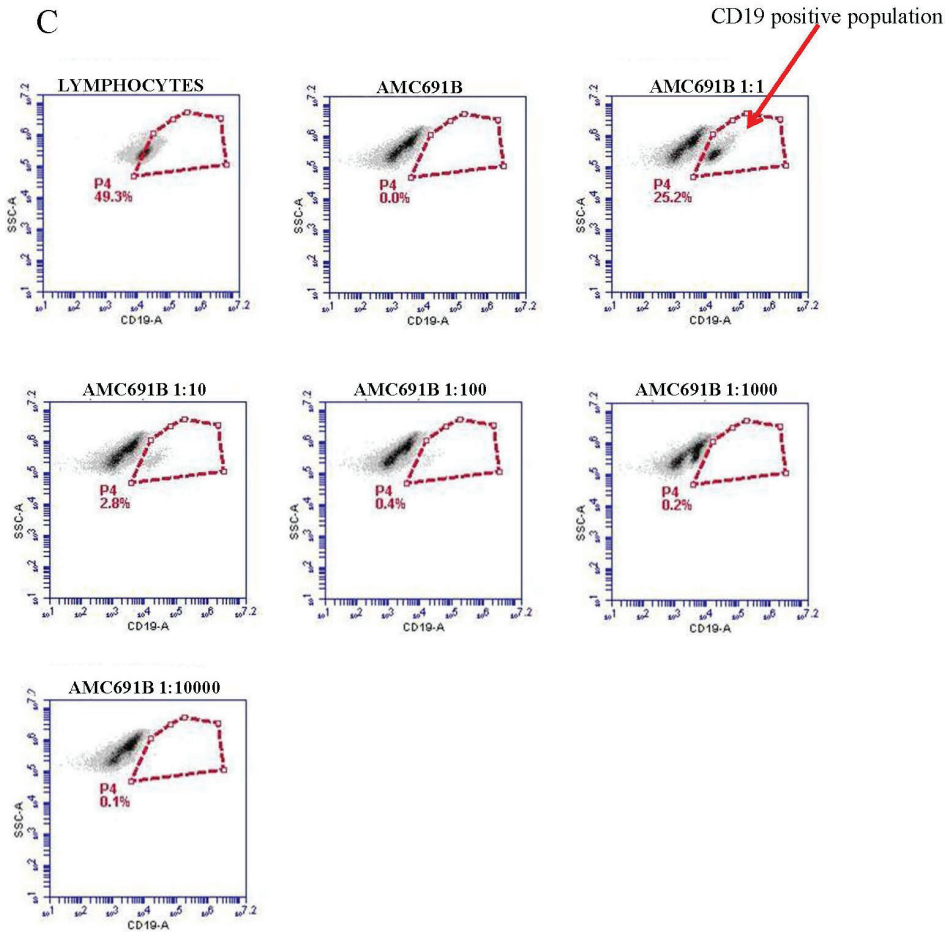
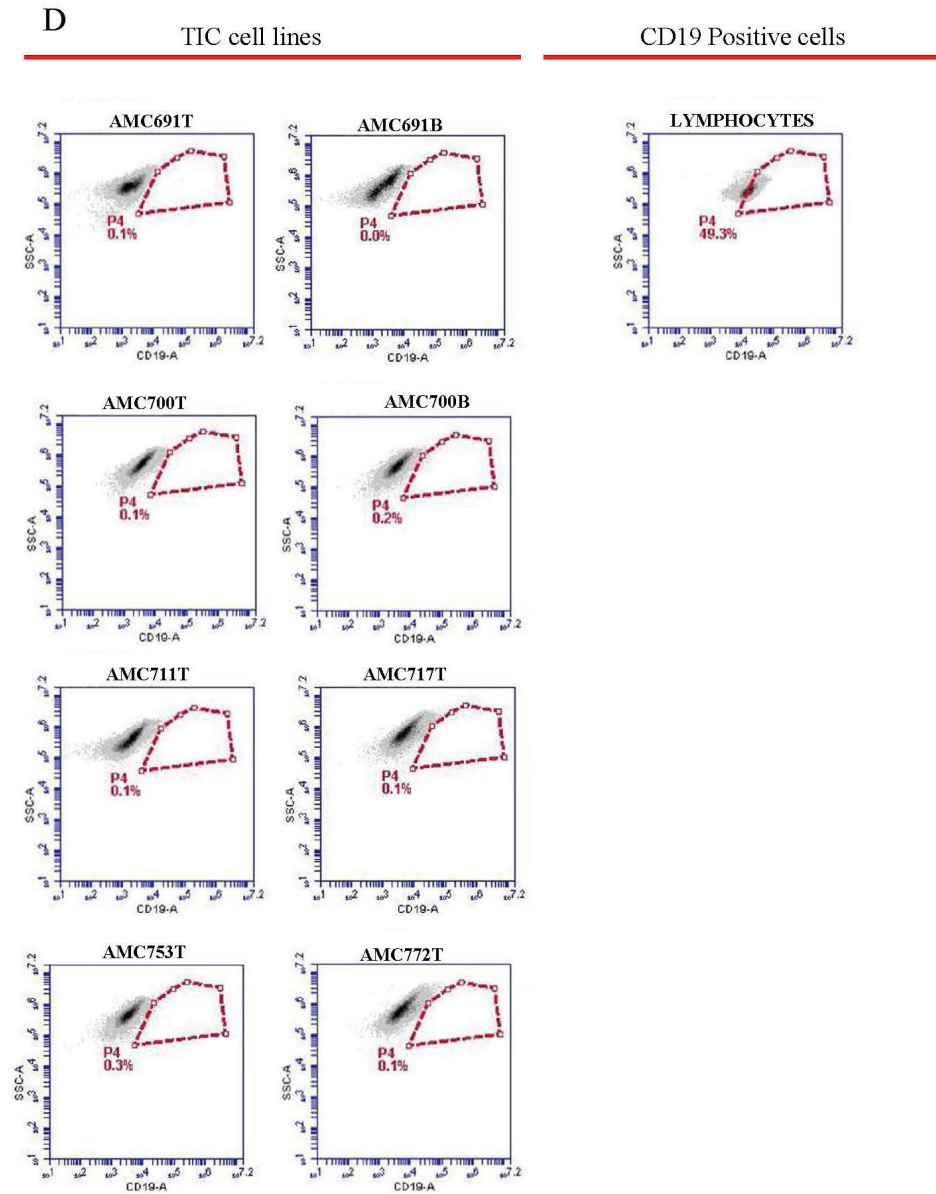### CD11b Positive cells

Supplementary Figure 2: MYCN amplification, identification of unique chromosomal breakpoints of neuroblastoma TIC cell lines and flow cytometry analysis of hematopoietic cell surface markers on neuroblastoma TIC cell lines. (E). Flow cytometry analysis of the level of expression of CD11b cell surface marker on TIC cell lines. CD11b lymphocytes served as positive controls.

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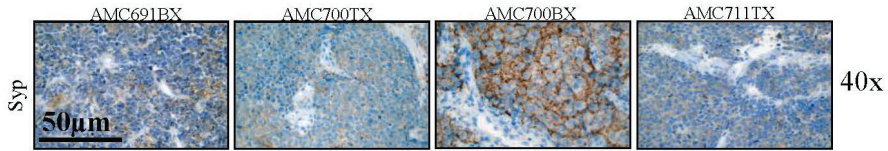
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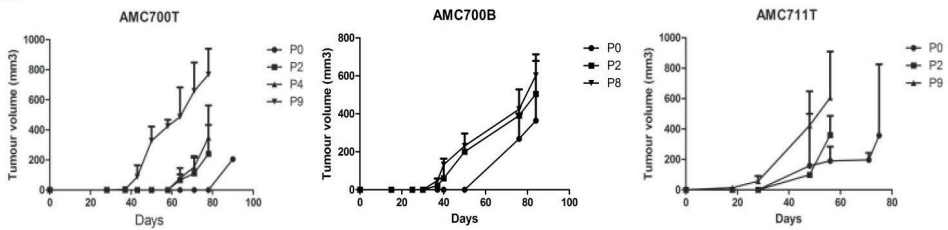
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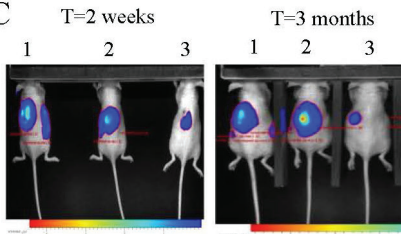
Supplementary Figure 3: *In vivo* growth properties and characterization of neuroblastoma TIC mice xenografts. (A) Immunohistochemistry was performed for Syp for neuroblastomas from AMC691BX, AMC700TX, AMC700BX and AMC711TX. (B) Serial xeno-transplantation of mice tumor pieces of AMC700TX, AMC700BX and AMC711TX into secondary and tertiary recipients was performed to improve tumor engraftment. The y-axis represents tumor volume in mm^3. (C) Intensity of luc2 AMC711T cells in adrenal glands of mice viewed with a CCD camera.

D
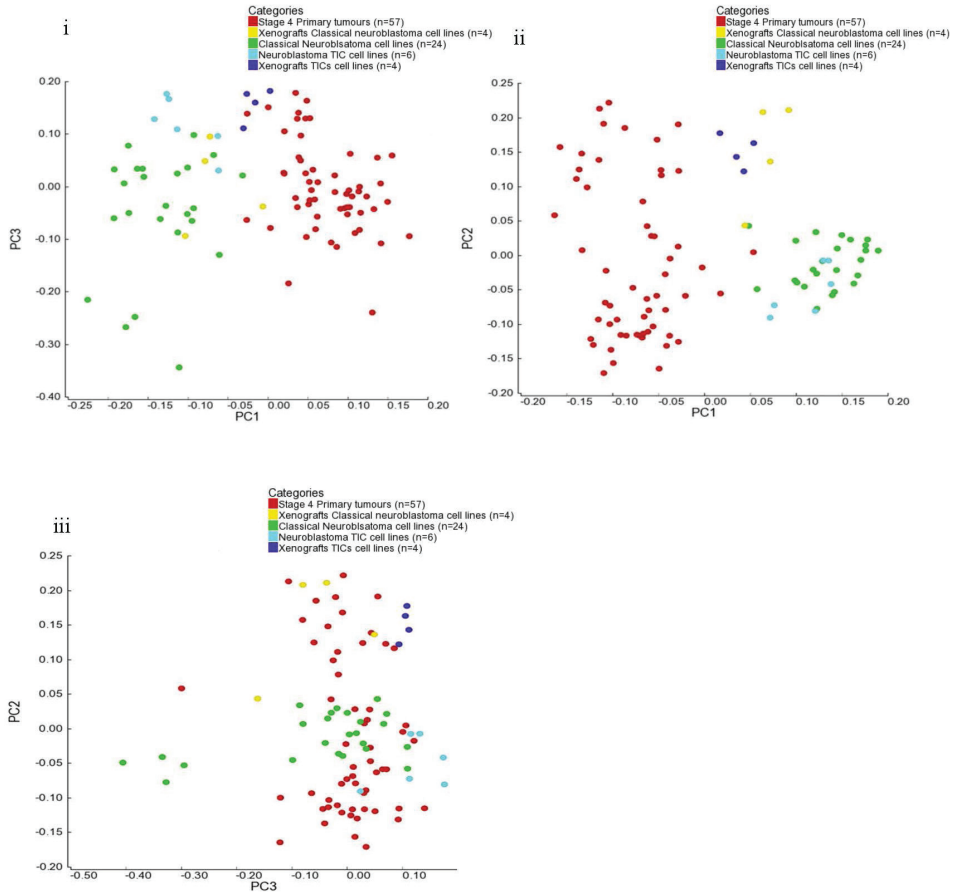
Supplementary Figure 3: *In vivo* growth properties and characterization of neuroblastoma TIC mice xenografts. (D) Principal component analysis of 57 stage 4 neuroblastoma tumors, xenografts from classical cell lines and xenografts from TIC cell lines (n=8) in three different components; PC2:PC1, PC3:PC1 and PC2:PC3(i-iii).

E
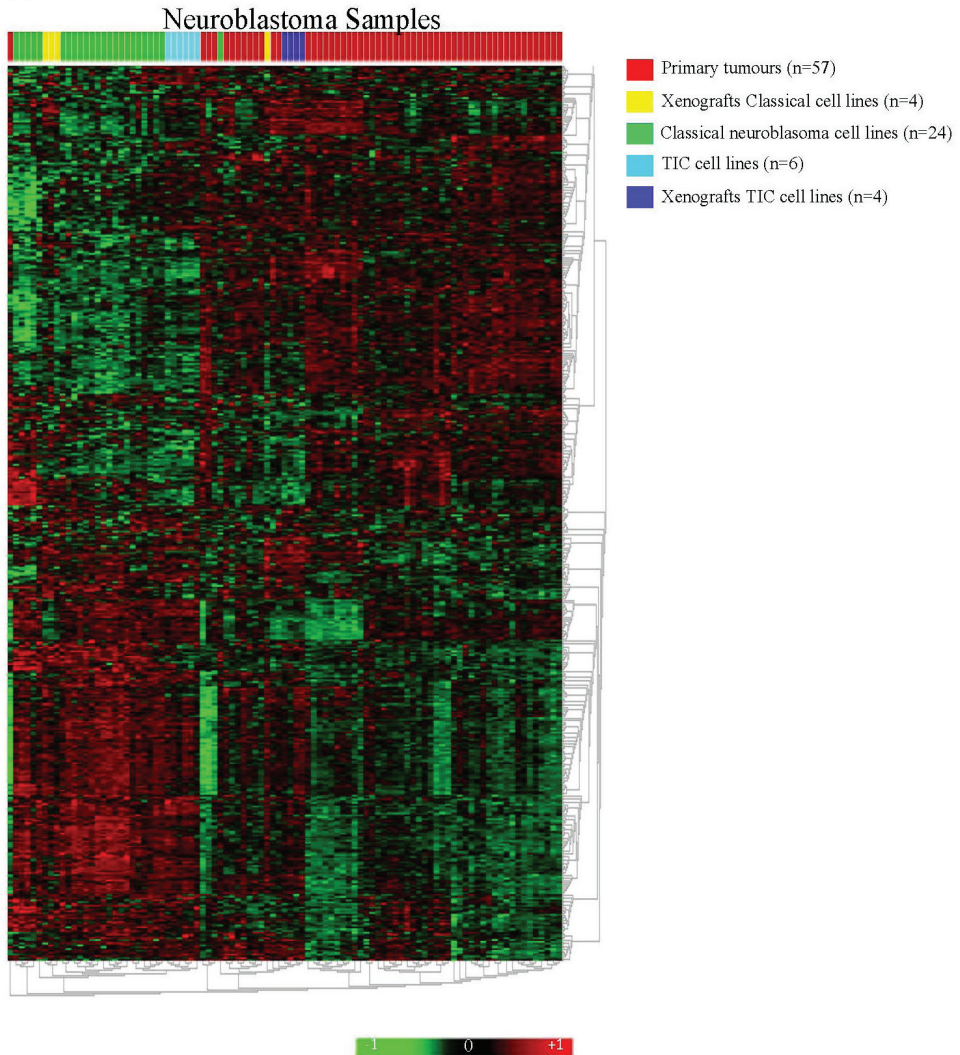
Supplementary Figure 3: *In vivo* growth properties and characterization of neuroblastoma TIC mice xenografts. (E) Euclidian distance cluster analysis of *in vivo* TICs and Classical neuroblastoma cell line xenografts (n=8), stage 4 primary neuroblastoma samples (n=57), neuroblastoma TIC cell lines (n=6) and classical neuroblastoma cells (n=24). The expression level of a given gene is indicated in red.

Cell line	Breakpoint	Nucleotide sequence	Product size
AMC691T	Deletion chromosome 11	5'-TCCCTTATAAGATGACCATCCC-3' 5'-CCAATTAAAAGGCAAAGATTGG-3'	199bp
AMC691B	Translocation (9;16)	5'-GTTAGCCTTTGGTCACGGAG-3' 5'-GGAGGCTGAGTCCTGGTGT-3'	194bp
AMC700T AMC700B	Translocation (1;17)	5'-TGCTCAAACACCAATACATGC-3' 5'-TGAGAATCGAGGTCTCAGCA-3'	244bp
AMC700B	Deletion chromosome 2	5'-GAAGTTGGGTGGAGGAGTCA-3' 5'-TGGGTTAGACTGGGATTTGTTC-3'	160bp
AMC711T	Translocation (1;6)	5'-CCTGGGCAACAAAGTGAGAC-3' 5'-TCCTTCTCCTCACTCCTCCA-3'	204bp
AMC717T	Translocation (7;17)	5'-CAGCACCTCTCCTGAAACAA-3' 5'-CTTTTCTCCACAGCCTCACC-3'	164bp
AMC753T	Translocation (11;17)	5'-TAGTGAGCACCAGGCAGTTG-3' 5'-CTGCGGTCTGTTCTCAACAT-3'	197bp
AMC772T	Translocation (11;21)	5'TAAAGATGTCCAACCAGGGC-3' 5'-GCTCAGTCTCCCTCACCCT-3'	189bp

Supplementary Table 1: Primer sequences used for breakpoint identification and chromosomal location of each breakpoint in each cell line.

	D3S1358	TH01	D21S11	D18S51	PentaE	D5S818	D13S317	D7S820	D16S539	CSF1PO	PentaD	Amel	Vwa	D8S1179	TPOX	FGA
AMC691T	14,15	7,9.3	29,31.2	14,18	7,13	10,11	9,14	9,12	9	10,11	12,13	X	18,19	16	8	21,22
AMC691B	14,15	7,9.3	29,31.2	14,18	7,13	10,11	9,14	9,12	9	10,11	12	X	18,19	16	8	21,22
AMC700T	14,17	7,9.3	29,31	12,16	13,17	10,12	11,12	8,10	9,11	12	10,11	X	14,15	12,16	8	18,20
AMC700B	14,17	7,9.3	29,31	12,16	13,17	10,12	11,12	8,10	9,11	12	10,11	X	14,15	12,16	8	18,20
AMC711T	16,17	7,9.3	31.2,32.3	14,18	7,12	12,13	11,12	8,9	11,12	10,11	10,13	X,Y	17,18	13,14	8	20,22
AMC717T	15,16	6,9.3	16,18	11,18	11,18	11	11,13	8,9	9,13	11,12	13,14	X,Y	16,18	11,14	8,9	20,21
AMC753T	11,18	9,9.3	28,31.2	14,18	17,20	12,13	12	11	10,11	11,12	13	X,Y	14,18	10,15	10,12	20,23
AMC772T	16,19	9.3	28	12,13	14,15	12	12,14	8,10	12,13	10,12	9	X,Y	16,17	10,13	8,10	19,23

Supplementary Table 2: Allelic determinants of neuroblastoma TIC cell lines relative to a reference DNA sample.

Cell line	Subcutaneous (% engrafted)	Orthotopic (% engrafted)
NMRI nu/nu		
AMC691T	0	ND
AMC691B	60	ND
AMC700T	41	ND
AMC700B	33	ND
AMC711T	67	ND
AMC717T	ND	ND
AMC753T	0	ND
AMC772T	ND	ND
NOD-SCIDS		
AMC691T	0	ND
AMC691B	ND	66
AMC700T	75	ND
AMC700B	66	ND
AMC711T	ND	ND
AMC717T	33	ND
AMC753T	0	ND
AMC772T	100	80

Supplementary Table 3: Overview of xenografting procedures and tumor take rates of all TIC lines in NMRI *nu/nu* and NOD-SCID mice.

SUPPLEMENTARY MATERIALS AND METHODS

1. Protein isolation and western blot and FACS analysis

Cell lysates were resuspended in laemmli lysis buffer (25ml H_2O, 5ml 1M Tris /HCl pH 6.8, 10ml 100% Glycerol, 10ml 20% SDS, with bromophenol blue) containing protease inhibitors. Lysates 15µg per lane were applied to SDS-PAGE. Immunoblotting of antibodies specific for Chromogranin A (1:10000 Abcam), Neural Cell Adhesion Molecule (1:10000 Millipore), Dopamine Beta Hydroxylase (1:1000 Cell Signalling), Tyrosine Hydroxylase (1:1000 Santa Cruz), CD11b (1:20000 Abcam), CD19 (1:1000 Dako), MYCN (1:5000 BD Pharmingen) and B-actin (1:5000 Abcam) were detected using HRP-conjugated anti-mouse or anti-rabbit Abs (Amersham) and Licor 800 mouse and rabbit secondary antibodies and visualized using the Odyssey LI-COR system (LI-COR BIOSCIENCES GmbH) and the Image Quant LAS 4000 mini system (GE Healthcare).

For FACs analysis, neuroblastoma TIC cell lines were harvested and rendered single celled using the Neurocult Chemical Dissociation Kit (Stem Cell Technologies). Cells were washed with FACs staining buffer (1% BSA, 0.5mM EDTA in PBS) and stained with CD11b (10µl BD Bioscience) and CD19 (2,5µl Abcam) and incubated at 4°C in the dark for 20 minutes. FACs analysis was carried out immediately using a BDAccuri flow cytometer (BD Biosciences).

2. Immunohistochemistry

Immunohistochemistry was performed on 4µM paraffin-embedded sections heated in an antigen retrieval solution (citrate buffer pH 6.0). Endogenous peroxidase activity was inhibited by incubation with 30% hydrogen peroxide (20 min, RT) and the non-specific sites blocked with PBS/BSA 0, 25% (10 mins, RT). CHGA (Abcam, USA) and NCAM (Abcam, USA) were detected by incubating tissue sections overnight at 4°C with primary antibodies. Sections were washed with TBS and incubated with anti-mouse or anti-rabbit immunoglobulins/horse rabbit peroxidase for 30 min at room temperature (DPVR-55HRP, DPVM-55HRP, Counterstaining of the slides was performed with haematoxylin and antibodies were detected using a DAB detection system (Ventana, USA).

3. Microsatellite typing and breakpoint PCR

DNA was harvested from primary neuroblastoma cell lines by the chloroform/ isopropanol method as previously described[18]. Microsatellite typing was performed

Newly-derived neuroblastoma cell lines propagated in serum-free medium
recapitulate the genotype and phenotype of primary neuroblastoma tumours

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with 10ng of DNA using the PowerPlex 16 system (Promega Corp) and results were analysed using the GeneMapper software (Applied Biosystems). The following Loci were amplified; Penta E, D18S51, D21S11, TH01, D3S1358, FGA, TPOX, D8S1179, vWA, Amelogenin, Penta D, CSF1P0, D16S539, D7S820, D13S317 and D5S818.

For breakpoint PCR, 10ng genomic DNA was used as templates for the amplification of breakpoint regions with 5ng of primers specific for each breakpoint PCR conditions were as follows: initial DNA denaturation for 3 minutes 95°C followed by 35 cycles consisting of 30 seconds of denaturation at 94°C, 30 seconds annealing at 58°C and 30 seconds of primer extension at 72°C. 10μl of PCR reactions were analysed by agarose gel electrophoresis (1% agarose) followed by ethidium bromide staining.

4. Sphere forming assays

For neurosphere culture, 2500 primary NB cells were seeded in ultra-low attachment 24 well plates (Corning) in 1% methylcellulose and spheres were counted as soon as they reached 25-200μM in diameter.

5. Data analysis

Segmentation by DNA copy using the bioconductor package was performed in-house before generation of a heat map for chromosome gains and losses. A copy number gain was defined as a 2log ratio >0,5 and a copy number loss was defined as a 2log ratio <0,5.

For the generation of WGS data, the somatic structural variants were compared between the TIC cell lines and the lymphocytes from the patients using the JunctionDiff and the Junction2Event tool from CGA tools. The somatic events were filtered based on the following criteria: events noted as artefacts, footprints less than 70 bases, smaller than 10 discordant mate pairs, under-represented repeats and present in a set of v2.0 baseline genomes.

WGS data for the primary tumours is deposited in the European Genome-phenome Archive and available under accession number EGAS00001000222.

PCA was performed in R based on mRNA expression data normalized with a MAS5.0 software and visualized with the R2 software program.

CHAPTER 3

ENHANCER OF ZESTE HOMOLOGUE 2 PLAYS AN IMPORTANT ROLE IN NEUROBLASTOMA CELL SURVIVAL INDEPENDENT OF ITS HISTONE METHYLTRANSFERASE ACTIVITY.

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ABSTRACT

Neuroblastoma is predominantly characterized by chromosomal rearrangements. Next to MYCN amplification, chromosome 7 and 17q gains are frequently observed. We identified a neuroblastoma patient with a regional 7q36 gain, encompassing the enhancer of zeste homologue 2 (EZH2) gene. EZH2 is the histone methyltransferase of lysine 27 of histone H3 (H3K27me3) that forms the catalytic subunit of the polycomb repressive complex 2 (PRC2). H3K27me3 is commonly associated with the silencing of genes involved in cellular processes such as cell cycle regulation, cellular differentiation and cancer. High EZH2 expression correlated with poor prognosis and overall survival independent of MYCN amplification status.

Unexpectedly, treatment of three EZH2-high expressing neuroblastoma cell lines (IMR32, CHP134 and NMB), with EZH2-specific inhibitors (GSK126 and EPZ6438) resulted in only a slight G1 arrest, despite maximum histone methyltransferase activity inhibition. Furthermore, colony formation in cell lines treated with the inhibitors was reduced only at concentrations much higher than necessary for complete inhibition of EZH2 histone methyltransferase activity.

Knockdown of the complete protein with three independent shRNAs resulted in a strong apoptotic response and decreased cyclin D1 levels. This apoptotic response could be rescued by overexpressing EZH2ΔSET, a truncated form of wild-type EZH2 lacking the SET transactivation domain necessary for histone methyltransferase activity.

Our findings suggest that high EZH2 expression, at least in neuroblastoma, has a survival function independent of its methyltransferase activity. This important finding highlights the need for studies on EZH2 beyond its methyltransferase function and the requirement for compounds that will target EZH2 as a complete protein.

INTRODUCTION

Overexpression of the Enhancer of zeste homologue 2 (EZH2) gene has been associated with tumourigenicity in numerous solid tumour types (1-7) and gain-of-function point mutations in the catalytically active SET domain of EZH2 has been recognized in B-cell and T-cell lymphomas(8-16). Genetic loss-of-function studies have demonstrated a crucial role of EZH2 in the establishment of cell fate decisions in the skin, heart and mammary glands (17). EZH2 together with suppressor of zeste 12 (SUZ12) and embryonic ectoderm development (EED) forms part of the Polycomb repressive complex 2 (PRC2), which mediates the silencing of genes by trimethylation of lysine 27 on histone H3 (H3K27me3) (18, 19). This H3k27me3 mark has been found in genes that play a key role in cellular processes such as cell differentiation, cell cycle regulation and oncogenesis (20-22). However, recent studies suggest that EZH2 directly binds to the promoter regions of certain genes and acts as a transcriptional co-activator independent of its histone methyltransferase enzymatic activity (23-25).

Neuroblastoma is a neuroendocrine tumour that arises from the peripheral nervous system (26). It is the most commonly diagnosed extracranial solid cancer in children, accounting for approximately 15% of all pediatric cancer deaths (27, 28). Chromosome 17q gain, partial loss of chromosome 1p or 11q and MYCN amplification are frequently observed genetic aberrations in neuroblastoma tumours (29). EZH2 is located on chromosome 7q35 and frequent gains of whole chromosome 7 have been observed in neuroblastoma (30, 31). A functional role for EZH2 in neuroblastoma was reported whereas EZH2 caused histone hypermethylation in the promoter regions of known tumour suppressor genes CASZ1, CLU, RUNX3 and NGFR resulting in the silencing and downregulation of these genes (32). In the current study, we show that pharmacological inhibition of EZH2 histone methyltransferase activity (33-36) only causes limited inhibitory effects on cell cycle progression, while silencing of the whole protein causes a strong apoptotic phenotype. We overcame apoptosis caused by EZH2 silencing by overexpressing a truncated form of wild-type EZH2 lacking histone methyltransferase activity. These findings highlight the importance of EZH2 for the survival of neuroblastoma cells independent of its histone methyltransferase activity and development of compounds that inhibit EZH2 protein as a whole might be beneficial for the treatment of neuroblastoma patients with high EZH2 expression.

2. MATERIALS AND METHODS

2.1. Patient samples, RNA isolation and profiling.

RNA was extracted from 88 tumours with TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer's protocols. RNA concentration and quality were determined using the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies). Fragmentation of cRNA, hybridization to Human Genome U133 Plus 2.0, microarrays and scanning were carried out according to the manufacturers protocol (Affymetrix inc. Santa Barbara, CA). mRNA gene expression data were normalized with the MAS5.0 algorithm within the GCOS program of Affymetrix Inc. Target intensity was set to 100. All data were analyzed using the bioinformatics platform R2 (http://r2.amc.nl). As a reference dataset, an RNAseq data set of 498 neuroblastoma tumors was used. Data was derived from GEO database under number gse 62564(37).

2.2 Array CGH analysis.

Array CGH was performed by hybridizing 100 ng genomic DNA to a 180K platform (Agilent Technologies). DNA was labelled by random priming with CY5-dCTP and CY3-dCTP respectively and hybridized at 65ºC for about 17 h. Chips were scanned on an Agilent G2565BA DNA microarray Agilent scanner. Data processing was performed using the bioinformatics platform R2. Circular binary segmentation (CGHcall package in R) was used for scoring the regions of gain, amplification and deletion. Testing for elevated EZH2 expression of tumours with 7q gain versus no gain tumours was determined using a one-tailed Student's t-test for equal variance.

2.3. Cell culture and compound exposure assays.

Classical human neuroblastoma cell lines and neuroblastoma tumour-initiating cell (TIC) lines were cultured as previously described (38). Cell culture protocols are described in detail in the Supplementary Materials and Methods.

Neuroblastoma cell lines were seeded in triplicate in 6-well plates using the most optimal confluency for each cell line. Cells were incubated overnight and treated with 1 nmol/L to 100 µmol/L GSK126 or EPZ6438. Control samples were treated with 0.5% DMSO. After 72 h, cells were transferred to 96-well plates (classical cell lines) or 48–well plates (TIC lines) and incubated with the compounds for another 72 h. Cell viability was determined prior to and after 144 h treatment using the 3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (39). Half maximal effective

concentration (IC_{50}) values were derived from dose-response curves. IC_{50} values at 144 h were calculated by determining the GSK126 or EPZ6438 concentrations needed to achieve a 50% reduction in cell viability observed for DMSO-treated cells at 144 h (set at 100%) using the GraphPad Prism software.

2.4. Western blotting.

Cells were lysed using Laemmli buffer (i.e. H_2O/glycerol/20% sodium dodecyl sulfate (SDS)/1 M Tris-HCl (pH 6.8) 5:2:2:1 (v/v/v/v)) containing protease inhibitors. Equal protein amounts (i.e. 40 µg) were separated by SDS- polyacrylamide gel electrophoresis (SDS-PAGE). The following primary antibodies were used: rabbit anti-human EZH2 (Clone 4905) monoclonal antibody (1:10,000, Cell Signaling Technology), rabbit anti-human tri-methyl histone lysine 27 (clone C3B6B11) monoclonal antibody (1:1,000, Cell Signaling Technology), rabbit anti-human total H3 (Clone 9715) polyclonal polyclonal (1:1,000, Cell Signaling Technology), rabbit anti-human PARP (Clone 9542S) monoclonal antibody (1:1,000, Cell Signaling Technology), mouse anti-human cyclin D1 monoclonal antibody (1:1,000, Thermo Scientific) and mouse anti-human β-actin (clone AC-15) monoclonal antibody (1:5,000, Abcam). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit (clone NA9340V) and goat anti-mouse (clone NXA931) secondary antibodies (1:10,000 GE Healthcare) were used prior to visualization with the Image Quant LAS 4000 mini system (GE Healthcare). (Amersham) IRDye 800CW goat anti-rabbit and goat anti-mouse secondary antibodies (1:5,000, Li-COR) were used prior to visualization on the Li-COR Odyssey.

2.5. FACS analysis.

Neuroblastoma cell lines IMR32, CHP134 and NMB were seeded in triplicates in 6-cm plates and incubated overnight. Cells were then treated for 72 h with 0.01% DMSO (control), GSK126 (62.5 nmol/L - 2 µmol/L) or EPZ6438 (62.5 nmol/L - 2 µmol/L). Floating and adherent cells were subsequently harvested for FACS analysis to determine the cell-cycle distribution and the apoptotic subG1 fraction. See Supplementary Materials and Methods for a detailed protocol.

2.6. Colony forming assays.

IMR32, CHP134 and NMB ($5x10^3$ cells per well) were resuspended in 500 µL DMEM containing 0.4% low melting point agar and seeded in duplicate in 24-wells plates coated overnight with 1% low melting point agar in DMEM containing 4% serum. GSK126 and EPZ6438 were diluted to final concentration ranges of 32.5 nM - 2 µM in 0.4%

low melting point soft agar and added to the corresponding wells. Control wells were treated with 0.1% DMSO in 0.4% low melting point soft agar. Colonies were allowed to form for 14 d and stained with 5 mg/mL MTT dissolved in 4% serum containing DMEM. Number of colonies were scored by the Image J quantification software (U.S. National Institute of Health).

2.7. Cell Transduction

IMR32, CHP134 and NMB were seeded onto 6-cm dishes ($2x10^5$ cells in 5 mL culture medium) and incubated overnight. Next, cells were transduced with non-targeting shRNA (AACAAGATGAAGAGCACCAA; negative control), EZH2 shRNA specifically targeting the coding sequence and the 3'UTR region of the gene (TRCN0000040073, TRCN0000040074, TRCN0000040076, TRCN0000293738 and TRCN0000286227) using the pLenti VI system or with EZH2ΔSET (which was donated by Dr. Marian Martínez-Balbás at the Department of Molecular Genomics of the Barcelona Molecular Biology Institute, Spain) using the pInd system according to the manufacturers protocol (Sigma Aldrich). After 72 h overexpression and knockdown, cells were harvested for FACS and Western blot analysis as described above.

3. RESULTS

3.1. Gain of 7q36 correlates to EZH2 over-expression in neuroblastoma.

Evaluation of the frequency distribution of all chromosomal gains and losses in 87 neuroblastoma showed that most frequent gains occur in chromosomes 17 and 7 (Fig. 1A). The gain of chromosome 7 mostly involved the complete chromosome, however we identified 1 neuroblastoma tumour with a 785 kb regional gain, forming a SRO harbouring only 10 genes located at chromosome 7q36 (Fig. 1B) including EZH2. Affymetrix mRNA expression profiling of the same neuroblastoma tumour series showed that the tumour with the regional gain harbouring EZH2 on 7q36 had the highest EZH2 gene expression (Fig. 1C). Neuroblastoma tumours harbouring increased chromosome 7 copy number and also shows significant higher expression of EZH2 than tumours with no gain in chromosome 7 ($P = 5.6x10^{-3}$) (Fig. 1D). These results suggest that the gain on DNA level contributed to EZH2 overexpression. Next we analyzed EZH2 Affymetrix mRNA expression data of 2,448 tumour samples representing 13 different tumour types and 504 samples of 9 types of normal tissues. Average EZH2 mRNA levels in neuroblastoma were significantly higher than the average EZH2 mRNA expression levels found in

multiple other tumour types ($P=1.9x10^{-22}$) and normal tissues ($P= 3.9x10^{-7}$) (Fig. 1E). In order to establish the clinical relevance of EZH2 in neuroblastoma, we analysed whether EZH2 expression correlates to prognosis in the 88 neuroblastoma series. Kaplan-Meier analysis of overall survival shows that EZH2 expression is significantly associated with poor prognosis ($P = 3.7x10^{-04}$) (Fig. 1F). These results were confirmed in a publicly available cohort of 493 tumours ($P=1.5x10^{-05}$) (Supplementary Fig. 1D). Previous studies have shown a correlation between MYCN amplification and EZH2 status which we could confirm in the larger cohort (p=0.41 and $1.5x10^{-05}$) (Supplementary Fig.1F). Independent of MYCN amplification EZH2 expression was still correlated to a poor prognosis in non-MYCN-amplified tumors (p=$5.2x10^{-05}$) (Supplementary Fig. 1E). However, EZH2 should not be considered as an independent prognostic factor in neuroblastoma. Together, these analyses show that EZH2 is highly expressed in neuroblastoma compared to other tumour types and normal tissues. Our data also suggests that gain of chromosome 7 contributes to overexpression of the gene.

3

A.

B.

Chr 7

EZH2

C.

Survival
INSS stage
MYCN amp

D.

EZH2 mRNA expression

No 7q Gain   7q Gain

E.

EZH2 mRNA Expression

F.

Overall survival probability

Low (n=71)

High (n=17)

Raw p 3.7e-04
Bonf p 0.027

Follow up in months
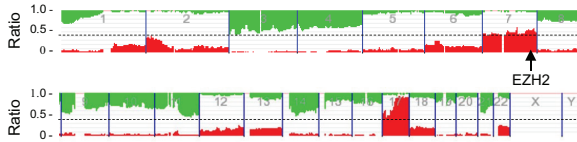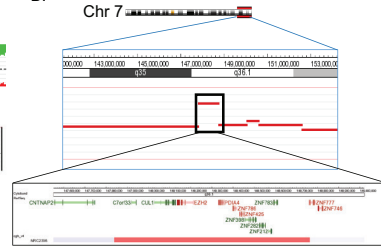
Figure 1. Array CGH and mRNA expression analysis of EZH2 in neuroblastoma tumours. (A) Summary of CGH analysis of 87 neuroblastoma tumours showing the frequency of gains (red) and losses (green) per chromosome (horizontal). The y-axis indicates the overall gains and losses per region. The arrow indicates the EZH2 gene locus. (B) Close-up view of CGH analysis of the neuroblastoma tumour N167T with a local gain of the EZH2 locus. A cytogenic map of the chromosome 7 is shown above with a blue box indicating the region shown below. CGH log-fold ratios are shown per probe (gray; no significant gain or loss; black significant gain or loss). The red line indicates the moving average over 5 probes. Below; chromosomal position in Mb and cytogenetic map. The blue box indicates the gained region of 785 kb encompass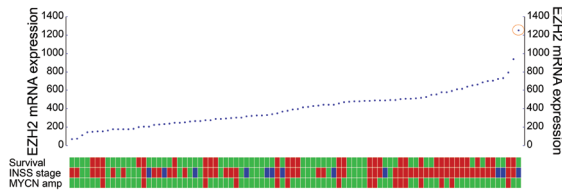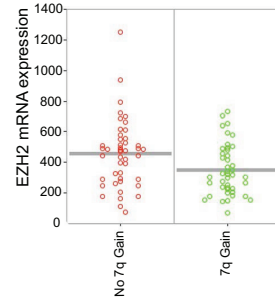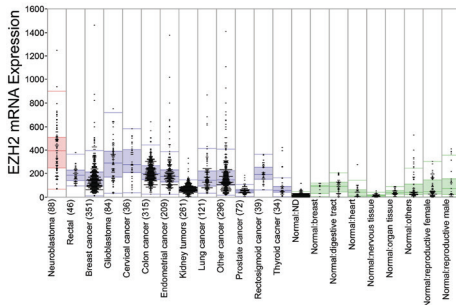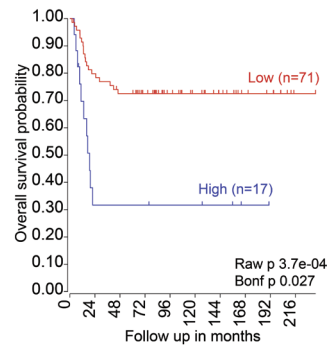ing EZH2. Below; a zoom-in of the gained region harbouring the EZH2 locus showing 10 genes including EZH2 in this region. (C) Overview of EZH2 mRNA expression levels measured by Affymetrix 133 plus 2.0 array analysis in 88 neuroblasoma tumours. Tumours are ranked by EZH2 expression (y-axis). The x-axis shows Survival (green: alive>5yrs red: death), INSS stage (light green: stage 1; dark green: stage 2; orange: stage 3; red: stage 4; blue: stage 4s) and MYCN amplification (red: >= 8copies of MYCN; green: < 8 copies of MYCN; gray: not informative). The circle indicates tumour N167T with local gain of EZH2. (D) EZH2 Affymetrix 133 plus 2.0 array mRNA expression levels in 87 neuroblastoma tumours (y-axis). Tumours were grouped according to CGH data for chromosome arm 7q as gain (red) or no gain (green) ($P = 5.6 \times 10^{-3}$). A copy number gain was defined as a 2log ratio >0.5 and a copy number loss was defined as a 2log ratio <0.5. (E) Combined box-dot plot of EZH2 Affymetrix 133 plus 2.0 array mRNA expression levels in 12 cancer datasets (blue), 1 neuroblastoma dataset (red) and normal tissues (green) divided in adrenal gland, central nervous system (CNS) tissues and non-CNS tissues. In a combined boxdot-plot every dot represents one sample. The number of tumour samples is given between brackets. The colored boxes represent the area between the 25th and the 75th percentile with a line indicating the median. (F) Kaplan-Meier analysis for overall survival of neuroblastoma patients divided into high and low EZH2 expression groups for all neuroblastoma patients (n = 88). Significance is denoted as *P*-value (p) and Bonferroni corrected *P*-value (bonf p).

3.2. Inhibition of the histone methyltransferase activity of EZH2 results in a slight G-1 arrest in neuroblastoma.

In order to explore the functional relevance of the histone methyltransferase activity of EZH2 in neuroblastoma, we first established the fitted IC_{50} values of 14 neuroblastoma cell lines treated with two EZH2 specific histone methyltransferase inhibitors EPZ6438 and GSK126 (Table 1 and Supplementary Fig. 1A).

Neuroblastoma cell lines IMR32 and CHP134 responded most potently to EPZ6438 with IC_{50} values in the nanomolar range (i.e. 570 and 670 nmol/L respectively) IMR32 responded most potently to GSK126 (i.e. IC_{50} = 740 nmol/L). Although IMR32 and CHP134 do express relatively high levels of EZH2 (Supplementary Fig 1B.), the IC_{50} values of EPZ6438 and GSK126 in the complete neuroblastoma cell line panel did not correlate with EZH2 mRNA expression levels (Supplementary Fig. 1C).

Cell lines	EPZ6438 (µmol/L)	GSK126 (µmol/L)
IMR32	0.6	0.7
CHP134	0.7	1.1
AMC691B	0.9	4.9
SKNAS	1.1	1.9
AMC106	1.5	1.6
AMC700B	2.1	1.5
AMC772T	2.3	1.1
KCNR	2.5	5.9
NMB	2.6	3.3
SHEP2	3.8	2.7
CAN	3.9	1.1
AMC700T	5.4	1.2
N206	7.4	1.1
AMC691T	8.3	8.0

Table 1. IC_{50} values of GSK126 and EPZ6438 for neuroblastoma cell lines.

To study the correlation between the phenotype after treatment with EPZ6438 and GSK126 and the histone methyltransferase activity of EZH2, neuroblastoma cell lines IMR32, CHP134 (with low IC50 values) and NMB (with highest expression of EZH2) were treated with increasing concentrations of both compounds and harvested for protein analysis. Treatment with low nanomolar concentrations of EPZ6438 (i.e. 62.5 nmol/L) and high nanomolar concentrations of GSK126 (i.e. 500 nmol/L) was sufficient to almost completely inhibit the methyltransferase activity of EZH2, as was shown by the downregulation of H3K27me3 (Fig. 2A). Strikingly, phenotypically the cells did not show obvious differences under the light microscope, even if cells were treated with high concentrations of the EZH2 inhibitors (supplementary figure 2A). To study the phenotype in more detail we performed flow cytometry for analysis of the cell cycle distribution and apoptotic sub G1 fraction after treatment with 125 nmol/L or 1 µmol/L EPZ6438 and GSK126. A slight increase in the fraction of cells in G1 phase was observed, but only after treatment with the highest dose of both compounds (Fig. 2B and Supplementary Fig. 2B). The minimal phenotypic effects obtained with micromolar concentrations of EPZ6438 and GSK126 were in sharp contrast with the strong inhibition of the EZH2 histone methyltransferase activity obtained with nanomolar concentrations of EPZ6438 and GSK126. We therefore hypothesized that the effect of inhibiting the histone methyltransferase activity on cell viability occurs only after prolonged treatment

of the cell lines with either EPZ6438 or GSK126. Therefore, we performed colony forming assays for 14 days on the three cell lines treated with concentrations whereby target specific inhibition of H3K27me3 is known to occur (125 nmol/L-1 μmol/L). In line with the effects on G1 arrest, the strongest inhibitory effect on colony formation was obtained after treatment with EPZ6438 and GSK126 only at concentrations much higher than necessary for complete EZH2 histone methyltransferase activity inhibition (i.e. 1 μmol/L) (Fig. 2C).

Taken together, these results indicate that, even though EZH2 is known to silence tumour suppressor gene activity through its methyltransferase activity, EZH2 might have other functionally relevant roles, independent of its histone methyltransferase activity, in neuroblastoma.

3.3. Downregulation of EZH2 causes a strong apoptotic response independent of the EZH2 histone methyl transferase activity.

It has previously been reported that the oncogenic role of EZH2 in diffuse B-cell lymphoma and prostate cancer can be independent of its histone methyltransferase activity (23-25). To investigate this for neuroblastoma, we first performed knockdown experiments (T=72h) targeting wild-type EZH2 with three independent shRNA's in the three EZH2 high expressing cells and studied its effect on the cell viability of these cells. Western blot analysis showed that knockdown of the complete EZH2 protein resulted in the downregulation of cyclin D1 and induction of cleaved PARP (Figure 3A). Light microscopy images showed a marked reduction in cell number and increased number of floating cells in all cell lines after EZH2 knockdown with all three shRNAs (Supplementary Fig. 3A). Subsequent cell cycle analysis using flow cytometry indicated a strong increase in the sub-G1 fraction in all three cell lines (Fig. 3B and Supplementary Fig 3B). Both PARP cleavage and sub G1 fraction were indicative for a strong apoptotic response. This was in contrast with the results using targeted compounds inhibiting the EZH2 methyltransferase activity. To determine whether the apoptotic response observed after knockdown of wild-type EZH2 was independent of EZH2 histone methyltransferase activity, we overexpressed an exogenous EZH2 mutant (EZH2ΔSET) lacking functional histone methyltransferase activity. This was then combined with a knockdown of wild-type EZH2 using two shRNAs specifically targeting the wild-type EZH2 transcript at the 3'UTR.

A.
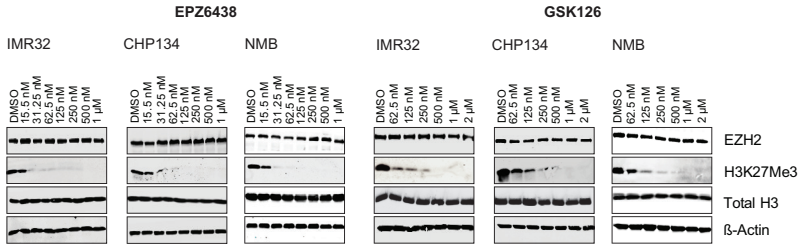
B.
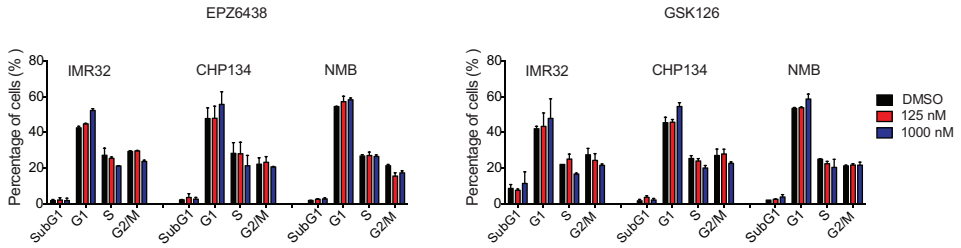
C.
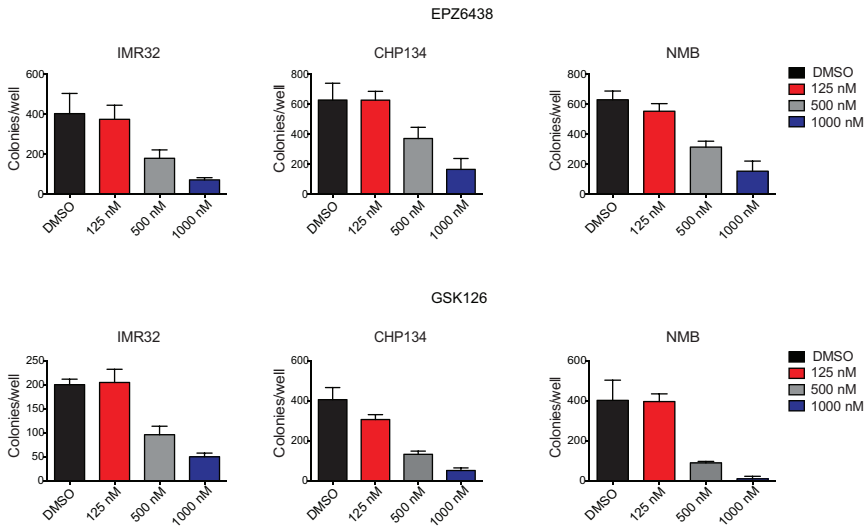
In cell line CHP134 we could show a decrease in cleaved PARP (Fig. 4A) and a reduction in the apoptotic sub-G1 fraction after overexpression of the EZH2ΔSET, indicative of a partial rescue of the apoptotic phenotype caused by knocking down wild-type EZH2 (Fig. 4B). We thereby concluded that the decrease in the amount of cleaved PARP and the sub-G1 fraction of apoptosis observed was as a result of EZH2ΔSET taking over the functional role of EZH2 on neuroblastoma cell survival upon depletion of wild-type EZH2 by shRNAs. This highlights an important role of EZH2 in neuroblastoma cell viability independent of its histone methyltransferase activity.

Figure 2. Pharmacological inhibition of EZH2 histone (4)(4)(4)(4)(4)(4)methyltransferase activity by EPZ6438 and GSK126 (A) Western blot analysis of EZH2 high expressing cell lines IMR32, CHP134 and NMB after 72 h treatment with increasing concentrations of EPZ6438 and GSK126. Blots were incubated with EZH2, H3K27Me3 and total H3 antibodies. β-Actin served as loading control for this experiment. (B) Flow cytometric analysis of PI stained nuclei of neuroblastoma cell lines IMR32, CHP134 and NMB. The effects on the cell cycle after 144h treatment with 125 nmol/L and 1 μmol/L of EPZ6438 and GSK126 are indicated. The data represents the mean percentage of cells ± SD of three replicate experiments. (C) Colony forming capacity of EZH2 high expressing cell lines IMR32, CHP134 and NMB treated for 14 d with 125nmol, 500nmol and 1μmol/L of EPZ6438 and GSK126. The data represents the mean number of colonies per well ± SD of two replicate wells per concentration of both compounds.

A.
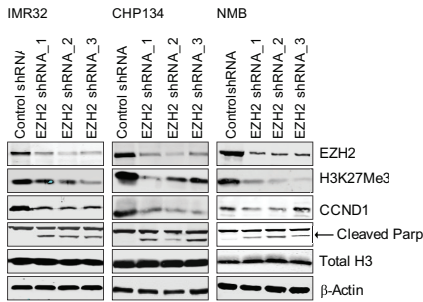
B.
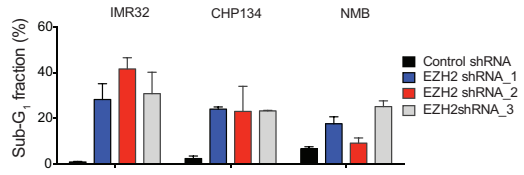
Figure 3. EZH2 knockdown by shRNA in EZH2 high expressing cell lines. (A) Western blot analysis of IMR32, CHP134 and NMB 72 h after transduction with lentivirus targeting EZH2 (i.e. EZH2 shRNA_1, EZH2 shRNA_2 and EZH2 shRNA_3) and control shRNA. Blots were incubated with EZH2, H3K27Me3, CCND1, total PARP and total H3 antibodies. β-actin served as loading control for this experiment. (B) Flow cytometric analysis of PI stained nuclei of neuroblastoma cell lines IMR32, CHP134 and NMB 96 h after lentiviral transduction with three shRNA's targeting EZH2 and a control shRNA. Data represent the mean percentages of cells in sub-G_1 ± SD of three replicate experiments.

3

A.

B.
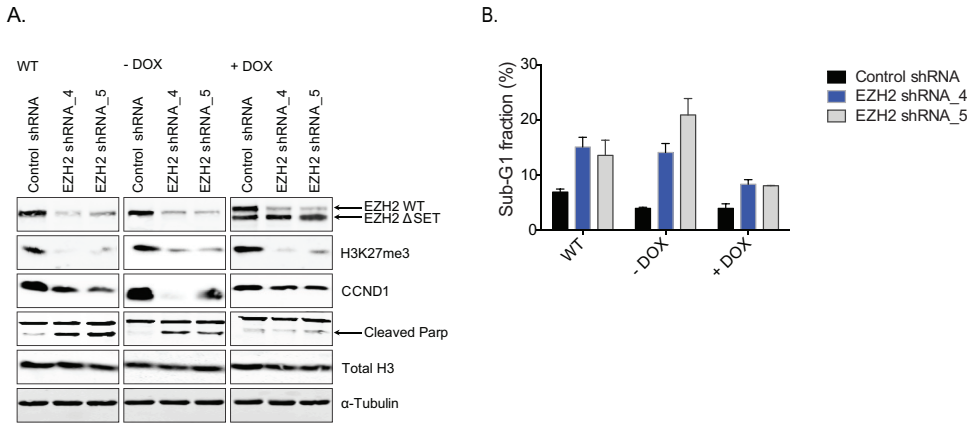
Figure 4. Overexpression of an exogenous EZH2ΔSET mutant upon depletion of wild-type EZH2. (A) Western blot analysis showing simultaneous overexpression of an exogenous EZH2 mutant form lacking histone methyltransferase activity and knockdown of wild-type EZH2 in CHP134 72 h after transduction with two shRNA specifically targeting wild-type EZH2 (EZH2 shRNA_4 and EZH2 shRNA_5) and control shRNA. Blots were incubated with EZH2, H3K27Me3, CCND1, total PARP and total H3 antibodies. α-tubulin served as a loading control for this experiment. (B) Flow cytometric analysis of PI stained nuclei of the neuroblastoma cell line CHP134 after 72 h overexpression of an exogenous EZH2ΔSET mutant and lentiviral transduction of CHP134 with two shRNA's targeting wild-type EZH2 and a control shRNA. Data represent the mean percentages of cells in sub-G_1 ± SD of three replicate experiments.

4. DISCUSSION

It has previously been reported that EZH2 plays a key role in the silencing of tumour suppressor genes through methylation of H3K27me3 in the promoter region of these genes in neuroblastoma (32, 40). In this study, we show that EZH2 is aberrantly gained and overexpressed in neuroblastoma tumours and that patients with high EZH2 expression have a poor prognosis. However, this is not independent of other prognostic factors as previously reported (41). In agreement with its described function, we found that the EZH2 specific histone methyltransferase inhibitors GSK126 and EPZ6438 strongly downregulated H3k27me3 in EZH2 high expressing cell lines. Interestingly, only a slight G1-arrest of the cell cycle and a mild reduction in colony formation was observed at biologically relevant compound concentrations. A more explicit inhibition of colony formation only occurred at higher concentrations. This suggests that this is due to off-target effects of both compounds at these concentrations. However, it has recently been reported that combined inhibition of DNA methylation with a DNA demethylating agent 5-aza-2'-deoxycytidine and the EZH2 histone methyltransferase-specific inhibitor EPZ6438 re-induced the expression of tumour suppressor genes suggesting that combining DNA demethylating agents with histone methyltransferase inhibitors might be a therapeutic effective option in neuroblastoma (41).

Additionally, we observed a strong apoptotic response in neuroblastoma cells upon inhibition of the total EZH2 protein which was largely independent of EZH2 methyltransferase activity. Alternative oncogenic functions of EZH2 have been described previously. First EZH2 was shown to function as a transcriptional activator but this required an intact methyltransferase domain (24). More recent papers have shown oncogenic functions of EZH2 independent of the methyltransferase activity (23, 25). In Natural Killer/T-cell lymphoma this required an intact PRC2 complex and functioned through direct transcriptional activation of CCND1 (Cyclin D1). In our study, we could show a downregulation of CCND1 after knockdown of EZH2 which suggests involvement of a similar mechanism. In SWI/SNF mutant cancers the majority of tumours showed EZH2 dependence which required an intact PRC2 complex but was independent of EZH2 methyltransferase activity. This resulted in insensitivity to EZH2 methyltransferase activity inhibitors. Neuroblastoma might mimic these tumour types where frequent mutations in SWI/SNF genes ARID1A and ARID1B have recently been shown to occur frequently in this tumour type (42).

Our findings of an oncogenic role of EZH2 in neuroblastoma independent of it's methyltransferase activity, highlights also the need for the development and testing of therapeutics which specifically target the EZH2 protein as a whole (43).

3

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SUPPLEMENTARY INFORMATION

3

Supplementary Figure 1. (A) Fitted IC_{50} curves of 14 neuroblastoma cell lines treated with EPZ6438 and GSK126. Fitted curves were obtained using Graphpad Prism software after treatment of the cells for 144h with both compounds. Each curve represents the mean of three wells per compound concentration. (B) mRNA expression (y-axis) of EZH2 in 14 neuroblastoma cell lines. Samples were ordered from high to low expression of EZH2. mRNA expression was obtained by affymetrix profiling of the cell lines using the u133 plus2 array platform and analysis was carried out with the R2 Software. (C) Correlation of EZH2 Affymetrix U133 plus 2 mRNA expression with the fitted IC_{50} values of EPZ6438 and GSK126. Significance is denoted as R^2 values and correlation coefficient was calculated by Linear Regression Analysis with $R^2 < 0.500$ as the minimal level of correlation and $R^2 > 0.500$ indicated as [***]. (D) Kaplan-Meier analysis for overall survival of neuroblastoma patients divided into high and low EZH2 expression groups for all neuroblastoma patients (n = 493). Significance is denoted as P-value (p) and Bonferroni corrected

P-value (bonf p). (E) Kaplan-Meier analysis for overall survival of neuroblastoma patients divided into high and low EZH2 expression groups for all non-MYCN-amplified neuroblastoma patients (n = 401). Significance is denoted as *P*-value (p) and Bonferroni corrected *P*-value (bonf p). (F) EZH2 Affymetrix 133 plus 2.0 array mRNA and RNAseq expression levels in 88 and 493 neuroblastoma tumours respectively (y-axis). Tumours were grouped according to MYCN-amplification (red) or non-MYCN-amplification (green) (P= 0.41 and 1.5×10^{-05}).
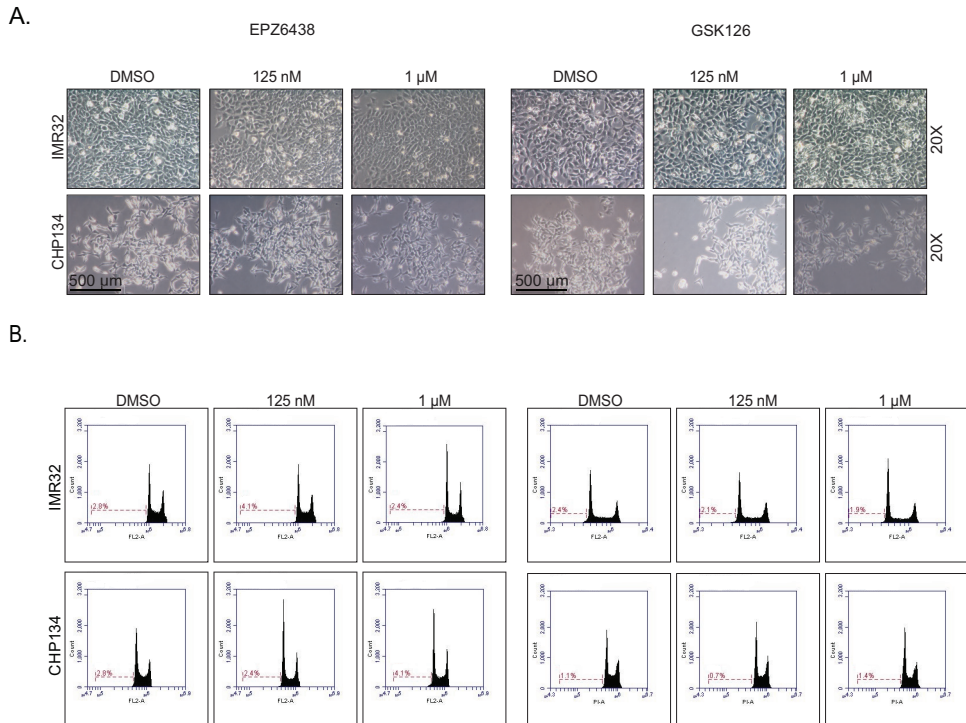
Supplementary Figure 2. (A) Light microscopy images of IMR32 and CHP134 treated for 144h with increasing concentrations of EPZ6438 and GSK126. Magnification of the images: 20x. Scale bar: 500 µm. (B) Flow cytometric DNA content histogram of IMR32 and CHP134 showing the effect of EPZ6438 and GSK126 on the cell cycle treated for 144h with DMSO, 125 nM and 1 µM.

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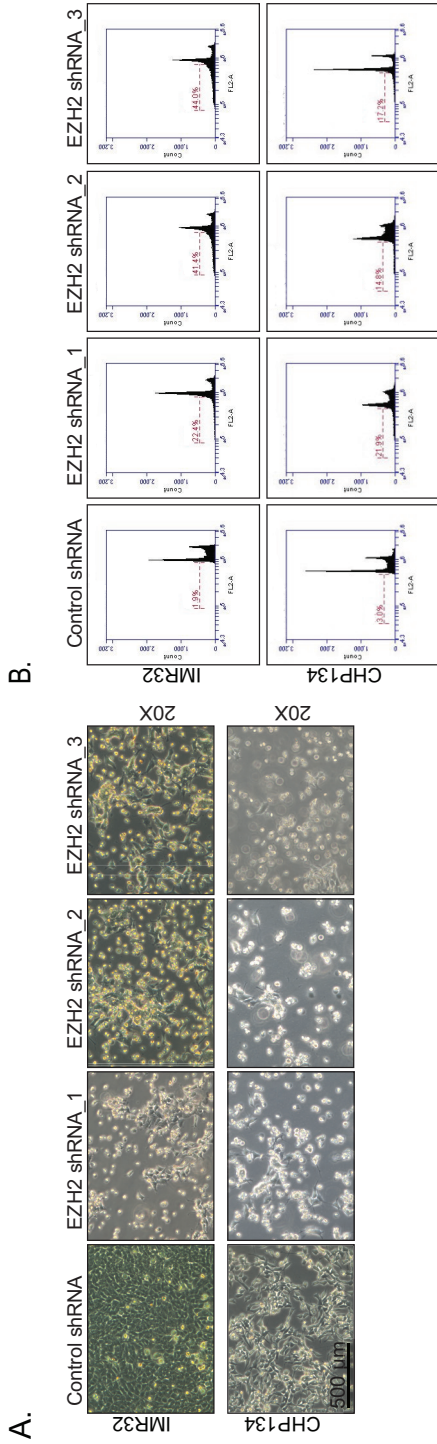
Supplementary Figure 3. (A) Light microscopy images of IMR32 and CHP134 transduced for 96h with a control shRNA and 3 shRNA's specifically targeting EZH2. Magnification of the images: 20x. Scale bar: 500 µm. (B) Flow cytometric DNA content histograms of IMR32 and CHP134 transduced for 96h with a control shRNA and 3 shRNA.

SUPPLEMENTARY MATERIALS AND METHODS

1. Cell culture.

Classical human neuroblastoma cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L D-glucose, glutamate and supplemented with 10% (v/v) foetal calf serum, 2 mM L-glutamine, 10 U/mL penicillin, 10 µg/mL streptomycin and MEM non-essential amino acids (1x). Neuroblastoma tumour-initiating cell (TIC) lines were grown in neural specific stem cell medium (400 mL DMEM GlutaMAXTM-1 containing 1g/L D-glucose and pyruvate, 133 mL F12 medium, 10 mL B27, 18 ng/mL EGF, 36 ng/mL FGF, 10 U/mL penicillin and 10 µg/mL streptomycin. Cells were maintained at 37°C under 5% CO_2 in humidified air. Penicillin and streptomycin were obtained from Sigma Aldrich, EGF from Corning Life Sciences and FGF from PeproTech. Other cell culture related materials were obtained from Life Technologies.

2. FACs Analysis.

Neuroblastoma cell lines IMR32, CHP134 and NMB were seeded in triplicates in 6-cm plates and incubated overnight. Cells were then treated for 72 h with 0.01% DMSO (control) or EPZ6438 and GSK126 using concentration ranges of 62.5 nmol/L to 2 µmol/L or with control shRNA and EZH2 shRNA. Adherent cells were washed once with PBS and the PBS solutions were pooled with the supernatants. After trypsinization of the adherent cells with 0.05% trypsin/EDTA, cells were resuspended in the pooled supernatant/PBS solution. Next, cells were centrifuged (5 min; 1,500 rpm), washed by resuspension in PBS and centrifuged again (5 min; 3,000 rpm). Cells were fixed with 100% ice-cold ethanol and stained with 0.05 mg/mL propidium iodide and 0.05 mg/mL RNAse A in PBS. After 1 h incubation in the dark at room temperature, cells were filtered through a 50 µm filter (BD Biosciences) and DNA contents of the nuclei were analyzed using a fluorescent activated cell sorter. A total of 20,000 nuclei per sample were counted. The cell cycle distribution and apoptotic Sub-G1 fraction were determined using the Accuri™ C6 flow cytometer with the CFlow plus software (BD Biosciences).

CHAPTER 4

HIGH EFFICACY OF THE BCL-2 INHIBITOR ABT199 (VENETOCLAX) IN BCL-2 HIGH-EXPRESSING NEUROBLASTOMA CELL LINES AND XENOGRAFTS AND RATIONAL FOR COMBINATION WITH MCL-1 INHIBITION.

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ABSTRACT

The anti-apoptotic protein B cell lymphoma/leukaemia 2 (*BCL-2*) is highly expressed in neuroblastoma and plays an important role in oncogenesis. In this study, the selective BCL-2 inhibitor ABT199 was tested in a panel of neuroblastoma cell lines with diverse expression levels of BCL-2 and other BCL-2 family proteins. ABT199 caused apoptosis more potently in neuroblastoma cell lines expressing high BCL-2 and BIM/BCL-2 complex levels than low expressing cell lines. Effects on cell viability correlated with effects on BIM displacement from BCL-2 and cytochrome c release from the mitochondria. ABT199 treatment of mice with neuroblastoma tumors expressing high BCL-2 levels only resulted in growth inhibition, despite maximum BIM displacement from BCL-2 and the induction of a strong apoptotic response. We showed that neuroblastoma cells might survive ABT199 treatment due to its acute upregulation of the anti-apoptotic BCL-2 family protein myeloid cell leukaemia sequence 1 (MCL-1) and BIM sequestration by MCL-1. *In vitro* inhibition of MCL-1 sensitized neuroblastoma cell lines to ABT199, confirming the pivotal role of MCL-1 in ABT199 resistance. Our findings suggest that neuroblastoma patients with high BCL-2 and BIM/BCL-2 complex levels might benefit from combination treatment with ABT199 and compounds that inhibit MCL-1 expression.

INTRODUCTION

Numerous cancer types have been associated with aberrations in genes encoding B cell lymphoma/leukaemia 2 (BCL-2) family proteins [1]. The BCL-2 family of proteins are key regulators of the intrinsic apoptotic pathway [2, 3], consisting of anti-apoptotic [e.g., BCL-2, BCL-extra large (BCL-X_L), BCL-2-like protein 2 (BCL-W) and myeloid cell leukaemia sequence 1 (MCL-1)] and pro-apoptotic members [e.g., BCL-2-like protein 11 (BIM) and BH3-interacting domain death agonist (BID)] [4-6]. Increased interactions between anti-apoptotic and pro-apoptotic proteins inhibits apoptosis by preventing mitochondrial outer membrane permeabilization by the essential effector family members BAX and BAK. Consequently, cytochrome c cannot be released into the cytosol where it activates caspase 9-induced proteolysis and cell death [7-13].

Neuroblastoma is the most commonly diagnosed extracranial solid cancer in children, accounting for approximately 15% of all pediatric cancer deaths [14]. A large subset of neuroblastoma patients has enhanced levels of the anti-apoptotic gene *BCL-2* [15, 16]. Previously, we showed that selective *BCL-2* inhibition using RNA interference caused an apoptotic response in cell lines with moderate to high *BCL-2* levels. These findings could be confirmed with the small-molecule BCL-2 family inhibitor ABT263, which inhibits the anti-apoptotic activity of BCL-2, BCL-X_L, BCL-W and MCL-1 with inhibition constant (K_i) values of 0.044, 0.055, 7 and 224 nmol/L, respectively [17]. Neuroblastoma cell lines expressing high *BCL-2* levels responded better to ABT263 treatment than low *BCL-2*-expressing cell lines. ABT263 furthermore delayed the onset of tumor formation in mice injected with high *BCL-2*-expressing neuroblastoma cells [16]. These observations supported the potential benefit of BCL-2 family inhibitors for the future treatment of high *BCL-2*-expressing neuroblastoma tumors. Unfortunately, the administration of ABT263 in phase I/II clinical studies for adult cancers was associated with dose-limiting thrombocytopenia due to concomitant inhibition of anti-apoptotic BCL-X_L, a key survival factor for circulating platelets [18-22]. Therefore, the more specific ABT263-derivative ABT199 was developed [17, 23, 24].

Compared with ABT263, ABT199 displays less activity against BCL-X_L (K_i of 48 nmol/L), BCL-W (K_i of 245 nmol/L) and MCL-1 (K_i of >444 nmol/L), while maintaining its activity against BCL-2 (K_i of <0.01 nmol/L) [17, 25]. ABT199 has shown preclinical and clinical efficacy against lymphoma, while sparing platelets [24, 26, 27]. In the current study, we explored the preclinical therapeutic potential of ABT199 for the treatment of BCL-2-dependent neuroblastoma tumors.

MATERIALS AND METHODS

Chemicals. ABT199, ABT263 and A-1210477 were purchased from Selleck Chemicals while QVD-OPH was purchased from Sigmaaldrich. For *in vivo* studies ABT199 and ABT263 were formulated in 10% ethanol/30% polyethylene glycol (PEG) 400/60% phosal 50 propylene glycol (PG) (v/v/v) in final concentrations of 10 mg/mL.

Cell culture. Classical human neuroblastoma cell lines and neuroblastoma tumor-initiating cell (TIC) lines were cultured as previously described [41, 42]. Cell culture protocols are described in detail in the Supplementary Materials and Methods.

IC_{50} and LC_{50}. Neuroblastoma cell lines were seeded in triplicate in 96-well (classical cell lines) or 48-well (TIC lines) plates using the most optimal confluency for each cell line [42]. Cells were incubated overnight and treated with 1 nmol/L to 50 µmol/L ABT199. Control samples were treated with 0.5% DMSO. Cell viability was determined prior to and after 72-hour treatment using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay [43]. Half maximal effective concentration (IC_{50}) and half lethal concentration (LC_{50}) values were derived from dose-response curves. IC_{50} values at 72 hours were calculated by determining the ABT199 concentrations needed to achieve a 50% reduction in cell viability observed for DMSO-treated cells at 72 hours (set at 100%). LC_{50} values at 72 hours were calculated by establishing the ABT199 concentrations needed to attain a 50% reduction in the cell viability compared to time point 0.

FACS analysis. Cells were treated with 0.1% DMSO (control) or ABT199 using concentration ranges of 7.8 nmol/L to 10 µmol/L. After 72-hour treatment, floating and adherent cells were harvested for FACS analysis to determine the cell-cycle distribution and the apoptotic sub-G_1 fraction. See Supplementary Materials and Methods for a detailed protocol.

Cell fractionation. CHP126, KCNR, SJNB12, SKNAS and SHEP2 were 24-hour treated with 0.04% DMSO (control) or 62.5 nmol/L to 10 µmol/L ABT199. Floating and adherent cells were harvested to determine cytochrome c in the cytosolic and organelle fractions. See Supplementary Materials and Methods for a detailed protocol.

***In vitro* western blotting.** The following antibodies were used: rabbit anti-human BCL-2 (clone D55G8) monoclonal antibody (1:1,000, Cell Signaling Technology);

4

rabbit anti-human BCL-X$_L$ (clone 54H6) monoclonal antibody (1:1,000, Cell Signaling Technology); rabbit anti-human MCL-1 monoclonal antibody (1:1,000, Cell Signaling Technology); rabbit anti-human BCL-W (clone 31H4) monoclonal antibody (1:1,000, Cell Signaling Technology); rabbit anti-human Noxa (clone EPR9735B) monoclonal antibody (1:1,000, Abcam); rabbit anti-human BIM (1:1,000, Cell Signaling Technology); mouse anti-human cytochrome c (clone 7H8.2C12) monoclonal antibody (1:1,000 BD pharmingen); rabbit anti-human PARP (clone 9542S) monoclonal antibody (1:1,000, Cell Signaling Technology); rabbit anti-human cleaved caspase 3 (clone 5AE17) monoclonal antibody (1:1000, Cell Signaling Technology); mouse anti-human α-tubulin (clone DM1A) monoclonal antibody (1:10,000, Cell Signaling Technology) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit (clone NA9340V) and goat anti-mouse (clone NXA931) secondary antibodies (1:10,000 GE Healthcare). See Supplementary Materials and Methods for detailed protocol.

***In vitro* co-immunoprecipitation and immunoblotting.** Cell lines were seeded onto 14-cm culture dishes. For the detection of basal BIM/BCL-2 complex levels, untreated cells were harvested after 72 hours incubation at normal culture conditions. For the detection of BIM displacement from BCL-2 and BIM complexation with MCL-1 and BCL-X$_L$, cells were treated with 0.25% DMSO (control) or 62.5 nmol/L or 1.25 µmol/L ABT199 at 24 hours after seeding and harvested after 24-hour treatment. Co-immunoprecipitation studies have been performed as described in detail in the Supplementary Materials and Methods.

Cell transfection. CHP126, KCNR, SJNB12 and SY5Y were seeded in 6-cm culture dishes (2x10^5 cells in 4 mL culture medium) and incubated overnight. Next, cells were transfected with non-targeting shRNA (AACAAGATGAAGAGCACCAA; negative control) or MCL-1 shRNA (TRCN0000199070 and TRCN0000005518) for the BCL-2 high-expressing cell lines using the PLenti VI system according to the manufacturers protocol (Sigma Aldrich), while SY5Y was transfected with either a Luc2 control or a TERT-on vector constitutively overexpressing the BCL-2 protein. After 72 hours, cells were transferred into 96-well plates for *in vitro* MTT assays and synergy studies (see below).

***In vitro* synergy assays.** CHP126, KCNR and SJNB12 transiently transfected with non-targeting shRNA or *MCL-1* shRNA were seeded in duplicates in 96-well plates and incubated overnight. Cells were then treated with ten-fold serial dilutions of ABT199 (0-10 µmol/L). Effects on cell viability were studied after 72-hour treatment with ABT199, using the MTT cell proliferation assay.

For synergy studies between ABT199 and the MCL-1 inhibitor A-1210477, non-transfected cells in 96-well plates were co-treated with ten-fold serial dilutions of ABT199 (0-10 µmol/L) and five-fold serial dilution of A-1210477 (0-50 µmol/L).

***In vivo* efficacy in neuroblastoma mouse models.** Female NMRI *nu⁻/nu⁻* mice (6-15 weeks old; 20-30 g) were obtained from Harlan and experiments were performed with permission from and according to the standards of the Dutch animal ethics committee (DAG 102776, 102830 and 102690). NMRI *nu⁻/nu⁻* mice with KCNR neuroblastoma xenografts of approximately 268 mm^3 were orally treated with 100 mg/kg/d ABT199 (n = 5), 100 mg/kg/d ABT263 (n = 5), or vehicle (n = 6) for 21 days. Tumor sizes were measured by an external caliper. See Supplementary Materials and Methods for a more detailed protocol.

***In vivo* western blotting.** Per mouse sample, 10 tumor sections of 50 µm were homogenized in 2% ChAPS buffer as previously described. Western blot detection of protein levels of BCL-2 like family members was carried out as described for *in vitro* western blotting.

***In vivo* co-immunoprecipitation.** Sections of treated and untreated KCNR tumors harvested at 4 hours after administration of the last dose were homogenized using the Ultra Turrax T25 tissue homogenizer (Janke & Kunkel) and lysed (overnight at 4 °C) in 2% CHAPS buffer. Co-immunoprecipitation was carried out as described above.

***In vivo* immunohistochemistry.** The following antibodies were used: rabbit anti-human Ki-67 (clone SP6) monoclonal antibody (1:1,000, Thermo Scientific), rabbit anti-human cleaved caspase 3 (Asp175) polyclonal antibody (1:100, Cell Signaling Technology) and BrightVision horseradish peroxidase-conjugated goat anti-rabbit polyclonal secondary antibody (undiluted; 30 min; Immunologic). See Supplementary Materials and Methods for a detailed protocol.

mRNA expression profiling. RNA was extracted from tumors with TRIzol (Invitrogen, Carlsbad, CA) following the manufacturers protocols. RNA concentration and quality were determined using the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies). Fragmentation of cRNA, hybridization to hg-u133 plus 2.0, microarrays and scanning were carried out according to the manufacturers protocol (Affymetrix Inc. Santa Barbara, CA). The mRNA gene expression data were normalized with the MAS5.0 algorithm within the GCOS program of Affymetrix Inc. Target intensity

was set to 100. All data were analyzed using the R2 genomic analysis and visualization platform (http://r2.amc.nl).

RESULTS

BCL-2 and BIM/BCL-2 complex levels predict sensitivity of neuroblastoma cells to ABT199.

IC_{50} and LC_{50} values of ABT199 were established for 21 classical neuroblastoma cell lines and 3 tumor-initiating cell lines (TIC) (Table 1). Cell lines CHP126, KCNR and SJNB12 responded most potently to ABT199, with IC_{50} and LC_{50} values in the nanomolar range (i.e. 10-210 and 16-338 nmol/L, respectively) versus micromolar IC_{50} and LC_{50} values (i.e. 4.9-19.3 and 6.9-32.3 µmol/L, respectively) for the other cell lines tested. Evaluation of the BCL-2, MCL-1, BCL-X_L, BCL-W and BIM protein levels (Fig. 1A) showed significantly higher BCL-2 levels in the sensitive neuroblastoma cell lines CHP126, KCNR and SJNB12 compared with the insensitive cell lines (Fig. 1B). No significant differences in expression of the anti-apoptotic proteins MCL-1, BCL-X_L, BCL-W and the pro-apoptotic protein BIM were found between the sensitive and insensitive cell lines (Fig. 1B). The same pattern was observed when looking at the mRNA level (Supplementary Fig. S1A). Sensitive neuroblastoma cell lines expressed significantly higher *BCL-2* mRNA levels as compared to the insensitive cell lines, while no expression differences were observed for the other *BCL-2* family genes. Of note, BCL-2 protein levels better predict the sensitivity of neuroblastoma cell lines to ABT199 than *BCL-2* mRNA levels, as shown by the factor difference in average expression between the sensitive and insensitive cell lines (i.e. ~14 versus ~4). As ABT199 acts by displacing pro-apoptotic BCL-2 family members including BIM from BCL-2 [17], we also studied if levels of BIM bound to BCL-2 could be used as a predictive biomarker for sensitivity to ABT199. BCL-2 immunoprecipitation followed by immunoblotting for BIM showed that BIM/BCL-2 complex levels were indeed significantly higher in the sensitive neuroblastoma cell lines (Fig. 1B and C). Results were confirmed by reciprocal co-immunoprecipitation experiments in which BIM/BCL-2 complex levels were determined by BIM immunoprecipitation followed by BCL-2 immunoblotting (Supplementary Fig. S1A and B). We also tested if there was a correlation between ABT199 sensitivity and *MYCN* status. *MYCN*-amplified neuroblastoma cell lines responded more potently to ABT199 than *MYCN* single copy cell lines, with IC_{50} values ranging from 10 nmol/L-20 µmol/L versus 10-18 µmol/L, respectively (Supplementary Fig. 1C).

Cell lines	IC$_{50}$ (µmol/L)	LC$_{50}$ (µmol/L)
CHP126	0.010	0.016
KCNR	0.026	0.153
SJNB12	0.210	0.338
LAN5	4.90	20.50
IMR32	6.01	8.80
AMC106	6.30	6.90
LAN1	6.40	7.10
N206	7.01	8.21
TR14	7.65	11.90
SJNB6	8.20	10.33
CHP134	9.40	17.70
SJNB1	9.40	9.50
700B	9.54	16.62
SKNFI	9.79	10.97
SKNSH	11.04	13.10
700T	11.29	17.50
GIMEN	14.01	21.80
SKNAS	14.20	19.10
NMB	14.60	17.40
SKNBE	14.70	18.60
SY5Y	15.32	17.71
691T	15.80	17.01
SHEP2	18.30	21.60
SJNB10	19.30	32.30

Table 1. IC$_{50}$ and LC$_{50}$ values of ABT199 for neuroblastoma cell lines.

ABT199 causes cell death in BCL-2-dependent neuroblastoma cells by activation of the intrinsic apoptotic program.

High BCL-2-expressing neuroblastoma cell lines CHP126, KCNR and SJNB12 and low BCL-2-expressing cell lines SKNAS and SHEP2 were treated with increasing doses of ABT199 to study effects on apoptosis. PARP cleavage induction in the high BCL-2-expressing cell lines was already observed after treatment with only 7.5 nmol/L ABT199, while for the low BCL-2-expressing neuroblastoma cell lines PARP cleavage was only detected in

SKNAS after treatment with 10 µmol/L ABT199 (Fig. 2A). Similar results were obtained when investigating the effects of ABT199 on cleaved caspase 3 (Supplementary Fig. S2A). Apoptotic effects of ABT199 were further validated by flow cytometry. In line with the effects on PARP and caspase 3 cleavage, ABT199 treatment of the high BCL-2-expressing neuroblastoma cell lines resulted in more pronounced increases in sub-G_1 fraction than observed for the low BCL-2-expressing cell lines (Fig. 2B and Supplementary Table S1) Treatment with only 7.5 nmol/L ABT199 resulted in increases in sub-G_1 fraction of 8% (CHP126), 13% (KCNR) and 5% (SJNB12) for the high BCL-2-expressing cell lines versus 0% (SKNAS) and 1% (SHEP2) for the low BCL-2-expressing cell lines. Effects on sub-G_1 were dose-dependent, with maximum increases observed after treatment with 10 µmol/L ABT199 (i.e. 44%, 25% and 37% for CHP126, KCNR and SJNB12, respectively, versus 7% and 3% for SKNAS and SHEP2, respectively). Next, *in vitro* effects of ABT199 on the activation of the intrinsic apoptosis pathway were studied. As ABT199 inhibits the activity of BCL-2 by displacement of pro-apoptotic proteins, we first studied the effects of ABT199 on BIM displacement from BCL-2. Treatment of the high BCL-2-expressing neuroblastoma cell lines with 62.5 nmol/L ABT199 was already sufficient for almost complete displacement of BIM from BCL-2 (Fig. 2C). No or only moderate increases in BIM displacement were observed after treatment with higher ABT199 concentrations (i.e. 1.25 µmol/L). BCL-2-dependent activation of the intrinsic apoptotic program was further studied by evaluation of the effects of ABT199 on cytochrome c release. Dose-dependent cytochrome c release from the mitochondria into the cytoplasm was observed after ABT199 treatment of CHP126, KCNR and SJNB12 (Fig. 2D). In line with the effects on BIM displacement from BCL-2, cytochrome c release was already observed after treatment with nanomolar concentrations of ABT199. No cytochrome c release was observed after ABT199 treatment of the low BCL-2-expressing cell lines, even at the highest concentration of 10 µmol/L (Fig. 2D). Together, these findings confirm that ABT199 causes apoptosis in BCL-2-dependent neuroblastoma cells via activation of the intrinsic apoptotic pathway. This was strengthened by the observation that the effects of ABT199 on PARP and caspase 3 cleavage and sub-G_1 fraction could be completely rescued in KCNR by combination treatment with the pan-caspase inhibitor QVD-OPH (Supplementary Fig. S2D and E).

In addition, overexpression of BCL-2 in the low BCL-2-expressing neuroblastoma cell line SY5Y resulted in a strong increase in sensitivity to SY5Y (Supplementary Fig. S2B and C). The remarkable decrease in IC_{50} value observed after BCL-2 overexpression (i.e. from 16.3 µM to 2.6 µM) shows that the effects of ABT199 obtained in CHP126, KCNR and SJNB12 are indeed caused by BCL-2 inhibition rather than off-target effects.

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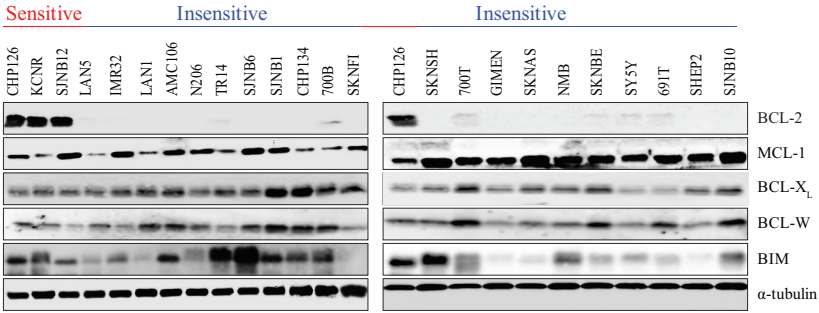
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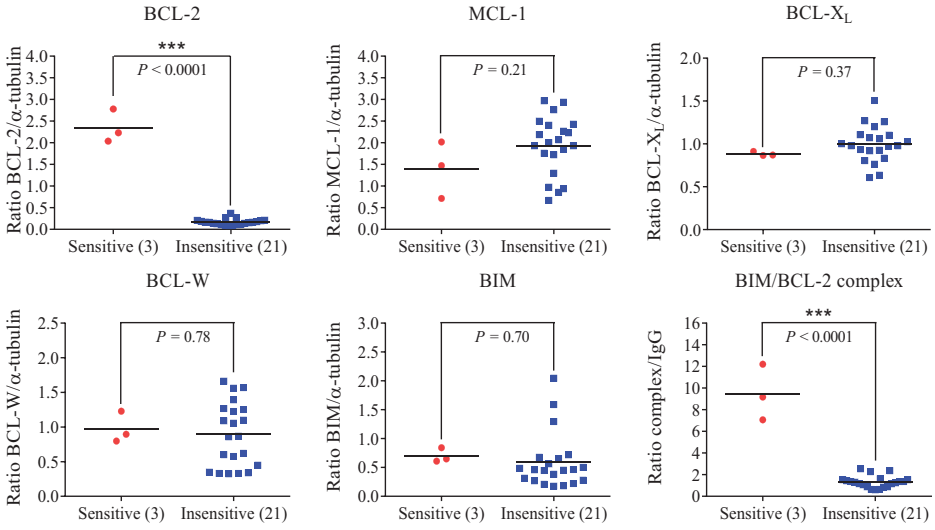
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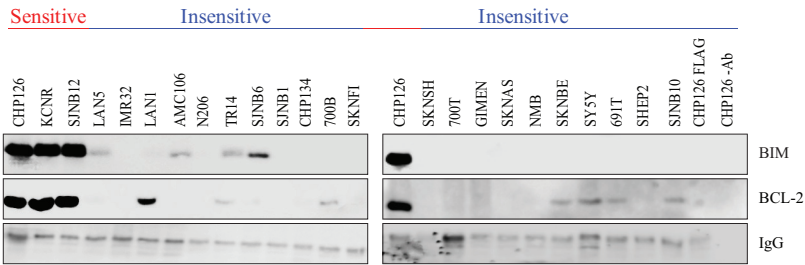
Figure 1. BCL-2 protein and BIM/BCL-2 complex levels predict sensitivity of neuroblastoma cell lines to ABT199. A, Western blot analysis of the protein expression levels of anti-apoptotic proteins BCL-2, MCL-1, BCL-X$_L$ and BCL-W and the pro-apoptotic protein BIM in neuroblastoma cell lines. α-tubulin was used as household protein. B, Protein levels of BCL-2, MCL-1, BCL-X$_L$, BCL-W and BIM and BIM/BCL-2 complex levels in sensitive (i.e., CHP126, KCNR, and SJNB12) versus insensitive neuroblastoma cell lines. Protein levels shown in Fig. A were quantified by calculating the protein/α-tubulin band intensity ratios. BIM/BCL-2 complex levels were established by anti-BCL-2 immunoprecipitation of whole cell lysates, followed by Western blotting for BIM. BIM band intensities were normalized to the IgG heavy chain of the BCL-2 antibody. Statistical differences between the sensitive and insensitive cell lines were calculated using a one-tailed (for BCL-2 and BIM/BCL-2 complex) or two-tailed unpaired Student t test, with $P < 0.05$ as the minimal level of significance and $P < 0.0001$ indicated as ***. Horizontal lines represent the mean of the relative intensities of the cell line panel. C, Western blot analysis of BIM/BCL-2 complex levels in 24 neuroblastoma cell lines, ordered from ABT199 sensitive (left) to ABT199 insensitive (right). The IgG heavy chain of the BCL-2 antibody served as a loading control. Total protein levels of the BCL-2-like family member proteins of the cell line panel in Figure 1A served as whole cell lysates for this experiment.
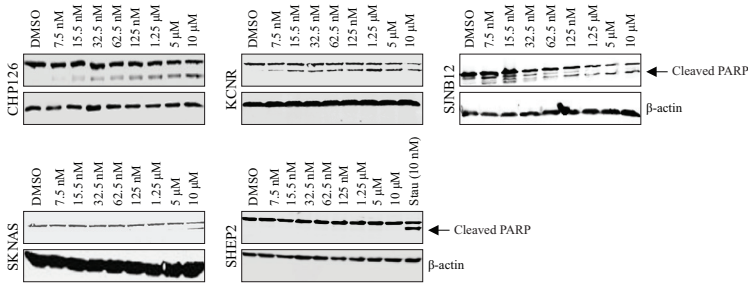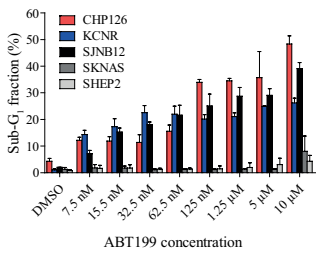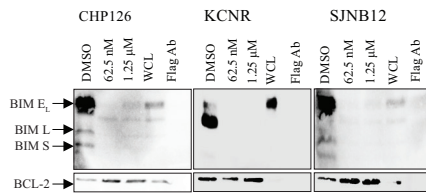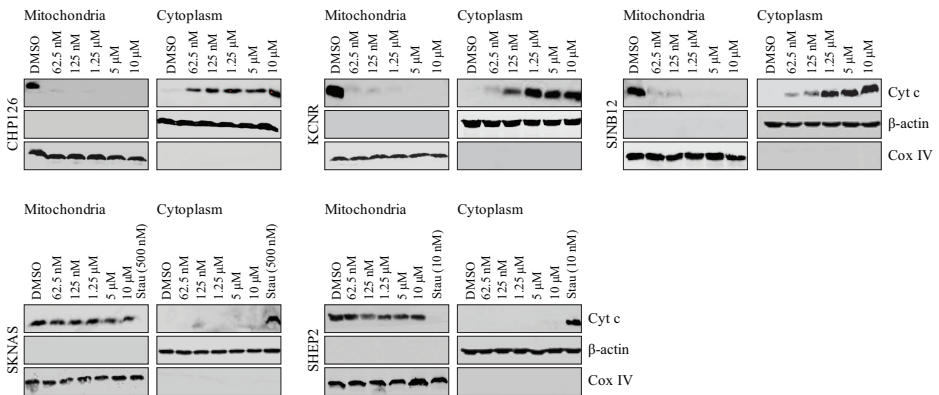
Figure 2. ABT199 induces cell death in BCL-2 dependent neuroblastoma cell lines through activation of the intrinsic apoptotic pathway. A, Western blot analysis of the *in vitro* effects of ABT199 on PARP cleavage after 72-hour treatment of sensitive neuroblastoma cell lines CHP126, KCNR and SJNB12 and insensitive cell lines SKNAS and SHEP2 with increasing ABT199 concentrations. β-actin served as loading control. As ABT199 failed to induce PARP cleavage in SHEP2, staurosporine (10 nmol/L, 24 hours; indicated as Stau) was used as a positive control for this cell line. B, FACS analysis of the *in vitro* effects on sub-G_1 induction after 72-hour treatment with increasing ABT199 concentrations. Data represent the mean percentages of cells in sub-G_1 ± SD of three replicate experiments. C, *In vitro* effects on BIM displacement from BCL-2 after 24-hour treatment of CHP126, KCNR and SJNB12 with 62.5 nmol/L or 1.25 μmol/L ABT199. BIM displacement was established by detecting BIM/BCL-2 complex levels by anti-BCL-2 immunoprecipitation, followed by western blotting for BIM. BCL-2 levels served as loading control. (BIM E_L = BIM extra-large, BIM L = BIM large and BIM S = BIM small) WCL= whole cell lysate. D, Western blot analysis of the *in vitro* effects of ABT199 on cytochrome c release from the mitochondria into the cytosol. Cytochrome c levels (indicated as Cyt c) in the mitochondrial and cytoplasmic cell fractions were established after 24-hour treatment of the cell lines with increasing ABT199 concentrations. COX IV and β-actin were used as loading controls for the mitochondrial and cytoplasmic fractions, respectively. As ABT199 failed to induce cytochrome c release in SKNAS and SHEP2, cell lines were 24-hour treated with 500 and 10 nmol/L staurosporine, respectively, as a positive control.

ABT199 causes apoptosis in high BCL-2-expressing neuroblastoma xenografts by BIM displacement from BCL-2.

The efficacy of ABT199 was subsequently studied *in vivo* in mice with high BCL-2-expressing KCNR neuroblastoma xenografts. Once-daily oral treatment with 100 mg/kg ABT199 for three consecutive weeks resulted in significant tumor growth inhibition relative to the vehicle treated controls (Fig. 3A). However, comparison studies in the same animal model demonstrated superior antitumor activity of ABT263 over ABT199, i.e. complete tumor regression versus tumor growth inhibition (Fig. 3A).

The difference in efficacy between ABT199 and ABT263 might be the result of the concomitant inhibition of other anti-apoptotic BCL-2 family members by ABT263 and/or incomplete BCL-2 inhibition by ABT199. We therefore studied the *in vivo* effects of ABT199 on BIM displacement from BCL-2. Co-immunoprecipitation studies at 4 h after administration of the last ABT199 dose showed almost complete release of BIM, while in the control mice BIM was still firmly complexed to BCL-2 (Fig. 3B). This indicates that sufficient high intratumoral ABT199 levels were achieved for maximum target inhibition. In line with ABT199 effects on BIM displacement from BCL-2, a strong apoptotic response was observed. Treatment with 100 mg/kg/day ABT199 caused an increase in cleaved caspase 3-positive cells (Fig. 3C and D). No clear phenotypic changes and effects on cell proliferation were observed, as shown by hematoxylin-eosin and Ki67 staining of tumor tissues, respectively (Supplementary Fig. S3A). Together, these results show that the differential efficacy between ABT199 and ABT263 is not the result of insufficient target inhibition by ABT199, but might result from simultaneous inhibition of multiple anti-apoptotic BCL-2 family members by ABT263. Discontinuation of treatment with ABT199 and ABT263 resulted in tumor growth and tumor recurrence, respectively (Supplementary Fig. S3B).

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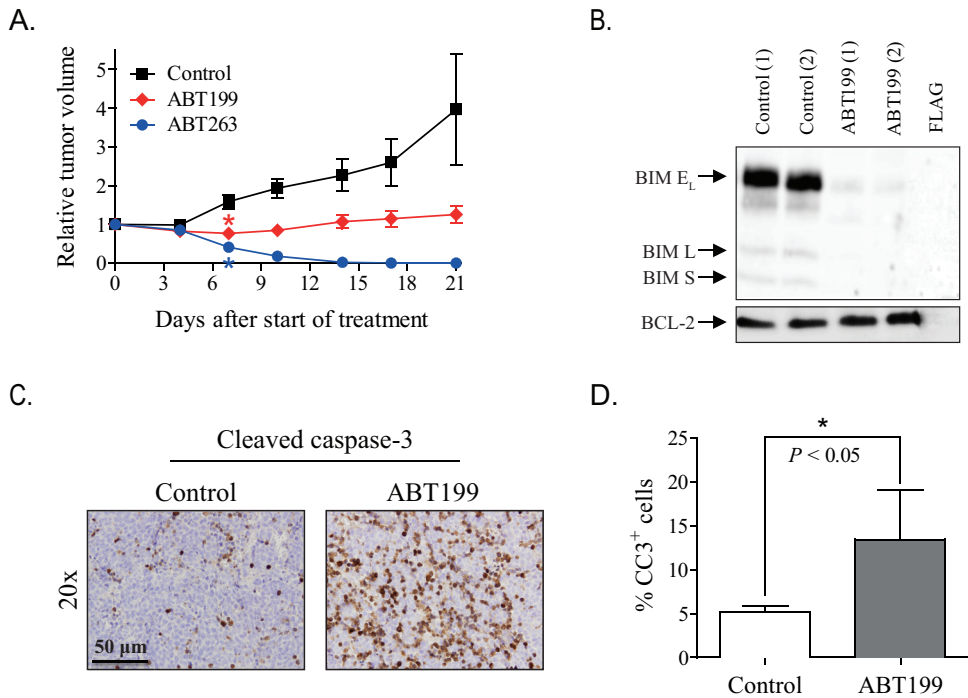
Figure 3. ABT199 and ABT263 cause apoptosis in mice with KCNR neuroblastoma xenografts expressing high levels of BCL-2 and BIM/BCL-2 complex. A, Inhibitory effects of ABT199 and ABT263 on the growth of KCNR neuroblastoma xenografts in mice. Relative tumor volume was calculated as the volume at the indicated day after start of treatment divided by the volume prior to treatment initiation. Data represent the mean relative tumor volume ± SEM [Group sizes: n = 10 (control; 10% ethanol/30% PEG 400/60% phosal 50 PG (v/v/v)), n = 5 (100 mg/kg ABT199), and n = 5 (100 mg/kg ABT263)]. Statistical differences between treated and control groups were calculated using one-way ANOVA with Bonferroni adjustment and are indicated on the first day after treatment initiation at which a statistically different effect was observed (*). P values < 0.05 were considered significant. B, *In vivo* effects of ABT199 on BIM displacement from BCL-2. Effects were studied by detecting BIM/BCL-2 complex levels by anti-BCL-2 immunoprecipitation, followed by western blotting for BIM (BIM E_L = BIM extra-large, BIM L = BIM large and BIM S = BIM small). Levels of BIM/BCL-2 complex were established for n = 2 mice per group at 4 hours after administration of the last dose of ABT199. BCL-2 levels served as loading control. Total protein levels of the BCL-2 family members in figure 4D served as input levels for this experiment. C, Representative microscopic images of cleaved caspase 3 stained paraffin-embedded tumor sections of control and ABT199-treated tumor samples collected at 4 hours after administration of the last dose. Magnification of the images: 20x. Scale bar: 50 μm. D, Quantification of the stimulatory effect of ABT199 on cleaved caspase 3 was performed by manual counting of the total number of cells and the number of cleaved caspase 3-positive (CC3$^+$) cells in 20 microscopic fields per mouse sample at 40x magnification (30% of each field). The average percentage of CC3$^+$ cells for each mouse was used to determine the effect of ABT199. Statistical differences between the control group (n = 6) and ABT199-treated group (n = 2) were calculated using a one-tailed unpaired Students *t* test and are indicated as * (P < 0.05).

MCL-1 stabilization and upregulation and BIM sequestration by MCL-1 provide rationale for combination treatment with MCL-1 inhibitors.

Upregulation and increased activity of the non-targeted anti-apoptotic BCL-2 family proteins MCL-1 and BCL-X$_L$ have been reported as key mechanisms of resistance to BCL-2 inhibitors including ABT199 [28-33]. We observed that ABT199 treatment of the high BCL-2-expressing neuroblastoma cell lines CHP126, KCNR and SJNB12 indeed resulted in strongly increased MCL-1 levels, while the other anti-apoptotic proteins and the pro-apoptotic protein BIM were not affected (Fig. 4A). As Noxa is a negative regulator of MCL-1 [33], effects of ABT199 on Noxa protein levels were also studied. Noxa protein levels were decreased after ABT199 treatment of CHP126, KCNR and SJNB12 (Fig.4A), which might explain the increased MCL-1 levels. Real-time quantitative PCR analysis after 24-hour treatment of KCNR cells treated with increasing ABT199 concentrations showed no significant changes in *MCL-1* and *Noxa* mRNA levels (Supplementary Fig. S3C and D). *In vitro* observations were confirmed in the *in vivo* experiments using tumor materials of the ABT199-treated KCNR xenografts and control samples. MCL-1 protein levels were again strongly increased after ABT199 treatment, while Noxa was decreased (Fig. 4B). No effects of ABT199 on anti-apoptotic proteins BCL-2 and BCL-X$_L$ and the pro-apoptotic protein BIM were observed *in vivo*. Affymetrix mRNA profiling of the *in vivo* tumor samples revealed no significant changes in *BCL-2*, *MCL-1*, *BCL-X$_L$* and *BIM* mRNA levels after ABT199 treatment (Supplementary Fig. S3E). The lack of effects on *MCL-1* mRNA indicates that ABT199-induced MCL-1 upregulation is caused at the protein rather than mRNA level, making the increased stabilization of MCL-1 by reduced Noxa protein levels more plausible. While also not statistically significant, *in vivo Noxa* mRNA levels appeared to be lower after ABT199 treatment. Next, the *in vitro* sequestration of released BIM by other anti-apoptotic proteins was studied. After 24-hour treatment of neuroblastoma cell lines CHP126, KCNR and SJNB12 with nanomolar concentrations ABT199, shifting of BIM from BCL-2 to the anti-apoptotic protein MCL-1 was observed while no sequestration of released BIM by the anti-apoptotic protein BCL-X$_L$ was observed (Fig. 4C and Supplementary Fig. S2F). BIM displacement from BCL-2 to MCL-1 also occurred *in vivo* (Fig. 4D), confirming the pivotal role of MCL-1 in the biological mechanism by which neuroblastoma cells can escape from ABT199-induced apoptosis.
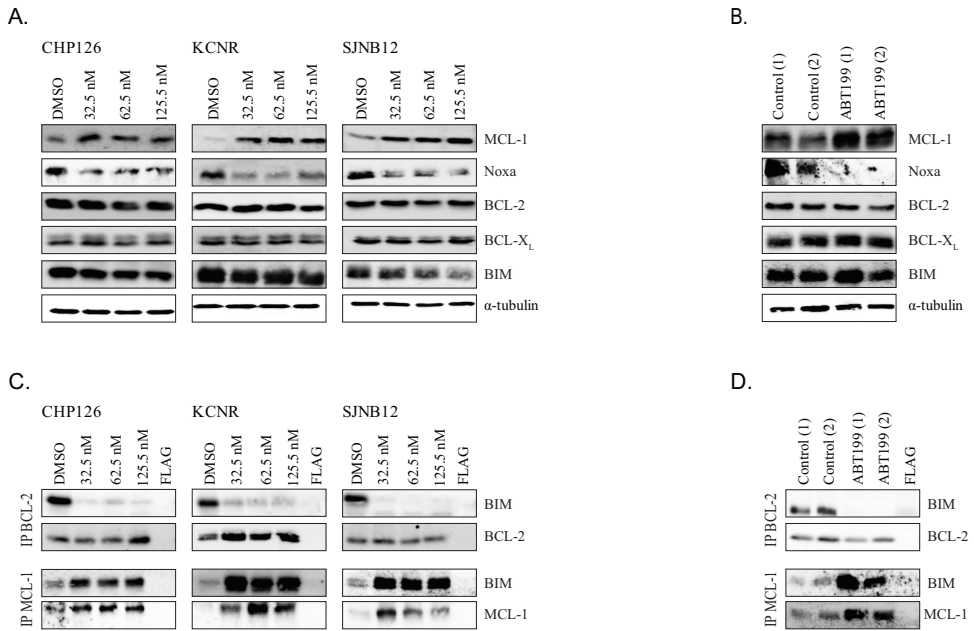
Figure 4. ABT199 causes MCL-1 upregulation and Noxa downregulation, leading to increased sequestration of released BIM from BCL-2. A, *In vitro* effects of ABT199 on total protein levels of MCL-1, Noxa, BCL-2, BCL-X$_L$ and BIM in CHP126, KCNR and SJNB12 after 24-hour treatment with increasing doses. Effects were determined by western blot analysis using α-tubulin as a loading control. B, *In vivo* effects of ABT199 on total protein levels of MCL-1, Noxa, BCL-2, BCL-X$_L$ and BIM. Mice with KCNR neuroblastoma xenografts were daily treated with vehicle or 100 mg/kg ABT199 for 3 consecutive weeks. Tumor samples were collected at 4 hours after administration of the last dose and analyzed by western blot analysis. α-tubulin served as a loading control. C, *In vitro* effects of ABT199 on BIM/BCL-2, and BIM/MCL-1 complex levels in CHP126, SJNB12 and KCNR after 24-hour treatment with increasing doses. BIM/BCL-2 and BIM/MCL-1 complex levels were established by immunoprecipitation of BCL-2 and MCL-1 followed by western blot analysis of BIM. Immunoprecipitated levels of BCL-2 and MCL-1 were used as loading controls. D, *In vivo* effects of ABT199 on BIM/BCL-2 and BIM/MCL-1 complex levels. Tumor samples (*n* = 2 per group) were collected after 3 weeks treatment with vehicle or 100 mg/kg/day ABT199, at 4 hours after administration of the last dose. BIM/BCL-2 and BIM/MCL-1 complex levels were subsequently determined as described above.

To further demonstrate the importance of MCL-1 in neuroblastoma resistance to ABT199, combined effects of *MCL-1* knockdown and ABT199 on cell viability were studied. *MCL-1* knockdown in CHP126, KCNR and SJNB12 using two different shRNAs sensitized the neuroblastoma cell lines to ABT199 treatment (Fig. 5A). Effects of MCL-1 inhibition on the sensitivity of neuroblastoma cell lines CHP126, KCNR and SJNB12 to ABT199 were additionally studied using the selective MCL-1 inhibitor A-1210477. Combined treatment of CHP126, KCNR and SJNB12 with ABT199 and A-1210477 caused additive to slightly synergistic inhibitory effects on cell viability (Fig. 5B and Table 2). In line with the effects on cell viability, combination treatment of KCNR with ABT199 and A-1210477 resulted in a significantly larger increase in sub-G_1 fraction than treatment with either of the inhibitors alone (Fig. 5C). BCL-2/BIM co-immunoprecipitation studies confirmed the occurrence of BIM displacement from BCL-2 after treatment with ABT199 alone or in combination with A-1210477. In a similar manner, MCL-1/BIM co-immunoprecipitation studies showed that the increased binding of BIM to MCL-1 observed after monotherapy with ABT199 was prevented by combination treatment with A-1210477 (Fig. 5D). Taken together, these results demonstrate that the efficacy of ABT199 can be potentiated by preventing sequestration of released BIM by MCL-1.

Figure 5. MCL-1 inhibition sensitizes high BCL-2 and BIM/BCL-2-expressing neuroblastoma cells to ABT199. A, *In vitro* effects of MCL-1 knockdown on the sensitivity of neuroblastoma cell lines CHP126, KCNR and SJNB12 to ABT199. Cells were 72-hour transfected with non-targeting control shRNA or MCL-1 shRNA (i.e., MCL-1 shRNA_1 or MCL-1 shRNA_2) prior to treatment with DMSO (control) or indicated ABT199 concentrations. Cell viability was assessed after 72-hour treatment using the MTT colorimetric assay. For each cell line, the viability of non-transfected cells treated with DMSO was set to 100%. B, *In vitro* effects of the small molecule MCL-1 inhibitor A-1210477 on the sensitivity of CHP126, KCNR and SJNB12 to ABT199. Cells were co-treated with 0-10 μmol/L ABT199 and 0-50 μmol/L A-1210477. Effects on cell viability were established after 72-hour treatment using the MTT colorimetric assay. For each cell line, the viability of untreated cells was set to 100%. C, FACs analysis of KCNR after 72-hour treatment with 10 nmol/L ABT199 alone or in combination with 10 μmol/L A-1210477. Data represent the mean percentages of cells in sub-G_1 ± SD of three replicate experiments. D, *In vitro* effects on BIM displacement from BCL-2 and MCL-1 after 24-hour treatment of KCNR with 10 nmol/L of ABT199 or 10μmol/L of A-1210477 and a combination of both compounds. BIM displacement was established by detecting BIM/BCL-2 and BIM/MCL-1 complex levels by anti-BCL-2 and anti-MCL-1 immunoprecipitation, followed by western blotting for BIM. BCL-2 and MCL-1 levels served as loading control.

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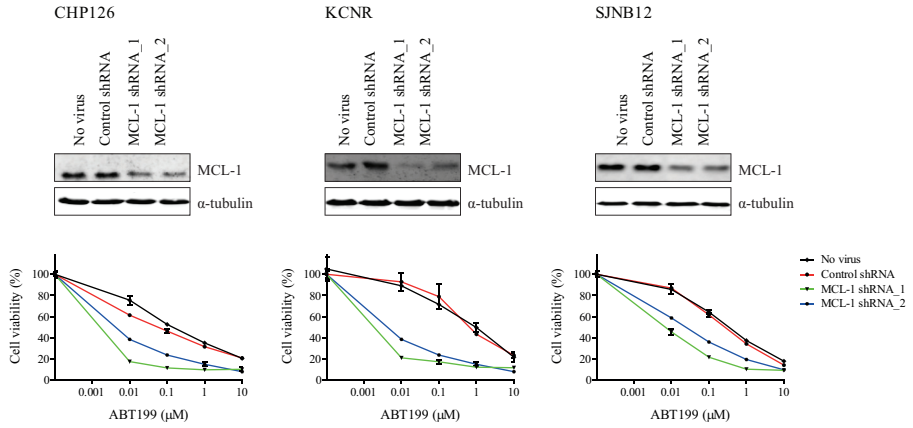
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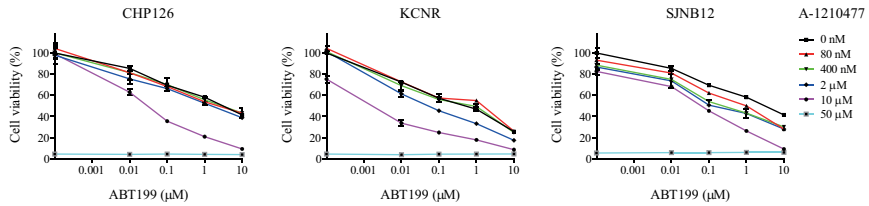
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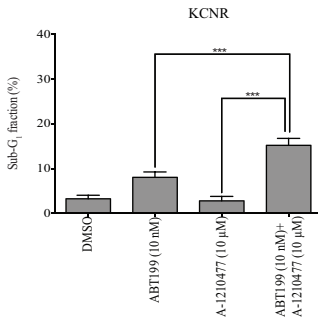
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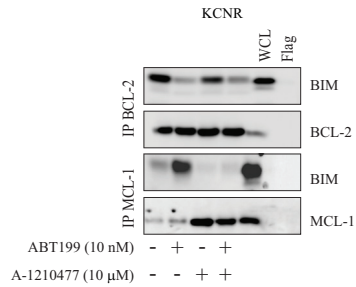
	CHP126	KCNR	SJNB12
A-1210477	++++ (0.18)	+++ (0.28)	+ (0.74)

Table 2. Synergy between BCL-2 inhibitor ABT199 and MCL-1 inhibitor A-1210477 in high BCL-2-expressing neuroblastoma cell lines. Combination indices (between brackets) were determined according to Chou and Talalay [44]2,3…n is the velocity of reaction in the simultaneous presence of n inhibitors, vi is the velocity observed in the presence of each individual inhibitor, and v0 is the velocity in the absence of inhibition. The derivation is based on the assumption that each enzyme species can combine with no more than one of the inhibitors (i.e. the inhibitors are mutually exclusive.

DISCUSSION

Based on previously performed preclinical studies [16], BCL-2 inhibitor ABT263 was considered to have high potential for testing in neuroblastoma patients. Unfortunately, the clinical implementation of ABT263 was hampered because of dose-limiting thrombocytopenia due to concomitant inhibition of the anti-apoptotic protein BCL-X_L. The current manuscript describes the preclinical evaluation of the novel selective BCL-2 inhibitor ABT199. In line with previous results obtained with ABT263, neuroblastoma cell lines expressing high *BCL-2* mRNA levels responded more potently to ABT199 than low *BCL-2*-expressing cell lines, with an over 90-fold difference in average LC_{50} value (i.e., 0.17 μM versus 15.46 μM, respectively). *In vitro* efficacy studies furthermore showed that BCL-2 protein and BIM/BCL-2 complex levels are better predictive biomarkers for ABT199 sensitivity than *BCL-2* mRNA levels. Although less strong, MYCN amplification status was also observed to be a predictive biomarker for sensitivity to ABT199.

The current manuscript describes the first side-by-side *in vivo* comparison of ABT199 with ABT263 for solid cancer treatment. Although ABT199 induced a strong apoptotic response, comparison of the efficacy of equal doses of ABT199 and ABT263 in mice with KCNR neuroblastoma xenografts expressing high levels of BCL-2 showed superior antitumor activity for ABT263. As maximum BIM displacement from BCL-2 was observed after ABT199 treatment, the most plausible explanation for the superior efficacy of ABT263 is its additional activity against anti-apoptotic proteins BCL-X_L and BCL-W. Results indicate that the simultaneous inhibition of multiple anti-apoptotic proteins is more effective for treatment of BCL-2-dependent neuroblastoma than inhibition of BCL-2 alone.

The importance of inhibiting multiple anti-apoptotic proteins is strengthened by the observations that *in vitro* and *in vivo* treatment with ABT199 resulted in upregulation of the anti-apoptotic protein MCL-1 and sequestration of released BIM by MCL-1. Upregulation of non-targeted anti-apoptotic BCL-2 family proteins taking over the function of BCL-2 has earlier been described as one of the key mechanisms of tumor resistance to BCL-2 inhibitors including ABT199 [28-32, 34]. Long-term ABT199 treatment of non-Hodgkin lymphoma cells resulted in upregulated levels of anti-apoptotic proteins MCL-1 and BCL-X$_L$ that sequestered BIM [29]. No changes in the expression and activity of BCL-X$_L$ were observed after ABT199 treatment, indicating that MCL-1 plays a more important role in neuroblastoma resistance to ABT199 than BCL-X$_L$.

MCL-1 upregulation after ABT199 treatment could be explained by Noxa downregulation and MCL-1 stabilization by BIM. Binding of Noxa to MCL-1 triggers Mule-mediated ubiquitination and proteasomal degradation of the anti-apoptotic protein, resulting in decreased MCL-1 levels [33, 35-40]. We observed that Noxa was downregulated after *in vitro* and *in vivo* treatment with ABT199, which has not been reported for BCL-2 inhibitors before. Since complexation of BIM with MCL-1 stabilizes MCL-1 independent of Noxa [33], BIM displacement from BCL-2 to MCL-1 after ABT199 treatment might also contribute to upregulated MCL-1 levels.

Combining *MCL-1* knockdown with ABT199 treatment resulted in synergistic cell growth inhibition. Potentiating effects of MCL-1 inhibition were confirmed with the small molecule MCL-1 inhibitor A-1210477, but combined effects of ABT199 and A-1210477 were additive rather than synergistic. The less potentiating effects obtained with A-1210477 might be due to the incomplete displacement of BIM from MCL-1, which has been described before. In addition, MCL-1 might have other functions independent of its role in apoptosis such as maintenance of mitochondrial integrity and deregulation of mitochondrial integrity by *MCL-1* knockdown in combination with BCL-2 inhibitors might have a more lethal effect on neuroblastoma cells than inhibition of the pro-apoptotic function of MCL-1 alone. Of interest is the observation that several compounds in use or development for cancer treatment also cause MCL-1 inhibition (e.g., CDK inhibitors, MEK inhibitors and PI3K/mTOR inhibitors [24, 28, 29]. Specific combination studies with these types of compounds should be considered.

For the clinical use of ABT199 it is important to identify pharmacodynamic biomarkers for efficacy. In line with previously published results for ABT199 [17], our study demonstrated that BIM displacement from BCL-2 and cytochrome c release from the

mitochondria can be utilized as target-specific efficacy biomarkers for ABT199. *In vitro* dose-dependent stimulatory effects on PARP- and caspase 3 cleavage and the *in vivo* observed cleaved caspase 3 induction after ABT199 treatment furthermore indicate that both apoptotic markers are potential non-target specific biomarkers for ABT199 efficacy. Additional studies are required to validate the dose-dependency of all potential biomarkers *in vivo*.

Taken together, the results presented in this study strongly suggest that children with neuroblastoma tumors expressing high levels of BCL-2 and BIM/BCL-2 complex might benefit from combined treatment with ABT199 and compounds that inhibit MCL-1.

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SUPPLEMENTARY INFORMATION

	ABT199 concentration	Sub-G_1 (%)	G_1 (%)	S (%)	G_2 (%)
CHP126	0 nmol/L	4.3±1.1	58.9±3.6	19.6±2.2	19.4±3.8
	7.5 nmol/L	12.2±1.2	58.1±5.3	13.6±2.7	14.6±1.6
	15.5 nmol/L	11.9±1.6	57.3±3.1	14.5±1.6	17.6±1.4
	32.5 nmol/L	11.3±2.8	59.8±2.1	14.1±0.4	15.1±1.1
	62.5 nmol/L	15.6±2.3	56.6±2.1	13.8±2.4	15.6±1.9
	125 nmol/L	33.9±1.1	51.1±5.6	9.5±5.4	7.2±3.6
	1.25 µmol/L	34.5±0.9	46.3±7.1	9.8±3.4	9.1±2.7
	5 µmol/L	35.7±9.8	45.2±7.5	9.9±0.9	9.3±1.9
	10 µmol/L	48.3±3.1	39.3±1.4	7.3±0.9	6.6±0.7
KCNR	0 nmol/L	1.1± 0.5	58.7±0.9	21.5±0.8	18.3±0.9
	7.5 nmol/L	14.4±1.6	55.1±2.9	19.4±1.9	11.4±2.4
	15.5 nmol/L	17.3±3.0	49.9±0.5	21.2±0.02	12.1±3.5
	32.5 nmol/L	22.5±2.7	47.7±1.6	18.7±1.6	10.6±1.5
	62.5 nmol/L	22.0±2.9	49.7±1.1	17.8±0.3	9.8±0.04
	125 nmol/L	20.2±1.6	53.9±2.2	17.0±3.4	9.4±0.5
	1.25 µmol/L	21.1±1.3	52.5±6.4	17.2±4.1	10.5±3.3
	5 µmol/L	24.9±0.4	48.5±0.6	16.5±1.2	9.2±0.3
	10 µmol/L	26.3±1.8	46.1±0.8	17.6±0.5	9.8±0.1
SJNB12	0 nmol/L	2.0±0.01	70.3±0.6	8.1±5.9	19.2±8.8
	7.5 nmol/L	7.2±1.2	63.5±2.1	5.0±0.4	24.3±0.5
	15.5 nmol/L	15.3±1.5	57.4±0.9	5.3±1.6	21.7±0.6
	32.5 nmol/L	18.0±1.1	57.2±1.7	4.6±1.9	20.0±2.8
	62.5 nmol/L	21.7±3.6	54.8±3.8	6.7±1.1	17.2±0.3
	125 nmol/L	25.1±4.4	52.4±4.8	6.6±0.9	15.9±1.2
	1.25 µmol/L	28.8±3.3	49.3±4.6	7±1.2	15.6±1.3
	5 µmol/L	29.1±2.5	48.9±2.7	7.5±0.7	15.7±2.1
	10 µmol/L	39.1±2.3	45.1±1.2	4.6±1.7	11.3±1.7
SKNAS	0 nmol/L	1.3±0.6	59.4±6.3	17.9±1.6	21.4±4.3
	7.5 nmol/L	1.8±1.2	58.2±2.1	18.9±0.5	19.3±1.7
	15.5 nmol/L	1.7±0.7	58.3±4.1	19.9±1.1	20.9±4.6
	32.5 nmol/L	1.1±0.4	59.1±3.7	19.1±0.3	20.1±3.1
	62.5 nmol/L	1.3±0.2	60.1±3.3	19.6±0.2	19.3±3.4
	125 nmol/L	1.2±0.1	59.6±1.1	20.1±1.1	18.4±1.6
	1.25 µmol/L	1.3±0.2	58.7±1.2	19.5±1.2	18.7±2.6
	5 µmol/L	1.4±0.09	58.8±2.6	21.6±2.6	17.4±0.6
	10 µmol/L	8.1±5.6	59.7±4.9	15.3±4.9	16.5±4.7
SHEP2	0 nmol/L	0.8±0.4	75.6±2.1	13.7±2.4	10.7±3.6
	7.5 nmol/L	1.6±1.1	74.6±0.9	11.7±0.9	12.1±2.9
	15.5 nmol/L	1.7±1.2	76.1±3.7	12.0±0.8	12.1±3.2
	32.5 nmol/L	1.3±0.3	75.7±2.3	11.3±1.3	12.6±1.3
	62.5 nmol/L	1.4±0.4	77.1±1.5	10.4±1.2	11.6±1.8
	125 nmol/L	1.4±1.1	77.9±2.6	11.0±1.6	10.4±3.3
	1.25 µmol/L	1.9±1.8	75.4±2.9	10.3±1.9	11.9±3.6
	5 µmol/L	3.6±2.4	72.1±2.8	12.8±0.4	12.1±2.3
	10 µmol/L	4.3±2.2	70.8±4.4	12.6±1.1	11.1±3.9

**Supplementary Table 1.** *In vitro* effects on sub-G_1 induction and cell cycle progression in neuroblastoma cell lines expressing high BCL-2 and BIM/BCL-2 complex levels (i.e., CHP126, KCNR and SJNB12) versus low expressing neuroblastoma cell lines (i.e., SKNAS and SHEP2) after 72 h treatment with ABT199. Values represent the average percentage of cells in sub-G_1, G_1, S and G_2 +/- SD.

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A.

B.

C.
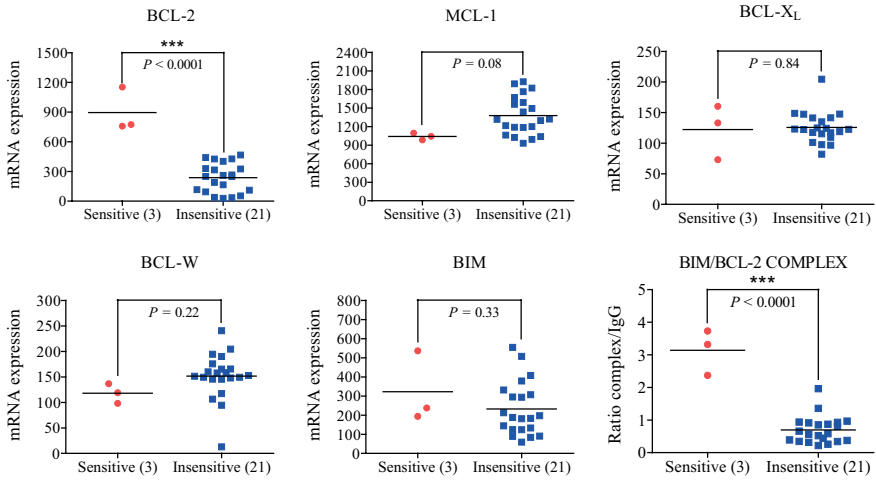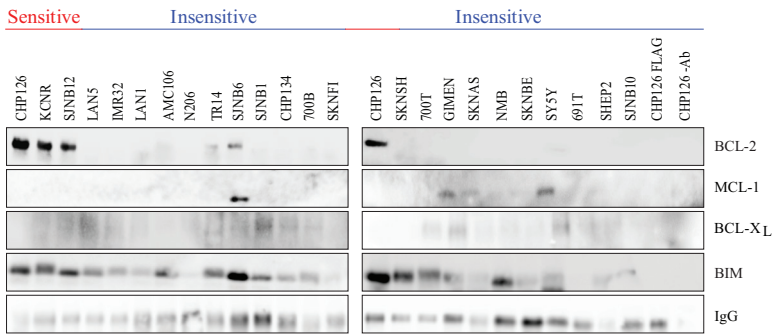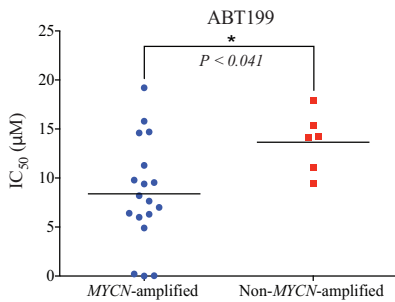
Supplementary Figure 1. *BCL-2* mRNA levels, protein levels of BCL-2/BIM complex and *MYCN* amplification status predict sensitivity to ABT199. A, mRNA expression levels of *BCL-2*, *MCL-1*, *BCL-X$_L$*, *BCL-W* and *BIM* and BCL-2/BIM protein complex levels in sensitive (i.e., CHP126, KCNR, and SJNB12) versus insensitive neuroblastoma cell lines. *BCL-2*, *MCL-1*, *BCL-X$_L$*, *BCL-W* and *BIM* mRNA levels have been established using the R2 bioinformatics platform (http://r2.amc.nl), which contains Affymetrix mRNA expression data of all cell lines studied in the current manuscript. BCL-2/BIM complex levels were established by anti-BIM immunoprecipitation of whole cell lysates, followed by western blotting for BCL-2. BCL-2 band intensities were normalized to the IgG heavy chain of the BIM antibody. Statistical differences between the sensitive and insensitive cell lines were calculated using a one-tailed (for *BCL-2* mRNA and BIM/BCL-2 protein complex) or two-tailed unpaired Student *t* test, with $P < 0.05$ as the minimal level of significance and $P < 0.0001$ indicated as [***]. Horizontal lines represent the mean of the mRNA expression of the BCL-2 family proteins and the BCL-2/BIM complex levels of the cell line panel. B, BCL-2/BIM, MCL-1/BIM and BCL-X$_L$/BIM complex levels in 24 neuroblastoma cell lines, ordered from ABT199 sensitive (left) to ABT199 insensitive (right). Complex levels were established by anti-BIM immunoprecipitation of whole cell lysates, followed by western blotting for BCL-2 and MCL-1. The IgG heavy chain of the BIM antibody served as a loading control. Total protein levels of the BCL-2-like family member proteins of the cell line panel in Figure 1A served as whole cell lysates for this experiment. C, IC$_{50}$ of *MYCN* amplified versus non-*MYCN* amplified cell lines treated with ABT199. IC$_{50}$ curves were obtained by MTT assays after 72-hour treatment of the cell lines with ABT199 and IC$_{50}$ values were calculated by the fitted-method using the Graphpad Prism software. Statistical differences in sensitivity to ABT199 between the *MYCN* and non-*MYCN* amplified cell lines were calculated using a one-tailed Student *t* test, with $P < 0.05$ as the minimal level of significance and $P < 0.0001$ indicated as [***].

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A.

B.

SY5Y

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D.

KCNR

E.

KCNR
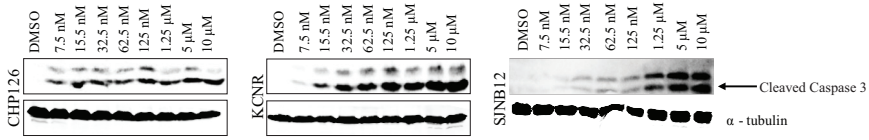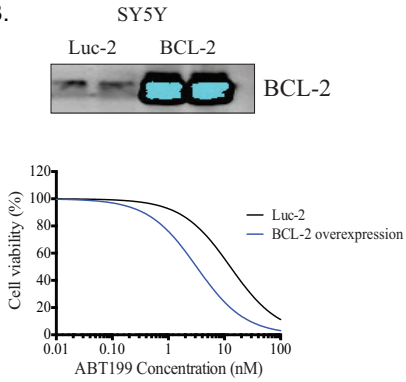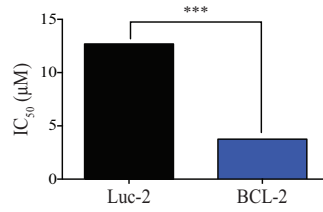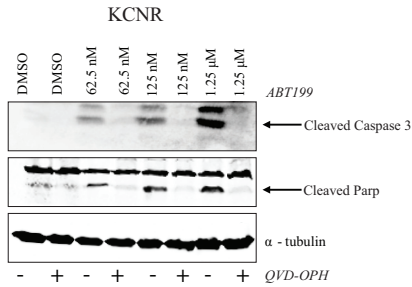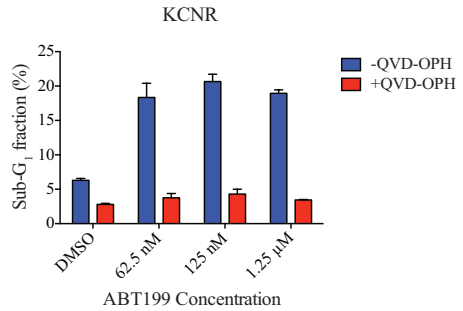
Supplementary Figure 2. ABT199 inhibits BCL-2 and causes apoptosis by induction of the intrinsic apoptotic pathway which can be rescued with caspase inhibitors. A, Western blot analysis of the *in vitro* effects of ABT199 on caspase 3 cleavage after 72-hour treatment of sensitive cell lines CHP126, KCNR and SJNB12 with increasing ABT199 concentrations. Alpha-tubulin served as loading control. B, Western blot analysis of the level of overexpression of BCL-2 in a BCL-2 low-expressing cell line SY5Y compared to an SY5Y cell lines expressing the Luc2 control construct and IC_{50} curves comparing the sensitivity of a BCL-2 low expressing cell lines SY5Y overexpressing BCL-2 in blue compared to an SY5Y line overexpressing a luciferase 2 construct in black. Cell viability was measured after 72-hour treatment of the cell lines with ABT199. The data are the mean of three replicate wells. C, Bar graphs comparing the IC_{50} values between SY5Y cells expressing the Luc2 construct and SY5Y overexpressing BCL-2. Data represents one experiment. D, Western blot analysis on the *in vitro* effects of the inhibition of caspase 3 and PARP cleavage after 4-hour treatment with the pan-caspase 3 inhibitor QVD-OPH and 24-hour treatment with the BCL-2 inhibitor ABT199 in the BCL-2 high expressing cell line KCNR. Alpha-tubulin served as loading control. E, FACS analysis of the *in vitro* effects on sub-G_1 induction after 4-hour treatment of KCNR with the pan-caspase inhibitor QVD-OPH and 24-hour treatment with increasing ABT199 concentrations. Data represent the mean percentages of cells in sub-G_1 ± SD of three replicate experiments.

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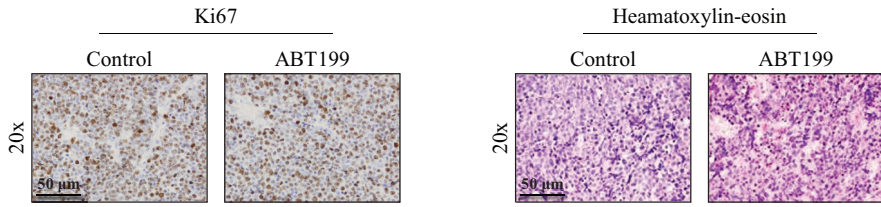
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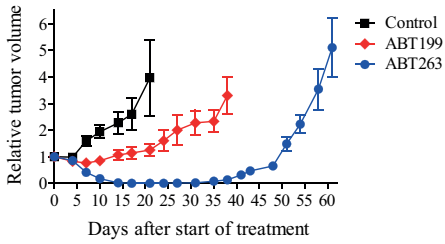
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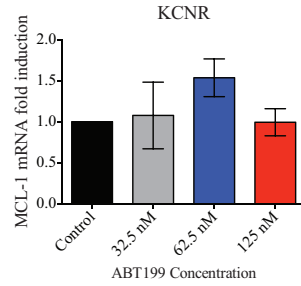
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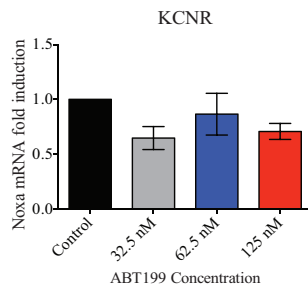
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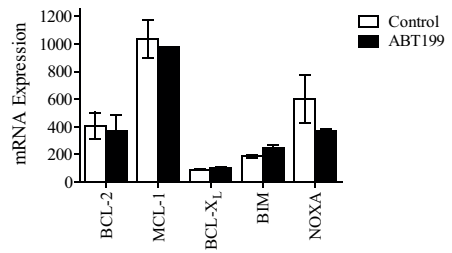
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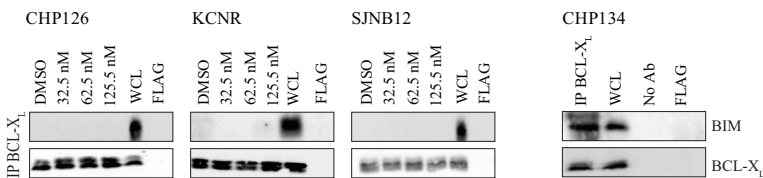
Supplementary Figure 3. ABT199 and ABT263 elicit anticancer activity in mice with KNCR neuroblastoma xenografts expressing high BCL-2 and BIM/BCL-2 complex levels. Mice with KCNR xenografts of approximately 268 mm^3 were daily treated with vehicle (control), 100 mg/kg ABT199 or 100 mg/kg ABT263 for 3 consecutive weeks. A, Representative microscopic images of Ki67 and heamatoxylin-eosin stained paraffin-embedded sections of control tumors versus tumors treated with ABT199. Tumor materials were collected at 4 hours after administration of the last dose. ABT199 effects on cell proliferation and phenotypic changes caused by ABT199 were studied by immunohistochemistry analysis of Ki67 and conventional heamatoxylin-eosin staining, respectively. Magnification of the images: 20x. Scale bar: 50 µm. B, Long-term effects of ABT199 and ABT263 on the growth of KCNR neuroblastoma xenografts after three weeks treatment. Relative tumor volume was calculated as the volume at the indicated day after start of treatment divided by the volume prior to treatment initiation. Data represent the mean relative tumor volume ± SEM. [Group sizes at the start of treatment: n = 10 (control), n = 5 (ABT199), and n = 5 (ABT263)]. C, D, Effects on *in vitro* mRNA levels of *MCL-1* and *NOXA* in the BCL-2 high expressing cell line KCNR treated for 24-hour with increasing concentrations of ABT199. *MCL-1* and *NOXA* fold change was analyzed by real-time quantitative-PCR. RNA levels of the control treated samples were set to 1 for analysis. Data represent average mRNA expression values ± SD. E, ABT199 effects on *in vivo* mRNA levels of *BCL-2*, *MCL-1*, *BCL-X$_L$*, *BIM* and *NOXA*. Tumor materials of control and ABT199-treated KCNR xenografts (n = 2 per group) were collected at 4 hours after administration of the last dose and analyzed by Affymetrix mRNA profiling. Data represent average mRNA expression values ± SEM. F, *In vitro* effects of ABT199 on BIM/BCL-X$_L$ complex levels in CHP126, SJNB12 and KCNR after 24-hour treatment with increasing doses. BIM/BCL-X$_L$ complex levels were established by immunoprecipitation of BCL-X$_L$ followed by Western blot analysis of BIM. Immunoprecipitated levels of BCL-X$_L$ were used as loading controls. As no BIM/BCL-X$_L$ complex could be detected in the cell lines tested, neuroblastoma cell line CHP134 was included as a positive control for BIM/BCL-X$_L$ complex.

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MATERIALS AND METHODS

Cell culture. Classical human neuroblastoma cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L D-glucose, glutamate and supplemented with 10% (v/v) foetal calf serum, 2 mmol/L L-glutamine, 10 U/mL penicillin, 10 µg/mL streptomycin and MEM non-essential amino acids (1x). Neuroblastoma tumor-initiating cell (TIC) lines were grown in neural specific stem cell medium (400 mL DMEM GlutaMAX™-1 containing 1g/L D-glucose and pyruvate, 133 mL F12 medium, 10 mL B27, 18 ng/mL EGF, 36 ng/mL FGF, 10 U/mL penicillin and 10 µg/mL streptomycin. Cells were maintained at 37°C under 5% CO_2 in humidified air. Penicillin and streptomycin were obtained from Sigma Aldrich, EGF from Corning Life Sciences and FGF from PeproTech. Other cell culture related materials were obtained from Life Technologies.

In vitro **FACS analysis.** High BCL-2-expressing neuroblastoma cell lines CHP126, KCNR, and SJNB12 and low BCL-2-expressing cell lines SKNAS and SHEP2 were seeded in triplicate onto 6-cm plates and incubated overnight. Cells were then 72-hour treated with 0.1% DMSO (control) or ABT199 using concentration ranges of 7.8 nmol/L to 10 µmol/L. Supernatants containing floating cells were collected from the culture dishes. Adherent cells were washed once with PBS and PBS solutions were pooled with the supernatants. After trypsinization of the adherent cells with 0.05% trypsin/EDTA, cells were resuspended in the pooled supernatant/PBS solution. Next, cells were centrifuged (5 min; 1,500 rpm), washed by resuspension in PBS and centrifuged again (5 min; 3,000 rpm). Cells were fixed with 100% ice-cold ethanol, stained with 0.05 mg/mL propidium iodide and supplemented with 0.05 mg/mL RNAse A in PBS. After 1-hour incubation in the dark at room temperature (RT), cells were filtered through a 50 µm filter (BD Biosciences) and DNA contents of the nuclei were analyzed using a fluorescence-activated cell sorter. A total of 20,000 nuclei per sample were counted. The cell cycle distribution and apoptotic Sub-G_1 fraction were determined using the BD Accuri™ C6 flow cytometer with the CFlow plus software (BD Biosciences).

Cell fractionation. CHP126, KCNR, SJNB12, SKNAS and SHEP2 were seeded onto 14-cm culture dishes and incubated overnight. Cell lines were then 24-hour treated with 0.04% DMSO (control) or 62.5 nmol/L to 10 µmol/L ABT199. Supernatants containing floating cells were collected from the culture dishes. Adherent cells were washed once with PBS and the PBS solutions were pooled with the supernatants. After trypsinization of the adherent cells with 0.05% trypsin/EDTA, cells were resuspended in the pooled supernatant/PBS solution. Next, cells were centrifuged (5 min; 1,500 rpm), twice washed

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by resuspension in PBS and centrifuged again (5 min; 1,500 rpm). Cytosolic and organelle fractions were subsequently separated using the ProteoExtract® Subcellular Proteome Extraction Kit according to the manufacturers protocol (Calbiochem, 539790). Cell fractions were used for cytochrome c detection by western blot analysis (see below).

***In vitro* western blotting.** Cells were seeded onto 9- or 14-cm dishes and treated with DMSO (control) or up to 10 µmol/L ABT199. Treated cells were harvested at indicated time points and lysed using Laemmli buffer [i.e., H_2O/glycerol/20% sodium dodecyl sulfate (SDS)/1 M Tris-HCl (pH 6.8) 5:2:2:1 (v/v/v/v)]. Lysates were homogenized by hydrodynamic shearing through a 23 G needle, followed by 10 min incubation at 50ºC. Protein concentrations were determined using the Bio-Rad *DC* Protein Assay (Bio-Rad, Veenendaal, the Netherlands).

Equal protein amounts (40 µg) were diluted in 5x reducing sampling buffer (i.e., Laemmli buffer/β-mercaptoethanol 3:1 (v/v) with bromophenol blue sodium salt). Diluted samples were boiled for 5 min at 95℃ and centrifuged (1,500 rpm 5 min). Proteins were separated by SDS-polyacrylamide gel electrophoresis on 12% Mini-Protean® Tris-glycince extended (TGX) precast gels (Bio-Rad) and transferred on hybond nitrocellulose membranes (0.45 µm) by 1.5-hour wet blotting (200 mA; 4ºC). Transfer buffer consisted of 20% (v/v) methanol, 3.025 g/L Tris and 14.4 g/L glycine in demineralized water. Membranes were blocked in 2% ECL Prime™ blocking agent (GE Healthcare) in PBS with 0.1% (v/v) Tween-20 (= blocking buffer) for 1-hour at RT. After blocking, membranes were incubated with the primary (overnight; 4ºC) and secondary (1-hour; RT) antibodies in blocking buffer and scanned using the Image Quant LAS 4000 detection system (GE Healthcare Life Science).

***In vitro* co-immunoprecipitation and immunoblotting.** For all samples, total cell lysates were prepared in 2% CHAPS buffer (i.e., 1 mol/L HEPES, 150 mmol/L NaCl, 5 mmol/L EDTA, 5% sodium glycerol phosphate (w/v) and 2% (w/v) CHAPS). Equal protein amounts (1 mg) were added to antibody-matrix complex Protein A-Agarose beads (Roche) for 24 hours at 4℃. Immunoprecipitated proteins were released from the matrix using RIPA buffer (1x) and analyzed by western blot analysis as described above, with minor modifications. MCL-1 detection has been performed using mouse anti-human MCL-1 (clone RC13) monoclonal antibody (Merck Millipore). Other antibodies were similar to the antibodies used for *in vitro* western blotting and all antibodies have been used in a 1:100 dilution. Flag control samples were immunoprecipitated with an anti-Flag rabbit DYDDDDK tag antibody (clone 2368) (1:100 Cell Signaling Technology).

In vivo efficacy in neuroblastoma mouse models. Female NMRI *nu⁻/nu⁻* mice (6-15 weeks old; 20-30 g) were subcutaneously injected with 1-5x10^6 cells/flank of KCNR. The size of the tumors was recorded twice weekly and when the tumors reached a size of approximately 1,000 mm^3, tumor pieces were serially xenotransplanted in recipient mice. Formalin-fixed and paraffin-embedded sections of the serially xenotransplanted tumors were routinely checked by hematoxylin-eosin staining. For the efficacy studies, recipient mice with KCNR neuroblastoma xenografts of approximately 268 mm^3 were orally treated with 100 mg/kg/d ABT199 (n = 5), 100 mg/kg/d ABT263 (n = 5), or vehicle (n = 6) for 21 days. Tumor sizes were measured by an external caliper.

In vivo immunohistochemistry. Paraffin-embedded sections of 4 μm were deparaffinized in xylene and hydrated in a series of alcohol baths. Endogenous peroxidase activity was inactivated by 10 minutes incubation with 0.3% (v/v) hydrogen peroxide in methanol. After rinsing in running tap water (5 min), tumor sections were pre-treated with 10.1 mmol/L Tris/EDTA buffer pH 9.1 (10 min; wet autoclaving). Sections were rinsed in Tris buffered saline (TBS) pH 7.8 (5 min) and 1-hour incubated with the primary antibodies. Anti-Ki67- and anti-cleaved caspase 3 antibodies were diluted in ScyTek Antibody Diluent (ScyTek Laboratories). Sections were washed with TBS (3x 2 min), incubated with the secondary antibody, rinsed again in TBS and incubated using the 3,3-diaminobenzidine (DAB) + Substrate Chromogen System (10 min; Dako, Heverlee, Belgium). Nuclei were stained by successively rinsing with running tap water, counterstaining with hematoxylin and rinsing again with running tap water. Sections were dehydrated in an ascending graded series of ethanol baths and xylene and mounted with Pertex and coverslip. Prior to visualization with an Olympus BX51 Microscope and DP70 Digital Camera System, sections were overnight incubated at 50ºC. All incubations were performed at RT, unless indicated otherwise.

RNA isolation and real-time quantitative PCR. RNA was isolated using the Trizol-chloroform method from the KCNR cell line treated for 24-hour with 32.5-125 nmol/L concentrations of ABT199. 1 μg of mRNA was reversed transcribed using the Taqman reverse transcription kit and amplified using the SYBR Green Master Mix (Applied Biosystems, N808234) and examined on a MyiQ Real-time PCR systems (Bio-Rad). Quantitative, real-time PCR was performed using the following specific primers: *MCL-1* 5'-ATGCTTCGGAAACTGGACAT-3'(forward) and 5'-TCCTGATGCCACCTTCTAGG-3'(reverse); *Noxa* 5'-TGGAAGTCGAGTGTGCTACTCAA-3' (forward) and 5'-CAGAAGA

GTTTGGATATCAGATTCAGA-3' (reverse) and *B-Actin* 5'-AGAAAATCTGGCACCACACC-3' (forward) and 5'-AGAGGCGTACAGGGATAGCA-3' (reverse).

4

CHAPTER 5

HIGH-THROUGHPUT SCREENING IDENTIFIES IDASANUTLIN AS A RE-SENSITIZING DRUG FOR VENETOCLAX-RESISTANT NEUROBLASTOMA CELLS.

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TRANSLATIONAL RELEVANCE

High BCL-2 expression has been observed in neuroblastoma tumors compared to other malignant tumors and normal tissues. Previous preclinical studies using the BCL-2-specific inhibitor venetoclax showed superior activity in neuroblastoma tumors with high BCL-2 and BIM/BCL-2 complex levels. However, eventually resistance occurred as is seen with most targeted compounds with high specificity.

The current study describes the identification of drugs capable of re-sensitizing venetoclax-resistant neuroblastoma cell lines to venetoclax. Long-term continuous exposure of BCL-2-dependent neuroblastoma cell lines to high venetoclax concentrations resulted in venetoclax-resistance. A compound-wide screen with targeted compounds in (pre-) clinical development and cytostatics used in neuroblastoma treatment identified idasanutlin as the strongest re-sensitizer for venetoclax in venetoclax-resistant BCL-2-dependent neuroblastoma cells. Re-sensitization by idasanutlin could be attributed to p53-mediated upregulation of BAX, resulting in re-activation of the intrinsic apoptotic pathway. *In vivo* combination studies with venetoclax and idasanutlin showed superior efficacy over single-agent treatments, with significant regression of BCL-2-dependent neuroblastoma xenografts.

Taken together, our findings suggest that BCL-2 high-expressing neuroblastoma patients might benefit from combination treatment of venetoclax with the MDM2 inhibitor idasanutlin.

ABSTRACT

Purpose: The anti-apoptotic protein B cell lymphoma/leukaemia 2 (*BCL-2*) is highly expressed in neuroblastoma. In previous preclinical studies, we have shown that the BCL-2 specific inhibitor venetoclax causes a strong apoptotic phenotype in BCL-2-dependent neuroblastoma cells. However, *in vivo* treatment with venetoclax showed partial resistance. In the current manuscript, we describe the identification of re-sensitizing drugs for venetoclax-resistant BCL-2-dependent neuroblastoma.

Experimental Design: Non-resistant and venetoclax-resistant neuroblastoma cell lines in the absence and presence of venetoclax were exposed to a 209–compound library to identify compounds that were more effective in the venetoclax-resistant cell lines in the presence of venetoclax.

Results: Long-term continuous exposure of BCL-2-dependent neuroblastoma cell lines SJNB12 and KCNR to high venetoclax concentrations resulted in >50 times less sensitivity to venetoclax, as shown by differences in IC_{50} values. Resistance to venetoclax was caused by the increased expression of myeloid cell leukaemia sequence 1 (MCL-1) and sequestration of BIM released from BCL-2 by MCL-1. High-throughput drug screening identified idasanutlin as a re-sensitizing drug for venetoclax-resistant neuroblastoma cells, as shown by the 1.5-20-fold higher activity of idasanutlin in the venetoclax-resistant cells under venetoclax pressure compared to the non-resistant cells. Idasanutlin caused BAX-mediated apoptosis in venetoclax-resistant neuroblastoma cells in the presence of venetoclax, while causing p21-mediated growth arrest in the non-resistant cells and venetoclax-resistant cells in the absence of venetoclax. *In vivo* combination treatment of mice with BCL-2-dependent neuroblastoma tumors with venetoclax and idasanutlin showed superior efficacy over single-agent treatments, with significant tumor regression.

Conclusions: This study suggests that patients with BCL-2-dependent neuroblastoma tumors might benefit from combined treatment with venetoclax and the re-sensitizing drug idasanutlin.

5

INTRODUCTION

Aberrations in genes encoding B cell lymphoma/leukaemia 2 (BCL-2) family proteins have been observed in numerous cancer types (1). The intrinsic apoptotic pathway consists of the anti-apoptotic BCL-2 family members (2, 3), [e.g., BCL-2, BCL-extra-large (BCL-X_L), BCL-2-like protein 2 (BCL-W) and myeloid cell leukaemia sequence 1 (MCL-1)]. These anti-apoptotic proteins prevent apoptosis from occurring by sequestering their BH3-only counterparts [e.g., BCL-2-like protein 11 (BIM), p53 upregulated modulator of apoptosis (PUMA), NOXA and BH3 interacting domain death agonist (BID)]. Displacement of the pro-apoptotic proteins during cellular stress activates the pro-apoptotic proteins BAX (BCL-2-associated X protein) and BAK (BCL-2 antagonist/killer 1) (4-6). Oligomerization of BAX/BAK causes mitochondrial outer membrane permeabilization, followed by cytochrome c release into the cytosol and activation of caspase-3-induced proteolysis and cell death (7-13).

Neuroblastomas are pediatric solid tumors that arise from the sympathetic nervous system. Despite extensive treatment regimens these tumors still account for approximately 15% of all pediatric cancer deaths (14). A large subset of neuroblastoma tumors express enhanced levels of the anti-apoptotic gene *BCL-2* (15, 16). Mitochondrial profiling of neuroblastoma cell lines expressing high BCL-2 level reveal a BH3 profile which predicts sensitivity to BCL-2 antagonists *in vitro*. BCL-2-dependent cell lines in which BIM was bound to the BH3 domain of BCL-2 were shown to respond potently to the BCL-2 inhibitor navitoclax, while neuroblasoma cell lines with an MCL-1 profile were deemed resistant to the compound (15). In previous studies, it was shown that navitoclax induced a strong apoptotic response in BCL-2-dependent neuroblastoma cell lines (16). Unfortunately, the clinical use of navitoclax was associated with dose-limiting thrombocytopenia due to concomitant targeting of BCL-X_L. Therefore, the BCL-2-specific inhibitor venetoclax was developed. *In vitro* studies have shown that also venetoclax caused strong apoptosis in BCL-2-dependent neuroblastoma cells through BIM displacement from BCL-2 and cytochrome c release from the mitochondria into the cytoplasm (17, 18). These findings could be confirmed *in vivo* in a BCL-2-dependent neuroblastoma xenograft mouse model. However, partial resistance to venetoclax was observed, which could be attributed to MCL-1 upregulation and sequestration of BIM displaced from BCL-2 by MCL-1. Combined inhibition of MCL-1 and BCL-2 abrogated the partial resistance observed in BCL-2 high-expressing cells treated with venetoclax. However, MCL-1 inhibitors are still in early preclinical development and combination

treatment of patients with MCL-1 and BCL-2 inhibitors have been reported to cause myelo-suppressive related side effects.

In the current study, we therefore aimed at identifying strategies to re-sensitize venetoclax-resistant neuroblastoma tumor cells to ventoclax.

MATERIALS AND METHODS

Chemicals. The Sequoia anti-neoplastic library containing 157 approved drugs used in cancer treatment and the SCREEN-WELL® epigenetics library containing 43 epigenetic compounds were purchased from Sequoia Research Products (Pangbourne, UK) and Enzo Life Sciences (Farmingdale, USA), respectively. Other targeted inhibitors used in the current study were purchased from Selleck Chemicals while the regularly used cytostatics in neuroblastoma treatment were purchased from Sigma Aldrich. Both compound libraries were reformatted to a single 384-well plate at a concentration of 200 µM in DMSO. Venetoclax (ABT199), navitoclax, AT7519, JQ1, ceritinib, ribociclib, idasanutlin (RG7388), trametinib and YM155 were added to the reformatted library plate. For *in vivo* studies, venetoclax was formulated in 10% ethanol/30% polyethylene glycol (PEG) 400/60% phosal 50 propylene glycol (PG) (v/v/v) in final concentrations of 10 mg/ml. Idasanutlin was formulated in 2% hydroxypropyl cellulose/0.1% polysorbate 80/0.09% methyl paraben/0.01% propyl paraben in final concentrations of 15mg/ml.

Cell culture. Human neuroblastoma cell lines SJNB12 and KCNR were cultured as previously described (19). Cell culture protocols are described in detail in the Supplementary Materials and Methods.

Generation of venetoclax-resistant neuroblastoma cell lines. Neuroblastoma cell lines SJNB12 and KCNR were made resistant to venetoclax by long-term continuous exposure to venetoclax doses equal to the IC_{85} concentrations for both cell lines (i.e. 2.75 µmol/L for SJNB12 and 7.5 µmol/L for KCNR). Venetoclax-containing medium was refreshed twice a week. This was done by allowing the cells to recover for 24-hours in normal culture medium, prior to venetoclax addition. Venetoclax resistance was confirmed by investigating differences in venetoclax dose-response curves and venetoclax-induced sub-G_1 accumulation between resistant and non-resistant cell lines after 72-hour treatment (see below).

BCL-2 DNA sequence analysis. Genomic DNA from non-resistant SJNB12 and KCNR cells and venetoclax-resistant SJNB12 and KCNR cells under venetoclax pressure (i.e. 2.75 µmol/L for SJNB12 and 7.5 µmol/L for KCNR) was obtained using the chloroform-isopropanol method. DNA was then purified using a QIAamp DNA Mini Kit (Qiagen), PCR amplified with Taq polymerase (Invitrogen), and sequenced in the ABI PRISM 3730 sequencer (Applied Biosystems). Primers used for the gene sequence analysis are: Exon 1: Forward primer: GTCCAAGAATGCAAAGCACA, Reverse primer: GAACGCTTTGTGGAGAGGAG. Exon 2: Forward primer: GCAGGATGCCTCTTTCTCTG, Reverse primer: AGCCTGCAGCTTTGTTTCAT.

mRNA expression profiling. RNA was extracted from non-resistant SJNB12 and KCNR cells and venetoclax-resistant SJNB12 and KCNR cells in the presence of venetoclax using TRIzol (Invitrogen, Carlsbad, CA) following the manufacturers protocols. RNA concentration and quality were determined using the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies). Fragmentation of cRNA, hybridization to Human Genome U133 plus 2.0, microarrays and scanning were carried out according to the manufacturers protocol (Affymetrix inc. Santa Barbara, CA). The mRNA gene expression data were normalized with the MAS5.0 algorithm within the GCOS program of Affymetrix Inc. Target intensity was set to 100. All data were analyzed using the R2 genomic analysis and visualization platform (http://r2.amc.nl).

High-throughput drug screening and hit selection. Non-resistant and venetoclax-resistant SJNB12 and KCNR neuroblastoma cell lines were seeded in 384-well plates (i.e. 10000 cells/well for SJNB12 lines and 8000 cells/well for KCNR lines). Resistant cell lines were plated both without venetoclax and in the presence of venetoclax doses equal the IC_{85} for the matching non-resistant cell lines (i.e. 2.75 µmol/L for SJNB12 and 7.5 µmol/L for KCNR). Cells were incubated overnight and treated with 10 nmol/L, 100 nmol/L and 1 µmol/L of the compounds on the library plate using the Sciclone ALH 3000 liquid handling robot (Perkin Elmer). Control samples were treated with DMSO. Cell viability was determined prior to and after 72-hour treatment using the 3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The cell viability of DMSO-treated cells was set to 100%. For each compound, the sum of the % viable cells observed after treatment with 10 nM, 100 nM and 1 µM was calculated for the non-resistant cells, venetoclax-resistant cells and venetoclax-resistant cells under venetoclax pressure. Compounds for which the sum of the percentages viable cells was lower for the venetoclax-resistant cells under venetoclax pressure than for the non-resistant

cells were considered re-sensitizing compounds. Hit selection was performed by taking the 40 top re-sensitizing compounds and subsequently excluding non-targeted compounds (except for the regularly used cytostatics in neuroblastoma treatment) and targeted compounds for which treatment with the highest tested concentration (i.e. 1 μM) resulted in ≥20% viable cells.

Validation re-sensitizing effects of compounds. Non-resistant and venetoclax-resistant SJNB12 and KCNR neuroblastoma cell lines were seeded in quadruplicate as described above. After overnight incubation, non-resistant SJNB12 and KCNR cells, venetoclax-resistant SJNB12 and KCNR cells and venetoclax-resistant SJNB12 and KCNR cells in the presence of venetoclax were treated with 8 concentrations of each compound and control samples were treated with DMSO using the Combi-Bulk Tecan HP D300 Digital Dispenser (Hewlett-Packard). Half maximal effective concentration (IC$_{50}$) values were derived from dose-response curves. IC$_{50}$ values at 72 hours were calculated by determining the compound concentrations needed to achieve a 50% reduction in cell viability observed for DMSO-treated cells at 72 hours (set at 100%).

FACS analysis. To confirm the generation of venetoclax-resistant SJNB12 and KCNR cells, non-resistant and venetoclax-resistant cells were treated with 0.1% DMSO (control) or venetoclax using concentration ranges of 7.8 nmol/L to 10 μmol/L. To study the effects of idasanutlin on sub-G$_1$ induction, non-resistant SJNB12 and KCNR cells, venetoclax-resistant SJNB12 and KCNR cells and venetoclax-resistant SJNB12 and KCNR cells in the presence of venetoclax were treated with 0.05% DMSO (control) or 25 nmol/L to 1 μmol/L idasanutlin. After 72-hour treatment, floating and adherent cells were harvested for FACS analysis to determine the cell-cycle distribution and the apoptotic sub-G$_1$ fraction. See Supplementary Materials and Methods for a detailed protocol.

Western blotting. The following antibodies were used: rabbit anti-human BCL-2 (clone D55G8) monoclonal antibody (1:1,000, Cell Signaling Technology); rabbit anti-human MCL-1 monoclonal antibody (1:1,000, Cell Signaling Technology); rabbit anti-human BCL-X$_L$ (clone 54H6) monoclonal antibody (1:1,000, Cell Signaling Technology); rabbit anti-human BCL-W (Clone 31H4) monoclonal antibody (1:1,000, Cell Signaling Technology); rabbit anti-human BIM (1:1,000, Cell Signaling Technology); rabbit anti-human PARP (Clone 9542S) monoclonal antibody (1:1,000, Cell Signaling Technolgy); rabbit anti-human p21 monoclonal antibody (1:1,000, Abcam); rabbit anti-human BAX polyclonal antibody (1:1,000, Cell Signaling Technology) mouse anti-human p53 monoclonal antibody (clone D0-7) (1:1,000, Neomarkers), rabbit anti-human MDM2

polyclonal antibody (clone N-20) (1:1,000, Santa Cruz Biotechnology), mouse anti-human α-tubulin (clone DM1A) monoclonal antibody (1:10,000, Cell Signaling Technology) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit (clone NA9340V) and goat anti-mouse (clone NXA931) secondary antibodies (1:10,000 GE Healthcare). See Supplementary Materials and Methods for detailed protocol.

***In vitro* co-immunoprecipitation and immunoblotting.** Non-resistant SJNB12 and KCNR cells, venetoclax-resistant SJNB12 and KCNR cells and/or venetoclax-resistant SJNB12 and KCNR cells in the presence of venetoclax were seeded onto T75 flasks. For the detection of BIM displacement from BCL-2 and BIM complexation with MCL-1 in the non-resistant cell lines and venetoclax-resistant cell lines, cells were harvested at normal culture conditions or treated with idasanutlin. For all samples, total cell lysates were prepared in 2% Chaps buffer (i.e., 1 mol/L HEPES, 150 mmol/L NaCl, 5 mmol/L EDTA, 5% sodium glycerol phosphate (w/v) and 2% (w/v) Chaps). Equal protein amounts (1 mg) were added to antibody-matrix complex Protein A-Agarose beads (Roche) for 24 hours at 4°C. Immunoprecipitated proteins were released from the matrix using RIPA buffer (1x) and analyzed by Western blot analysis as described above, with minor modifications. MCL-1 detection has been performed using mouse anti-human MCL-1 (clone RC13) monoconal antibody (Merck Millipore). Other antibodies were similar to the antibodies used for *in vitro* Western blotting and all antibodies have been used in a 1:100 dilution. Flag control samples were immunoprecipitated with an anti-Flag rabbit DYDDDDK tag antibody (clone 2368) (1:100 Cell Signaling Technology).

***In vivo* combination studies in neuroblastoma mouse models.** Female NMRI *nu*⁻/*nu*⁻ mice (6-15 weeks old; 20-30 g) were obtained from Charles River Laboratories and experiments were performed with permission from and according to the standards of the Dutch animal ethics committee (DAG 103059, 102776, 102830 and 102690). NMRI *nu*⁻/*nu*⁻ mice were subcutaneously injected with 1-5x10⁶ cells/flank of KCNR. The size of the tumors was recorded twice weekly and when the tumors reached a size of approximately 1,000 mm³, tumor pieces were serially xenotransplanted in recipient mice. Formalin-fixed and paraffin-embedded sections of the serially xenotransplanted tumors were routinely checked by hematoxylin-eosin staining and treatment started when the tumors of recipient mice with KCNR neuroblastoma xenografts reached an approximate size of about 268 mm³. Mice were randomly divided into five groups for the following treatment regimens: (1) three weeks treatment with vehicles venetoclax and idasanutlin (n=5), (2) three weeks treatment with 100 mg/kg/d venetoclax (n=6), (3)

three weeks treatment with 25 mg/kg/d idasanutlin (n=7), (4) three weeks combination treatment with 100 mg/kg/d venetoclax and 25 mg/kg/d idasanutlin (n=7) and (5) one week single treatment with 100 mg/kg/d venetoclax followed by two weeks combination treatment with venetoclax and 25 mg/kg/d idasanutlin (n=6). Compounds were administered orally and tumor sizes were measured by an external caliper.

***In vivo* western blotting.** Ten tumor sections of 50 μm were homogenized in 2% Chaps buffer as previously described. Western blot detection of protein levels of BAX was carried out as described for *in vitro* Western blotting.

***In vivo* co-immunoprecipitation.** Sections of treated and untreated KCNR tumors harvested at 4 hours after administration of the last dose were homogenized using the Ultra Turrax T25 tissue homogenizer (Janke & Kunkel) and lysed (overnight at 4 °C) in 2% Chaps buffer. Co-immunoprecipitation was carried out as described above.

RESULTS

Long-term continuous exposure of BCL-2-dependent neuroblastoma cells to high venetoclax concentrations results in MCL-1-mediated resistance

Normally sensitive BCL-2-dependent neuroblastoma cell lines SJNB12 and KCNR were made venetoclax-resistant by continuous exposure to high venetoclax doses equal to the IC_{85} concentrations for both cell lines (2.75 μmol/L for SJNB12 and 7.5 μmol/L for KCNR). After 3 months continuous exposure, SJNB12 and KCNR cell lines under venetoclax pressure divided as fast as the non-resistant cell lines. Resistance of SJNB12 and KCNR to venetoclax was first confirmed by differences in venetoclax dose-response curves (Fig. 1A), with 51 and 191 times higher IC_{50} values and 12 and 22 times higher LC_{50} values for resistant versus non-resistant SJNB12 and KCNR cell lines, respectively (Table 1). In addition, apoptotic responses in resistant cell lines were only observed for SJNB12 upon treatment with micromolar concentrations of venetoclax (i.e. ≥5μmol/L), while for both non-resistant cell lines nanomolar concentrations of venetoclax (i.e. 7.5 nmol/L) were already sufficient to induce a ≥2-fold induction in sub-G_1 (Fig. 1B).

Sequencing of venetoclax-resistant neuroblastoma cell lines showed no mutations in the BH3 domain of BCL-2 (data not shown). Gene expression profiling (Affymetrix U133 plus 2.0 Arrays) of non-resistant KCNR cells and venetoclax-resistant KCNR cells under venetoclax pressure showed significantly increased *MCL-1* gene levels in the resistant

5

cells (Fig. 1C). This was in line with the increased MCL-1 protein levels observed in the resistant KCNR cells under venetoclax pressure (Fig. 1D). Also for SJNB12 MCL-1 protein was found to be higher expressed in the resistant cells under venetoclax pressure that in the non-resistant cells. Although at the gene level no significant changes were observed for the other anti-apoptotic BCL-2 family genes *BCL-2*, *BCL-X$_L$* and *BCL-W* between non-resistant and venetoclax-resistant KCNR cells, BCL-X$_L$ protein levels were higher in the resistant KCNR cells under venetoclax pressure. BCL-2 immunoprecipitation followed by immunoblotting for BIM confirmed (almost) complete displacement of BIM from BCL-2 in resistant SJNB12 and KCNR cells under venetoclax pressure (Fig. 1E). In line with earlier observations for venetoclax treated BCL-2-dependent neuroblastoma cells, analysis of BIM/MCL-1 complex levels showed that released BIM from BCL-2 was sequestrated by MCL-1 in the venetoclax-resistant cell lines. These results confirm that MCL-1 also plays an important role in resistance caused by long-term continuous treatment of BCL-2 dependent neuroblastoma cells with venetoclax.

High-throughput screening identifies idasanutlin as re-sensitizing agent for venetoclax-resistant neuroblastoma cells

As the aim of the current study was to identify compounds that re-sensitize venetoclax-resistant neuroblastoma cells to venetoclax, we first performed a high-throughput drug screen. Non-resistant SJNB12 and KCNR cells, venetoclax-resistant SJNB12 and KCNR cells and venetoclax-resistant SJNB12 and KCNR neuroblastoma cells in the presence of venetoclax were included in the screen. Cells were treated for 72 hours with a chemical library (10 nmol/L, 100 nmol/L and 1 μmol/L) containing 157 approved drugs used in cancer treatment, 43 epigenetic compounds and 9 targeted compounds in (pre-)clinical development for neuroblastoma treatment. Compounds that were more effective in the resistant cell lines under venetoclax pressure than in the non-resistant cell lines were considered re-sensitizing drugs. Hit selection was performed by selecting the 40 top re-sensitizing compounds and subsequently excluding all non-targeted compounds (except for the regularly used cytostatics in neuroblastoma treatment) and targeted compounds for which the percentage viable cells after treatment with the highest tested concentration (i.e. 1 μmol/L) was still ≥20%. This yielded 13 and 11 hits for venetoclax-resistant SJNB12 and KCNR, respectively, of which idasanutlin (MDM2 inhibitor), omipalisib (PI3K/mTOR inhibitor), flavopiridol (CDK inhibitor) and vorinostat (HDAC inhibitor) were overlapping (Fig. 2A and B). For idasanutlin, omipalisib and flavopiridol the increase in efficacy between venetoclax-resistant cells and the non-resistant cells was larger in the presence of venetoclax, confirming the occurrence of re-sensitization

in addition to or instead of overcoming resistance. Of the overlapping hits, idasanutlin was the number 1 re-sensitizing hit for venetoclax-resistant SJNB12 and the number 8 re-sensitizing hit for venetoclax-resistant KCNR. The observation that the non-resistant cell lines were much more sensitive to BCL-2 inhibitors venetoclax and navitoclax than the venetoclax-resistant cell lines (both in the presence and absence of venetoclax), confirmed that SJNB12 and KCNR cell lines exposed to long-term venetoclax treatment were still resistant during screening.
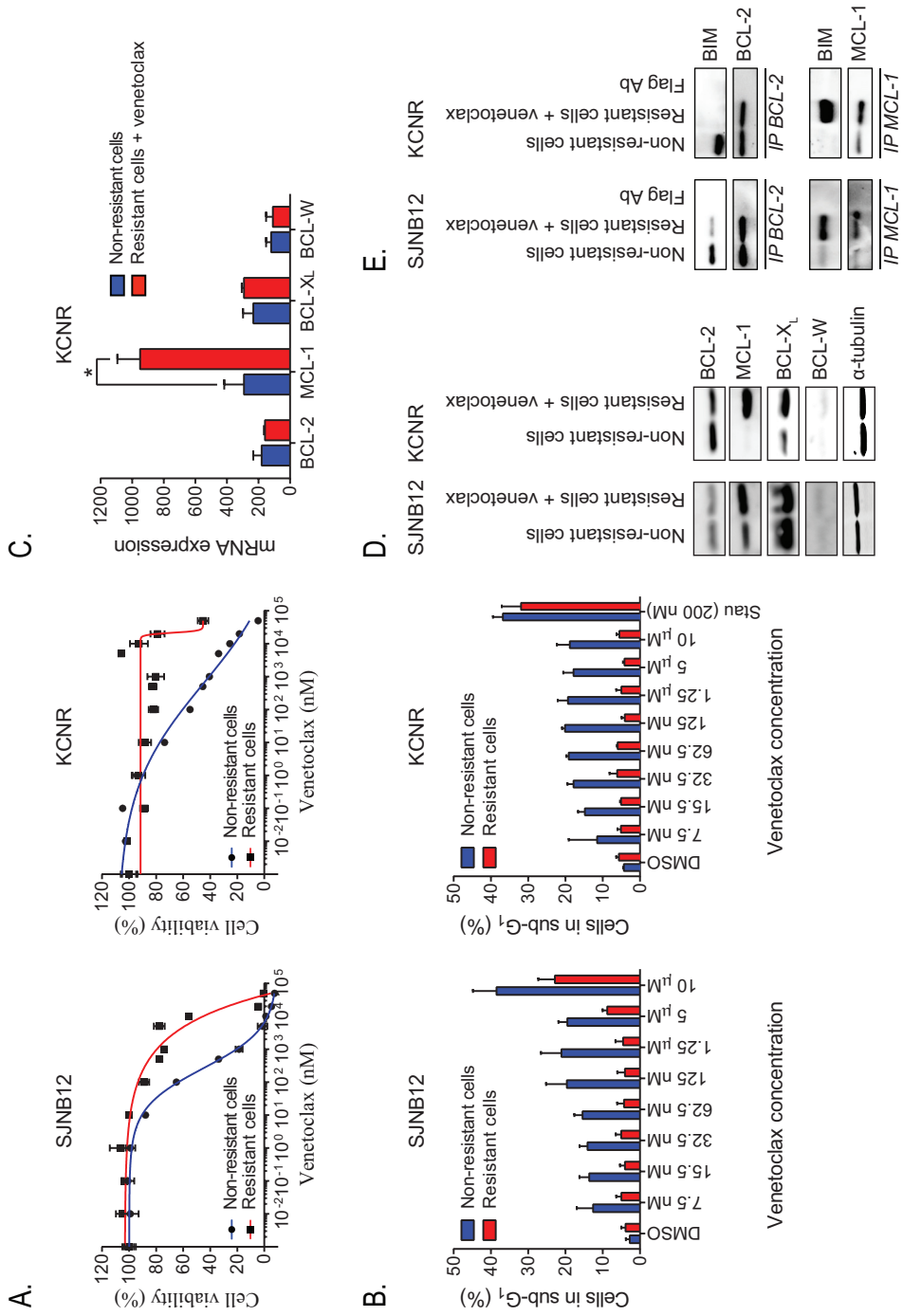
Figure 1. Long-term continuous exposure of BCL-2-dependent neuroblastoma cell lines to high venetoclax concentrations results in MCL-1-mediated venetoclax resistance. A, Dose-response curves of venetoclax for non-resistant (blue) versus venetoclax-resistant (red) SJNB12 and KCNR cells. Resistant neuroblastoma cell lines were generated by long-term continuous exposure of the normally sensitive SJNB12 and KCNR neuroblastoma cells to venetoclax doses equal to the IC_{85} concentrations for both cell lines (i.e. 2.75 μmol/L for SJNB12 and 7.5 μmol/L for KCNR). Venetoclax effects on cell viability were established at 72 hours after treatment using the MTT colorimetric assay. The cell viability of cells treated with DMSO for 72 hours was set to 100%. B, FACS analysis of the *in vitro* effects of venetoclax on sub-G_1 induction in non-resistant (blue) versus venetoclax-resistant (red) SJNB12 and KCNR cells. Effects on sub-G_1 induction were established after 72-hour treatment with increasing venetoclax concentrations. For venetoclax-resistant KCNR cells, staurosporine was included as a positive control. Data represent the mean percentages of cells in sub-G_1 + SD of three replicate experiments. C, Differences in gene expression levels of anti-apoptotic BCL-2 family members between non-resistant KCNR cells (blue) and venetoclax-resistant KCNR cells in the presence of 7.5 μM venetoclax (red). *BCL-2, MCL-1, BCL-X_L, and BCL-W* mRNA levels were established by Affymetrix microarray profiling. Data represent mean expression levels of triplicate samples + SD. Statistical differences between non-resistant and resistant KCNR cells in the presence of venetoclax were calculated using a one-tailed unpaired Student *t* test, with $P<0.05$ as the minimal level of significance (indicated as *). D, Western blot analysis of the protein expression levels of anti-apoptotic proteins BCL-2, MCL-1, BCL-X_L, and BCL-W in non-resistant SJNB12 and KCNR cells versus venetoclax-resistant SJNB12 and KCNR cells under ABT199 pressure (i.e. 2.75 μmol/L for SJNB12 and 7.5 μmol/L for KCNR). α-Tubulin was used as household protein. E, BIM/BCL-2 and BIM/MCL-1 complex levels in non-resistant versus venetoclax-resistant cells in the presence of venetoclax. BIM/BCL-2 and BIM/MCL-1 complex levels were established by anti-BCL-2- an anti-MCL-1 immunoprecipitation, respectively, followed by Western blotting for BIM.

Cell lines	IC_{50} (μmol/L)	LC_{50} (μmol/L)
Non-resistant KCNR cells	0.23	2.50
Venetoclax-resistant KCNR cells	44.00	55.00
Non-resistant SJNB12 cells	0.21	1.00
Venetoclax-resistant SJNB12 cells	10.80	11.70

Table 1. IC_{50} and LC_{50} values of venetoclax in non-resistant and venetoclax-resistant SJNB12 and KCNR neuroblastoma cells.

A.
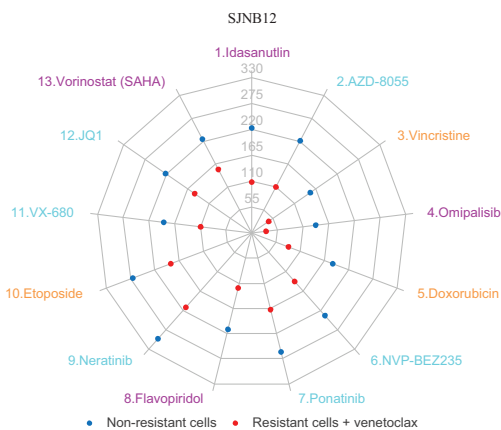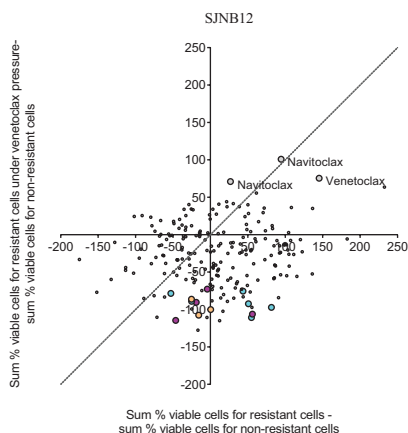
B.
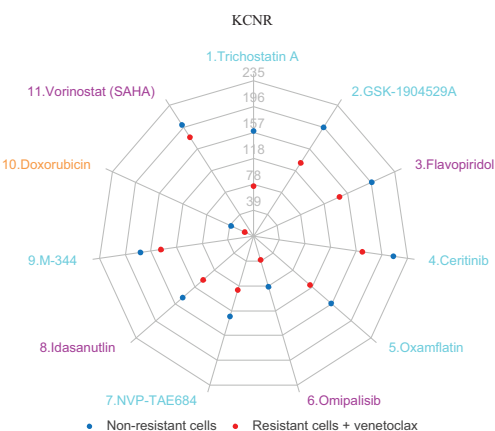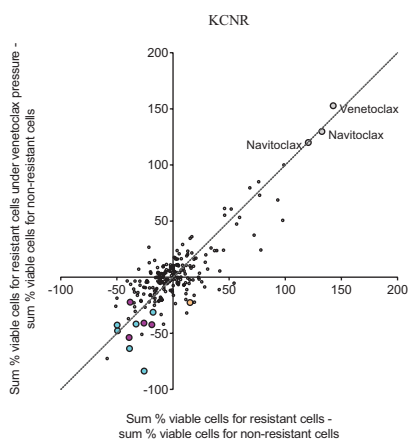
Figure 2. High-throughput screening (HTS) identifies drugs re-sensitizing neuroblastoma cells to venetoclax treatment. Non-resistant SJNB12 and KCNR cells, venetoclax-resistant SJNB12 and KCNR cells and venetoclax-resistant SJNB12 and KCNR cells in the presence of venetoclax (i.e. 2.75 μmol/L for SJNB12 and 7.5 μmol/L for KCNR) were treated with a 209-compound library using concentrations of 10 nmol/L, 100 nmol/L and 1 μmol/L. Effects on cell viability were established at 72 hours after treatment using the MTT colorimetric assay. The cell viability of DMSO-treated cells was set to 100%. For each compound, the sum of the % viable cells observed after treatment with 10 nmol/L, 100 nmol/L and 1 μmol/L was calculated for the non-resistant cells, venetoclax-resistant cells and venetoclax-resistant cells under venetoclax pressure. Compounds for which the sum of the percentages viable cells was lower for the venetoclax-resistant cells under venetoclax pressure than for the non-resistant cells were considered re-sensitizing compounds. Hit selection was performed by taking the 40 top re-sensitizing compounds and subsequently excluding non-targeted compounds (except for the regularly used cytostatics in neuroblastoma treatment) and targeted compounds for which treatment with the highest tested concentration (i.e. 1 μmol/L) resulted in ≥20% viable cells.

Graphs on the left show the difference in the sum of % viable cells between venetoclax-resistant cells and non-resistant cells (X-axis) versus the same difference between venetoclax-resistant cells under venetoclax pressure and non-resistant cells (Y-axis) for SJNB12 (A) and KCNR (B). Each dot represents a single compound and re-sensitizing hits are marked purple (hit for SJNB12 and KCNR), light blue (selective hit for SJNB12 or KCNR) or orange (regularly used cytostatic in neuroblastoma treatment). Radar charts on the right show the sum of % viable cells for the non-resistant cells versus the venetoclax-resistant cells in the presence of venetoclax obtained with the top re-sensitizing hits for SJNB12 (A) and KCNR (B), respectively. Re-sensitizing hits are numbered from most potent to least potent.

In vitro validation confirms re-sensitizing potential of idasanutlin

All re-sensitizing hits from the screen were tested more extensively to validate the screening results. We also included targeted compounds for which synergy with BCL-2 inhibition has been previously described in the literature (20-28) and the regularly used cytostatics in neuroblastoma treatment (Table 2). For all compounds (i.e. 29), IC_{50} values in non-resistant SJNB12 and KCNR cells, in venetoclax-resistant SJNB12 and KCNR cells and in venetoclax-resistant SJNB12 and KCNR cells in the presence of venetoclax were determined from dose-response curves after 72 hours treatment (Supplementary Table S1). Fig. 3A and B show the top 15 of compounds with the largest fold decrease in IC_{50} value between the venetoclax-resistant cells under venetoclax pressure and the non-resistant cells for SJNB12 and KCNR, respectively. Results confirm the re-sensitizing potential of idasanutlin, with an almost 20-fold decrease in IC_{50} value for SJNB12 (i.e. 1011 versus 52 nmol/L) and a 1.5-fold decrease in IC_{50} value for KCNR (i.e. 30 versus 20 nmol/L) (Fig. 3C). For both neuroblastoma cell lines only limited differences were observed in IC_{50} values between the venetoclax-resistant cells in the absence of venetoclax and the non-resistant cells. This supports that idasanutlin re-sensitizes venetoclax-resistant neuroblastoma cells to venetoclax, rather than overcomes venetoclax resistance. In line with the screening results, omipalisib and vorinostat were also found in the top 15 list of most potent re-sensitizing drugs for venetoclax-resistant SJNB12 and KCNR neuroblastoma cells.

A.
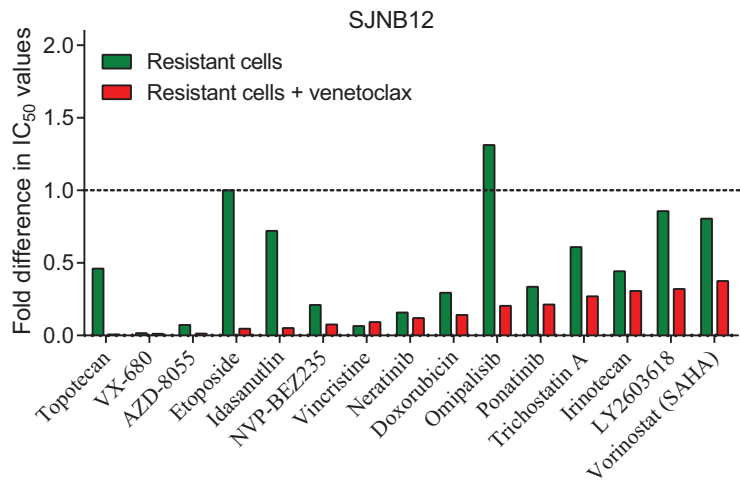
B.
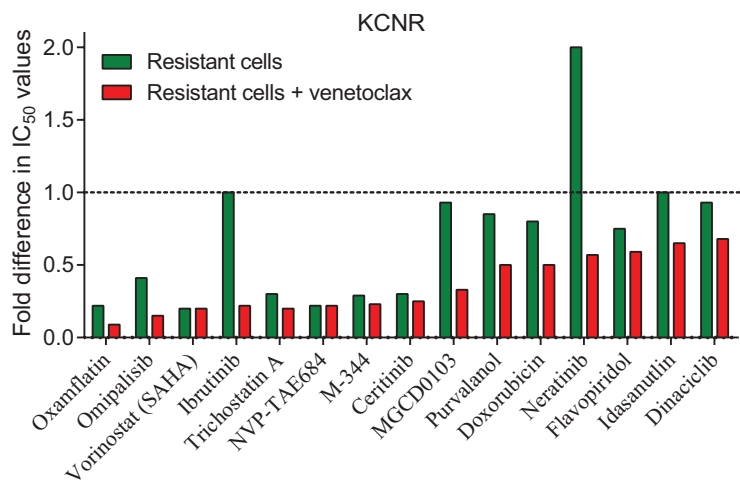
C.
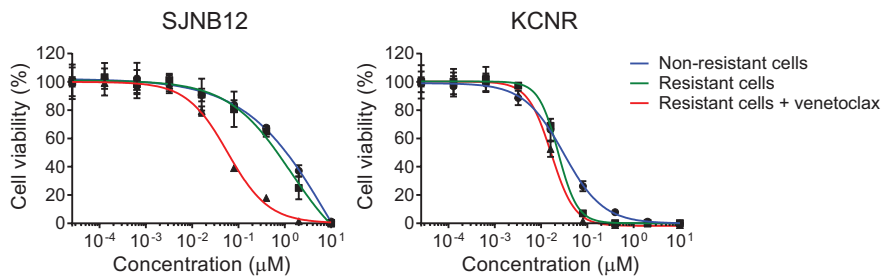
Figure 3. *In vitro* validation studies confirm re-sensitization of venetoclax-resistant neuroblastoma cells to venetoclax by idasanutlin treatment. For combined hits from the HTSs as well as targeted compounds for which synergy with BCL-2 inhibition has been described in literature and the regularly used cytostatics in neuroblastoma treatment, dose-response curves were established in non-resistant SJNB12 and KCNR cells, venetoclax-resistant SJNB12 and KCNR cells and venetoclax-resistant SJNB12 and KCNR cells under venetoclax pressure to establish IC_{50} values. A and B, Fold difference in IC_{50} values between venetoclax-resistant cells and non-resistant cells (green bars) and venetoclax-resistant cells under venetoclax pressure and non-resistant cells (red bars). Results are shown for the 15 top compounds inducing the largest difference in sensitivity between venetoclax-resistant cells under venetoclax pressure and non-resistant cells for SJNB12 (A) and KCNR (B), respectively. C, Dose-response curves of MDM2 inhibitor idasanutlin in non-resistant SJNB12 and KCNR cells (blue lines), venetoclax-resistant SJNB12 and KCNR cells (green lines) and venetoclax-resistant SJNB12 and KCNR cells in the presence of venetoclax (red lines).

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Compound (Target)	Hit HTS for KCNR	Hit HTS for SJNB12	Potential hits from literature	Regularly used cytostatic in neuroblastoma treatment
AZD-8055 (mTORC1/2)		X		
Ceritinib (ALK)	X			
Cisplatin (DNA synthesis)				X
Dinaciclib (CDK1/2/5/9)			X	
Doxorubicin (DNA topoisomerase II)	X	X	X	X
Etoposide (DNA topoisomerase II)		X		X
Flavopiridol (CDK1/2/4/6/7)	X	X		
GS1101 (PI3Kδ)			X	
GSK-1904529A (IGFR)	X			
Ibrutinib (BTK)			X	
Idasanutlin (MDM2)	X	X	X	
Irinotecan (DNA topoisomerase II)				X
JQ1 (BRD4 (1/2))		X	X	
LY2603618 (CHK1)			X	

Table 2. Compounds selected for more extensive *in vitro* testing of their effects on re-sensitization of venetoclax-resistant neuroblastoma cells to venetoclax. In addition to the re-sensitizing hits identified in the HTSs, we investigated the re-sensitizing effects of targeted compounds for which synergy with BCL-2 inhibition has been described in the literature as well as of the regularly used cytostatics in neuroblastoma treatment. Hit selection from the high-throughput screens (HTS) was performed by establishing the 40 top compounds more effective in the venetoclax-resistant cells under venetoclax pressure than in the non-resistant cells and subsequently excluding all non-targeted compounds (except for the regularly used cytostatics in neuroblastoma treatment) as well as targeted compounds for which the percentage viable cells after treatment with the highest tested concentration (i.e. 1 μmol/L) was still ≥20%.

Compound (Target)	Hit HTS for KCNR	Hit HTS for SJNB12	Potential hits from literature	Regularly used cytostatic in neuroblastoma treatment
M-344 (HDAC)	X			
MGCD0103 (HDAC)			X	
Neratinib (HER2, EGFR)		X		
NVP-BEZ235 (PI3Kα/β/δ/γ, mTORC1/2)		X	X	
NVP-TAE684 (ALK)	X			
Omipalisib (PI3Kα/β/δ/γ, mTORC1/2)	X	X	X	
Oxamflatin (HDAC)	X			
Ponatinib (Abl, PDGFRα, VEGFR2, FGFR1, Src)		X		
Purvalanol (CDK1/2/4/5)			X	
Temozolomide (DNA alkylation)				X
Topotecan (DNA topoisomerase l)				X
Trichostatin A (HDAC)	X			
Vincristine (Microtubule polymerization)		X		X
Vorinostat (SAHA) (HDAC)	X	X	X	
VX-680 (Aurora kinase A/B/C)		X	X	

Table 2. (continued)

Idasanutlin induces growth arrest in non-resistant cells and apoptosis in venetoclax-resistant cells in the presence of venetoclax

As idasanutlin acts by inhibiting the interaction between MDM2 and the tumor suppressor p53 - thereby preventing p53 degradation by the proteasome (29, 30) we first studied idasanutlin effects on p53 and MDM2 protein levels. For SJNB12 and KCNR, idasanutlin dose-dependently increased p53 and MDM2 in the non-resistant cells as well as the venetoclax-resistant cells both in the absence and presence of venetoclax (Fig. 4A). Increased MDM2 levels could be explained by p53-mediated transcriptional activation of MDM2. Because p53 is involved in cell cycle arrest and apoptosis, we also studied idasanutlin effects on the bonafide p53 target genes p21 and BAX (31-33). Interestingly, in the non-resistant cells and venetoclax-resistant cells in the absence of venetoclax a stronger p21 upregulation was observed, while in the venetoclax-resistant SJNB12 and KCNR cells in the presence of venetoclax a stronger BAX upregulation was observed upon idasanutlin treatment. These findings correspond to a higher induction of apoptosis in the venetoclax-resistant lines under venetoclax pressure as marked by the induction in PARP cleavage and a higher sub-G_1 fraction on DNA content analysis using cytometry (Fig. 4B and Supplementary Fig. S1). These results show that idasanutlin re-sensitizes venetoclax-resistant BCL-2-dependent neuroblastoma cells to undergo apoptosis.

A.
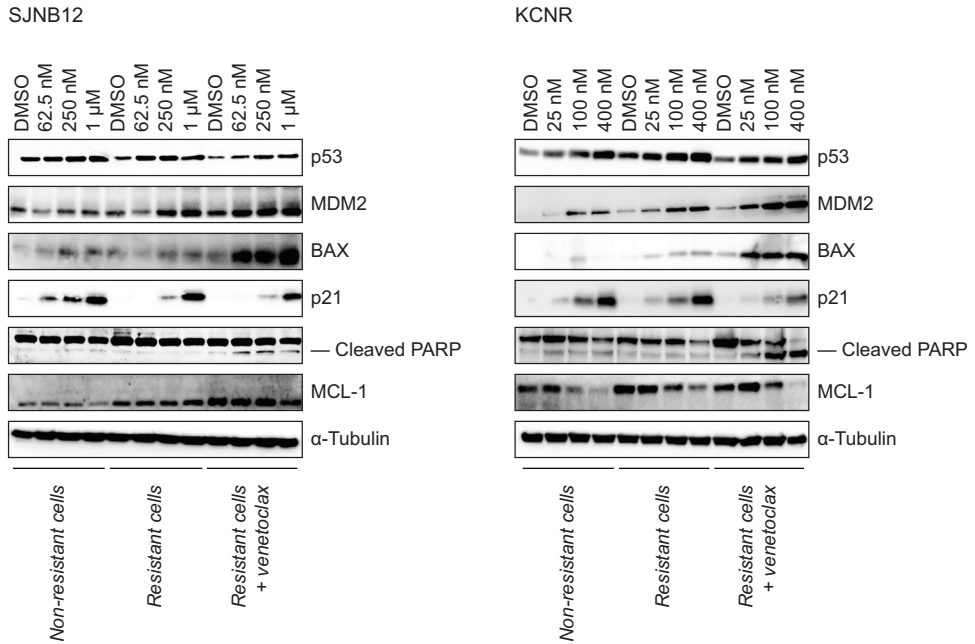
B.
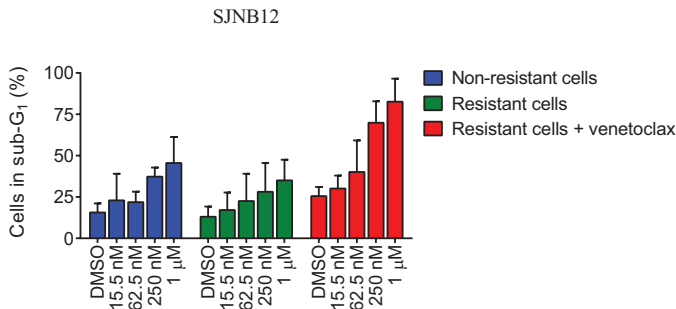
Figure 4. Idasanutlin treatment of venetoclax-resistant neuroblastoma cells in the presence of venetoclax causes BAX-mediated apoptosis. A, Western blot analysis of the *in vitro* effects of idasanutlin on p53, MDM2, BAX, p21, cleaved PARP and MCL-1 protein levels in non-resistant SJNB12 (left) and KCNR (right) cells versus venetoclax-resistant SJNB12 and KCNR cells and venetoclax-resistant SJNB12 and KCNR cells under venetoclax pressure after 72 hours treatment. α-Tubulin was used as a loading control. B, FACS analysis of the *in vitro* effects of idasanutlin on sub-G_1 induction in non-resistant SJNB12 cells (blue) versus venetoclax-resistant SJNB12 cells (green) and venetoclax-resistant SJNB12 cells in the presence of venetoclax (red). Effects on sub-G_1 induction were established after 72-hour treatment with increasing idasanutlin concentrations. Data represent the mean percentages of cells in sub-G_1 + SD of three replicate experiments.

Combination of venetoclax with idasanutlin shows superior efficacy in a BCL2 high-expressing xenograft model.

The efficacy of combining venetoclax with MDM2 inhibitor idasanutlin was subsequently studied *in vivo* in mice with BCL-2-dependent KCNR neuroblastoma xenografts. Direct combination treatment with venetoclax and idasanutlin for three consecutive weeks resulted in significant tumor regression (i.e. average tumor size reduction of 80±21%) and superior efficacy over single-agent treatments. As expected, tumor reduction was also observed in the delayed combination group, though less significant compared to the group receiving the combination from the start of treatment (i.e. average tumor size reduction of 53±39%) (Fig. 5A). The improved tumor regression observed after combination treatment with venetoclax and idasanutlin might be the result of a dual effect on the intrinsic apoptotic pathway: i.e. BIM release from BCL-2 caused by venetoclax and p53-mediated activation of the pro-apoptotic BCL-2 family protein BAX (34). While BIM was still complexed to BCL-2 in the control group, co-immunoprecipitation studies at 4 hours after administration of the last dose(s) showed (almost) complete BIM release in the venetoclax-only treated group and the group directly receiving the combination. Surprisingly, residual levels of BIM bound to BCL2 were observed in the delayed combination group. Additional Western blot analysis revealed upregulation of BAX in both combination groups as well as in the idasanutlin-only treated group (Fig 5B and C). Together, these results indicate that the significant tumor regression observed upon combination treatment with venetoclax and idasanutlin might be the result of simultaneous inhibition of BCL-2 and activation of p53-mediated induction of BAX.
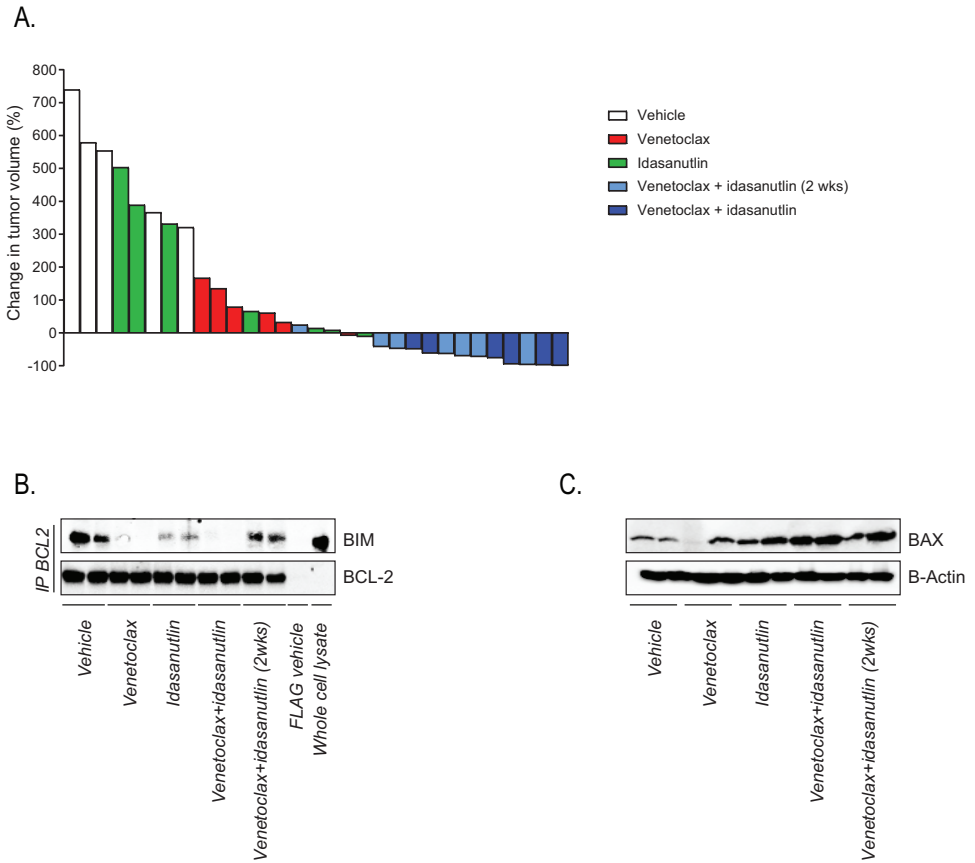
Figure 5. Combination of venetoclax with idasanutlin results in superior anticancer activity mice with BCL2 high-expressing neuroblastoma xenografts. A, Inhibitory effects of combination treatment with venetoclax and idasanutlin versus single-agent treatments on the growth of KCNR neuroblastoma xenografts. Percentages change in tumor volume at the end of treatment were calculated using the following formula: ((tumor volume at the end of treatment   tumor volume at the start of treatment) / tumor volume at the start of treatment) x 100%. Mice were daily treated with: (1) vehicles venetoclax and idasanutlin, (2) 100 mg/kg/d venetoclax, (3) 25 mg/kg/d idasanutlin, (4) one week monotherapy with 100 mg/kg/d venetoclax followed by combination treatment with 25 mg/kg/d idasanutlin or (5) 100 mg/kg/d venetoclax plus 25 mg/kg/d idasanutlin. All mice were treated for a total of three consecutive weeks. B, *In vivo* effects on BIM displacement from BCL-2. Effects were studied by detecting BIM/BCL-2 complex levels by anti-BCL-2 immunoprecipitation, followed by Western blotting for BIM. Levels of BIM/BCL-2 complex were established for n=2 mice per group at 4 hours after administration of the last drug dose(s). BCL-2 levels served as loading control. C, *in vivo* effects on BAX protein levels. Effects were studied by Western blot analysis for n=2 mice per group at 4 hours after administration of the last drug dose(s). β-actin served as a loading control.

DISCUSSION

Previous preclinical studies using the BCL-2-specific inhibitor venetoclax showed superior anti-tumor activity in neuroblastoma tumors with high BCL-2 and BIM/BCL-2 complex levels (17, 18). Eventually resistance occurred as is seen with most targeted compounds with high specificity.

Several mechanisms for resistance to BCL-2 inhibitors have been described. Acquired resistance after long-term exposure of lymphoma cells to venetoclax resulted in clonal selection of cells with mutations in the BH3 domain of BCL-2, leading to decreased affinity for venetoclax (35). However, sequencing of venetoclax-resistant neuroblastoma cell lines showed no mutations in the BCL-2 BH3 domain. Alternatively, resistance to venetoclax can occur via upregulation of the anti-apoptotic BCL-2 family protein MCL-1(17, 21, 36). We indeed observed a striking upregulation of MCL-1 in venetoclax-resistant neuroblastoma cells. Upregulated MCL-1 sequestered BIM displaced from BCL-2 by venetoclax, thereby preventing apoptosis from occurring in the resistant neuroblastoma cells.

The observation that MCL-1 upregulation is involved in preventing apoptosis in venetoclax-resistant cells, suggests the possibility to combine selective MCL-1 inhibitors with selective BCL-2 inhibitors. However, MCL-1 inhibitors are still in the early phases of development. In addition, the expected toxicity when combining two compounds with myelo-suppressive side effects might be hampering clinical development. We thus sought to identify compounds that can re-sensitize venetoclax-resistant neuroblastoma cells. Because we aimed at clinical implementation we performed a compound-wide screen with targeted compounds in clinical development and cytostatics used in the treatment of neuroblastoma patients. Targeted compounds idasanutlin, omipalisib, flavopridol and vorinostat and the cytoststatic doxorubicin demonstrated superior anti-cell viability activity in venetoclax-resistant BCL-2 high expressing cell lines compared to their respective mother lines. These results confirm previous reported synergistic combinations (21-28). DNA damaging agents as doxorubicin have shown to give strong synergistic responses with venetoclax and navitoclax (16, 37). The combination with PI3K inhibitors, like omipalisib, was found in several tumor types to potentiate BCL-2 inhibition through downregulation of MCL-1 (21, 38). Finally, AURKA inhibitors were identified in neuroblastoma to combine efficiently with venetoclax (36), which we only confirmed in the SJNB12 cell line.

The synergistic combination of venetoclax with p53 reactivating, nutlin-like compounds was only reported for hematologic malignancies. We now show that, from an unbiased screening approach, idasanutlin selects as the strongest re-sensitizer for venetoclax in venetoclax-resistant neuroblastoma cells. This re-sensitization is mediated through re-activation of p53, which results in upregulation of the p53 target gene BAX. BAX then oligomerizes and forms pores in the outer membrane of the mitochondria, thereby causing cytochrome c release and apoptosis. Upregulation of BAX was only seen in venetoclax-resistant neuroblastoma cells in the presence of venetoclax and idasanutlin. This suggests that p53-mediated BAX activation by idasanutlin and BCL2 inhibition by venetoclax might result in a potent apoptotic phenotype in the venetoclax-resistant cells, which was observed in these cells. In venetoclax-resistant cells without venetoclax pressure, p53 release from MDM2 does not activate BAX but increases the expression of the p53 target gene p21. p21 is known to mediate cell cycle arrest, which we indeed observed in these cells.

In vivo studies confirmed the beneficial effects of combining BCL-2 inhibitor venetoclax with MDM2 inhibitor idasanutlin, as was shown by the remarkably improved regression of BCL-2-dependent neuroblastoma tumors. Unexpectedly, improved combined effects were more pronounced after immediate combination compared to combination after short term monotherapy with venetoclax. This could be because of the higher residual levels of BIM bound to BCL-2 observed in the delayed combination group. The underlying cause of the higher residual BIM/BCL-2 complex levels after delayed combination of venetoclax with idasanutlin is unclear. For future experiments it would be interesting to study if complete tumor regression can be achieved when directly combining venetoclax with higher doses of idasanutlin. The complete or almost complete release of BIM from BCL-2 observed after direct combination of venetoclax with idasanutlin suggests that there is no much rational to use higher venetoclax doses.

Our results indicate idasanutlin as a potential compound for the re-sensitization of venetoclax-resistant neuroblastoma cells to venetoclax. We predict that the clinical testing of single compound venetoclax in children with neuroblastoma will induce tumor responses. Various pre-clinical studies have confirmed that neuroblastoma tumors have an expression profile that is related to high efficacy of this compound (17, 18). However, we also predict that resistance will occur in response to venetoclax treatment and therefore combination treatments should be anticipated. Our work and that of others suggest that a combination with DNA damaging agents would potentiate the efficacy of BCL-2 inhibition. A backbone therapy including cyclophosphamide or topoisomerase inhibitors would be an option for early clinical trials. Our current findings also suggest that a clinical combination of venetoclax with idasanutlin might prevent

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resistance to the BCL-2 inhibitor. Preclinical studies using this combination in adults is currently ongoing (ClinicalTrials.gov Identifier: NCT02670044) and a similar combination should be further explored in neuroblastoma.

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SUPPLEMENTARY INFORMATION
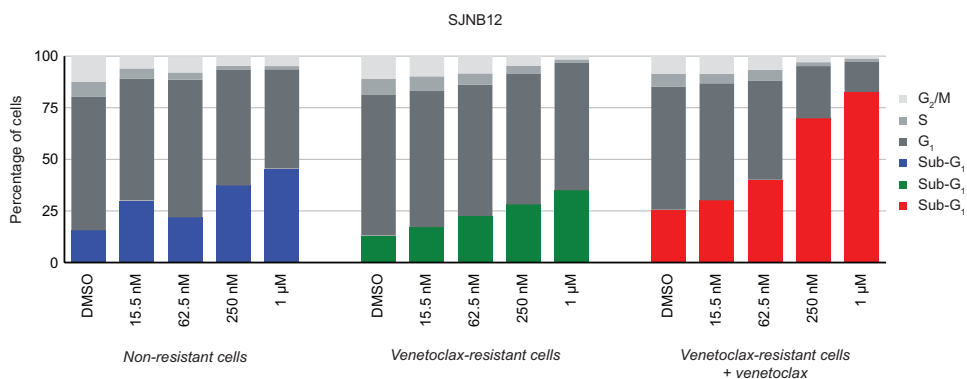
Supplementary Figure 1. Idasanutlin re-sensitizes venetoclax-resistant neuroblastoma cells to venetoclax by inducing apoptosis. FACS analysis of the *in vitro* effects of idasanutlin on the fraction of cells in G_2/M, S, G_1 and sub-G_1 in non-resistant SJNB12 cells (blue/gray) versus venetoclax-resistant SJNB12 cells (green/gray) and venetoclax-resistant SJNB12 cells in the presence of venetoclax (red/gray). Effects were established after 72-hour treatment with increasing idasanutlin concentrations. Data represent the mean percentages of cells in G_2/M, S, G_1 and sub-G_1 of three replicate experiments.

SUPPLEMENTARY TABLES

Compound (Target)	Non-resistant (nmol/L)	Resistant (nmol/L)	Resistant + venetoclax (nmol/L)	Non-resistant (nmol/L)	Resistant (nmol/L)	Resistant + venetoclax (nmol/L)
	SJNB12			KCNR		
AZD-8055 (mTORC1/2)	>10000	729	134	89	140	92
Ceritinib (ALK)	2655	2180	1041	94	28	23
Cisplatin (DNA synthesis)	>10000	>10000	5903	966	1772	1270
Dinaciclib (CDK1/2/5/9)	11	28	14	8	8	6
Doxorubicin (DNA topoisomerase II)	>10000	2940	1410	31	26	16
Etoposide (DNA topoisomerase II)	>10000	>10000	473	363	716	261
Flavopiridol (CDK1/2/4/6/7)	237	151	109	188	141	111
GS1101 (PI3Kδ)	>10000	>10000	8821	>10000	>10000	>10000
GSK-1904529A (IGFR)	>10000	>10000	>10000	>10000	>10000	>10000
Ibrutinib (BTK)	6230	>10000	4735	>10000	>10000	2152
Idasanutlin (MDM2)	1011	729	52	30	30	20
Irinotecan (DNA topoisomerase II)	>10000	4430	3068	1207	1660	1161
JQ1 (BRD4 (1/2))	374	418	176	291	884	770

Supplementary Table 1. IC_{50} values for non-resistant SJNB12 and KCNR neuroblastoma cells, venetoclax-resistant SJNB12 and KCNR cells and venetoclax-resistant SJNB12 and KCNR cells in the presence of venetoclax (i.e. 2.75 µmol/L for SJNB12 and 7.5 µmol/L for KCNR). Compounds tested include re-sensitizing hits identified in the high-throughput drug screens, targeted compounds for which synergy with BCL-2 inhibition has been previously described in the literature and regularly used cytostatics in neuroblastoma treatment.

Compound (Target)	Non-resistant (nmol/L)	Resistant (nmol/L)	Resistant + venetoclax (nmol/L)	Non-resistant (nmol/L)	Resistant (nmol/L)	Resistant + venetoclax (nmol/L)
	SJNB12			KCNR		
LY2603618 (CHK1)	>10000	8567	3203	808	956	959
M-344 (HDAC)	312	269	140	175	50	40
MGCD0103 (HDAC)	201	259	152	448	418	149
Neratinib (HER2, EGFR)	1772	281	212	1745	3495	998
NVP-BEZ235 (PI3Kα/β/δ/γ, mTORC1/2)	618	130	47	41	320	53
NVP-TAE684 (ALK)	288	259	176	27	6	6
Omipalisib (PI3Kα/β/δ/γ, mTORC1/2)	43	56	9	13	5	2
Oxamflatin (HDAC)	594	612	258	215	47	20
Ponatinib (Abl, PDGFRα, VEGFR2, FGFR1, Src)	600	201	128	516	1700	1699
Purvalanol (CDK1/2/4/5)	>10000	9231	3921	3828	3235	1933
Temozolomide (DNA alkylation)	>10000	>10000	>10000	>10000	>10000	>10000
Topotecan (DNA topoisomerase I)	6850	3160	42	32	32	29
Trichostatin A (HDAC)	497	303	134	122	32	27
Vincristine (Microtubule polymerization)	5	0.3	0.5	0.001	0.005	0.002
Vorinostat (SAHA) (HDAC)	697	561	262	490	117	94
VX-680 (Aurora kinase A/B/C)	3023	50	36	14	38	22

Supplementary Table 1. (continued)

SUPPLEMENTARY MATERIALS AND METHODS

Cell culture. Human neuroblastoma cell lines used in the current study were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L D-glucose, glutamate and supplemented with 10% (v/v) foetal calf serum, 2 mmol/L L-glutamine, 10 U/mL penicillin, 10 μg/mL streptomycin and MEM non-essential amino acids (1x). Cells were maintained at 37°C under 5% CO_2 in humidified air. Penicillin and streptomycin were obtained from Sigma Aldrich, Other cell culture related materials were obtained from Life Technologies.

FACS analysis. Non-resistant SJNB12 and KCNR cells, venetoclax-resistant SJNB12 and KCNR cells and/or venetoclax-resistant SJNB12 and KCNR cells in the presence of venetoclax (i.e. 2.75 μmol/L for SJNB12 and 7.5 μmol/L for KCNR) were seeded onto 6-cm plates and incubated overnight. To confirm the generation of venetoclax-resistant SJNB12 and KCNR cells, non-resistant and venetoclax-resistant cells were treated with 0.1% DMSO (control) or venetoclax using concentration ranges of 7.8 nmol/L to 10 μmol/L. To study the effects of idasanutlin on sub-G_1 induction, non-resistant SJNB12 and KCNR cells, venetoclax-resistant SJNB12 and KCNR cells and venetoclax-resistant SJNB12 and KCNR cells under venetoclax pressure were treated with 0.02% DMSO (control) or 25 nmol/L to 1 μmol/L RG7388. After 72-hour treatment, supernatants containing floating cells were collected from the culture dishes. Adherent cells were washed once with PBS and PBS solutions were pooled with the supernatants. After trypsinization of the adherent cells with 0.05% trypsin/EDTA, cells were resuspended in the pooled supernatant/PBS solution. Next, cells were centrifuged (5 min; 1,500 rpm), washed by resuspension in PBS and centrifuged again (5 min; 3,000 rpm). Cells were fixed with 100% ice-cold ethanol, stained with 0.05 mg/mL propidium iodide and supplemented with 0.05 mg/mL RNAse A in PBS. After 1-hour incubation in the dark at room temperature (RT), cells were filtered through a 50 μm filter (BD Biosciences) and DNA contents of the nuclei were analyzed using a fluorescence-activated cell sorter. A total of 30,000 nuclei per sample were counted. The cell cycle distribution and apoptotic Sub-G_1 fraction were determined using the BD Accuri™ C6 flow cytometer with the CFlow plus software (BD Biosciences) and the FlowJo LLC software (FlowJo LLC).

Western blotting. Non-resistant SJNB12 and KCNR cells, venetoclax-resistant SJNB12 and KCNR cells and/or venetoclax-resistant SJNB12 and KCNR cells in the presence of venetoclax (i.e. 2.75 μmol/L for SJNB12 and 7.5 μmol/L for KCNR) were seeded onto 6-cm plates and incubated overnight. To study the effects of idasanutlin on p53, MDM2,

BAX, p21, cleaved PARP and MCL-1, non-resistant cells, venetoclax-resistant cells and venetoclax-resistant cells in the presence of venetoclax were 72-hour treated with 0.02% DMSO (control) or up to 1 μmol/L idasanutlin. For all experiments, cells were lysed using Laemmli buffer [i.e., H_2O/glycerol/20% sodium dodecyl sulfate (SDS)/1 M Tris-HCl (pH 6.8) 5:2:2:1 (v/v/v/v)]. Lysates were homogenized by hydrodynamic shearing through a 23 G needle, followed by 10 min incubation at 50°C. Protein concentrations were determined using the Bio-Rad *DC* Protein Assay (Bio-Rad, Veenendaal, the Netherlands).

Equal protein amounts (20 μg) were diluted in 5x reducing sampling buffer (i.e., Laemmli buffer/β-mercaptoethanol 3:1 (v/v) with bromophenol blue sodium salt). Diluted samples were boiled for 5 min at 95°C and centrifuged (1,500 rpm 5 min). Proteins were separated by SDS-polyacrylamide gel electrophoresis on 12% Mini-Protean® Tris-glycince extended (TGX) precast gels (Bio-Rad) and transferred on hybond nitrocellulose membranes (0.45 μm) by 1.5-hour wet blotting (200 mA; 4°C). Transfer buffer consisted of 20% (v/v) methanol, 3.025 g/L Tris and 14.4 g/L glycine in demineralized water. Membranes were blocked in 2% ECL Prime™ blocking agent (GE Healthcare) in PBS with 0.1% (v/v) Tween-20 (= blocking buffer) for 1 hour at RT. After blocking, membranes were incubated with the primary (overnight; 4°C) and secondary (1 hour; RT) antibodies in blocking buffer and scanned using the ChemiDoc™ Touch Imaging detection system (Bio-Rad).

CHAPTER 6

A SYSTEMATIC REVIEW ON TARGETING BCL2 IN PEDIATRIC SOLID TUMORS.

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ABSTRACT

The aim of this study was to perform a systematic review on the pre-clinical evidence of targeting BCL2 in pediatric solid tumors. An elaborative literature search identified 3189 hits from which 74 papers were finally selected for further analysis. Papers included in this study were selected based on data on BCL2 expression patterns, *in vitro* and *in vivo* validation of the relevance of targeting BCL2, predictive biomarkers for patient stratification and efficacy of BCL2 inhibition, strategies to overcome resistance to BCL2 inhibition and clinical trials involving small molecule BCL2 inhibitors for pediatric solid tumor treatment. BCL2 protein and mRNA expression as well as BIM/BCL2 complex levels were identified as relevant predictive biomarkers for sensitivity to small molecule BCL2 inhibitors in only a few pediatric solid tumor types. BCL2 was adequately reported and highly expressed in neuroblastoma, synovial sarcoma, Ewing sarcoma, osteosarcoma and retinoblastoma and correlated with a poor survival outcome in several of these tumor types. An absence of the protein was reported in rhabdoid and inflammatory myofibroblastic tumors. ABT-199 (venetoclax) and ABT-263 (navitoclax) were the two most widely pre-clinically evaluated small molecule BCL2 inhibitors reported and were most effective in neuroblastoma model systems. The most frequently reported mechanism of resistance to BCL2 inhibitors was via MCL1 upregulation and its sequestration of BIM, preventing further apoptosis from occurring. Consequently, the primarily used strategy to overcome resistance to small molecule BCL2 inhibitors was by concomitant inhibition of MCL1.

Taken together, the current systematic review provides a resource for summarized knowledge on targeting BCL2 in pediatric solid tumors. Neuroblastoma was the pediatric solid tumor with the most validated data categories. This warrants future trials with BCL2 inhibitors to assess predictive responses in neuroblastoma patients and to investigate the clinical implementation of combination strategies to overcome resistance. Target presence was also found for limited other pediatric tumor types, but further research is essential to determine the relevance of targeting BCL2 in these tumor types (especially for synovial sarcoma, Ewing sarcoma, osteosarcoma and retinoblastoma).

INTRODUCTION

The B-cell lymphoma 2 (BCL2) protein is highly expressed in numerous adult and pediatric tumor types (1). The anti-apoptotic BCL2 family proteins BCL2, BCL-X$_L$ (BCL2L1), MCL1, BCL-W (BCL2L2) and A1 (BCL2A1) together with its pro-apoptotic counterparts BIM (BCL2L11), BID (FP497), BAX (BCL2L4), BAK (BAK1), PUMA (BBC3) and NOXA (PMAIP1) are key players in the activation of the intrinsic apoptotic pathway (2). During cellular stress or redrawal of growth factors, activation of the intrinsic pathway is initiated upon BIM displacement from BCL2. Displaced BIM triggers the oligomerization of BAX/BAK, leading to pore formation at the outer membrane of the mitochondria. This then results into cytochrome c release from the interstitial space of the mitochondria into the cytoplasm. Cytochrome c together with apoptotic protease-activating factor I (APAF1) forms the apoptosome. The apoptosome is responsible for the recruitment and cleavage of effector caspases 3 and 9, thereby triggering apoptosis. (3). Several small molecule inhibitors targeting BCL2 have been developed and pre-clinically tested in various pediatric malignancies. ABT-263 (navitoclax) is one of the first line pan-BCL2 family of proteins inhibitors and was shown to induce a strong apoptotic phenotype at the *in vitro* level in various tumors types (4, 5). At the *in vivo* level, potent regression of tumors expressing high BCL2 levels was observed upon navitoclax treatment, demonstrating the great promise of this compound for the treatment of BCL2-dependent malignancies (3, 6-10). However, the clinical use of navitoclax was associated with dose-limiting thrombocytopenia due to the concomitant inhibition of the anti-apoptotic protein BCL-X$_L$ (11, 12). Given the toxicity exhibited by patients treated with navitoclax at the higher doses, the BCL2-specific inhibitor ABT-199 (venetoclax) was developed and pre-clinically tested in various malignancies (13, 14). A phase I dose-escalation study of venetoclax in patients with relapsed or refractory chronic lymphocytic leukemia showed a 79% response rate, paving the way for the implementation of venetoclax in the future treatment of children with BCL2-dependent solid tumors (15). The current systematic review aims to establish an evidence-based perspective on the relevance of targeting BCL2 in pediatric solid tumors. This should facilitate the prioritization of children with solid tumors that are most likely to benefit from clinical trials with small molecule BCL2 inhibitors. Additionally, we included available knowledge related to molecular mechanisms promoting resistance to BCL2 inhibitors and combination strategies to overcome resistance. This additional information can be used to design drug combination trials for pediatric cancer patients with acquired resistance to BCL2 inhibitors.

6

MATERIALS AND METHODS

Guidelines for this systematic review were based on the NHS Centre for Reviews and Dissemination (1996) (http://www.york.ac.uk/inst/crd) and the Cochrane method for systematic reviews (http://methodology.cochrane.org/).

Search strategies

The online bibliographic database PubMed was chosen to extract literature (published till 18th April 2016) relevant to the subject matter of the research. Search procedures were performed using an integrative approach, using the same nine important key words for each search (**Table 1**). Specific disease names were used as the last key word. The key words "BCL2" or "BCL-2" referred to the various abbreviations of the B-cell lymphoma protein 2 used in literature. Search terms "ABT199" or "ABT-199" and "ABT263" or "ABT-263" referred to the names given to the small molecule BCL2 inhibitors for research purposes, while "venetoclax" or "navitoclax" or "obatoclax" referred to their generic names. Investigators read the abstracts of each paper and classified the papers into 3 different categories: relevant, not relevant and uncertain. For the final inclusion criteria of papers, abstracts of all papers in the uncertain category were re-read by the investigator to make a final decision on whether to include these papers.

Inclusion criteria

In order to be included in the systematic review, the paper had to contain information about at least one of the following topics: (1) BCL2 expression patterns in primary tumors (the outcome of the expression analysis should be shown in table form and ≥40% of the study subjects should be children) and target dependency *in vitro* (BCL2 dependency should be validated *in vitro* in at least five cell lines by investigating phenotypic changes upon BCL2 knockdown), (2) target dependency *in vivo*, (3) *in vitro* efficacy of small molecule BCL2 inhibitors in at least one BCL2 high-expressing cell lines, (4) *in vivo* compound efficacy in xenograft mouse models, (5) predictive biomarkers for patient stratification and efficacy of BCL2 inhibition, (6) molecular mechanisms causing resistance to BCL2 inhibition, (7) combination strategies to improve the efficacy of BCL2 inhibitors and/or (8) phase I-III clinical trials with small molecule inhibitors targeting the BCL2 pathway in children (**Supplementary Table 1**).

Exclusion criteria

Papers were excluded when: (1) There was no evidence of *in vitro* experimental data reported in cell lines, (2) BCL2 and its small molecule inhibitors were cited without experimental data, (3) BCL2 was used as a biomarker for inhibition of apoptosis and/or (4) BCL2 expression levels in primary tumors have only been analyzed by histological staining without quantification or tabulated patient data.

Extracted information

From the included papers, the following information was extracted per pediatric tumor type: (1) the frequency of BCL2 overexpression and the correlation with survival data, (2) *in vitro* and *in vivo* responses to small molecule BCL2 inhibitors, (3) if biomarkers for sensitivity to and/or efficacy of small molecule BCL2 inhibitors were identified, (4) patient responses to BCL2 inhibitors in clinical trials, (5) genes and pathways mediating resistance to BCL2 inhibition and (6) *in vitro* and *in vivo* data on chemotherapeutics and targeted small molecule inhibitors that were successfully combined with small molecule BCL2 inhibitors. For each of the papers, color and number codes were given in order to score the papers in terms of reliability of the experimental information and the experimental setups. Color codes: red (negative experimental outcome), green (very positive experimental outcome) or yellow (inconsistent results). Number codes: 1 (excellent methodology and experimental setup), 2 (moderate experimental setup) or 3 (poor methodology and experimental setup).

RESULTS

Literature search results

This systematic review was performed in order to determine the clinical relevance of inhibiting BCL2 in pediatric solid tumor types, i.e. neuroblastoma, rhabdomyosarcoma, hepatoblastoma, Ewing sarcoma, synovial sarcoma, Wilms' tumors, ependymoma, medulloblastoma, retinoblastoma, inflammatory myofibroblastic tumors, osteosarcoma, glioblastoma multiforme, astrocytoma grade III, atypical teratoid rhabdoid tumors and malignant peripheral nerve sheet tumors. We used a series of search terms computed in the PubMed search database and identified 3189 papers. The total number of papers for each pediatric tumor type and the number of papers included in the review are given in **Table 1**. Eventually, 74 out of the 3189 papers fulfilled the in- and exclusion criteria to be included in the study. Papers included in the study were selected after appraisal

of the abstract, methodology and result sections. In order for a paper to be included for review, the methodology and result section should contain sufficient information on the number of samples used for each study. A scoring system of 1-3 and a color code of green, orange and yellow was used in order to assess whether a paper contained sufficient information based on the number of samples used for the study and the manner of reporting (**Supplementary Table 2**).

Target patterns identified in primary tumors

BCL2 expression patterns were found to be described either by RNA or protein analysis. RNA expression patterns were mostly determined by mRNA profiling of tumor materials. Protein expression analysis was performed by immunohistochemistry staining of tumor material and subsequent scoring of the number of BCL2-positive cells in each sample. Out of the 17 tumor types studied, the BCL2 expression pattern was well validated for only 5 types: neuroblastoma, synovial sarcomas, Ewing sarcoma, osteosarcoma and retinoblastoma (3, 16-22). The expression pattern of BCL2 in neuroblastoma was determined by both RNA and protein expression analysis of patient materials, while protein expression analysis was mostly reported for the other tumor types. Based on the data reported in the papers included in this study, BCL2 was found to be highly expressed in 57 and almost 100% of the neuroblastoma tumors (154 and 42 neuroblastoma tumors were scored for BCL2 expression in two different studies). In retinoblastoma, Ewing sarcoma, osteosarcoma and synovial sarcoma, BCL2 was reported to be highly expressed in, respectively, 66, 70, 84 and 100% of the tumors. Correlations between BCL2 expression and tumor stage and prognosis were also addressed in these papers. In neuroblastoma, Ewing sarcoma and osteosarcoma, there was no prognostic significance and correlation between BCL2 expression and survival in high risk patients (3, 16, 18). Synovial sarcoma patients presenting with metastatic patterns and short-term survival rates had a higher BCL2 expression pattern, despite the lack of a significant correlation between overall survival and BCL2 expression (17). In retinoblastoma patients, BCL2 expression levels correlated with poor differentiation, metastasis and high risk (19). BCL2 might serve as a biomarker of poor prognosis in synovial sarcoma and retinoblastoma and targeting this gene in tumors with high expression of the protein might prove beneficial in the long-term survival of these patients. In four of the tumors (i.e. Wilms' tumor, astrocytoma, ependymoma and rhabdomyosarcoma), the expression pattern of BCL2 was scored inconclusive because of the small sample size of patients (23-27). Expression of BCL2 was completely negative in atypical teratoid rhabdoid and inflammatory myofibroblastic tumors, while no studies were conducted reporting BCL2

expression patterns in glioblastoma, medulloblastoma, malignant peripheral nerve sheet tumors and hepatoblastoma (28).

Target validation *in vitro* and *in vivo*

Target validation *in vitro* was scored based on the phenotypic responses of cell lines to BCL2 depletion. As described in two papers, shRNA knockdown of BCL2 resulted in clear apoptotic responses in multiple BCL2-high expressing neuroblastoma cell lines (3, 16). Published *in vitro* results were clearly sufficient to validate BCL2 as a potential drug target for neuroblastoma. Due to the limited number of cell lines used, there was insufficient evidence to suggest *in vitro* BCL2 dependency in hepatoblastoma. In glioblastoma, target dependency was deemed negative because BCL2 overexpression did not potentiate the sensitivity of glioblastoma cell lines to small molecule BCL2 inhibitors. For all other pediatric tumor types addressed in the current systematic review, no literature was available reporting on the *in vitro* target dependency. *In vivo* validated tumor dependency was scored based on information about the effect of BCL2 overexpression on the tumor-forming potential of pediatric tumor cells in mouse models. In medulloblastoma, the tumor-forming potential of BCL2 was only observed upon co-expression of the protein with the *sonic hedgehog* gene. This finding suggests that the tumor-driving events of BCL2 only occur in the presence of other oncogenic partner genes in this tumor type (29). Additionally, downregulation of BCL2 by loss of function of estrogen receptor β lead to tumor reduction and increased apoptosis in medulloblastoma xenograft models (30), supporting a strong *in vivo* correlation to BCL2 dependency. There were no studies reported for BCL2 dependency *in vivo* for the rest of the tumor types (**Table 2**).

In vitro and *in vivo* compound efficacy

ABT-263 or navitoclax is a first generation BH3 mimetic which showed great promise in lymphoid malignancies and non-small cell carcinoma, with strong apoptotic responses *in vitro* and complete regression of BCL2 high-expressing xenografts *in vivo* (4). However, navitoclax additionally targets BCL-W as well as BCL-X$_L$ leading to thrombocytopenia in patients treated with the compound (11, 31). This led to the development and pre-clinical evaluation of ABT-199 (venetoclax), a BCL2-specific inhibitor with improved oral bioavailability (13). A strong apoptotic response was observed upon venetoclax treatment of BCL2-high expressing neuroblastoma cell lines, while tumor growth inhibition was observed *in vivo* (13, 32). The most commonly studied biomarkers for efficacy of small molecule BCL2 inhibitors are BIM displacement from BCL2 and

the downstream effectors of the intrinsic apoptotic pathway, i.e. cleaved PARP and caspase-3 as well as cytochrome c release from the mitochondria into the cytoplasm (32). Evaluation of each marker *in vitro* was reliably reported in 37% of the included papers (3, 6-8, 10, 14, 32-46), with *in vivo* efficacy being reported in only 9% of the papers. *In vitro* BIM displacement from BCL2, PARP cleavage and/or caspase-3 cleavage by BCL2 inhibitors navitoclax or venetoclax was reported in neuroblastoma, glioblastoma, hepatoblastoma, Ewing sarcoma, osteosarcoma, retinoblastoma and synovial sarcoma. Biomarkers for efficacy were most extensively validated in neuroblastoma. At the *in vivo* level, the efficacy of BCL2 inhibitors was determined by their effects on tumor growth reduction or inhibition, BIM displacement from BCL2 and the presence or absence of cleaved PARP and/or caspase-3. Tumor regression or growth inhibition was observed upon treatment of xenograft mouse models in neuroblastoma, glioblastoma and hepatoblastoma. Growth inhibition was strongly correlated to increased BIM displacement from BCL2 and cleaved caspase-3 (7, 10, 14, 32, 34, 37, 38). The promising pre-clinical results obtained with BH3 mimetics in these tumor types highlight the relevance of carrying out clinical trials with BCL2 inhibitors in pediatric neuroblastoma, glioblastoma and hepatoblastoma patients.

Selection biomarkers

Predictive biomarkers for sensitivity can be described as genomic, epigenetic or proteomic events that predict which patients are likely to benefit from treatment with the inhibitor of interest. Based on the *in vitro* and *in vivo* pre-clinical evaluation of small molecule BCL2 inhibitors in neuroblastoma, four predictive biomarkers for sensitivity were identified: BCL2 protein levels, BCL2 mRNA levels, BIM/BCL2 complex levels and BCL-X_L/BCL2 ratio levels (3, 14, 32). At the *in vitro* and *in vivo* levels, neuroblastoma was the only tumor whereby sensitivity to small molecule BCL2 inhibitors was shown to strongly correlate to BCL2 protein levels, BCL2 mRNA expression and BIM/BCL2 complex levels (32). No studies on the correlation of the sensitivity biomarkers to BH3 mimetics has been performed in the other solid pediatric tumor types. Currently, predictive biomarkers for sensitivity of pediatric solid tumor patients to small molecule BCL2 inhibitors have not yet been validated in a clinically relevant setting.

Resistance mechanisms and combinations

Several mechanisms inducing resistance to BCL2 inhibitors have been described. (16, 47). Missense mutations in the BH3 domain of BCL2 preventing venetoclax binding to BCL2 and subsequent BIM displacement and apoptosis induction has been reported in

various adult hematological malignancies (47). Resistance was also reported to occur due to upregulation of the anti-apoptotic BCL2 family proteins BCL-X$_L$ and MCL1. Increased levels of both proteins were shown to sequestrate BIM displaced from BCL2, thereby preventing apoptosis from occurring (6, 48, 49). In neuroblastoma, rhabdomyosarcoma and glioblastoma, MCL1 upregulation and sequestration of BIM was the principal route through which resistance to BH3 mimetics occurred, while in malignant peripheral nerve sheath tumors (RMS:MPNST), upregulated BCL-X$_L$ was the primary cause of resistance (14, 16, 32, 45, 50). Several strategies to overcome MCL1-mediated resistance mechanisms were studied and reported. Firstly, targeted downregulation of MCL1 by HDAC inhibitor vorinostat (SAHA), PI3K/mTOR inhibitors and aurora kinase inhibitors potentiated the efficacy of BCL2 inhibitors in neuroblastoma, rhabdomyosarcoma and in glioblastoma (8, 9, 14, 16, 32, 36, 50-52). Synergism between BCL2 inhibitors and chemotherapeutic and DNA damaging agents doxorubicin, cyclophosphamide, paclitaxel and 6-diazo-5-oxo-L-norleucineine and the proteasome inhibitor bortezomib was also reported in both neuroblastoma and glioblastoma as well as in hepatoblastoma, synovial sarcoma, Ewing sarcoma, medulloblastoma and atypical teratoid rhabdoid tumors. (**Table 2**). The ability to overcome resistance to BH3 mimetics by combination strategies with other small molecule targeted inhibitors and chemotherapeutic agents highlights the potential benefit of combination treatment over single-agent treatment with BH3 mimetics.

Clinical trials

Despite of the extensive documentation on the clinical relevance of targeting BCL2 in pediatric solid tumor types, none of the 74 papers included in the current review reports on (ongoing) clinical trials studying small molecule BCL2 inhibitors in these patients. The set-up of a phase I dose-escalation study in neuroblastoma patients is currently being carried out and, based on the results obtained in these patients, this phase I study can serve as a platform for initiation of clinical trials in other pediatric solid tumor types with a proven track record of high BC2 expression and BCL2 dependency (neuroblastoma, synovial sarcoma, Ewing sarcoma, osteosarcoma and retinoblastoma).

DISCUSSION

The anti-apoptotic protein BCL2 is aberrantly expressed in numerous cancer types (3, 16-22). This systematic review was performed to get a broad perspective on the clinical relevance and feasibility of targeting BCL2 for the treatment of pediatric solid tumor patients. Appraisal of the search methodology led to a selection of 74 papers which were finally included for reviewing based on the inclusion and exclusion criteria. These papers showed a diversity in methodology, data analysis and the quality of reporting. Papers included in the study were scored and ranked using color and number codes based on the methodology and experimental procedures reported in each paper. Interpretation of the expression pattern of BCL2 in the included papers showed a strong BCL2 expression pattern in several malignancies (neuroblastoma, synovial sarcoma, Ewing sarcoma, osteosarcoma and retinoblastoma. Navitoclax and venetoclax were the most extensively validated BH3 mimetics in this study(3, 6, 9, 14, 34, 35, 41, 50, 52). Several predictive biomarkers for sensitivity and efficacy have been reported for BCL2 inhibition by BH3 mimetics. The most widely validated biomarkers for sensitivity are the BCL2 protein and mRNA expression levels and the ratio of the BIM/BCL2 complex levels (32). The efficacy to BCL2 small molecule inhibitors was exclusively determined by BIM displacement from BCL2 by the compounds and activation of downstream effectors of the intrinsic apoptotic pathway such as cleaved PARP and caspase 3 (3, 14, 32). The excellent results obtained *in vitro* and *in vivo* on the pre-clinical evaluation of these compounds highlights the relevance of targeting BCL2 in pediatric solid tumors with high BCL2 expression. However, several mechanisms of resistance to BCL2 inhibition by small molecule inhibitors have been reported (47, 49). MCL1 upregulation and its sequestration of BIM displaced from BCL2 by the compounds is the most extensively studied mechanism of resistance to BCL2 small molecule inhibition (49). To date, several strategies to elucidate and overcome this resistance mechanism has been studied. Combined targeted depletion of MCL1 protein levels or inhibition of the gene function with MCL-specific inhibitors and targeted BCL2 inhibition yielded a strong synergistic effect (6, 8, 16, 32, 52). Excellent results have also been obtained upon combination studies with chemotherapeutics and BCL2 specific inhibitors (3, 10, 33, 34, 39, 41, 42, 53).

Based on the results obtained from the review process, neuroblastoma was the pediatric solid tumor type with the most sufficient data on the validation of BCL2 as a therapeutic target. Validation of *in vitro* target dependency with shRNA was an important first step in

determining the relevance of targeting this pathway in this tumor type. The subsequent pre-clinical evaluation of BH3 mimetics *in vitro* and *in vivo* yielded promising results with sensitivity to BH3 mimetics being strongly correlated to BCL2 mRNA and protein levels as well as the BIM/BCL2 complex levels. At the *in vitro* level, the strong apoptotic effects as well as tumor growth regression and inhibition linked to BIM displacement from BCL2 was a strong indicator of the exquisite efficacy of small molecule BCL2 inhibitors in neuroblastoma. The identification of MCL1 as one of the key routes through which resistance to BH3 mimetics might occur has been extensively reported and studied in neuroblastoma. The successful abrogation of this resistance mechanism by targeted downregulation of MCL1 in combination with BH3 mimetics highlights a strategy to improve neuroblastoma patient survival. Neuroblastoma patients are therefore excellent candidates for the testing of small molecule BCL2 inhibitors in a clinical setting and currently, a phase I dose-escalation study is being set-up for this tumor type.

Additional studies are needed to extensively validate biomarkers for sensitivity and efficacy of BH3 mimetics in other pediatric tumors shown to have high expression of BCL2 (i.e. synovial sarcoma, Ewing sarcoma, osteosarcoma and retinoblastoma). This systematic review also highlighted the fact there is a severe lack of knowledge on the validation of BCL2 dependency in most BCL2 high-expressing pediatric solid tumors. Based on this review, resistance to BH3 mimetics by MCL1 upregulation was the most validated resistance mechanism in pediatric solid tumors. Most combination studies laid emphasis on the targeted downregulation of MCL1. Other mechanisms of resistance to BH3 mimetics and strategies to overcome resistance need to be addressed. The ultimate goal will be the setting up of clinical trials for the testing of BH3 mimetics as a single agent and subsequently in combination with other therapeutic agents with known synergism with these compounds.

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Tumor type	Search terms (Pubmed database)	Items found	Articles Included
Neuroblastoma	((BCL2 or BCL-2) or (ABT199 or ABT-199) or (ABT263 or ABT-263) or venetoclax or navitoclax or obatoclax) and neuroblastoma.	851	11
Rhabdomyosarcoma	((BCL2 or BCL-2) or (ABT199 or ABT-199) or (ABT263 or ABT-263) or venetoclax or navitoclax or obatoclax) and rhabdomyosarcoma	72	3
Synovial sarcoma	((BCL2 or BCL-2) or (ABT199 or ABT-199) or (ABT263 or ABT-263) or venetoclax or navitoclax or obatoclax) and synovial sarcoma	146	3
Ewing Sarcoma	((BCL2 or BCL-2) or (ABT199 or ABT-199) or (ABT263 or ABT-263) or venetoclax or navitoclax or obatoclax) and Ewing sarcoma	44	2
Osteosarcoma	((BCL2 or BCL-2) or (ABT199 or ABT-199) or (ABT263 or ABT-263) or venetoclax or navitoclax or obatoclax) and osteosarcoma	366	4
Wilms' tumor	((BCL2 or BCL-2) or (ABT199 or ABT-199) or (ABT263 or ABT-263) or venetoclax or navitoclax or obatoclax) and Wilms' tumor	73	2
Retinoblastoma	((BCL2 or BCL-2) or (ABT199 or ABT-199) or (ABT263 or ABT-263) or venetoclax or navitoclax or obatoclax) and retinoblastoma	511	3
Inflammatory myofibroblastic tumors	((BCL2 or BCL-2) or (ABT199 or ABT-199) or (ABT263 or ABT-263) or venetoclax or navitoclax or obatoclax) and Inflammatory myofibroblastic tumors	13	7
High grade glioma and glioblastoma multiforme	((BCL2 or BCL-2) or (ABT199 or ABT-199) or (ABT263 or ABT-263) or venetoclax or navitoclax or obatoclax) and glioblastoma multiforme	508	8
STS non-RMS:malignant peripheral nerve sheath tumors	((BCL2 or BCL-2) or (ABT199 or ABT-199) or (ABT263 or ABT-263) or venetoclax or navitoclax or obatoclax) and malignant peripheral nerve sheath tumors	33	4
Astrocytoma grade III	((BCL2 or BCL-2) or (ABT199 or ABT-199) or (ABT263 or ABT-263) or venetoclax or navitoclax or obatoclax) and astrocytoma grade III	403	4
Ependymoma	((BCL2 or BCL-2) or (ABT199 or ABT-199) or (ABT263 or ABT-263) or venetoclax or navitoclax or obatoclax) and ependymoma	14	4
Medulloblastoma	((BCL2 or BCL-2) or (ABT199 or ABT-199) or (ABT263 or ABT-263) or venetoclax or navitoclax or obatoclax) and medulloblastoma	88	6
Atypical peripheral rhabdoid tumors	((BCL2 or BCL-2) or (ABT199 or ABT-199) or (ABT263 or ABT-263) or venetoclax or navitoclax or obatoclax) and atypical teratoid rhabdoid tumors	5	3
Hepatoblastoma	((BCL2 or BCL-2) or (ABT199 or ABT-199) or (ABT263 or ABT-263) or venetoclax or navitoclax or obatoclax) and hepatoblastoma	47	6

Table 1: Search results for Medline/PubMed: 18-04-2016.

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Tumor types	Expression patterns of BCL2	BCL2 (% positive/ high expression)	In vitro BCL2 dependency	In vivo BCL2 dependency	In vitro compound efficacy	In vivo compound efficacy	Biomarkers for sensitivity	Biomarkers for efficacy	Resistance mechanisms	Combination strategies	Clinical trials
Neuroblastoma	n=185 n=42	31% (16) 100% (3)	Apoptotic response (cleaved PARP, caspase-3) (3)	-	Apoptotic response (cleaved PARP, caspase-3) (3, 32, 34)	Tumor regression and growth inhibition (14, 32-34).	BCL2 protein& mRNA, BIM/ BCL2 complex levels (3, 14, 16, 32)	BIM displacement from BCL2 (32)	MCL1 upregulation (16, 32)	Chemotherapeutics (Doxorubicin, cisplatin, etoposide, cyclophosphamide) (3, 33, 34). Targeted therapy (MCL1, BET and AURKA inhibitors) (14, 32, 54)	-
Rhabdomyosarcoma	n=6 n=11	90% (55) 45% (56)	-	-	-	-	-	-	MCL1 upregulation (50)	Targeted therapy (mTOR inhibitors) (50)	-
Synovial sarcoma	n=32	100% (17)	-	-	Apoptotic response (cleaved PARP, caspase-3) (46)	-	-	-	-	Chemotherapeutics (Doxorubicin) (46)	-
Ewing Sarcoma	n=72	70% (18)	-	-	Apoptotic response (cleaved PARP, caspase-3) (57)	-	-	-	-	Targeted therapy (6-Diazo-5-oxo-L-norleucineine) (57)	-
Osteosarcoma	n=202 n=49 n=29	84% (20) 43% (21) 53% (22)	-	-	Apoptotic response (cleaved PARP caspase-3) (43)	-	-	-	-	-	-
Wilms' tumor	n=10	90% (23)	-	-	-	-	-	-	-	-	-
Retinoblastoma	n=60	66% (19)	-	-	Apoptotic response (cleaved PARP, caspase-3) (44, 58)	-	-	-	-	-	-

Table 2: Summary of results obtained from reviewed papers.

Tumor types	Expression patterns of BCL2 (% positive/ high expression)		*In vitro* BCL2 dependency	*In vivo* BCL2 dependency	*In vitro* compound efficacy	*In vivo* compound efficacy	Biomarkers for sensitivity	Biomarkers for efficacy	Resistance mechanisms	Combination strategies	Clinical trials
Inflammatory myofibroblastic tumor	Negative		-	-	-	-	-	-	-	-	-
High grade glioma			Lack of apoptotic response upon BCL2 KD (9)	-	Apoptotic response (cleaved PARP, caspase-3) (7)	Tumor regression (7)	-	-	MCL1 upregulation (8, 36, 51, 52)	Targeted therapy (PI3K, bortezomib, YM155, SAHA) (9, 36, 51, 52)	-
STS non-RMS:MPNST	-		-	-	-	-	-	-	BCL-X$_L$ upregulation (48)	-	-
Astrocytoma grade III	n=21	55% (24)	-	-	-	-	-	-	-	-	-
	n=104	15% (25)									
Ependymoma	n=31	45% (26, 27)	-	Tumor forming potential (59)	-	-	-	-	-	-	-
Medulloblastoma	-		-	-	-	-	-	-	-	Chemotherapeutics (Etoposide) (59)	-
ATRT:Rhabdoid	Negative (28)		-	-	-	-	-	-	-	Targeted therapy (Debromohyme-nialdisine) (60)	-
Hepatoblastoma	-		Apoptotic response (cleaved PARP, caspase-3) (39)	-	Apoptotic response (cleaved PARP, caspase-3) (40)	Tumor regression (10, 42)	-	-	-	Chemotherapeutics (Cisplatin, paclitaxel) (41, 42). Targeted inhibitors (TRAIL) (10).	-

Table 2: (continued)

	Target expression	In vitro target dependency	In vivo target dependency	In vitro compound efficacy	In vivo compound efficacy	Biomarkers for sensitivity	Biomarkers for efficacy	Resistance mechanisms	Combination strategies	Clinical • Phase 1 • Phase 2 • Phase 3
Neuroblastoma	Green	Green	Grey	Green	Green	Green	Yellow	Yellow	Yellow	Grey
Rhabdomyosarcoma	Yellow	Grey	Grey	Grey	Grey	Grey	Grey	Green	Green	Grey
Synovial sarcoma	Green	Grey	Grey	Yellow	Grey	Grey	Yellow	Grey	Yellow	Grey
Ewing sarcoma	Green	Grey	Grey	Yellow	Grey	Grey	Grey	Grey	Yellow	Grey
Osteosarcoma	Green	Grey	Grey	Yellow	Grey	Grey	Yellow	Grey	Grey	Grey
Wilms' tumor	Yellow	Grey	Grey	Grey	Grey	Grey	Grey	Grey	Grey	Grey
Retinoblastoma	Green	Grey	Grey	Yellow	Grey	Grey	Yellow	Grey	Grey	Grey
Inflammatory myofibroblastic tumor	Red	Grey	Grey	Grey	Grey	Grey	Grey	Grey	Grey	Grey
High grade glioma	Grey	Red	Grey	Yellow	Yellow	Grey	Yellow	Green	Yellow	Grey
STS-non RMS:MPNST	Dark grey	Grey	Grey	Grey	Grey	Grey	Grey	Yellow	Grey	Grey
Astrocytoma Grade III	Yellow	Grey	Grey	Grey	Grey	Grey	Grey	Grey	Grey	Grey
Ependymoma	Yellow	Grey	Grey	Grey	Grey	Grey	Grey	Grey	Grey	Grey
Medulloblastoma	Grey	Grey	Yellow	Grey	Grey	Grey	Grey	Dark grey	Yellow	Grey
ATRT: Rhabdoid	Red	Grey	Grey	Grey	Grey	Grey	Grey	Grey	Yellow	Grey
Hepatoblastoma	Grey	Yellow	Dark grey	Yellow	Yellow	Grey	Yellow	Dark grey	Green	Grey

Legend:
- Green = Sufficient
- Yellow = Inconclusive
- Red = Negative
- Grey = Not tested

Table 3: Target actionability literature score.

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SUPPLEMENTARY INFORMATION

Module	Subcategories	Scoring	
1. Target pattern	Number of samples	1	n>25
		2	n>10<25
		3	n<10
	Type of analysis	1	Two or more different methods were used or WGS/WES
		2	qPCR, differential PCR, Southern/Northern blot, array CGH
		3	IHC, FISH
	Expression vs overexpression vs amplification	1	Well-defined amplification (>4 copies) or shown correlation between high expression and outcome
		2	Gain, overexpression
		3	Expression/immunoreactivity
	Adult vs pediatric patients	1	>80% pediatric patients
		2	40-80% pediatric patients
		3	<40% pediatric patients
2. Target validation in vitro	Knockdown/knockout	1	Different methods to induce knockdown/knockout or >3 cell lines
		2	Use of 1-3 shRNAs or siRNAs
		3	Questionable knockdown/knockout
	Confirmation and analysis of knockdown	1	Phenotypic analysis and analysis of effects of knockdown
		2	One of the two
		3	None of the two

Table S1: Scoring system PoC modules.

Module	Subcategories	Scoring	
3. Target validation in vivo	Type of in vivo model used	1	Transgenic mouse model, >2 different xenografts
		2	Xenografts with inducible genomic modification
		3	Xenografts constitutive genomic modification
	Validation of in vivo model	1	Comprehensive study of developed tumors (characterization, mRNA expression, protein expression, etc.)
		2	Minor validation of the developed tumors
		3	No validation of the developed tumors
4. Drug efficacy in vitro	Number of cell lines	1	5 cell lines or more + at least two appropriate controls
		2	2-5 cell lines + at least one appropriate controls
		3	1 cell line and/or lack of control
	Validation of compound efficacy	1	2 or more methods are used for validation (protein expression, cell cycle distribution, apoptosis assay, etc.)
		2	1 method used for validation
		3	No validation of compound efficacy
5. Drug efficacy in vivo	Number and type of in vivo models used	1	2 or more xenograft models or one transgenic mouse model with appropriate control
		2	1 xenograft models with appropriate control
		3	1 xenograft models without appropriate control
	Validation of compound efficacy	1	2 or more methods are used for validation (protein expression, cell cycle distribution, apoptosis assay, etc.)
		2	1 methods are used for validation
		3	No validation of compound efficacy

Table S1: (continued)

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Module	Subcategories	Scoring	
6. Biomarkers	Confirmation of correlation	1	Correlation molecularly confirmed in 2 or more models (e.g. silencing, overexpression, etc.)
		2	Correlation confirmed in one model
		3	Corerlation not confirmed
	Number of models used to find correlation	1	>10
		2	3-10
		3	<3
7. Resistance		1	Reported resistance + comprehensive analysis + reversing/overcoming resistance
		2	Reported resistance + analysis of molecular changes underlying or due to resistance
		3	Only reporting resistance
8. Combinations	Concentrations tested	1	>4 concentrations of each compound are tested
		2	1-4 concentrations of each compound are tested
		3	1 concentration of each compound is tested
	Definition of synergy	1	Combination index <0.5
		2	Combination index 0.5-0.9 or >5-fold decrease in IC50
		3	Combination index 0.9-1.1 or other methods used
9. Clinical trials		1	Not applicable
		2	Not applicable
		3	Not applicable

Good methodology, convincing evidence (1)
Reasonably good methodology (2)
Evidence not convincing, flaws in methodology (3)

Table S1: (continued)

Table S2: Summaries and scoring of papers included in the review study.

This table can be found at:

https://docs.google.com/spreadsheets/d/1G8pL21BdZ4KToCdZ7zAtOXIeEwffMVD3FK
dm-XDJcCw/edit?usp=sharing

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CHAPTER 7

DISCUSSION

DISCUSSION

Neuroblastoma remains one of the deadliest forms of childhood cancers with only about 30-40% long-term survival in high-risk patients. Studies of the genomic landscape of neuroblastoma patients by whole genome sequencing (WGS) have identified only a few targetable genomic aberrations. Thus far, treatment options for these patients with more precise targeted therapies are limited. Currently, the treatment of neuroblastoma entails the use of chemotherapeutic agents with a wide range of toxic side-effects and minimal responses from the patients. Additionally, *in vitro* and *in vivo* systems to carry out pre-clinical drug studies that can be better translated to the clinical situation are still in development.

In the first part of this thesis, we reported on the isolation and establishment of *in vitro* organoid systems and TIC line xenografts models. We show that these organoids recapitulate the phenotype and genotype of the primary tumors from which they were derived. Next, we identified frequent gain and overexpression of the EZH2 gene in neuroblastoma tumors and we attempted to elucidate the clinical relevance of targeting the histone methyltransferase activity of EZH2 with small molecule inhibitors. We identified a histone methyltransferase-independent function of EZH2 in neuroblastoma. Lastly, we carried out the *in vitro* and *in vivo* pre-clinical evaluation of the BCL2-specific inhibitor venetoclax in neuroblastoma. We attempted to elucidate mechanisms of resistance to venetoclax and finally identified the small molecule MDM2 inhibitor idasanutlin as a targeted inhibitor capable of re-sensitizing venetoclax-resistant high BCL2-expressing cells.

In Chapter 2, we describe the isolation and propagation of primary neuroblastoma cells termed "tumor-initiating cells" (TICs) or organoids. The proper terminology for these cell systems is still under debate. Organoids are defined as 3D structures that arise from stem cells and are composed of organ-specific cell types (1). This terminology was first used for non-malignant cell systems. In a later stage, cells derived from patient tumors that were cultured under the same conditions were defined as 'tumor organoids'. In this case, it is not clear if the same definition, as used for non-malignant cells, can be sustained. Tumor organoids might not have clear stem cell properties and do not have organ-specific cell types. Thus, the only characteristic that is retained is: growth in defined medium in a 3D structure. Tumor organoids and TIC lines are both short-term cultured cells derived from primary or relapsed patient tumor material that retain spheroid characteristics when cultured under non-adherent conditions. Based on this

definition, the term 'tumor organoid' can loosely be applied to define the neuroblastoma cell systems that we describe in chapter 2. But since we used the term TIC lines in the publication, we will sustain this terminology in the current thesis.

Classical cell lines cultured in 2D under serum conditions fail to recapitulate the phenotype of primary neuroblastoma tumors (2). Cluster analyses of classical cell lines with primary tumors in glioblastoma and neuroblastoma tumors have shown them to cluster far-away from primary tumors (2, 3). This result suggests that pathway activity in classical cell lines is distinctly different than that in primary tumors. Additionally, classical cell lines are thought to have acquired mutations that are not present in primary neuroblastoma tumors due to long-term propagation effects. For example, neuroblastoma cell lines have a high frequency of TP53 mutations, while these are not found in primary or relapsed neuroblastoma tumors. Thus, carrying out pre-clinical studies in such model systems might lead to false positive results in targetable pathways only activated under 2D cultured conditions and not in primary tumors.

Cell lines cultured in 3D under serum-free conditions on the other hand have been shown to more faithfully recapitulate the signaling pathway profile of the tumors from which they are derived (2). mRNA profiling and protein expression analysis of neuroblastoma TIC lines indeed suggest that the 3D grown TIC lines better reflect the phenotype of the primary tumors. For example, important neuroblastoma pathogenic markers such as dopamine beta-hydroxylase (DBH), chromogranin A (CHGA) and neural cell adhesion molecule (NCAM) were found to be highly expressed both in the primary tumors and their corresponding TIC lines. mRNA expression analysis showed that neuroblastoma TIC lines and their corresponding mice xenografts clustered together with the primary tumors from which they were derived. This suggests that neuroblastoma TIC lines might represent a better *in vitro* system than classical cell lines (3). Additionally, TIC lines exhibit a similar genomic landscape compared to their corresponding primary tumors. For example, mutations in the Ras-MAPK pathway in the form of a homozygous deletion of the NF1 allele were observed in both the primary tumors and the TIC lines that were derived from these tumors. The Ras-MAPK pathway has been shown to be an important driving genomic event in neuroblastoma relapse formation and propagation (4). Carrying out *in vitro* pre-clinical evaluation of MEK inhibitors using TIC lines from the NF1 mutated tumor might generate results which better represent the patient situation (5, 6).

 The success rate of obtaining neuroblastoma cell lines cultured under 3D conditions remains poor. This is most likely due to the fact that growth factors present in the primary tumors that are important for the maintenance of neuroblastoma TIC lines are not adequately supplied in the culture medium. (7). In order to improve the culture conditions of TIC lines, a first step will be to determine essential growth factors and cell survival pathways in primary neuroblastoma tumors by profiling of these tumors. Supplementing TIC line culture medium with these growth factors might improve the efficiency of culturing these cells, while preserving their genomic and phenotypic landscape. Another strategy for optimizing the culture conditions of TIC lines can be, for example, by co-culturing these cells with tumor-derived stromal cells. Growth factors secreted by these tumor-derived stromal cells might improve the growth of these TIC lines, thereby increasing the chances of maintaining these systems *in vivo*.

Epigenetic modulation of tumor suppressor and oncogenes is one of the key tumor-promoting events in a vast number of cancers. The concept of 'epigenetics' can be described as heritable changes in gene function that cannot be explained by changes in DNA sequence (8). There are several classes of epigenetic systems. The polycomb/trithorax system, for example, is involved in histone methylation processes. In **chapter 3,** we describe a local gain and high expression of EZH2. EZH2 is one of the key components of the PRC2 complex, which belongs to the polycomb/trithorax system. High expression of EZH2 correlates with a poor prognosis in neuroblastoma (9). Thus, targeting EZH2 might be a clinically relevant strategy in neuroblastoma tumors with high EZH2 expression.

Surprisingly, we found that targeting the histone methyltransferase activity of EZH2 with small molecule histone methyltransferase inhibitors was ineffective. Despite maximum target inhibition observed at sub-micromolar concentrations of EZH2-specific histone methyltransferase inhibitors, there were only very mild phenotypic effects. Still, EZH2 inhibition might have potential in neuroblastoma. Inhibition of the EZH2 methyltransferase activity alone might lead to molecular changes that result in a mild phenotype but that sensitize these cells for other inhibitors. Thus, combination strategies of histone methyltransferase inhibitors with other compounds might result in effective responses. Potential evidence for such combinations comes from recent studies that show transcriptional repression of tumor-suppressor genes by DNA methyltransferases and PRC2-mediated histone tail modifications in high-risk neuroblastoma patients. This led to the hypothesis that tumor suppressor genes hyper-

7

methylated and downregulated due to DNA methylation and histone methylation events by the PRC2 complex can be re-expressed upon combination treatment with DNA demethylating agents and EZH2-specific histone methyltransferase inhibitors (10). Combining DNA demethylating agents with the EZH2 histone methyltransferase inhibitor EPZ6438 resulted in a more pronounced re-expression of the tumor-suppressor genes compared to single agent treatment and a potent phenotypic effect of the cells. These results suggest that combining EZH2-specific histone methyltransferase inhibition with DNA demethylating agents might be effective in the future treatment of neuroblastoma. Other effective combinations of histone methyltransferase inhibitors with chemotherapeutic agents have yielded promising results in other tumor types (11).

A second alternative approach to target EZH2 will be by inhibition of its non-canonical functions. Targeted downregulation of EZH2 with shRNAs in EZH2 high-expressing neuroblastoma cell lines resulted in a strong apoptotic phenotype. This suggests that neuroblastoma cell lines might be dependent on EZH2 for their survival, independent of its methyltransferase activity (12). The apoptotic phenotype observed in neuroblastoma cell lines might be due to the downregulation of key anti-apoptotic proteins such as BCL2 and FOXM1 which are reported to be direct downstream targets of EZH2 (13). These results demonstrate the need for compounds that specifically target the non-canonical functions of EZH2 in neuroblastoma.

Resistance to apoptosis is a precursor to tumor formation and maintenance. In most cancer cell types, upregulation of key players of the extrinsic and intrinsic apoptotic pathway provide a mechanism of evasion to apoptotic cues, leading to uncontrollable proliferation of tumor cells. Hypermethylation of the promoter region of the *CASP8* gene, gain of the 17q25 locus housing the *BIRC5* gene (leading to its concomitant overexpression) and overexpression of the BCL2 protein are frequently observed anti-apoptotic events in neuroblastoma (14-16). Earlier attempts to specifically target BCL2 with the small molecule inhibitor navitoclax showed great promise *in vitro* and *in vivo*. However, due to the concomitant targeting of the anti-apoptotic proteins BCL-X$_L$ and BCLW, thrombocytopenia was observed in patients treated with this compound (17). This led to the development of the new BCL2-specific inhibitor venetoclax (18). In **chapter 4**, we describe the pre-clinical validation of venetoclax in neuroblastoma.

First, we identified *BCL2* mRNA, BCL2 protein and BIM/BCL2 complex levels as promising candidate selection biomarkers for sensitivity to venetoclax (19). Others have suggested that MYCN levels might also hold some predictive power for determining sensitivity

to venetoclax (20), but this still needs to be clearly defined in larger series of model systems. The upcoming trial of venetoclax in neuroblastoma patients might give more insight in the predictive value of MYCN.

Despite promising *in vitro* and *in vivo* responses to the compound, resistance to venetoclax was observed. Neuroblastoma resistance to venetoclax could be attributed to upregulation of the anti-apoptotic protein MCL1. Upregulated MCL1 sequestrates BIM displaced from BCL2 by venetoclax, thereby preventing further activation of downstream events in the intrinsic apoptotic pathway (19). Several strategies to overcome MCL1-mediated resistance to venetoclax have been explored. Firstly, by concomitant inhibition of pathways or proteins positively regulating MCL1 expression such as the PI3K/AKT and Ras/MAPK pathways and cyclin-dependent kinases (CDKs). Inhibitors of the PI3K/AKT and Ras/MAPK pathways and CDKs have been shown to effectively downregulate MCL1 protein levels and promising results were observed when directly combining these inhibitors with venetoclax (21, 22). Implementing such combination strategies in neuroblastoma treatment might be an effective strategy to overcome MCL1-mediated resistance to venetoclax.

The second strategy enacted to overcome MCL1-mediated resistance to venetoclax is by combination treatment with compounds directly inhibiting MCL1 by occupying its BH3 domain. In the current thesis, we report moderate synergistic and strong additive effects between venetoclax and MCL1 inhibitor A-1210477 across the BCL2 high-expressing cell lines. Additional studies need to be performed to validate the *in vitro* obtained results with this combination in *in vivo* model systems.

In **chapter 5**, we studied the re-sensitization of venetoclax-resistant BCL2 high-expressing cell lines to venetoclax by targeted and chemotherapeutic compounds. High-throughput drug screening of venetoclax-resistant cells and their corresponding motherlines with over 200 compounds revealed a select number of targeted and chemotherapeutic compounds capable of re-sensitizing venetoclax-resistant cell lines to venetoclax. Besides the above discussed P13K/mTOR and CDK inhibitors, histone deacetylase (HDAC) inhibitors and the MDM2 inhibitor idasanutlin were identified as promising targeted compounds for combination treatment with venetoclax, of which idasanutlin was the most promising re-sensitizing compound. P53-mediated BAX activation of apoptosis in venetoclax-resistant BCL2 high-expressing cell lines might be an attractive strategy to overcome resistance in these cells. At the mechanistic level, re-sensitization of the venetoclax-resistant neuroblastoma cell lines to venetoclax

7

by idasanutlin could be attributed to the potent upregulation of the pro-apoptotic protein BAX. Additional studies need to be performed in order to elucidate the exact mechanistic contribution of idasanutlin in activating the intrinsic apoptotic pathway in venetoclax-resistant BCL2 high-expressing cell lines.

Combined treatment with BCL2 and MDM2 inhibitors venetoclax and idasanutlin led to partial responses *in vivo*, with clear tumor regression and a strong upregulation of BAX. However, no complete regression was obtained and tumors regrew upon withdrawal of both compounds in mice. Several approaches could be explored to improve the efficacy of this combination. We can test more intensive treatment regimens both concerning dosage as well as duration. This is in line with the treatment protocols that will be implemented in the clinical trial. An additional strategy might be the implementation of the venetoclax/idasanutlin combination treatment in regular treatment protocols.

Numerous other malignancies have a high expression of BCL2 and targeting this protein with BH3 mimetics has shown promising results in various adult malignancies. The BCL2-specific small molecule inhibitor venetoclax has recently been clinically validated in chronic lymphocytic leukemia (23). For further clinical development of this compound for pediatric patients with solid tumors, a systematic summary of the relevant literature addressing this subject was needed. In **chapter 6**, we performed a systematic review on the expression patterns and pre-clinical evaluation of small molecule BCL2 inhibitors in pediatric solid tumors.

Based on the literature review, we found overexpression of BCL2 present in 5 pediatric solid tumor types (i.e. neuroblastoma, synovial sarcoma, retinoblastoma, osteosarcoma and Ewing sarcoma), while a negative BCL2 target presence was noted in two of the tumor types (atypical teratoid rhabdoid and inflammatory myofibroblastic tumors) (15, 24-31). For the tumors whereby expression analysis was deemed inconclusive, additional studies are needed in order to validate target presence. BH3 mimetics were extensively pre-clinically tested in several tumor types (i.e. neuroblastoma, synovial sarcomas, retinoblastoma, osteosarcoma, Ewing Sarcoma, glioma and hepatoblastoma) and showed strong apoptotic responses with tumor regression and growth inhibition *in vivo* (15, 19, 32-39). Till date, clinical trials of venetoclax as a single agent have only been performed in adult lymphoid malignancies (23), but the first trials in neuroblastoma patients are in the pipeline. Results obtained from clinical trials in neuroblastoma patients might pave the way for the future implementation in neuroblastoma patients as well as pediatric patients with other solid tumor types expressing high levels of BCL2.

FUTURE DIRECTIONS

1. Organoid systems in neuroblastoma

One of the most challenging aspects of isolating and maintaining neuroblastoma TIC lines is the determination of an adequate culture condition to improve the growth of these cell lines. Affymetrix profiling of the primary tumors from which the TIC lines are derived should pinpoint which growth factors are highly expressed in primary tumors. Subsequent addition of these growth factors to the culture medium of TIC lines might improve the efficiency of spheroid growth in this system. The TIC lines are currently grown without a supporting matrix. Methods to grow the TIC lines in a 3D matrix should also be explored. Additionally, studies need to be carried out to determine the advantages of using TIC lines for *in vitro* pre-clinical studies over classical neuroblastoma cell lines. Great emphasis should also be placed on the development of neuroblastoma PDX models, as they represent *in vivo* models that are phenotypically and genetically more identical to the primary tumors than the xenograft models from classic cell lines. Results obtained from pre-clinical studies in TIC lines and PDX models might improve the efficacy of testing of diverse therapeutic agents and the ease of translation of the results in a clinically relevant setting.

2. Hypothesis on the functional role of EZH2 in neuroblastoma

EZH2 is aberrantly expressed in neuroblastoma and despite widespread evidence of its tumor-promoting role due to its histone methyltransferase activity, a histone methyltransferase-independent function of the protein seems involved in neuroblastoma. This calls into question the relevance of targeting the histone methyltransferase function of EZH2 with small molecule inhibitors in neuroblastoma. Additional studies are needed to elucidate the non-canonical functions of this protein. We observed that *in vitro* targeted depletion of EZH2 results in an apoptotic phenotype and consequently hypothesize that EZH2 might exist upstream of neuroblastoma survival pathways. These could then be inhibited by small molecule inhibitors specifically targeting the complete EZH2 protein. Previous attempts to specifically target EZH2 non-canonical functions with the first generation EZH2 inhibitor DZNeP did not prove successful due to off-target effects and *in vivo* toxicity caused by the concomitant downregulation of other histone methyltransferases (40, 41). The recently discovered stabilized-α-helix of EZH2 peptide (SAH-EZH2) downregulates EZH2 protein by disrupting the PRC2 complex and has shown great promise in CLL cells (42). Pre-clinical evaluation of this peptide

in neuroblastoma might shed light on the therapeutic implication and feasibility of targeting the EZH2 protein in neuroblastoma.

3. Effective targeting of BCL2 in neuroblastoma

BCL2 mRNA, BCL2 protein and BIM/BCL2 complex levels have extensively been validated as predictive biomarkers for sensitivity to venetoclax in neuroblastoma. A phase I/II clinical trial to determine PK and PD in neuroblastoma patients is planned. However, based on *in vitro* and *in vivo* evidence of MCL1 upregulation and the occurrence of resistance to venetoclax, treatment of neuroblastoma patients with venetoclax as a single agent might not be an effective therapeutic strategy. Combination strategies of venetoclax with chemotherapeutic agents, MCL1 inhibitors and targeted therapies with known synergistic effects might be more effective than single treatment with the compound. Targeted MDM2 inhibitor idasanutlin has been identified as an excellent candidate re-sensitizing agent, with good partial responses obtained in mice with BCL2 high-expressing neuroblastoma xenografts. A clinical trial with venetoclax in combination with idasanutlin might be an effective therapeutic strategy to prevent resistance to venetoclax in neuroblastoma patients. Additional studies still need to be carried out to identify other candidate re-sensitizing agents as well as other routes through which neuroblastoma resistance to venetoclax can occur.

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APPENDICES

ENGLISH SUMMARY
NEDERLANDSE SAMENVATTING
ACKNOWLEDGEMENTS
CURRICULUM VITAE
PORTFOLIO
PUBLICATION LIST

ENGLISH SUMMARY

Neuroblastoma is a childhood cancer that arises from the sympathetic nervous system and accounts for about 6% of all pediatric tumors. An overall survival rate of about 30% is observed in infants less than one year of age and only about 15% in older children. Classification of neuroblastoma is based on the INSS system with local disease observed in stage 1-3 and distal metastatic patterns observed in stage 4 patients. MYCN amplification is a strong predictor of aggressive disease and poor survival. The treatment regimen of neuroblastoma is based on the DCOG 2009 protocol using myeloablative chemotherapeutics, autologous stem cell transplantation and MIBG treatment. However, due to a low success rate and recurrence of the disease after conventional chemotherapeutic treatment, a more precise therapy based on the molecular and genomic profile of the patients is needed.

Most often, *in vitro* pre-clinical evaluation of targeted therapies involves the use of 2D-cultured classical cell lines. However, spontaneous genomic mutations in 2D-cultured cells might arise due to the long-term culture of these cells. These cells thus fail to recapitulate the phenotype and genotype of primary tumors from which they are derived from. Adequate *in vitro* model systems that best represent primary tumors are thus needed. In **chapter 2**, we described the isolation and characterization of neuroblastoma TIC lines or organoids. We show that TIC lines recapitulate the phenotype and genotype of primary tumors from which they are derived from. However, the success rate of isolating and maintaining such organoid systems remains low due to sub-optimal culture conditions. Tumor cells co-exist in a 3D architecture consisting of extracellular matrix and stromal derived cells. The secretion of paracrine and growth factors by these stromal cells is necessary for the maintenance and growth of the tumor cell niche. Thus, identification of tumor stromal cell-derived growth factors is imperative. Supplementing TIC medium with such growth factors might improve the efficacy of the *in vitro* growth conditions of such cells. Additionally, co-culturing TIC lines directly with tumor-derived stromal cells might be another strategy to improve the maintenance of such cells *ex vivo*.

Several somatic and germline genomic events have been observed in neuroblastoma. MYCN amplification, 17q gain and loss of 11q and LOH 1p are key genomic events in neuroblastoma. A local gain of the 7q36 locus harboring the *EZH2* gene has previously been reported in neuroblastoma. EZH2, a histone methyltransferase is overexpressed in neuroblastoma. In **chapter 3**, we described the pre-clinical evaluation of the EZH2-

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specific histone methyltransferase inhibitors EPZ6438 and GSK126. Targeted inhibition of the histone methyltransferase function of EZH2 with the small molecule inhibitors surprisingly led to only a slight G1 arrest in EZH2 high-expressing neuroblastoma cell lines. Inhibition of the histone methyltransferase function of EZH2 with EZH2 specific inhibitors in neuroblastoma cells might lead to molecular changes within these cells rendering them susceptible to other small molecule inhibitors. An effective strategy to improve the efficacy of EZH2 small molecule inhibitors was based on the enhanced phenotypic effect observed upon combining DNA demethylating agents with EZH2 specific histone methyltransferase inhibitors. Based on these results, combining EZH2 specific inhibitors and DNA demethylating agents might be an effective strategy in the treatment of neuroblastoma.

A second effective strategy might be by targeting the non-canonical functions of EZH2. A strong apoptotic response was indeed observed upon targeted downregulation of the EZH2 protein with shRNA. These results clearly demonstrate the need for compounds that specifically target the histone methyltransferase independent functions of EZH2 in neuroblastoma.

Uncontrolled proliferation of tumor cells most often occurs as a result of resistance to apoptotic cues in these cells. Overexpression of members of the extrinsic and intrinsic apoptotic pathway has frequently been observed in numerous malignancies. The *BCL2* gene, an important member of the intrinsic apoptotic pathway is overexpressed in neuroblastoma. Thus, targeting this protein with small molecule inhibitors might prove an effective strategy for the treatment of neuroblastoma. In **chapter 4**, we described the *in vitro* and *in vivo* pre-clinical evaluation of the BCL2-specific small molecule inhibitor venetoclax in neuroblastoma. Sensitivity to venetoclax was strongly correlated to *BCL2* mRNA, protein and BIM/BCL2 complex levels. Despite the promising results obtained from the *in vitro* and *in vivo* evaluation of the compound, resistance to venetoclax was observed. Resistance to the compound could be attributed to the upregulation of the anti-apoptotic protein MCL1. Upregulated MCL1 sequestrated BIM displaced from BCL2 by venetoclax, preventing further activation of the intrinsic apoptotic pathway. Several strategies to overcome MCL1-mediated resistance to venetoclax were studied. Targeted downregulation of MCL1 in combination with BCL2 inhibition proved an effective strategy in overcoming resistance. BIM displacement from MCL1 by MCL1-specific small molecule inhibitors in combination with BCL2 inhibition by venetoclax proved an effective strategy as well. It is thus crucial to validate such results in *in vivo* systems.

In **chapter 5**, we studied strategies to resensitize venetoclax-resistant BCL2-high expressing cell lines to the compound by targeted therapy and chemotherapeutic compounds. High-throughput screening of these resistant cell lines with compound libraries identified the MDM2 inhibitor idasanutlin as the most promising candidate resensitizing agent in the panel of compounds. Resensitization of these cells to venetoclax could be attributed to p53-mediated upregulation of BAX by idasanutlin and activation of apoptosis. At the *in vivo* level, a partial response was observed upon combined inhibition of MDM2 by idasanutlin and BCL2 by venetoclax. However, complete regression was not observed and tumors regrew upon redrawal of both compounds. Several strategies could be explored in order to improve the efficacy of this combination. Increasing the dosage as well as the duration of treatment with both compounds might be an effective strategy. An additional approach might be by implementation of the idasanutlin/venetoclax combination in the neuroblastoma treatment protocol.

Numerous malignancies have high expression of BCL2. Pre-clinical evaluation of BH3 mimetics has shown promising results in various adult tumors. The BCL2-specific inhibitor venetoclax has recently been validated in lymphoid malignances. In order to determine the relevance of targeting BCL2 with this small molecule inhibitor, in **chapter 6**, we performed a systematic review on the expression patterns and pre-clinical evaluation of BH3 mimetics in pediatric solid tumors. Based on the systematic review, BCL2 was reported to be highly expressed in five pediatric solid tumors (neuroblastoma, osteosarcoma, synovial sarcoma, Ewing sarcoma and retinoblastoma) while a negative expression was observed in two tumors (atypical teratoid rhabdoid and inflammatory myofibroblastic tumors). Extensive studies on the pre-clinical validation of BH3 mimetics was reported in several tumor types (i.e. neuroblastoma, synovial sarcomas, retinoblastoma, osteosarcoma, Ewing Sarcoma, glioma and hepatoblastoma) with a strong apoptotic response and tumor regression being observed *in vivo*. Till date, clinical trials with venetoclax as a single agent have only been performed in adult lymphoid malignancies. A clinical trial with venetoclax as a single agent in neuroblastoma is in the pipeline. Results obtained from the clinical trials in neuroblastoma might pave the way for future implementations in other pediatric solid tumors with high BCL2 expression.

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NEDERLANDSE SAMENVATTING

Neuroblastoom is een vorm van kinderkanker die ontstaat uit het sympathisch zenuwstelsel en ongeveer 6% van alle kindertumoren uitmaakt. Neuroblastoma patiënten met hoog risicoziekte hebben slechts een overleving kans van 40% of minder. Neuroblastoom tumoren worden ingedeeld in verschillende stadia op basis van het zogenoemde "International Neuroblastoma Staging System (INSS)". Bij stadium 1, 2 en 3 neuroblastoom tumoren gaat het om lokale tumoren, terwijl stadium 4 tumoren worden gekenmerkt door de aanwezigheid van metastasen naar andere plaatsen in het lichaam. Amplificatie van het MYCN gen, heeft een sterke negatieve invloed op de prognose van neuroblastoom patiënten. De huidige behandeling van neuroblastoom patiënten in Nederland is gebaseerd op een protocol dat in 2009 is opgesteld door de zogenoemde "Dutch Childhood Oncology Group (DCOG)". Volgens dit protocol worden neuroblastoom patiënten behandeld met een combinatie van radiotherapie, chemotherapie (al dan niet gevolgd door autologe stamceltransplantatie) en chirurgie. Maar deze zware behandeling is in een groot aantal van de patiënten niet curatief en daarom is er een nauwkeurigere therapie op basis van de genomische afwijkingen in de tumor van de patiënten nodig.

Meestal impliceert de in vitro preklinische evaluatie van deze nieuwe therapieën, het gebruik van 2D-gekweekte klassieke cellijnen. Het nadeel is echter dat dat deze kweek modellen niet representatief zijn voor de tumoren waar ze van zijn afgeleid. Zowel fenotypisch als genotypisch kunnen er significante verschillen ontstaan. *In vitro* modelsystemen die meer overeenkomen met de primaire tumoren zijn dus nodig. In **hoofdstuk 2** beschrijven we het isoleren en karakteriseren van neuroblastoom Tumor Initiërende Cellijnen (TICs) of organoids. We laten zien dat deze organoids zowel wat fenotype als genotype lijken op de primaire tumoren waarvan ze zijn afgeleid. Maar de succes frequentie waarmee organoids daadwerkelijk kunnen worden gekweekt uit primaire tumoren is nog laag door suboptimale kweekomstandigheden. Tumorcellen, groeien normaal in een 3D architectuur omringd door een extracellulaire matrix en stromale cellen. Excretie van groeifactoren door die stromale cellen is essentieel voor de groei van neuroblastoma cellen. Supplementeren van het organoid groeimedium met deze groeifactoren zou de efficiëntie en slagingspercentage van het groeien van organoids uit primaire tumoren kunnen verbeteren. Daarnaast zou het co-kweken van neuroblastoma tumorcellen, met stromale cellen een andere methode kunnen zijn om de groei van organoiden te verbeteren.

In neuroblastoma worden diverse genomische afwijkingen regelmatig gezien. Amplificatie van het MYCN oncogen, gain van de 17q, verlies van 1p en 11q zijn zulke typische neuroblastoma afwijkingen. Daarnaast wordt gain van chromosoom 7 regelmatig gezien. In een tumor zagen wij een lokale gain van het 7q36 locus met het EZH2 gen. Dit gen komt ook erg hoog tot expressie in neuroblastoma hetgeen bij elkaar suggereert dat dit een drijvend oncogen is in neuroblastoma. In Hoofdstuk 3 beschrijven wij de preklinische evaluatie van de EZH2 specifieke methyltransferase remmers EPZ6438 en GSK126. Volledige remming van de histon methyltransferase activiteit van EZH2 met deze remmers resulteerde in slechts een geringe remming van de celcyclus in neuroblastoma cellen met hoge expressie van EZH2. Als mono behandeling zijn deze EZH2 specifieke remmers dus niet geschikt voor het remmen van neuroblastoma groei, maar het is wel mogelijk dat deze EZH2 remmers, neuroblastoma cellen gevoelig maken voor andere remmers. Dit is bijvoorbeeld aangetoond voor DNA demethylerende middelen in combinatie met EZH2 remmers. Dit zou dus een potentiele nieuwe behandeling voor neuroblastoma kunnen zijn. Naast deze benadering is de non-canonische functie van EZH2 een potentiële manier om neuroblastoma groei te remmen. Wij lieten zien dat het remmen van de expressie van EZH2 eiwit een sterke apoptotische reactie gaf. Remmers die werken op de methyltranferase onafhankelijke functies van EZH2 moeten dus evident verder ontwikkeld worden voor neuroblastoma.

Een van de cruciale eigenschappen van tumorcellen is het feit dat deze cellen de geprogrammeerde celdood (apoptose) geïnactiveerd hebben. Over-expressie van genen die zowel de intrinsieke als excentrieke apoptose remmen wordt frequent gezien in diverse soorten kanker. Het BCL2 gen is een belangrijke speler in de intrinsieke apoptotische route en dit gen komt sterk tot over expressie in neuroblastoma. Het remmen van dit genproduct met chemische inhibitors zou dus een strategie kunnen zijn voor het behandelen van neuroblastoma. In hoofdstuk 4 beschrijven we de in vitro en in vivo evaluatie van de BCL2 remmer Venetoclax. Gevoeligheid voor de remmer bleek sterk gecorreleerd met over expressie van het BCL2 gen en BCL2/BIM complex niveaus. Ondanks de veelbelovende in vitro en in vivo resultaten werd er ook resistentie tegen de remmer gezien. Resistentie tegen de BCL2 remmer werd met name veroorzaakt door over expressie van een ander lid uit de BCL2 familie; MCL1. MCL1 sekwestreert BIM als dat van BCL2 loskomt bij behandeling met de Ventoclax waardoor apoptotische activatie wordt voorkomen. We hebben diverse methodes getest om deze MCL1 gemedieerde resistentie tegen Ventoclax te voorkomen. Remmen van MCL1 met antisense RNA liet

zien dat de cellen weer gevoelig werden voor BCL2 remming. Chemische remmers van MCL1 lieten een soortgelijk effect zien. Het is cruciaal om dit verder in vivo te valideren.

In hoofdstuk 5 hebben we de bekeken hoe we de Venetoclax resistente neuroblastoma cellen met hoge BCL2 expressie weer gevoelig konden maken voor BCL2 remming. Screening met een groot aantal potentiële medicijnen liet zien dat de MDM2 remmer Idasanutlin de beste re-sensitisator was. Deze re-sensitisatie werd met name veroorzaakt door P53 gemedieerde op regulatie van BAX en dientengevolge re-activatie van de apoptose. In vivo werd een VGPR (zeer goede partiële response) gezien maar er bleven resttumoren aanwezig. Diverse strategieën worden nu getest om dit effect nog verder te optimaliseren. De dosering kan verder verhoogd worden, de behandelduur uitgebreid of beiden. Daarnaast kan overwogen worden om deze behandeling combinatie in de bestaande protocollen in te passen.

Verschillende andere tumoren laten over expressie van BCL2 zien en pre-klinische evaluatie van BCL2 remmers hebben positieve resultaten gegeven in diverse trials. Om te kunnen bepalen in welke andere kindertumoren de BCL2 remmer Venetoclax mogelijk effectief is, hebben we een systematisch literatuuronderzoek gedaan hetgeen beschreven staat in hoofdstuk 6. Uit dit onderzoek bleek dat er vijf soorten kindertumoren zijn waar het testen van de BCL2 remmers mogelijk effect zou kunnen hebben (neuroblastoma, osteosarcoma, synovial sarcoma, Ewing Sarcoma en Retinoblastoma). In een aantal andere tumoren leek er genoeg bewijs te zijn om BCL2 remming verder niet te testen (ATRT en myofibroblastische tumoren). Tot op heden is Venetoclax alleen getest in tumoren bij volwassenen. Een trial met Venetocalx in neuroblastoma is in de planning. Resultaten in neuroblastoma zouden mogelijk kunnen leiden tot het verder testen van Venetoclax in andere kindertumoren met hoge BCL2 expressie.

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To my darling fiancé, **Remi**, what a journey we have had together. Thank you for your support. I cannot wait for our next adventures. The best is yet to come.

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CURRICULUM VITAE

The author of this thesis was born on the 29th of July 1983 in Yaounde, the Republic of Cameroon. Her primary and secondary education was completed in 2001 after which she enrolled at the University of Yaounde I to study a BSc. in Animal Biology and Physiology which was completed in 2005. She spent 2 years working as a volunteer in the African Indigenous Women's Organization in Yaounde Cameroon and subsequently enrolled for a Post graduate certificate in International Project Management at the Rovaniemi University of Applied Sciences in Lapland, Finland which was completed in 2008. She started a Masters in Biosciences at the Åbo Akademi University in Turku, Finland. During this time, she did her thesis at the department of Biosciences under the supervision of Dr. Cecilia Sahlgren. In November 2010, she started her PhD in the group of Prof. Dr. Rogier Versteeg at the department of Oncogenomics under the supervision of Dr. Jan Molenaar, Dr. Emmy Dolman and Prof. Dr. Huib Caron. In 2016, she moved to the Princess Maxima Centre for Pediatric Oncology where her PhD studies were completed at the department of Translational Medicine under the supervision of Dr Jan Molenaar and Dr. Emmy Dolman. The data presented in this thesis is a summary of the main research subjects undertaken during her PhD.

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PORTFOLIO

Name PhD student: Laurel T. Bate-Eya
PhD period: November 2010-March 2017
Name PhD supervisor: Prof. Huib N. Caron
Name PhD co-supervisor (s): Dr. Jan J. Molenaar
                           Dr. Emmy Dolman

PhD training	Year	Workload (Hours/ ECTS)
General courses		
• Project management	2016	0.6
• Clinical data management	2016	0.3
• Ebrok	2016	1.0
• Gene targeting	2014	0.4
• Bioinformatics	2013	1.1
• Biostatistics	2013	0.3
Presentations		
• The histone methyl transferase activity of EZH2 in neuroblastoma: Friend or Foe, Advances in Neuroblastoma Research.		
• EZH2 is highly expressed in neuroblastoma and plays an important role in neuroblastoma cell survival independent of its histone methyltransferase activity, Advances in Neuroblastoma Research.		
• High efficacy of BCL-2 inhibitor venetoclax (ABT-199) in neuroblastoma and rational for combination therapy, Advances in Neuroblastoma Research.		
(Inter)national conferences		
• Advances in Neuroblastoma Research (Cairns, Australia).	2016	0.5
• Advances in Neuroblastoma Research (Cologne, Germany).	2014	0.5
• Annual oncology Graduate School (OOA) PhD retreat (Texel, NL, Poster).	2014	0.5
• Annual oncology Graduate School (OOA) PhD retreat (Texel, NL, Poster).	2011	0.5

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PUBLICATION LIST

Publications	Year
Peer reviewed	
Publication in thesis	
• **Bate-Eya LT**, Gierman HJ, Ebus ME *et al*. EZH2 is highly expressed in neuroblastoma and plays an important role in neuroblastoma cell survival independent of its histone methyltransferase activity**.** European Journal of Cancer	2017
• **Bate-Eya LT,** Den Hartog IJM, Van der Ploeg I *et al*. High efficacy of the BCL-2 inhibitor ABT199 in neuroblastoma and rational for combination with MCL-1 inhibition. *Oncotarget. 2016;* **7:** 27946-27958.	2016
• **Bate-Eya LT**, Ebus ME, Koster J *et al*. Newly-derived cell lines propagated in serum-free media recapitulate the genotype and phenotype of primary neuroblastoma tumours. *European Journal of Cancer*. 2014; **50:** 628-637.	2014
• **Bate-Eya LT**, Schubert NA, Alles LK *et al*. High-throughput screening identifies idasanutlin as a re-sensitizing drug for venetoclax-resistant neuroblastoma cells. Submitted.	
• **Bate-Eya LT**, Schubert NA, Caron HN, *et al*. A systematic review on targeting BCL2 in pediatric solid tumors. Manuscript in preparation.	
Other Publications	
• M.Emmy.M Dolman, Van Der Ploeg I, Koster J, **Bate-Eya LT** *et al*. DNA-dependent protein kinase as molecular target for radiosensitization of neuroblastoma cells. *Plosone*; **10:** 1-18	2015
• Mamaeva V, Rosenholm JM, **Bate-Eya LT** *et al*. Mesoporous Silica Particles as Drug Delivery Systems for Targeted Inhibition of Notch Signalling in Cancer *Mol Ther*. 2011; **19:** 1538-46.	2011
• Rosenholm JM, Pehu E, **Bate-Eya LT** *et al*. Cancer-Cell-Specific Induction of Apoptosis using Mesoporous Silica Nanoparticles as Drug Delivery Systems. *Small* 2010; **6:** 1234-41	2010

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