New therapeutic targets in the intrinsic apoptotic pathway in neuroblastoma

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Introduction
1. Neuroblastoma

1.1 Symptoms and diagnosis
Neuroblastoma are tumors that originate from the embryonal precursor cells of the sympathetic nervous system in early childhood. This tumor can arise anywhere throughout the sympathetic nervous system and the tumors are very heterogeneous in site, symptoms and outcome. Most neuroblastoma are localized in the abdomen (65%) of which the adrenal gland is the most common primary site with a prevalence of at least 30% of the tumors, followed by abdominal, thoracic, cervical and pelvic sympathetic ganglia (fig. 1). Neuroblastoma may metastasize to the lymph nodes, bone marrow, bone, liver and skin. Paraspinal located tumors tend to invade the neuronal foramina and can cause compression of the spinal cord. Bone metastases tend to appear in the orbit and therefore periorbital ecchymosis is a classical sign of disseminated neuroblastoma.

About half of the patients present with evidence of metastatic disease, ranging from loco regional spread in lymph nodes to distant metastases. Children with metastases mostly have extensive tumor burden and are very ill. About 5% of the patients are infants with a phenotype of small tumors that metastasize to skin, liver and bone marrow that tend to disappear spontaneously without treatment.\textsuperscript{1-4}

Neuroblastoma diagnosis requires either a positive histological analysis or evidence of neuroblastoma cells in the bone marrow with positive catecholamines in the urine.\textsuperscript{1-4} A diagnostic tool is the Metaiodobenzylguanidine (MIBG) scan. MIBG is a neurotransmitter-like substance that can be radioactively labeled with $^{131}$I for treatment or $^{123}$I for diagnostics. The compound

Fig 1. Common regions of primary neuroblastoma localization (www.cancer.net; American Society of Clinical Oncology)
is actively transported into the cell by the norepinephrine transporter, which is specifically expressed in 90% of the neuroblastoma tumors.\textsuperscript{5-8}

\subsection*{1.2 Tumor biology}
Neuroblastic tumors typically show a lack of differentiation and therefore histological markers of early developmental lineages can still be seen in the tumor.\textsuperscript{1,3,4} The classification of neuroblastic tumors into ganglioneuroma, ganglioneuroblastoma or neuroblastoma is based on the proportion of neural type cells (neuroblasts and ganglion cells) and Schwann type cells. Neuroblastoma are the most undifferentiated and the most aggressive of the neuroblastic tumors. These can be classified as differentiating, poorly differentiated and the most aggressive undifferentiated. Neuroblastoma are composed almost entirely of neuroblasts which appear as small round blue cells. The ganglioneuroblastoma contain neuroblasts with a more mature appearance that are clustered in small foci surrounded by Schwannian stroma. Ganglioneuroma are predominantly composed of Schwann cells with mature ganglion cells.\textsuperscript{9}

\subsection*{1.3 Risk stratification}
Tumors are staged according to the International Neuroblastoma Staging System (INSS). Stage 1, 2 and 3 represent regional tumors with or without positive lymph nodes. Stage 4 tumors have distant metastases. The specific subgroup of stage 4S tumors is reserved for patients less than 1 year of age with dissemination limited to skin, liver and/or bone marrow.\textsuperscript{3} Stage 1-3 neuroblastoma have an excellent prognosis, but the majority of patients with stage 4 tumors die. The word ‘staging’ suggests that each neuroblastoma starts as a stage 1 tumor, which can progress to a high stage tumor. If so, early diagnosis and treatment of neuroblastoma would result in lower incidence of high stage tumors. However, in large population screening programs where young infants were screened for catecholamines in the urine, more children were diagnosed with low stage neuroblastoma but the screening did not have any effect on the incidence of high stage neuroblastoma.\textsuperscript{10,11} Only screening in older infants resulted in diagnosis of more neuroblastoma with genetic unfavorable markers. However, this did not result in a significantly decreased mortality either.\textsuperscript{12} Therefore, it seems that the different stages of neuroblastoma are in fact different types of neuroblastoma tumors that cannot change into one another.

Risk stratification is based on tumor stage according to the INSS, age of the patient and genetic risk factors such as MYCN amplification and deletion of 1p (fig. 2). In Europe all neuroblastoma and ganglioneuroblastoma are included in the treatment protocols
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Ganglioneuroma on the other hand are classified as a benign disease and are not included.\textsuperscript{1,3} Recently several high throughput methods have shown additional value in risk stratification. Specifically Comparative Genomic Hybridization adds critical prognostic information to conventional markers and will be included in future treatment stratification.\textsuperscript{13} Also the development of Whole Genome Sequencing can give a lot of information about individual tumors and may therefore be an interesting tool for personalized medicine.

1.4 Prognosis and Therapy

Patients with low-risk tumors can be treated by surgery alone and have a very good prognosis. However, patients with high risk disease are treated with intensive chemotherapy, surgery and high-dose myeloablative therapy to eradicate minimal residual disease. Despite this extensive treatment, children with high stage neuroblastoma have a poor prognosis with 35% overall survival. Even though neuroblastoma is a very rare disease with 30 to 40 new patients in the Netherlands for malignant neuroblastoma.
every year, it is still the second cause of cancer related death in children. The Dutch Childhood Oncology Group (DCOG) Neuroblastoma Disease Committee has launched a new treatment protocol in 2007, which is based on the German Pediatric Oncology Group (GPOG) treatment strategy (fig. 3) 14,15. In the Dutch protocol twice radioactive $^{131}$I-MIBG treatment was added up front to the German high risk protocol 6,7. After initial radiation 3 cycles N5 chemotherapy (cisplatinum, etoposide, vindesine) and 3 cycles N6 chemotherapy (vincristine, dacarbazine, ifosfamide, doxorubicin) are given. Resection is performed if substantial tumor remains after induction therapy. This resection should be attempted after the 4th or 6th chemotherapy cycle. After

the primary induction therapy and surgery a myeloablative high-dose-chemotherapy with melphalan, etoposide and carboplatin is given, followed by autologous stem cell reinfusion. Finally retinoic acid is given in 6 cycles followed by a short break and 3 more cycles (fig. 3) \textsuperscript{14,15}.

1.5 Common genetic aberrations
Various types of genetic aberrations occur in neuroblastoma. DNA content aberrations are very frequent and divide neuroblastoma into two categories: near-diploid and hyper-diploid. Hyper-diploid tumors are thought to have defects in mitosis, associated with whole chromosome gains and losses. These tumors tend to be less aggressive. In contrast, more malignant neuroblastoma show chromosomal rearrangements and unbalanced translocations. These tumors maintain their diploid DNA content. Near-diploid as well as hyper-diploid tumors have gains or losses, only in the near-diploid tumors these events concern chromosome arms or regions while hyper-diploid tumors tend to have gains or losses of whole chromosomes.\textsuperscript{1}

Gain in neuroblastoma is most frequently found in chromosome 17. The 17q arm is gained in almost all high stage neuroblastoma. Because breakpoints vary, gain from 17q21 to the telomere suggests a dosage effect of several genes in that region.\textsuperscript{1,16} One of the candidate tumor driving genes is \textit{BIRC5} (Survivin), which will be discussed in this thesis. Partial loss is most frequently seen in chromosome 1p and 11q. Both are associated with poor prognosis but only 1p deletions are strongly correlated with \textit{MYCN} amplification. For chromosome 1 the Smallest Region of Overlap (SRO) is located at 1p36 in which several potential tumor suppressor genes have been identified. On chromosome 11 the SRO is located around 11q23, but no tumor suppressor genes in this region have been identified yet.\textsuperscript{1,2,16}

The chromosome 2p arm also shows frequent gain. This region encompasses \textit{MYCN} and \textit{ALK}, the two best known oncogenes in neuroblastoma. \textit{MYCN} is amplified in 20\% of neuroblastoma, which strongly correlates with a bad prognosis. The Myc oncogene family members, \textit{MYC} (c-Myc), \textit{MYCN} and \textit{MYCL}, are transcription factors that are involved in cell growth through protein synthesis, transcriptional regulation of ribosomal RNA processing, cell adhesion and tumor invasion \textsuperscript{1-4,17}. The other oncogene on 2p is \textit{ALK} (anaplastic lymphoma kinase), a receptor tyrosine kinase involved in neuronal differentiation. Potentially activating \textit{ALK} mutations located in the kinase domain were recently found in 6-10\% of the neuroblastoma. In most cases of familiar neuroblastoma and in a few cases of sporadic neuroblastoma \textit{ALK} was mutated.\textsuperscript{18-23} A correlation was found between \textit{ALK} mutations and high stage tumors.\textsuperscript{19} Moreover, when \textit{ALK} was also over-expressed or amplified, the correlation
to a poor outcome was stronger.\textsuperscript{23}

In addition, the \textit{PHOX2B} homeobox transcription factor, which functions in the differentiation of the sympatho-adrenal lineage, was found to be sporadically mutated.\textsuperscript{24} Studies indicate that this mutation can be one of the possible defects in cell maturation and differentiation that may be cancer predisposing.\textsuperscript{24,25} The functional consequences are still being studied.

\textbf{1.6 New neuroblastoma therapies}

Until a few years ago the survival of high stage neuroblastoma patients was 20\%. The first more specific neuroblastoma targeting compounds are currently implemented in treatment protocols and have started to improve survival rates. The first one is an antibody treatment targeting GD2. Recently, randomized phase 3 clinical trials using the anti-GD2 antibody ch14.18 alternating with cycles of GM-CSF or interleukin-2 showed a significant improvement in 2-year event-free survival in the immunotherapy group.\textsuperscript{4,26-29} \textsuperscript{131}I-MIBG is another neuroblastoma specific therapy that is currently being used for neuroblastoma treatment. It showed to be effective in newly diagnosed, high risk neuroblastoma patients with a large tumor mass and a high uptake and storage of the radio-pharmaceutical \textsuperscript{6,7}.

Targeted therapies, specific for genes with a role in neuroblastoma tumors, aim at \textit{ALK} and \textit{AURKA}. \textit{AURKA} has been identified as a potential target for therapy. The gene was found to be sporadically amplified in neuroblastoma and showed an extensive over-expression. Moreover, a synthetic lethal-like relation with \textit{MYCN} was found.\textsuperscript{30} A clinical phase I/II trial in neuroblastoma patients with the AURKA inhibiting small molecule MLN8237 is ongoing. (http://clinicaltrials.gov) Since the identification of \textit{ALK} as a potential tumor-driving gene in neuroblastoma, \textit{ALK} targeting compounds have been extensively tested in vitro and in vivo. At this time, Crizotinib (Pfizer) is the most successfully developed compound. Crizotinib is currently in phase I/II clinical trial in neuroblastoma patients.\textsuperscript{20,31} Mutated genes are found to be the most interesting genes to target in cancer therapy. However, besides \textit{ALK}, no other genes were frequently mutated in neuroblastoma\textsuperscript{32}, and it seems that copy number defects are more likely to be of major importance in the development of neuroblastoma. Therefore, the best option in neuroblastoma is to target pathways, and for this thesis we chose the intrinsic apoptotic pathway as a drug target.
2. Apoptosis

The most widely used classification of mammalian cell death recognizes three types: apoptosis, necrosis and autophagy. Apoptosis is programmed cell death, which will be explained in more detail further on in this paragraph. Cells that undergo necrosis are characterized by cell and organelle swelling or rupture of surface membranes with spillage of intracellular contents. The compromise of organellar membranes allows proteolytic enzymes to escape from lysosomes, enter the cytosol, and cause cell demolition. Autophagy is a process in which cells generate energy and metabolites by digesting their own organelles and macromolecules. One of the hallmarks of cancer is the mechanism by which tumors evade apoptosis. This is the focus of this thesis and it will be further explained in the next paragraphs.

2.1 Morphology of apoptosis

Activation of the apoptotic cascade results in shrinking of the cell and its nucleus. Cytoskeletal proteins are cleaved by aspartate-specific proteases, and thereby subcellular components collapse. Other characteristic features are chromatin condensation and nuclear fragmentation. Unlike in necrosis, the plasma membranes remain intact and participate in the process of apoptosis. DNA fragments and other small particles such as organelles and bits of cytoplasm, are enclosed by a membrane to form the so called ‘apoptotic bodies’. These apoptotic bodies are engulfed by macrophages and thereby apoptotic cells are removed from the tissue without an inflammatory response. This is unlike necrosis, where the cell membrane becomes leaky so that its contents are released into the surrounding tissue, causing inflammation.

2.2 Extrinsic apoptosis pathway

An apoptotic signal from outside the cell can induce members of the Tumor Necrosis Factor (TNF) superfamily to bind the receptors on the cell membrane (TRAIL, TNFR, DR4, DR5 and FAS). This initiates the formation of the multiprotein death-inducing signaling complex, which triggers the catalytic activity of CASP8. CASP8 can activate CASP3 directly, resulting in apoptosis, or it can activate the mitochondrial (intrinsic) apoptotic pathway via cleavage of tBID, which eventually also results in CASP3 cleavage and apoptosis (fig. 4). The NF-κB pathway is also regulated by cell death receptors. After activation by the receptors, signaling to IKK proceeds either through the canonical or through the non-canonical pathway, which results in activation of NF-κB and transcription of both pro-death and pro-survival target genes.
2.3 **Intrinsic apoptosis pathway**

The intrinsic or mitochondrial apoptotic pathway can be activated after an apoptotic signal from inside the cell. Initiators include increased intracellular reactive oxygen species, DNA damage, the unfolded protein response, and the deprivation of growth factors. Upon an apoptotic signal the mitochondrial permeability is increased by BAX and BAK. These proteins form complexes with themselves and thereby form pores in the membrane of the mitochondria through which pro-apoptotic proteins such as Cytochrome C and DIABLO are released. When released to the cytoplasm Cytochrome C forms a complex with APAF1 and inactive pro-CASP9. This complex is called the apoptosome. By the formation of this complex CASP9 is activated, which activates cleavage of CASP7 and CASP3. The other protein released from the mitochondria is DIABLO which can bind and antagonize 'Inhibitor of Apoptosis Proteins' (IAPs). Release of Cytochrome C and DIABLO from the mitochondria is regulated by the BCL2 family proteins. These proteins have at least one of the four BCL2 homology (BH) domains. Anti-apoptotic members of the BCL2 family include BCL2, MCL1, BCL2L1, BCL2L2, BCL2A1 and BCL2L10 and have all four BH domains. These proteins are located on the outer mitochondrial membrane where they bind to BAX and BAK (both multi-domain members of the BCL2 family).
resulting in inhibition of pore formation and thereby preventing the release of caspase activating proteins from the mitochondria into the cytosol. There are two types of pro-apoptotic members of the BCL2 family, also called BH3-only proteins. One set of proteins (BBC3, BCL2L11 and BID) can either induce BAX and BAK or bind to the anti-apoptotic BCL2 family members and thereby inhibit them. The other set of proteins (PMAIP1, BAD, BIK, HRK, BMF and BCL2L14) can only inhibit the anti-apoptotic BCL2 family members.\textsuperscript{33,35,38}

Another important group of apoptosis inhibitors are the IAPs, defined by the presence of a baculovirus IAP repeat (BIR) protein domain.\textsuperscript{39} BIRC2 and BIRC3 can suppress TNFα stimulated cell death by preventing formation of the TNFR1 pro-apoptotic signaling complex. They can also regulate the NF-κB pathway by ubiquitination of NF-κB-inducing kinase (NIK).\textsuperscript{40,41} BIRC6 can induce ubiquitination and degradation of DIABLO.\textsuperscript{42-44} BIRC5 can bind and inhibit DIABLO functionally by blocking its BIR-domain or it can bind and stabilize XIAP, another IAP. This results in inhibition of caspase activation and apoptosis.\textsuperscript{45-47} In addition BIRC5 has a function in the nucleus. BIRC5 was found to be a chromosomal passenger protein that forms a complex with CDCA8 (Borealin), AURKB (Aurora Kinase B) and INCENP by which it regulates microtubule dynamics at the kinetochores. If the microtubule-kinetochore dynamics are disturbed, mitotic catastrophe occurs. This results in P53 and CASP2 activation after which the mitochondrial apoptotic pathway is activated (fig. 4).\textsuperscript{48-52}

\textbf{2.4 Apoptosis and neuroblastoma}

The apoptotic pathway is often deregulated in neuroblastoma. Mutations of the established tumor suppressor \textit{TP53} do occur in tumors that relapse after treatment. However, \textit{TP53} mutations are very rare in primary neuroblastoma.\textsuperscript{1,2,53} \textit{TP53} is shown to be functionally inactivated through sequestration in the cytoplasm in undifferentiated neuroblastoma.\textsuperscript{1,2} Also, the p53/MDM2/p14\textsuperscript{ARF} pathway is often inactivated by \textit{MDM2} amplification or p14\textsuperscript{ARF} inactivation specifically in \textit{MYCN} amplified cells. MDM2-p53 antagonist Nutlin-3 resulted in TP53-mediated growth arrest and apoptosis in these cells.\textsuperscript{54,55} Recent studies also showed convincingly that miR-380-5p suppresses \textit{TP53} and that it is associated with a poor outcome in patients with \textit{MYCN} amplification. Treatment with miR-380-5p antagonist induced p53-dependent cell death in neuroblastoma cells and decreased tumor growth in vivo.\textsuperscript{56,57} \textit{CASP8} is hypermethylated and thereby inactive in some neuroblastoma resulting in an inactive extrinsic apoptotic pathway.\textsuperscript{1,16} \textit{BIRC5} is highly expressed in neuroblastoma, which correlates to a bad prognosis.\textsuperscript{58-61} Also, the expression of
**BCL2** is often increased in neuroblastoma tumors and cell lines. BIRC5 and BCL2 are both found to be good potential targets for therapy for which compounds are clinically available. However, no consistent genomic aberrations in the intrinsic apoptotic pathway in neuroblastoma are known.

3. **Drug development**

3.1 *Mechanism of currently used cytostatics*

Neuroblastoma are the second cause of cancer related death in children. In children that survive, over 70% suffer from severe side effects of the therapy. Currently used cytostatics in neuroblastoma treatment are aspecific compounds with severe toxic effects on normal cells as well. Because of the lack of efficacy and the extended toxicity it is necessary to find more specific targeted compounds which will affect neuroblastoma tumor cells and leave normal cells unharmed. We follow a standardized preclinical procedure that will be further clarified in the coming paragraphs (fig. 5).

![Fig 5. Flow diagram of the preclinical steps in drug development research.](image)

The projects of this thesis are presented on the right with arrows.
3.2 Target identification

Target gene selection is based on identification of aberrations in DNA and mRNA expression in neuroblastoma tumor samples. In addition correlations of expression levels and DNA aberrations to clinical properties are used as selection criteria. For that purpose, a large series of neuroblastoma samples were analyzed using various high throughput assays. To analyze these high-throughput data Jan Koster and colleagues have designed the bioinformatic platform R2. Affymetrix mRNA profiles of 143 neuroblastoma and 24 neuroblastoma cell lines, array CGH and SNP array data of 110 neuroblastoma tumors and 24 cell lines have been generated in our lab and can be analyzed by R2.\(^{66-68}\) Moreover, we can compare our data with public available profiles of over 25,000 tumor samples of 50 different tumor types. R2 facilitates an integrated analysis and allows identification of important regulatory pathways. This platform has been used extensively for this thesis. Recently we sequenced the complete genomes of 87 primary neuroblastoma.\(^ {32,69}\)

3.3 Target validation

The next step in targeted drug development is the validation of a gene or pathway as drug target in neuroblastoma cell line systems. We have used a panel of 24 neuroblastoma cell lines and 6 primary neuroblastoma cultures with diverse genetic properties such as \(\text{MYCN}\) amplification and \(\text{ALK}\) mutations.\(^ {70-72}\) In these models the expression levels of potential drug targets are manipulated. Silencing is mainly performed using the RNA interference technique. We used plasmids that express short hairpin RNA molecules combined with a lentiviral transfection system (Sigma TRC). In addition we use a stable doxycycline inducible lentiviral system if knock down of a certain gene is studied in more detail.\(^ {73}\) Over-expression analysis is studied with a lentiviral stable inducible plasmid with a Tet operator system (Invitrogen). If knock-down or over-expression of a certain target gene leads to a significant phenotype like apoptosis or differentiation, further analysis is performed using targeted compounds.

3.4 In vitro and in vivo compound efficacy

Preferably, targeted compounds are selected that have already passed Phase I/II clinical trials in adults. Phase I trials in children are difficult to establish and pharmaceutical companies are reluctant to test targeted compounds solely for development in children. The core questions in this phase of research are whether this compound induces apoptosis at low concentrations and whether apoptosis is caused by specifically interfering with the gene or protein of interest. These findings can also lead to the identification of biomarkers of efficacy that can be used in further
clinical development of the compound in neuroblastoma. We test if the compound works specifically against the tumor and not against normal tissue by using non-malignant fibroblasts. Finally we test if the compound works synergistically with the currently used cytostatics. After this, the compound is considered for testing in mouse models. For this we use a neuroblastoma xenograft model, in which a classical cell line or primary culture of neuroblastoma tumors is implanted subcutaneously in both flanks of the mice. Serial transplantation improves the reproducibility of the model. Mice are treated with the compound of interest and tumor growth is measured. If a compound is found to be effective in vitro and in vivo and the mechanism of action is validated, the next step is to test the compound in Phase I/II clinical trial.

4. Drug targets in the intrinsic apoptotic pathway

In this thesis, we identified and validated two targets in the intrinsic apoptosis pathway that can be inhibited by new compounds that are currently in Phase I/II clinical trial in adults.

In chapter 2 and 3 we identified BIRC5 as a potential target for therapy. We found BIRC5 to be highly expressed in neuroblastoma compared to normal tissues and other kinds of cancer and this high expression correlated to a bad prognosis. Validation of BIRC5 as a target revealed that its function in the chromosomal passenger complex is of major importance in neuroblastoma cells and knock down of BIRC5 by shRNA induced apoptosis via mitotic catastrophe. We validated the efficacy of a novel small molecule BIRC5 inhibitor, YM155. YM155 inhibited BIRC5 specifically and induced apoptosis in most neuroblastoma cell lines. However a subset of cell lines was resistant to YM155, which was caused by high ABCB1 expression. This ‘Multi Drug Resistance pump’ can be inhibited by cyclosporine or ABCB1 shRNA prior to treatment with YM155, which sensitized the resistant cell lines. We concluded that YM155 is an effective BIRC5 inhibitor in vitro.

Chapter 4 describes BCL2 as a new target for neuroblastoma therapy. Affymetrix micro-array expression data revealed that BCL2 is highly expressed in neuroblastoma tumors. Most neuroblastoma cell lines however, did not have this high expression except for KCNR and SJNB12, which we both used as a model. These cell lines were found to undergo massive apoptosis after BCL2 knockdown by shRNA or inhibition by ABT263, a small molecule BCL2 inhibitor. ABT263 also had an anti-tumor effect in a neuroblastoma mouse model. Combination assays of ABT263 with cytostatics that are regularly used in neuroblastoma patients revealed strong synergistic effects.
Validation of BIRC6 in chapter 5 gave us potential future opportunities for therapy, since knockdown of this gene resulted in increased DIABLO protein levels, inducing apoptosis. However, compounds directly targeting BIRC6 are currently not yet available.

Both YM155 and ABT263 are currently under evaluation for Phase I/II clinical trial in neuroblastoma patients with a relapse.
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