New therapeutic targets in the intrinsic apoptotic pathway in neuroblastoma
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Summary and Discussion
Summary of research

In this thesis we have focused on the systematic validation of potential drug targets in the intrinsic apoptotic pathway in neuroblastoma. For targeted drug development we follow a stepwise procedure. First a potential drug target gene is identified based on genomic and expressional aberrations. Subsequently, the target is validated by silencing and over-expression experiments. Finally, a clinically applicable compound can be tested for in vitro efficacy and in vivo efficacy. Targets and targeted compounds that show consistent results and a favorable phenotype (e.g. apoptosis or growth inhibition) in all these steps can be used for further development towards a Phase I/II clinical trial.

In chapter 2 we showed that Survivin (BIRC5) was highly expressed in neuroblastoma, which correlated to a poor prognosis. We functionally validated BIRC5 by targeted silencing using lentiviral shRNA. BIRC5 knockdown induced apoptosis in neuroblastoma cells via mitotic catastrophe, indicating that BIRC5 may indeed be a viable target for therapy.

In chapter 3 we therefore tested the efficacy of YM155, a novel small molecule BIRC5 inhibitor\(^1\)-\(^5\), which induced apoptosis in a large subset of neuroblastoma cell lines as a result of specific BIRC5 inhibition. However, we also identified a subgroup of cell lines to be resistant to YM155, even though these cell lines were sensitive to lentiviral BIRC5 knockdown. Resistance to YM155 was strongly related to \(ABCB1\) over-expression. \(ABCB1\) is a protein that functions as a multidrug resistance pump\(^6\)-\(^8\) and we were able to sensitize resistant cell lines to YM155 by pre-treatment with cyclosporine, a known \(ABCB1\) inhibitor.\(^7\),\(^9\)

In chapter 4 we identified a high expression of \(BCL2\) in neuroblastoma. \(BCL2\) knockdown induced apoptosis in neuroblastoma cells with high \(BCL2\) expression, but not in neuroblastoma cells without \(BCL2\) expression or in normal fibroblasts. We validated ABT263, a small molecule \(BCL2\) inhibitor\(^10\)-\(^13\), which showed the same \(BCL2\)-dependent efficacy in neuroblastoma cells compared to \(BCL2\) shRNA. ABT263 was also very effective in our neuroblastoma xenograft model and worked synergistically with doxorubicin, etoposide and vincristin in vitro.

Finally, in chapter 5, we investigated the intrinsic apoptotic genes as a group and concluded that BIRC5, BiRC6 and BCL2 are the most promising drug targets in the
intrinsic apoptotic pathway in neuroblastoma. Since BIRC5 and BCL2 were already validated in the previous chapters, we investigated the potency of BIRC6 as a drug target. BIRC6 is highly expressed in neuroblastoma and knockdown induced apoptosis. Functional analysis indicated that BIRC6 functions in neuroblastoma cells as an inhibitor of apoptosis protein in the cytoplasm where it can bind and degrade DIABLO. We hypothesized that BIRC6 is a viable target for neuroblastoma therapy especially in combination with ABT263. However, no clinically applicable BIRC6 inhibitor is currently available.

Discussion

Clinical implementation of YM155 in neuroblastoma treatment
Phase I/II clinical trials with YM155 show promising results. Also in vitro and in vivo BIRC5 knock-down experiments and experiments with BIRC5 inhibitors have shown consistent apoptotic responses. The next step is in vivo testing of the efficacy and pharmacokinetics of YM155. Preliminary data showed that this compound is very effective in a neuroblastoma xenograft mouse model and therefore we aim at a Phase I/II clinical trial in pediatric neuroblastoma. A preclinical evaluation of patient selection biomarkers and biomarkers for efficacy is needed.

Almost all high risk neuroblastoma tumors present with gain of 17q and high BIRC5 levels. Therefore, neither BIRC5 DNA copy number nor mRNA expression are adequate biomarkers for neuroblastoma patient selection. However, we showed in chapter 3 that ABCB1 can induce resistance to YM155. ABCB1 could be a biomarker for patient selection if expression can be detected in tumor material before starting YM155 treatment. ABCB1 detection methods such as qPCR or immunohistochemistry have to be developed and optimized for diagnostics. Because BIRC5 expression is inhibited by YM155, BIRC5 mRNA and protein expression are potential biomarkers for efficacy. Therefore multiple tumor biopsies are required during treatment for detection of BIRC5 inhibition. BIRC5 levels could be measured using Western blot or immunohistochemistry on tumor biopsies. Alternatively, analysis in surrogate tissue (hair) could be used to prevent multiple biopsies.

New compounds can be implemented in therapy either as a single compound or in combination with currently used cytostatics if there is rationale for combined treatment. It is therefore necessary to perform in vitro and in vivo combination assays especially with compounds that are used in current treatment protocols. In addition
to the cytostatics that are currently used, new compounds could be tested for synergy. In chapter 2 we have provided evidence for BIRC5 as a microtubule stabilizing protein during cell division, which could guide combinatorial treatment of a BIRC5 inhibitor with other compounds. Most interestingly, preliminary data showed that ABT263 and YM155 combined are synergistic in neuroblastoma cell lines with high BCL2 expression (data not shown). Mitotic catastrophe induced apoptosis by YM155 activates apoptosis by Cytochrome C release from the mitochondria. This can be blocked by high BCL2 levels. It would be of major interest to test this combination in an in vivo assay as well.

We have shown that ABCB1 induced resistance to YM155 in neuroblastoma cells. We were able to resensitize cell lines with high ABCB1 expression with cyclosporine. However, cyclosporine concentrations needed for this application in patients were toxic in combination treatment, presumably because cyclosporine induced sensitization of the bone marrow to chemotherapy. Next-generation ABCB1 inhibitors are currently in clinical trial. PSC833 is a non-immunosuppressant derivative ABCB1 inhibitor with promising in vitro and in vivo results. Clinical trials with PSC833 show mixed results. In patients with Acute Myeloid Leukemia (AML) PSC833 did not improve outcome, however some patients with pediatric acute leukemia showed complete remission or a partial response. In vitro testing of this compound in neuroblastoma cells is currently ongoing.

Clinical implementation of ABT263 in neuroblastoma treatment

ABT263, a small molecule BCL2 inhibitor, was also validated as a potential target for intervention in neuroblastoma. Targeted BCL2 inhibition has been investigated in a neuroblastoma model earlier by Lestini et al. They concluded that BCL2 knockdown in neuroblastoma cell lines did not induce apoptosis. Our BCL2 knockdown experiments are in line with their results, as SKNAS was resistant to knockdown or inhibition of BCL2. However, they concluded that this cell line is resistant to BCL2 knockdown, possibly because of a high MCL1 expression, whereas we concluded that the resistance in SKNAS results from the absence of BCL2 expression. Our studies show that only two neuroblastoma cell lines have BCL2 and MCL1 expression levels comparable to neuroblastoma tumors. Both cell lines were very sensitive to BCL2 shRNA. Therefore we concluded, unlike Lestini et al, that BCL2 alone can be a good target for neuroblastoma therapy.

We are currently further validating ABT263 in vivo. We have shown that this com-
pound delays tumor growth, but we also have to demonstrate growth inhibition in more established tumors. In addition, we will verify if the synergy that we detected in vitro between ABT263 and commonly used cytostatics can be confirmed in vivo. Since new compounds in Phase I/II clinical trial will be implemented in relapse patients first, in vivo synergy assays will include cytostatics that are currently used in these patients, such as etoposide, topotecan and temozolomide.

In parallel, we will start collaborations with Abbott to explore the possibilities for a combined Phase I/II clinical trial for ABT263 in neuroblastoma. Since neuroblastoma with low BCL2 expression will probably not respond to treatment with this compound, an assay to predict sensitivity based on BCL2 expression levels has to be optimized for diagnostics. In chapter 4 we showed that BCL2 RNA levels showed a very strong correlation with BCL2 protein expression. This suggests that BCL2 protein levels could be used as a biomarker for patient selection. Goldsmith et al\textsuperscript{19} previously published an assay to predict sensitivity of cell lines to AT-101 and ABT-737. BCL2 and NOXA were elevated in ABT263 sensitive cell lines, whereas \textit{MCL1} expression was higher in resistant cell lines.\textsuperscript{20} However it is impossible to use this assay in a clinical setting since viable cells are needed. We conclude that BCL2 protein expression analysis has to be optimized as a patient selection biomarker. Patients with low BCL2 expression levels should be excluded from the ABT263 trial.

\textit{BIRC6 as a potential drug target}

In chapter 5 we validated BIRC6 as a potential new drug target for neuroblastoma treatment. We observed that knockdown of BIRC6 induced apoptosis and we showed that BIRC6 is functional as an IAP in the cytoplasm in neuroblastoma cells.\textsuperscript{21,22} We have shown that neuroblastoma have extensively increased levels of DIABLO in the mitochondria. BIRC6 seems crucial in this specific setting where DIABLO in the cytoplasm has to be degraded very effectively by BIRC6 to prevent an apoptotic response. We hypothesize that cells with high levels of DIABLO are therefore extremely sensitive for BIRC6 inhibition. Targeted inhibition of BIRC6 using an inhibiting compound is therefore a potential new intervention that could specifically affect neuroblastoma cells and not other cells. However, a BIRC6 inhibitor is currently not available. Alternatively BIRC6 could be inhibited using bortezomib, a proteasome inhibitor that may inhibit the DIABLO degradation by BIRC6. This compound can indeed induce apoptosis in neuroblastoma cell lines.\textsuperscript{23,24}
Lack of mutations in the intrinsic apoptotic pathway in neuroblastoma

Activating or inactivating mutations in the intrinsic apoptotic pathway are rare in primary neuroblastoma tumors. Recent analysis of 87 primary neuroblastoma in our lab using whole genome sequencing by Complete Genomics\textsuperscript{25,26}, also revealed an absence of somatic events in the coding sequence of these genes. Therefore we concluded that inhibition of the intrinsic apoptotic pathway results from upstream transcriptional deregulation, epigenetic events and/or copy number alterations as a result of gains or losses of larger chromosomal regions. The latter point is analyzed in chapter 5 using CGH analysis and SNP arrays. Apoptosis does occur in untreated neuroblastoma as is clearly visible on cleaved caspase 3 stainings of neuroblastoma tumors. This is however fully compensated by the extensive tumor growth. We conclude that apoptosis is not completely inactivated in neuroblastoma, but it is rather unbalanced by a combination of events that lead to a change in expression of genes.

The extrinsic apoptotic pathway

This thesis focuses on the intrinsic apoptotic pathway and we validated two clinically available inhibitors as potential drugs in neuroblastoma treatment. However, the extrinsic apoptotic pathway has also been studied in neuroblastoma. \textit{CASP8} is hypermethylated and thereby inactive in some neuroblastoma resulting in an inactive extrinsic apoptotic pathway.\textsuperscript{27,28} Also the NF-κB pathway, which has many target genes that are involved in cell death and survival, has been widely investigated.\textsuperscript{29-32} Combinations of drugs targeting both the intrinsic and extrinsic apoptotic pathway should be explored, since this may lead to a synergistic effect.
References


