Aberrant DNA hypermethylation and apoptotic defects in pediatric neuroblastomas
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Chapter 6

General discussion and future directions
Summary

Pediatric neuroblastomas are important contributors to cancer-related mortality in children. Neuroblastomas are malignant tumors of the sympathetic nervous system and adrenal medulla. The clinical spectrum and outcome are variable. Approximately half of all neuroblastoma patients suffer from high-risk neuroblastoma disease with bulky tumors and/or distant metastases at diagnosis. The outcome in this group is poor, 20-30% 5 years event-free survival (EFS) despite the use of intensive and multi-modality anti-tumor therapy, high-dose chemotherapy with peripheral blood stem cell rescue. However, there is also a group of neuroblastoma patients with limited disease that have an acceptable to favorable prognosis of 70-95% 5 yrs EFS and even patients with spontaneous tumor regression without treatment. This variable clinical spectrum correlates with variable genetic and molecular findings in each clinical group. It supports the idea that the underlying biology of neuroblastomas is not uniform, but includes several different biological entities. Neuroblastomas should therefore better be regarded as a group of malignant diseases, originating from the sympatho-adrenal lineage.

At the time when this thesis was undertaken, there was a growing interest in the role of regional methylation of DNA and the silencing of tumor suppressor genes in (adult) cancer. A stream of data supported the idea that DNA methylation (the addition of a methyl group on the C5 position of cytosines) of cytosine- and guanine-rich areas in gene promoters (CpG islands) was involved in the silencing of these genes. It was evident from the beginning that this regional hypermethylation of gene promoters was especially found in cancer cells or other pathological cells, but not in normal tissue cells. The availability of demethylating agents, which could demethylate such gene promoters in vitro and restore the gene-expression and -function excited the field of cancer biology. It held the promise that demethylating agents could be used in vivo to restore tumor suppressor activity and be used in the treatment of cancer. In neuroblastomas, little was known about a possible role of changes in methylation in the biology of the tumor cell. At that time, the few available data suggested that loss of chromosome 1p36, a common genetic defect in neuroblastoma cells, induced methylation changes of the chromosomal region of HLA-C leading to the loss of HLA class I, a common defect in aggressive neuroblastomas and other tumors (R. Versteeg, unpublished data). This thesis originally intended to follow up on these data, but immediately shifted attention towards aberrant methylation of candidate tumor suppressor genes in neuroblastomas. In brief, chapter 1 of this thesis reviews the common genetic and molecular defects in neuroblastomas, and attempts to correlate these to the variable clinical entities. Chapter 2 describes the aberrant methylation pattern of 4 members of the family of Tumor Necrosis Factor Receptors (TNFR), namely Death Receptor 4 (DR4), Death Receptor 5 (DR5), Decoy Receptor 1 (DcR1) and Decoy Receptor 2 (DcR2). In chapter 3, we focused on the influence of overexpression of the oncogene MYCN on both caspase 8- and caspase 9 dependent apoptosis and gene methylation of several genes of the apoptotic pathways. Chapter 4 shows that in a large group of candidate tumor-
suppressor-genes methylation seems to cluster in specific chromosomal regions. Chapter 5 describes the serendipitous finding that differential methylation of the tissue-specific gene Death Receptor 3 (DR3) is associated with its specific expression pattern in adult tissues. Furthermore, the data suggest a role of aberrant methylation in the genesis of pediatric Burkitt's lymphoma. These results and future directions for research and possible new avenues for targeted neuroblastoma treatment are summarized and discussed below.

Chapter 1: Introduction into the biology of neuroblastoma. The first part aims to integrate the most important genetic alterations into the different clinical stages and prognoses. For instance hyperdiploid/near-diploid tumor cell genomes are strongly associated with low risk disease, stage 1-2 and a favorable prognoses. Diploid and tetraploid genomes are frequently associated with high-risk disease as seen in clinical stage 3-4. The latter group also exhibits frequent genetic alterations, such as amplification of the oncogene MYCN, loss of heterozygosity (LOH) of chromosome 1p36, gain of chromosome 17q, t(1;17) and LOH of several other chromosomal regions. The second part describes the alterations in biological pathways in neuroblastomas and aims to correlate this to the earlier described genetic/clinical subgroups. Multiple different aberrations in neuroblastomas (including the research described in this thesis) lead to defects in apoptosis. A model surfaces in which MYCN amplification acts as an enhancer of both cell growth and apoptosis. Additional defects in the apoptotic pathways then shift the balance towards increased cell growth and a cancerous phenotype.

Chapter 2: Neuroblastomas are characterized by defects in TRAIL (Tumor Necrosis Factor-Related Apoptosis Inducing Ligand) induced apoptosis. This strongly contrasts with the increased TRAIL sensitivity of most other tumor types. We addressed the question of the TRAIL sensitivity by studying the tumor-specific down-regulation of the TRAIL receptor genes DcR1 and DcR2, as well as DR4 and DR5 in a group of neuroblastoma and control tumor cell lines. Lack of expression of DcR1 and DcR2 mRNA was widespread, both in the adult tumor cell lines as well as in the pediatric tumor lines. DR4 and DR5 were expressed in the majority of cell lines. Promoter methylation analysis of these four receptor genes showed that they were completely unmethylated in normal tissues, but frequently hypermethylated in tumor cell lines. For DcR1 and DcR2, hypermethylation was found in 69% and 90% of non-expressing cell lines, respectively. For DR4 and DR5 hypermethylation was found in ~70% non-expressing cell lines. Demethylation assays resulted in partial demethylation and restored mRNA expression. Mutation analysis excluded mutations in the DR4 or DR5 genes in any of the cell lines. In fresh neuroblastomas, down-regulation of DcR1 and DcR2 was found in ~80% and hypermethylation was observed in 21-25%. DR4 and DR5 were both expressed in 80% of tumors, and no promoter methylation was observed. We concluded that hypermethylation of the promoters of DcR1 and DcR2, but not DR4 and DR5 is important in the down-regulation of expression in neuroblastomas.
Chapter 3: Amplification of MYCN in neuroblastomas is strongly associated with both unresponsiveness to TRAIL induced apoptosis and down-regulation and methylation of CASP8. However, MYCN has also been implicated in induction of apoptosis, especially activation of CASP9 mediated apoptosis. We found that ectopic MYCN expression induces TRAIL susceptibility, both by CASP8 and CASP9 mediated apoptosis in neuroblastoma cell lines. MYCN did not modify CASP8 expression and methylation. We concluded that CASP8 defects therefore represent an independent event in neuroblastoma, counteracting the MYCN induced susceptibility to apoptosis. The analysis of the CASP9 mediated route showed normal expression and no aberrant methylation of four apoptotic intermediates, including CASP9 itself.

Chapter 4: It describes the methylation pattern in 22 neuroblastoma cell lines of 34 genes on 12 different chromosomal regions, including 18 genes on chromosome 1p36, a region commonly deleted in neuroblastomas. Besides the earlier described methylation of the TRAIL receptors, FLIP at 2q33 was additionally methylated in 8/22 and CASP8 in 20/22 cell lines. The 6 genes methylated in neuroblastomas appeared to occur in pairs: FLIP and CASP8 are strongly homologous genes and map both to chromosome 2q33. The 4 TRAIL receptor genes are also strongly related and map as a group to chromosome 8p21. Not only were the methylation patterns of the grouped genes very similar, but interestingly, the expression pattern of FLIP and CASP8 was almost identical and similarly, the mRNA expression of DcR1 and 2 was almost identical. We found no evidence for regional spreading of methylation, as we did not observe de novo methylation in additional local CpG islands. The data suggested a mechanism of co-regulated transcriptional silencing of the gene pairs, followed by a methylation event that is less penetrating. It supports a model in which CpG islands are not randomly targeted by methylation in neuroblastoma.

Chapter 5: Describes a serendipitous finding concerning the DR3 gene on chromosome 1p36. DR3 is a member of the TNFR (Tumor Necrosis Factor Receptor) Super Family of pro-apoptotic receptors and related to DR4 and DR5. DR3 is a tissue-specific gene since it is only expressed in normal peripheral blood leukocytes, and not in any other tissue. We showed that DR3 is unmethylated in normal leukocytes, which express the gene. However, in other non-expressing tissues we found consistent hypermethylation ranging from 50-100% per tissue sample. This was even more pronounced in tumor cell lines, in which the methylation pattern of the cell lines reflected the situation in normal tissues: hematological malignancies were almost invariably unmethylated, whereas cell lines from different solid tumors were all completely methylated. In tissues from human embryos between 3-9 weeks post conception, we observed a pattern of undermethylation as compared to adult tissues, suggesting that methylation patterns are not fully established at 9 weeks. Interestingly, frequent methylation was not found in common haematological malignancies, such as ALL and AML, but was clearly present in a significant number of non-Hodgkin's lymphomas, especially pediatric Burkitt's lymphomas. The data suggest a possible role for loss of DR3 in the genesis of non-Hodgkin's lymphomas or pediatric Burkitt's lymphomas.
Discussion

The data described here and elsewhere\(^1\) indicate that multiple genes of the apoptotic pathways are aberrantly methylated in neuroblastoma. This is mostly seen in high-risk neuroblastomas and is associated with over-expression of the oncogene MYCN. The emerging phenotype is one of loss of signaling through the so-called 'extrinsic', caspase 8-dependent pathway to apoptosis in combination with overexpression of MYCN. This is a familiar theme in cancer biology, since several cancer types are characterized by apoptotic defects and overexpression of an oncogene. The presumed mechanism is that overexpression of MYCN increases both the potential for growth as well as apoptosis. In combination with apoptotic defects the balance is disrupted and tips over towards cell growth and proliferation, leading to a cancerous phenotype (Fig. 1).

![Figure 1. MYCN increases both proliferation and apoptosis](image)

However, the increased sensitivity for apoptosis induced by MYCN protein is induced through the mitochondria as part of the so-called 'intrinsic' apoptotic pathway. In the mitochondria several pro- and anti-apoptotic proteins from the Bcl-2 protein family reside in a delicate balance. Upon apoptotic signaling, the balance can shift and increases the activation of the pro-apoptotic proteins. This will induce the release of several proteins into the cytosol, such as cytochrome C and other pro-apoptotic factors. Cytochrome C will form a complex with APAF-1 and pro-caspase 9. The complex then activates caspase 9, which can activate the effector caspases 3, 6 and 7 (Fig. 2). Therefore, MYCN activation will lead to caspase 9 dependent apoptosis. However, this general model of apoptosis-induction by MYCN does not explain why defects in the caspase 8 dependent pathway in neuroblastomas lead to a reduction of the MYCN-induced apoptosis. To understand this, two additional factors should be considered. First, in neuroblastoma cell lines we showed that MYCN overexpression stimulated both the caspase 8 and caspase 9 dependent apoptotic pathway (chapter 3). Second, it has often been suggested that there is probably a considerable cross talk between the two different pathways. The intrinsic and extrinsic apoptosis signaling pathways communicate with each other. Caspase-8 has been shown to cleave the pro-apoptotic Bcl-2 family member Bid. The cleavage of BID by caspase-8 and the translocation of truncated Bid to the mitochondria to promote cytochrome c release through interaction with BAX and BAK provide a plausible mechanistic link between the extrinsic and intrinsic pathways\(^2\) (Fig. 2). This
apparently amplifies the apoptotic signal following death receptor activation, and different cell types may be more reliant on this amplification pathway than others\(^3\). This would explain why defects in one route induce a considerable down-regulation of the total apoptotic potential.

An additional aspect of the association of MYCN overexpression and the loss of especially caspase 8 is the suggestion that MYCN might induce the caspase 8 defect. We were able to show in chapter 3 that silencing or methylation of CASP8 was never caused by MYCN overexpression. Therefore, MYCN amplification and methylation of CASP8 should be considered as two separate events in tumor cell progression.

Our observation that multiple genes can be aberrantly methylated in the same tumor is not unique (chapters 2 and 4). In fact, most studies have indicated that aberrant methylation follows a cancer type-specific methylation pattern, involving several genes per cancer type. Chapter 4 renders support for this tumor type-specific methylation. In a group of 22 neuroblastoma cell lines, only 6 of 34 genes on 12 different chromosomal regions tested for hypermethylation were methylated. 28 genes were never methylated in any of the cell lines. The 6 hypermethylated genes belonged to two groups of related genes and co-localized per group on chromosomes 2q33 and 8p21.
respectively. It has often been suggested that methylation can spread from one methylated hotspot to surrounding chromosomal regions or CpG islands. Although the co-localization of the methylated genes seemed to support this hypothesis, further analysis showed that additional de novo methylation had not occurred in these regions and therefore it is unlikely that the observed pattern was the effect of loco-regional spreading of methylation. Also, down-regulation of the genes did not always involve methylation of the promoter. We concluded that structurally related and neighboring genes can be subjected to gene silencing by regulatory regions or mechanisms that seemed to precede the methylation. An additional aspect of the awareness that multiple genes can be subjected to aberrant methylation is that we may not know all the hypermethylated genes in neuroblastomas. The only additional gene reported is hypermethylation of RASSF1A, a RAS-family related gene, hypermethylated and silenced in many different tumor types (see chapter 1). It will therefore be important to conduct genome-wide methylation assays and correlate the outcome with expression of the genes and the patient’s prognosis. It is expected that such an approach will lead to the uncovering of additional genes and pathways involved in the etiology of neuroblastomas. Tissue-specific methylation has been a controversial topic for many decades. The initial observation in the gamma-globin gene and others that silencing was associated with hypermethylation first stirred the opinions. However, these observations were done in CpG poor promoters and exons and the role of their methylation in relation to transcriptional regulation was unclear. Additional testing showed that demethylation of the sequences did not alter the expression of the complete gene. Therefore, the remaining opinion is mainly skeptical towards methylation as a mechanism for tissue-specific gene silencing. However in mice, examples of tissue-specific methylation of CpG rich promoter areas (CpG islands) were shown for the HOXA5 and Galectin-1 gene. In chapter 5, we described a similar finding for the human DR3 gene, coding for the pro-apoptotic receptor expressed in normal leukocytes. It suggests that the expression of a selected group of genes with tissue-specific expression can in fact be guided by methylation. The expression of a lineage specific gene is considered to have great relevance for the involved tissue. Therefore, silencing of this gene in lineage-specific cancer cells probably strongly bears on the phenotype of the aberrant cell. It is likely that such a gene acts as a lineage-specific tumor suppressor gene. We therefore considered it highly significant that DR3 was methylated in multiple non-Hodgkin’s lymphoma (NHL) samples, as opposed to other hematological malignancies like ALL and AML (chapter 5). The DR3 protein is involved in the negative selection of thymocytes, and silencing of the DR3 gene could therefore very well increase the cell survival of hematological precursor cells. Further studies are warranted to better understand the physiological role of DR3 in leukocytes and the prognostic relevance of hypermethylated DR3 in NHL.

Future Directions
The majority of high-risk neuroblastomas have proven to be resistant to conventional anti-tumor
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therapies, even when applied in high dose or intensive combinations. There is a strong need for novel strategies in the treatment of this disease. Future research in neuroblastomas should therefore not only be aimed at a better understanding of the biology, but more specifically at understanding those defects or mechanisms which could serve as potential targets for therapy. The research described in chapters 2-4 connects with two novel avenues in neuroblastoma research, which carry the possibilities for new treatments. Firstly, epigenetic changes, such as promoter hypermethylation are highly tumor specific and the methylation is potentially reversible by demethylating agents. Secondly, the apoptotic defects in neuroblastomas suggest that neuroblastoma treatment could benefit from strategies to repair or substitute apoptosis. Of particular interest is the observation that the epigenetic defects in neuroblastomas overlap with the apoptotic defects, which suggests the possibility to combine the different approaches for treatment purposes. Possible new strategies include:

1. Demethylating treatment

DNA cytosine methylation takes part in the cascade of heterochromatin formation and gene inactivation. DNA methylation can inhibit transcription directly by hindering transcriptional activators, or by the attraction of transcriptional repressor protein complexes such as methyl-DNA binding proteins (MBD’s like MeCP2 and MBD2), which eventually recruit other enzymes like histone deacetylases (HDAC). These HDACs remove acetyl groups from lysine residues of histone H3 and H4 and attract repressor proteins like ISWI ATPase and initiate chromatin condensation. DNA methylation and histone deacetylation, especially of lysine 9 on histone H3 (H3K9) are strongly connected to each other and are both inducers of gene silencing (fig 3).

DNA methylation can be reversed by demethylating agents, such as the analogues of cytosine, 5-aza-cytidine and the more potent 5-aza-2'-deoxycytidine (decitabine). Reactivation of silenced genes by using these compounds was shown in vitro for many different situations, for instance in the hypermethylated CGG expanded repeats in the fragile X syndrome, in the silenced copy of the SNRPN and neighboring genes in cell lines of Prader-Willi patients, but also in many aberrantly hypermethylated genes in different cancer types. In neuroblastoma cell lines, we showed in chapter 2 that gene silencing by DNA methylation of Dcr1, 2 and DR4 and 5 was reversible by decitabine. Clinical testing of these agents already started in the 1970s, since they were originally synthesized as cytosine analogues. A large body of clinical trials particularly with 5-azacytidine in acute lymphoblastic leukemia showed effectiveness that was comparable to equitoxic doses of cytarabine. However, a revival in the interest for these agents for their demethylating effect has recently started. Clinically significant results were obtained in myelodysplastic syndrome (MDS), acute myeloid leukemia (AML) and chronic myeloid leukemia (CML). Demethylation of the tumor suppressor gene p15 was observed in several patients and correlated with clinical response. Vice versa, the increase in methylation of p15 in one patient correlated with disease progression. Interestingly, dose de-
Figure 3. Chromatin remodeling. Flowchart of interactions between histone acetylation, histone methylation, and DNA methylation. HMT: histone methyltransferases, HP1: heterochromatin protein 1, DNMT: DNA methyltransferases, MBD: Methyl-binding domain proteins, HDAC: histone deacetylases, HAT: histone acetyltransferases (adapted from ref 5).

Escalating testing showed that decitabine was most effective in the lower or middle doses, with low or minimal non-hematologic toxicity. Although these early clinical trials are promising for future testing in many different tumor types, several key questions remain. Important is to evaluate which gene or genes can be reactivated in tumors with a ‘methylation phenotype’ (defined by the presence of one or more methylated genes). Equally important will be to investigate if the presence of methylated genes is predictive of a clinical response to demethylating agents. Very little, if anything is known about the response to these types of agents in pediatric cancer patients. An important issue to address in this age group will be influence of demethylating agents on imprinted (hemi-methylated) genes or loci. Several imprinted genes are important in growth and development and loss of imprint (LOI) is associated with the development of cancer, especially in the pediatric age group. For instance, LOI of chromosome 11p15 and overexpression of the imprinted IGF2 gene (Insulin Growth Factor-2) is associated with overgrowth syndromes like Beckwith-Wiedemann syndrome, the development of nephroblastoma (Wilms’ tumor) and other pediatric malignancies. It has been shown that fewer than 1% of 10,000 genes increase their expression level in response to decitabine treatment\(^9\), suggesting that only aberrantly methylated genes respond to demethylating agents. However, the response of imprinted genes to demethylating treatment has not been studied in detail and should be considered carefully in the pediatric age group.
2. HDAC inhibitors

Histone tails can be acetylated by histone acetyltransferases (HATs), and acetylated histones are usually associated with transcriptionally active chromatin. Vice versa, removing acetyl groups from histones, especially of the H3K9 (lysine residue 9 on the tail of the histone 3 protein) is essential in the formation of heterochromatin and silencing of genes. The deacetylation of histone proteins by histone deacetylase enzymes (HDACs) can pharmacologically be inhibited (fig 3). Several HDAC inhibitors have been identified and tested in vitro, including trichostatin (TSA), sodium phenyl butyrate (SPB), 4-phenylbutyrate (4-PBA), procainamide, and suberoylanilide hydroxamic acid (SAHA) for their effect on gene transcription reactivation. An important study by Cameron et al. showed a synergistic effect of histone deacetylase inhibition and demethylation. TSA and decitabine were both able to reverse gene silencing of several hypermethylated genes in colon cancer cell lines. However, the combination of the two agents showed a much more significant demethylation and reactivation of the genes. This synergistic effect of demethylating agents and HDAC inhibitors was confirmed in further studies. Clinical testing of HDAC inhibitors was first applied in acute promyelocytic leukemia (APL), a disease characterized by the inactivation of the retinoic acid receptor-beta (RARβ) by a HDAC repressive complex. The APL specific fusion protein PML/RARα has the ability to suppress RARβ by recruitment of a nuclear co-repressor -histone deacetylase (NCOR/HD) complex, resulting in local chromatin remodeling. This silencing of RARβ can be reversed by SPB in a transgenic mouse model and in clinical studies with refractory APL patients. Currently a wide range of different HDAC inhibitors alone or in combination with demethylating agents are in pre-clinical and clinical phase I/II trials, and although the effectiveness has not been established so far, toxicity seems to be very limited, without unexpected toxicity reported. It should also be noted that these drugs have a strong anti-proliferative effect and induce differentiation. In neuroblastoma cell lines and xenografts, the HDAC inhibitor m-carboxycinnamic acid bis-hydroxamide (CBHA) was able to induce apoptosis and had a synergistic effect with All-Trans retinoic acid treatment in inducing apoptosis and differentiation.

3. TRAIL/Apo2L

Most tumors can be characterized by an increased sensitivity for the apoptosis inducing ligand TRAIL/Apo2L, compared to normal human tissues. TRAIL has therefore gained interest as a possible anti-tumor agent that can selectively induce apoptosis in tumor cells, but not in normal tissues. Apoptosis induction in response to most DNA-damaging drugs usually requires the function of the tumor suppressor p53, which engages primarily the intrinsic apoptotic-signaling pathway. TRAIL induces apoptosis through the extrinsic, p53-independent pathway and can induce apoptosis in a variety of cancer cell lines regardless of p53 status, thus helping to circumvent resistance to chemo- and radiotherapy. The combination of TRAIL with chemotherapeutic agents has indeed been found to be particularly effective in killing cancers, but most abundantly in tumors with wild-
type p53\textsuperscript{21}. Importantly, administration of soluble recombinant TRAIL in experimental animals, including mice and nonhuman primates, induces significant tumor regression without systemic toxicity\textsuperscript{22, 23}. However, an alarming finding showed that normal human hepatocytes were also efficiently killed by TRAIL\textsuperscript{24}, leading to liver necrosis. This finding was similar to earlier reports on hepatotoxicity when using FasL as an anti-tumor agent. This serious cytotoxicity has prevented phase I/II studies with recombinant TRAIL so far. Attempts to circumvent the problem have led to shifting from recombinant TRAIL to gene therapy approaches with TRAIL. A TRAIL-expressing adenoviral vector has been recently shown to cause direct tumor cell killing, as well as a potent bystander effect through prolonged presentation of TRAIL by transduced normal cells\textsuperscript{25}. In combination with a tumor-specific promoter this approach could potentially avoid severe toxicity. The human telomerase reverse transcriptase (hTERT) gene is active in more than 85% of human cancers, but quiescent in most somatic cells. Lin et al. demonstrated that an adenoviral vector expressing the GFP/TRAIL fusion gene from the hTERT promoter elicited high levels of transgene expression and apoptosis in a variety of breast cancer cell lines. Furthermore, treatment with Ad/gTRAIL effectively induced apoptosis in malignant cells but not in normal human primary hepatocytes \textit{in vitro}, suppressed tumor growth and prolonged duration of survival \textit{in vivo}\textsuperscript{26}. Although these are promising therapies, they largely depend on the efficient infection of the tumor and avoidance of immune clearance to be effective. Toxicity to human hepatocytes is still present in adenoviral vectors with TRAIL and clinical use of adenoviral vectors appears to be limited to local therapy or controlled by a tumor-specific promoter (hTERT) to avoid severe liver damage\textsuperscript{27, 28}. An important factor to consider in neuroblastomas is that the TRAIL-induced route to apoptosis is defective in the majority of tumors, as explained in chapters 3 and 4. TRAIL treatment for neuroblastomas would therefore be dependent on concurrent strategies to circumvent or repair apoptotic defects, e.g. in combination with demethylating agents or HDAC inhibitors\textsuperscript{29}.

4. Intrinsic pathway to apoptosis
Enhancement of the mitochondrial pathways to apoptosis offers immediate ways of increasing apoptosis and possibilities to circumvent defects in the extrinsic apoptotic pathway. Up-regulation of Bcl\textsubscript{2} has been found in neuroblastomas and correlated with a decreased prognosis (see chapter 1). Antisense molecules to Bcl\textsubscript{2} are now in phase III clinical trials. Pre-clinical studies demonstrated that the antisense Bcl\textsubscript{2} oligonucleotide alone was superior to standard chemotherapy for malignant melanoma cells and enhanced apoptosis in other tumor models when used in combination with chemotherapy\textsuperscript{30}. Another approach to enhance the mitochondrial death pathway is to increase the expression of APAF-1, which could be achieved by the demethylating agent 5-aza-2-deoxycytidine, which suggests that the expression of APAF-1 is under the epigenetic control of a trans-acting factor\textsuperscript{31}. Since XIAP blocks apoptosis at the effector phase, a point where multiple signaling pathways converge, strategies targeting XIAP may also prove effective to overcome
resistance. Smac was identified as a protein that is released from mitochondria in response to apoptotic stimuli, and it has been reported that Smac agonists sensitize TRAIL-induced apoptosis and induce the regression of malignant glioma \textit{in vivo}\textsuperscript{32}.

**Conclusions**

One of many features characteristic of neuroblastomas is the silencing of genes as a result of DNA hypermethylation and the subsequent loss of apoptotic potential. Novel strategies are being conducted to repair or circumvent these tumorigenic events. DNA silencing by methylation and the formation of heterochromatin can be reversed by demethylating agents and other chromatin remodeling agents, particularly HDAC inhibitors. This holds the promise of new treatments based on the reversal of silenced genes, such as \textit{CASP8} and other intermediates of the apoptotic cascade. A second promising future application of new drugs could be through enhanced signaling of the intrinsic, mitochondria-dependent route to apoptosis. Possible avenues include silencing of the anti-apoptotic protein Bcl2 by antisense-Bcl2 or siRNA or circumventing the mitochondria completely by administration of the post-mitochondrial pro-apoptotic Smac protein. Future testing in pre-clinical and clinical trials will teach the value of these new agents, alone or as a combination for a group of vulnerable neuroblastoma patients.
References


