

REVIEW ARTICLE

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Targeting of epigenetic regulators in neuroblastoma

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Abstract

Approximately 15,000 new cases of pediatric cancer are diagnosed yearly in Europe, with 8–10% corresponding to neuroblastoma, a rare disease with an incidence of 8–9 cases per million children <15 years of age. Although the survival rate for low-risk and intermediate-risk patients is excellent, half of children with high-risk, refractory, or relapsed tumors will be cured, and two-thirds of the other half will suffer major side effects and life-long disabilities. Epigenetic therapies aimed at reversing the oncogenic alterations in chromatin structure and function are an emerging alternative against aggressive tumors that are or will become resistant to conventional treatments. This approach proposes targeting epigenetic regulators, which are proteins that are involved in the creation, detection, and interpretation of epigenetic signals, such as methylation or histone post-translational modifications. In this review, we focused on the most promising epigenetic regulators for targeting and current drugs that have already reached clinical trials.

Introduction

Cancer therapy underwent a drastic change in the 20th century. The spread of anesthesia in the 1840s eased surgical procedures and added to the introduction of radiotherapy in the early 1900's, and the discovery of chemotherapeutics during World War II caused this field to grow exponentially¹. Most of these approaches act by targeting DNA or DNA-related proteins, which produce alterations that become lethal, particularly in dividing cells. However, the efficacy of these strategies is not optimal because cancer remains one of the main causes of death in developed countries, and the toxicity and high mutagenic potential of many of these therapeutic agents render them highly uncomfortable with many undesired side effects^{2–4}. These deficiencies have prompted the search for targeted therapies that aim to inhibit elements that are involved in signaling pathways or mechanisms

that are specific to the tumor and responsible for its tumorigenic features. However, in many cases, cancer cells are able to evade the effect of a specific targeted therapy using independent mechanisms, eventually resulting in drug resistance⁵. To overcome this challenge, cancer research also focuses on multi-target therapies aimed at disrupting multiple cancer pathways with combinations of specific drugs^{6–8}.

Epigenetic therapies are an emerging option for overcoming drug resistance. This approach proposes targeting of epigenetic regulators, which are proteins involved in the creation, detection and interpretation of epigenetic signals. The term epigenetics refers to all of the chemical changes that can modulate gene expression and can be transmitted through mitosis and meiosis without altering the nucleotide sequence⁹. The main epigenetic signals are DNA methylation, histone modifications and RNA-associated silencing. These processes are responsible for the specific expression of certain sets of genes that must be transcribed at a certain dose and at a particular time. The inhibition of one epigenetic regulator could have the same effect on several cell processes as if all of these

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pathways were individually targeted with a specific drug. A further advantage of epigenetic therapies is that they act at the transcriptional level, which enables the repression of certain genes or the transcriptional reactivation of genes epigenetically silenced in cancer^{10, 11}. In the recent two decades, interest in development and validation of drugs that target epigenetic regulators has continued to increase. Selected compounds have already been approved for treatment of certain tumors, and many other compounds are currently at a pre-clinical stage or already under clinical trials^{12–17}. All of these advances render epigenetic therapies a promising alternative for cancers in which survival rates are still poor due to resistance to current treatments.

High-risk neuroblastoma is one of the malignancies that often become refractory to current therapies and for which epigenetic therapies could be useful. Neuroblastoma (NB) is an embryonal tumor of the sympathetic nervous system and is the most common extracranial solid tumor of childhood, causing 12–15% of pediatric cancer deaths in European populations. This disease appears mainly in the adrenal glands, and in advanced stages, it can disseminate to distant lymph nodes, bone, bone marrow, liver, and skin. Neuroblastoma patients are classified according to disease stage and molecular alterations into three groups: low, intermediate, and high risk. Although the first two groups show five-year survival rates greater than 90%, the survival of high-risk patients remains poor at approximately 40%. Despite aggressive treatment consisting of surgery and a combination of high-dose chemotherapy, radiotherapy and immunotherapy, the survival rate of high-risk neuroblastoma remains notably low^{18, 19}. Therefore, high-risk NB is a good candidate for epigenetic therapies to overcome drug resistance.

Currently, most epigenetic drugs act at three main levels (Fig. 1): (i) DNA methylation, which can be modulated by targeting of DNA methyltransferases (DNMT); (ii) histone modifications, such as acetylation and methylation, which can be targeted by inhibiting the enzymes responsible for these chemical changes; and (iii) blockage of the interpretation of these modifications by targeting epigenetic readers, among which proteins containing bromodomains are the most thoroughly characterized. In this review, we offer an accurate compilation of the current status of epigenetic therapy research for neuroblastoma treatment and highlight the most promising therapeutic targets and potential drugs involved in these three epigenetic levels.

DNA methylation

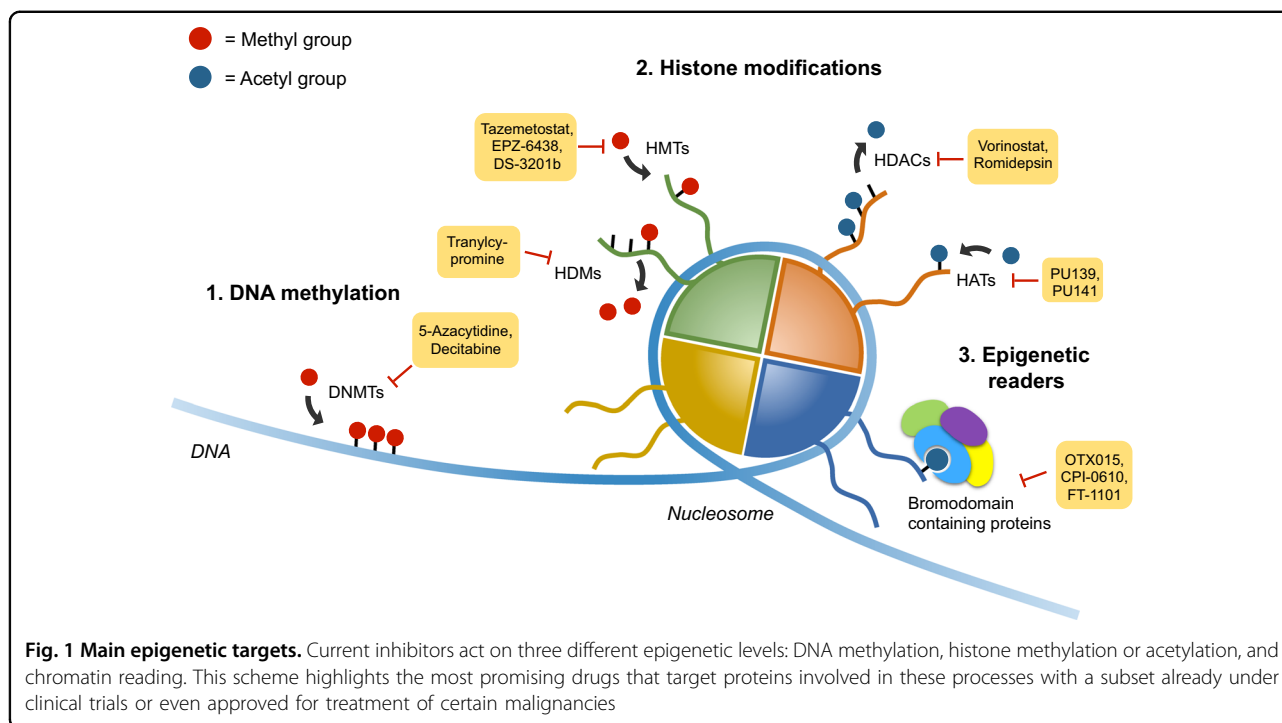
The bases of the epigenetic field were founded on the study of DNA methylation in the 1960s. DNA methylation leads to stable long-term transcription repression,

whereas unmethylated DNA tends to remain in a more relaxed structure, thereby facilitating entry of the replicative and transcription machinery²⁰. For methylation to occur, four DNA methyltransferases (DNMT1, DNMT3a, DNMT3b, and DNMT3L) exist in mammals, and the activity of these methyltransferases consists of transfer of a methyl group from S-adenosyl-L-methionine to the C5 position of cytosine residues (Fig. 2a). DNMT are capable of performing de novo methylation when the initial pattern is set during embryogenesis and perpetuating this methylation throughout the individual's life. DNMT3A and B are considered the de novo DNMT. However, DNMT1 is responsible for maintaining methylation in the daughter DNA strand during replication. Finally, DNMT3L is a related protein lacking catalytic activity that stimulates de novo methylation by DNMT3A and is required for the establishment of maternal genomic imprints (previously reviewed²¹).

The association between DNA methylation and cancer was established soon after discovery. In 1965, Craddock and Magee analyzed DNA methylation in the liver during carcinogenesis²², and one year later, Silber et al. described methylation in normal and leukemic leukocytes²³. Currently, aberrant DNA methylation patterns have been observed in many different cancers.

The expression of DNMTs have been shown to be altered in neuroblastoma. Particularly, DNMT3A/B expression was observed to be higher in high-risk NB tumors and overexpressed in cisplatin-resistant NB cells²⁴. Recently, a truncated form of DNMT3B, i.e., DNMT3B7, was identified in primary NB tumors. Interestingly, although DNMT3B expression correlates with NB with poor outcome, DNMT3B7 expression was associated with better clinical behavior. In fact, ectopic expression of DNMT3B7 in NB cells inhibited tumor growth *in vivo* by reducing cell proliferation and increasing apoptosis. Furthermore, reduced tumor vascularity was also observed. Genomic and transcriptomic analyses revealed that DNMT3B7-overexpressing cells had higher levels of genomic methylation and increased expression of genes related to the retinoic acid pathway. Consistent with these findings, treatment of DNMT3B7-overexpressing cells with all-trans retinoic acid enhanced NB differentiation²⁵. Why DNMT3A/B and DNMT3B7 have opposite roles and whether these DNMT target different genomic regions remain to be elucidated.

Nevertheless, general increased genomic methylation is associated with poor outcome in NB²⁶. Therefore, the use of DNMT inhibitors (DNMTi) might offer new alternatives for patients who do not respond to current therapies. One of the first DNMTi tested in NB cells was 5-aza-deoxycytidine (5-aza or decitabine), a chemical analog of the nucleoside cytidine. Treatment of NB cells with 5-aza showed induced cell differentiation²⁷ and



reduced proliferation and colony formation^{27, 28}. Further studies demonstrated that 5-aza can potentiate the cytotoxic effects of current chemotherapies, such as doxorubicin, cisplatin and etoposide²⁹, thereby suggesting that a combination of 5-aza with standard therapies could lead to more effective and safer treatments. However, a phase I clinical study of decitabine with doxorubicin showed that only low-doses of decitabine with this combination were tolerable and that those capable of producing clinically significant biologic effects were not well tolerated³⁰.

These results suggest that more specific DNMT-inhibitors might offer better safety profiles. Recently, two new DNMT inhibitors, i.e., SGI-1027 (selective for DNMT1, DNMT3A/B) and nanaomycin A (DNMT3B-specific), displayed higher cytotoxic effects alone or in combination with doxorubicin but without alteration of general genome methylation³¹. The use of these new inhibitors is expected to result in fewer side effects.

Histone modifications

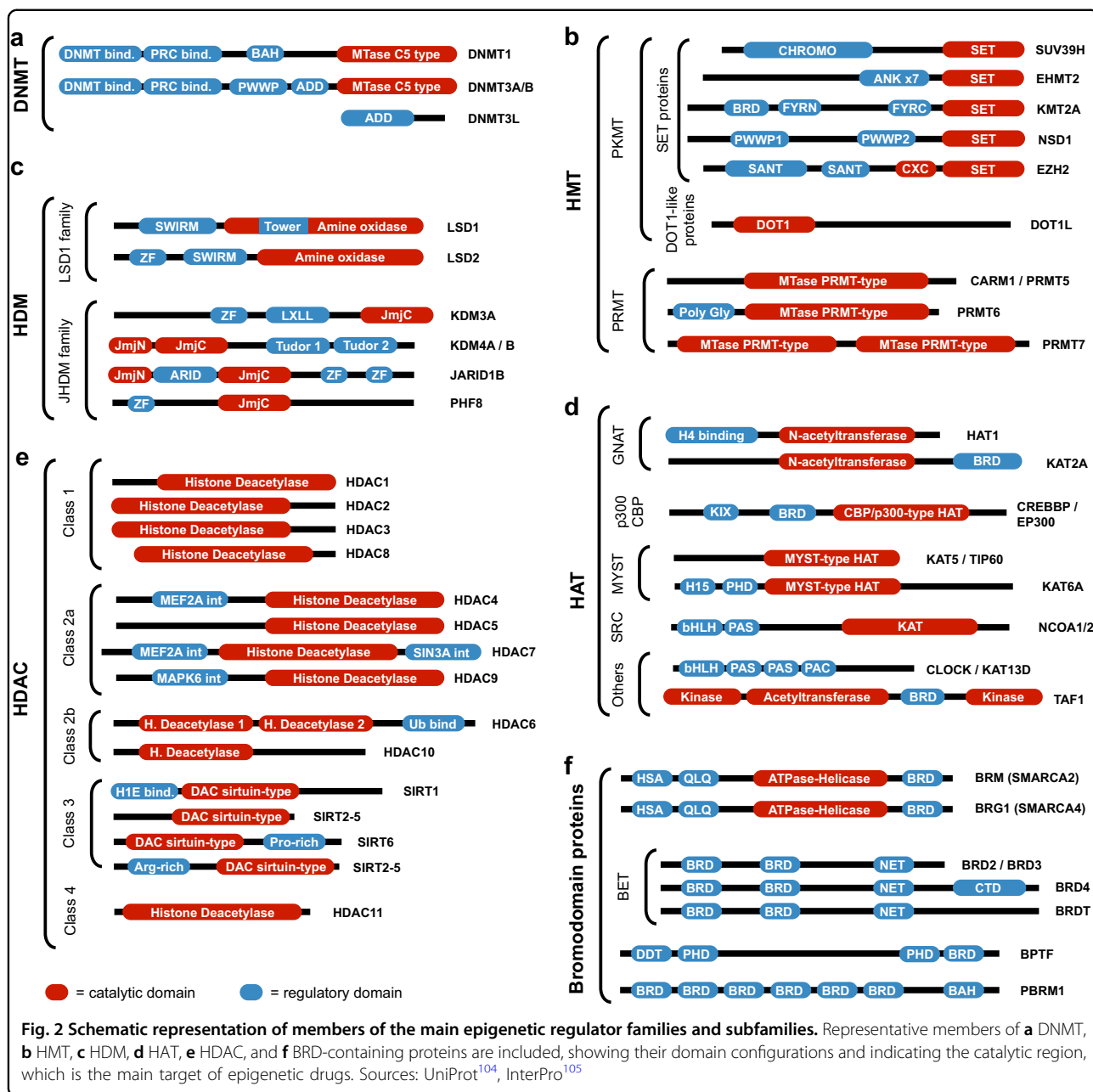
Histones are the evolutionary solution to compaction of large amounts of DNA in the nucleus of eukaryotic cells. Approximately 147 bp of DNA are wrapped in histone octamers (formed by H2A, H2B, H3, and H4) to form a nucleosome. Nucleosomes are assembled in successively higher-order structures to eventually form a chromosome. Nucleosomes build chromatin, which can exist as euchromatin (decondensed and transcriptionally active) or heterochromatin (condensed and transcriptionally

inactive). The compaction of chromatin is regulated by modifications on the histone tails. The N-terminal and C-terminal domains protrude from the nucleosome and are subjected to different covalent post-transcriptional modifications, such as methylation, acetylation, phosphorylation, and sumoylation. The enzymes responsible for these covalent modifications are known as “writers”, whereas the enzymes that remove these marks are referred to as “erasers”. Finally, enzymes capable of recognizing histone marks are denoted as “readers” (reviewed in ref. 32–34) (Fig. 1).

Histone methyltransferases

Histone methyltransferases (HMT) are a class of histone writers that transfer methyl groups from S-adenosyl methionine to histone-specific lysine or arginine residues on the histones^{35, 36}. Histone methylation is involved in different processes, such as chromatin compaction, X-chromosome inactivation, genomic imprinting and repression, or activation of transcription, among other tasks. These functions are influenced by the site and degree of methylation on specific residues (reviewed in previous work^{36, 37}). Histone methylation usually occurs on the H3 and H4 tails.

To date, approximately 60 HMTs have been identified. HMT are classified depending on the histone amino acid that is methylated, and lysine methyltransferases (PKMT) modify lysine residues by mono-methylation, di-methylation, or tri-methylation and are classified in a SET



domain-containing or a non-SET domain-containing PKMT^{35, 36}. Arginine residues are mono-methylated and symmetrically or asymmetrically di-methylated by arginine methyltransferases (PRMT)³⁸ (Fig. 2b).

In the last decade, certain SET-PKMS have been associated with prognostic factors, although the functional significance of these alterations remain to be determined.

Nonetheless, PKMT inhibitors show therapeutic potential in NB. This is the case of BIX-01294, a specific inhibitor of EHMT2 and a protein frequently over-expressed in several tumor types. Treatment of NB cells with BIX-01294 showed decreased cell proliferation,

inhibition of cell mobility and invasion³⁹, induction of apoptosis in vitro and reduced tumor growth in pre-clinical mouse models⁴⁰.

Histone demethylases

Histone methylation represents a balance resulting from the opposing activity of HMT and histone demethylases (HDM). KDM1 (also known as LSD1) was the first enzyme found to be capable of removing the methyl group from mono-methylated and di-methylated Lys 4 in histone 3 (H3K4me1/2)⁴¹. HDM can be divided into two lysine HDM families: (i) the KDM1 family and (ii) the

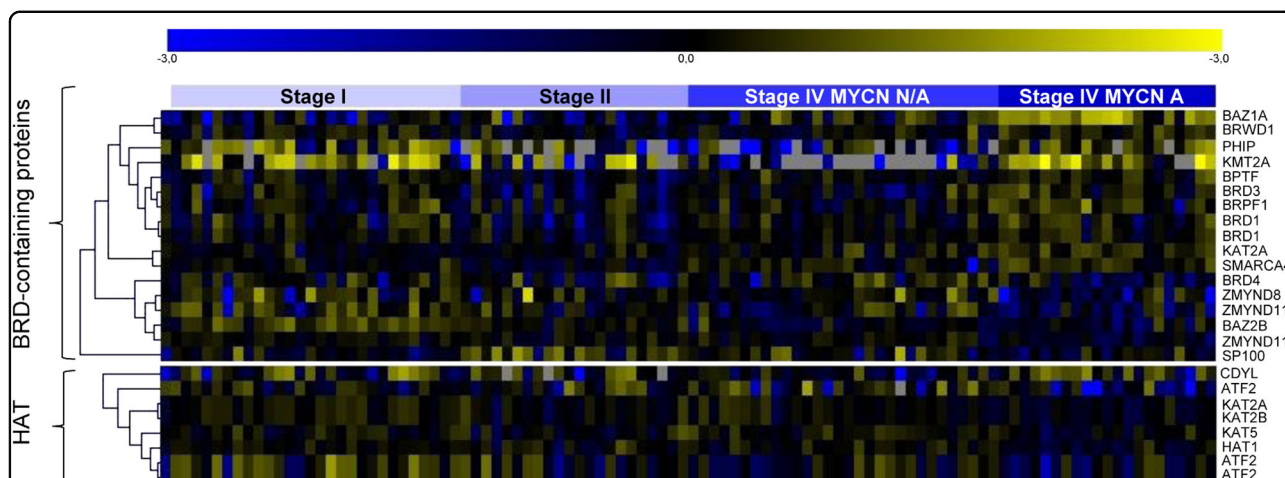


Fig. 3 In silico analysis of HAT and BRD-containing protein expression in NB. mRNA expression levels from 39 different BRD-containing proteins and 16 histone acetyltransferases were analyzed in neuroblastoma samples from the E-GEOD-3960 ($n = 101$) dataset. Samples were sorted according to disease stage and MYCN status. Heatmap represents the differentially expressed genes ($p < 0.05$) between the groups of patients with poor prognosis (stage 4, MYCN amplification) versus the remainder of patients (i.e., stage 1, 3, and 4 MYCN non-amplified)

JHDM family. Although proteins from the KDM1 family demethylate mono-methylated or di-methylated lysines, those of the JHDM family demethylate tri-methylated lysines. Of note, JMJD6 (a member of the JHDM family) is also an arginine-specific HDM that demethylates H3R2me1/2 and H4R3me1/2⁴² (Fig. 2c).

KDM1A was the first HDM, the expression of which was found to correlate with adverse outcome and undifferentiated tumors. Loss-of-function experiments showed that KDM1A silencing resulted in a reduction in cell proliferation, colony formation, migration and invasion of NB cell lines^{43, 44}.

Several KDM1A inhibitors have been designed, but only trans-2-phenylcyclopropylamine (TCP) derivatives have advanced into early phase clinical trials. However, the therapeutic potential of these compounds in neuroblastoma animal models or in clinical trials remains to be addressed.

The JHMD family is larger than the KDM1A family, and therefore, more members of the family are associated with NB. One of such examples is the KDM4B found in a search for mediators of oncogenic functions of MYCN. KDM4B knockdown reduced NB cell proliferation and induced differentiation in vitro and in vivo⁴⁵. Mechanistically, KDM4B physically interacts with MYCN, removes histone methylation marks at MYCN binding sites, and blocks the transcription of MYCN direct targets, such as the miR-17-92 cluster, CDC25A, TRIP13, and VCAN⁴⁵.

Histone acetyltransferases

A further histone modification is the addition of acetyl groups from acetyl-CoA to specific histone lysine

residues. This process can be performed by histone acetyltransferases (HAT)⁴⁶. HAT are capable of modulating gene transcription by altering histone acetylation patterns^{47, 48} or by acetylating non-histone substrates, such as transcription factors (reviewed in ref. 49). HAT are usually classified based on sequence similarity and structure to define five families: GNAT, p300/CBP, MYST, SRC (nuclear receptors coactivators) and others (reviewed in ref. 50) (Fig. 2d).

To date, no HAT expression or functional studies have been conducted either in clinical or preclinical NB models. Nevertheless, in silico analyses did show that several HAT are differentially expressed in advanced stages of NB. In most cases, HAT levels are expressed at lower levels in patients with poor prognosis (i.e., stage 4 with MYCN amplified), thereby indicating that strong criteria for selection of patients who could benefit from these therapies must be considered (Fig. 3).

Nevertheless, three HAT inhibitors have been tested in NB models: PU139 (a HAT pan-inhibitor⁵¹), PU141 (a CBP and p300 selective inhibitor⁵¹), and BF1 (an H3-acetylation protein inhibitor⁵²). All of these inhibitors reduced NB cell growth in vitro^{51, 52}, but only PU139 and PU140 were demonstrated to reduce tumor growth in vivo⁵¹. Furthermore, PU139 showed synergism with doxorubicin in vivo, thereby blocking tumor growth⁵¹.

Histone deacetylases

Histone acetylation and deacetylation exert a dynamic balance that controls gene transcription. Although histone acetylation is associated with active transcription, histone deacetylation is associated with transcriptional repression. Hypoacetylated nucleosomes usually result in

Table 1 HDAC inhibitors studied in Neuroblastoma

Name	Alias	Effective in vivo	Reference
m-Carboxycinnamic acid bis-hydroxamide	CBHA	+	106–108
Suberoyl-3-aminopyridineamide hydroxamic acid	Pyroxamine	n.d.	109
MS-275	Entinostat	+	110–113
Sodium butyrate	NaB	n.d.	114–119
BL1521	BL1521	n.d.	120–122
Trichostatin A	TSA	+	123–129
Glycerin tributryate	Tributyryn	n.d.	85
M344	M344	n.d.	111
HKI 46F08	HKI 46F08	n.d.	130
Helminthosporium carbonum-toxin	HC-toxin	n.d.	131
Romidepsin	Istodax	+	132
C149	C149	n.d.	133
LBH-589	Panobinostat	+	134, 135
PCI-24781	Abexinostat	+	136, 137
BRD8430	BRD8430	n.d.	138
CAS 14513-15-6	Cambinol	+	76
Salermide	Salermide	n.d.	77
PCI-35051	PCI-35051	+	70, 139
Tubacin	Tubacin	n.d.	79, 80
1-Naphthohydroxamic acid	Cpd2	+	70

n.d. not determined

tightly compacted chromatin, thereby restricting the access of transcription factors to their target DNA and leading to transcription repression (reviewed in ref. 53). An alteration in this acetylation balance might result in the development of diseases, such as cancer.

The 18 histone deacetylases (HDAC) encoded in our genome can be classified based on their homology with yeast HDAC⁵⁴ as follows: class I, which includes HDAC1, 2, 3 and 8; class II HDAC4, 5, 6, 7, 9 and 10; class III sirtuins (SIRT1-7); and class IV-only HDAC11⁵⁵.

All HDAC share a conserved histone deacetylase domain, but they vary in location, structure and expression patterns⁵⁶. Classes I, II, and IV share homology in structure and sequence and require a zinc ion for their catalytic activity. Class III HDAC share no similarities with the other classes and require nicotinamide adenine dinucleotide (NAD⁺) for their activity⁵⁷ (Fig. 2e).

Only two HDAC have been reported as associated with NB prognosis. Particularly, HDAC8 and HDAC10 were found to be overexpressed in high-risk NB, and their inhibition resulted in reduced NB cell proliferation in vitro^{58, 59} and in vivo⁶⁰. Moreover, the inhibition of HDCA8 and 10 was found to increase doxorubicin sensitivity^{58, 61}.

One of the most important genetic factors associated with NB outcome is the genomic amplification of the transcription factor MYCN, a driver oncogene in NB, which in turn regulates the expression of a myriad of genes associated with cell proliferation, survival and metastasis, among others. Selected HDAC have been shown to participate in a positive feedback loop with MYCN.

One of such examples was described for the Class III HDACs SIRT1. SIRT1 studies revealed that MYCN directly induced the transcription of SIRT1 and increased the stability of this oncogenic protein. Furthermore, pharmacologic inhibition of SIRT1 (cambinol) reduced tumorigenesis in a MYCN-driven neuroblastoma transgenic mouse model⁶².

Owing to the relevance of HDAC proteins in cancer, many inhibitors have been developed in recent decades. These inhibitors are classified depending on the targeted HDAC class. The first developed HDAC inhibitors (HDACi) were those targeting Classes I, II and IV and can be classified into six basic types depending on the structure of the inhibitor (reviewed in ref. 63). In contrast, Class III HDAC are inhibited with derivatives of NAD⁶⁴.

Multiple studies showed the therapeutic potential of HDACi in NB in preclinical studies (Table 1), but few reached clinical trials (Table 2).

One of the most studied HDACi in NB is valproic acid (VAP), which was discovered by B.S. Burton in 1882⁶⁵. This inhibitor has higher but not exclusive selectivity to Class I HDAC. Initially, this compound was used to treat seizures, bipolar disorders or migraines. Different studies later showed that VAP inhibited HDAC proteins (reviewed in ref. 66), thereby opening a door to cancer treatment.

When NB cells are treated with VAP, a strong inhibition of cell proliferation and induction of differentiation and apoptosis is observed^{67, 68}. Other studies showed the therapeutic potential of VAP in combination with current therapies, such as ABT-510 (an angiogenic inhibitor)⁶⁹ or with OGX-011⁷⁰ (inhibitor of clusterin), resulting in tumor growth impairment. However, in certain cases, VAP combination effects are subject to administration order. When VAP is combined with conventional chemotherapeutic agents, such as etoposide or cisplatin, these drugs must be administered before any other treatment^{71, 72}.

Table 2 Current epigenetic drugs clinical trials in Neuroblastoma

Name of the drug	Type of drug	Phase	Estate	Number
Decitabine	DNMT pan-inhibitor	Phase I	Complete	NCT01241162
		Phase I	Complete	NCT00075634
Genistein	DNMT pan-inhibitor	Phase II	Recruiting	NCT02624388
Vorinostat	HDACclass I and II inhibitor	Phase I	Complete	NCT00217412
		Phase I	Complete	NCT01132911
		Phase II	Ongoing but not recruiting	NCT02035137
		Phase I	Recruiting	NCT02559778
		Phase I	Complete	NCT01019850
VAP	HDACclass I and II inhibitor	Phase I	Complete	NCT01208454
4-PB	HDACpan-inhibitor	Phase I	Complete	NCT00001565
GSK525762	iBET	Phase I	Recruiting	NCT01587703

Another well-studied HDACi in NB is vorinostat (also known as SAHA). Vorinostat is a selective class I and II HDACi and is currently in use in multiple clinical trials in NB. Treatment of NB cells with vorinostat resulted in cell cycle arrest in G2/M phase followed by the activation of the intrinsic apoptotic pathway⁷³. Vorinostat was also shown to impair VEGF secretion by NB cells, thereby suggesting a potential antiangiogenic effect⁷⁴.

Vorinostat has also been shown to potentiate the anti-tumor activity of different drugs, such as flavopiridol (a pan-Cdk inhibitor)⁷⁵ and fenretinide (a synthetic retinoid)⁷⁶, and therapies, such as radiotherapy^{77, 78}.

The latest HDACi to reach clinical trials was 4PB (4-phenylbutyrate), which is also selective for HDAC class I and II. In 1998, Pelidis et al. described the effectiveness of 4PB in NB for the first time and demonstrated that 4PB reduced proliferation and induced differentiation of NB cell lines. Moreover, 4PB demonstrated additive cytotoxic effects when administered with the chemotherapeutic drug vincristine⁷⁹. Concurring with these results, Tang et al. showed that 4PB induced the expression of several genes associated with favorable outcome (i.e., EPHB6, EFNB2, EFNB3, NTRK1, and CD44) and impaired tumor growth and metastasis in vitro and in vivo⁸⁰.

Histone phosphorylation

Histone phosphorylation is widely known to be involved in chromatin condensation during cell division and apoptosis and also acts as an important signal for DNA damage response. These are transient processes that do not produce stable and heritable changes in gene expression. However, phosphorylation of certain histone residues has also been directly related to transcriptional regulation control. In fact, many

phosphorylated sites act by crosstalk with other histone modifications, such as methylation or acetylation. (reviewed in the literature^{81, 82}).

Phosphorylation and dephosphorylation of histones are performed by kinases and phosphatases, respectively. Most of these enzymes are not histone specific. For example, Aurora B kinase is known to phosphorylate histone H3 on serine 10 to induce chromosome condensation in the early phases of cell division, which is essential for cell cycle progression⁸³. Aurora B kinase has been found as a potential target for NB treatment, and its inhibition with barasertib promotes arrest in the G2/M phase followed by apoptosis in vitro and in vivo^{84, 85}. Selected histone phosphatases have also been reported to play a role in NB biology. EYA1, which dephosphorylates the tyrosine 142 of histone H2AX, is down-regulated in the advanced stages of NB⁸⁶. Phosphatase PP2A has also been observed to exert tumor suppressive effects on NB cells⁸⁷.

Although many kinases and phosphatases able to modify histone residues are under study as targets for NB treatment, the impact of histone phosphorylation in itself on NB progression is still not well characterized and should be investigated further.

Histone modifiers are by far the largest family of epigenetic regulators. Structural similarities have paved the way for the design of pan-inhibitors, such as those developed for HDAC. Despite a growing body of evidence showing therapeutic potential in preclinical studies, only modest results have been observed in clinical trials to date. A better understanding of the tumor-specific dependency on each of these epigenetic regulators might lead to the development of additional target-specific inhibitors and better patient selection.

Table 3 Potential new epigenetic targets in neuroblastoma at early preclinical research studies

Family/Class	Gene symbol	Prognostic value	Function	Ref.
HMT / PKMT	NSD1	Low is worse	Cell proliferation	134
HMT / PKMT	WHSC1	High is worse	n.d.	135
HMT / PKMT	EZH2	High is worse	Cell differentiation and gene expression regulator	136
HMT / PRMT	PRMT5	High is worse	Cell proliferation / survival	137
HDM / KDM1	KDM1A	High is worse	Cell proliferation / invasion	43, 44
HDM / JHDM	JMJD1A	n.d.	Migration / invasion	138
HDM / JHDM	JARID1B	High is worse	Invasion / chemoresistance	139
HDAC / Class I	HDAC2	n.d.	Increased proliferation / survival	140
HDAC / Class I	HDAC5	n.d.	Block differentiation / induce proliferation	141
HDAC / Class II	HDAC6	n.d.	Regulates cell survival	142
HDAC / Class III	SIRT2	n.d.	Increased proliferation	130
HDAC / Class IV	HDAC11	n.d.	Regulates cell survival	143

HMT histone methyl-transferase, n.d. not determined^{144–151}

Chromatin readers

To translate the pattern of histone modifications into a functional phenotype, these modifications must be recognized by proteins known as “readers”. These readers are bromodomain (BRD), chromodomain and tudor-domain containing proteins, which recognize histone marks and recruit other proteins required to start or inhibit transcription. Bromodomain-containing proteins are capable of recognizing the acetylation of histones, whereas chromodomains and tudor-domains recognize methylated histones^{88, 89}.

BRD-containing proteins are highly conserved throughout evolution and can perform various functions, such as histone acetylation, chromatin remodeling and transcriptional activation⁹⁰. The human homolog of the drosophila gene Brahma (Brm)⁹¹ was the first of 61 human BRD to be described, a subset of 46 BRD-containing proteins⁹². All known BRD have a central hydrophobic pocket with a highly conserved asparagine residue responsible for the binding to the acetylated lysines of histones⁹³ (Fig. 2f).

Only one study to date has demonstrated the correlation of a BRD-containing protein and NB outcome. BPTF was found to be amplified in 55% of NB cases due to gain of the 17q24.3 locus⁹⁴. In silico analysis of mRNA expression data sets in NB [EGEOD-3960, Fig. 3] shows that at least 15 BRD-containing proteins are differentially expressed in patients with advanced disease and poor prognosis (10 were upregulated, 5 were downregulated), thereby suggesting that they could be new potential therapeutic targets for NB.

The crystallization of the BRD structure and the feasibility of designing small molecules to target this domain

placed the BRD inhibitors in the spotlight as new therapeutic targets for cancer. In 2010, two small-molecule inhibitors against BET-bromodomains (a family of BRD known as bromodomain and extra-terminal domain, which consists of four different proteins) were described by two independent groups (JQ1 and I-BET^{95, 96}) with high affinity for BRD2, BRD3 and BRD4. Both compounds showed that BRD inhibition resulted in antitumor effects in mixed lineage leukemia⁹⁷, multiple myeloma⁹⁸, or lung adenocarcinomas⁹⁹.

The therapeutic potential of BRD inhibition in NB was first analyzed by Puissant et al. Treatment of NB cells with the BET inhibitor (iBET) JQ1 resulted in a reduction in MYCN levels, reduced cell growth and induction of apoptosis in vitro and in vivo^{100, 101}. The JQ1 inhibitor also showed synergistic effects when combined with the HDACi panobinostat. This drug combination showed reduced MYCN protein expression and impaired tumor growth in vivo¹⁰².

Recently, another BRD inhibitor (i.e., OTX015) was tested in NB. Administration of OTX015 produced a reduction in MYCN expression and loss of interaction of MYCN with the promoter of their target genes. Furthermore, BRD4 was also shown to be preferentially displaced from DNA super-enhancers regulated by MYCN¹⁰³.

Conclusions and future perspectives

Classically, pediatric oncology has mirrored the therapeutic strategies of adult oncology, and epigenetic therapies are not an exception. In addition to considering epigenetic modifications as diagnostic or prognostic tools, a large proportion of clinical trials (~40%) focus on

evaluating the therapeutic potential of DNMTi followed by HDACi (~10%), either as single agents or in combination with standard therapies. Therefore, a long list of potential new epigenetic targets remains to be explored. In particular, in NB, only three HDACi, two DNMTi and one iBET compounds have reached clinical trials, and of these, only two inhibitors have reached phase II: genistein and vorinostat (Table 2).

The low number of active clinical trials, despite pre-clinical evidence of the role of epigenetic regulators in NB, underlines the need for advanced preclinical studies in new therapeutic targets (Table 3) and the development of new compounds, probably more specific versions, which should be more effective at tolerable doses and with fewer side effects. The compounds that reached clinical trials have specificity for more than one target of the same family and are referred to as pan-inhibitors. Presumably, the development of more specific compounds (instead of pan-inhibitors) against chromatin remodeler proteins found to be altered in NB will be more cancer-specific and have potentially fewer side effects. Nevertheless, most of these epigenetic regulators are poorly characterized with few crystallized structures. Therefore, design and development of new small molecules remains a great challenge. Additional efforts must be invested in the definition of these structures to create better inhibitors for development of future treatments. Alternatively, blocking of protein-protein interactions or siRNA-mediated gene silencing could be considered.

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Conflict of interest

The authors declare that they have no conflict of interest.

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