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Review: Targeting EZH2 in neuroblastoma

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ABSTRACT

Neuroblastoma is one of the commonest extra-cranial pediatric tumors, and accounts for over 15% of all childhood cancer mortality. Risk stratification for children with neuroblastoma is based on age, stage, histology, and tumor cytogenetics. The majority of patients are considered to have high-risk neuroblastoma, for which the long-term survival is less than 50%. Current treatments combine surgical resection, chemotherapy, stem cell transplantation, radiotherapy, anti-GD2 based immunotherapy as well as the differentiating agent isotretinoin. Despite the intensive multimodal therapies applied, there are high relapse rates, and recurrent disease is often resistant to further therapy. Enhancer of Zeste Homolog 2 (EZH2), a catalytic subunit of Polycomb Repressive Complex 2 (PRC2), is a histone methyltransferase that represses transcription through trimethylation of lysine residue K27 on histone H3 (H3K27me3). It is responsible for epigenetic repression of transcription, making EZH2 an essential regulator for cell differentiation. Overexpression of EZH2 has been shown to promote tumorigenesis, cancer cell proliferation and prevent tumor cells from differentiating in a number of cancers. Therefore, research has been ongoing for the past decade, developing treatments that target EZH2 in neuroblastoma. This review summarises the role of EZH2 in neuroblastoma and evaluates the latest research findings on the therapeutic potential of targeting EZH2 in the treatment of neuroblastoma.

Introduction

Overview of neuroblastoma

Neuroblastoma is the most common extra-cranial solid tumor with up to 100 children newly diagnosed annually in the UK [1]. Around 40% of patients are diagnosed before 1 year old and the median age at diagnosis is 17–18 months [2]. Neuroblastoma is an embryonal tumor of the sympathetic nervous system arising from immature neural crest cells [3]. It generally originates in the adrenal glands (65%), however, it can arise from anywhere containing sympathetic nervous tissue, including the chest (20%), neck (5%) and pelvis (5%) [4]. The staging system for neuroblastoma is based on either localized or metastatic disease and a number of agreed imaged defined risk factors [5]. Stage 4S is a special form of metastatic disease which occurs only in children younger than 12 months old, and accounts for 5% of all cases, often showing spontaneous regression [6].

The International Neuroblastoma Risk Group (INRG) risk stratification model was developed to predict prognosis or response to treatment. Patients can be classified as having either low-risk, intermediate-risk or high-risk disease, based on a number of pre-treatment risk factors, including age, histology, cytogenetics (including MYCN oncogene amplification status), surgical resectability and metastatic status of the tumor [7]. MYCN amplification is seen in approximately 20% of neuroblastoma cases [8], and is associated with high-risk disease, and poor patient outcomes. MYCN is a transcription factor, and its amplification can lead to tumorigenesis via numerous pathways including enhanced expression of ornithine decarboxylase, suppression of cell cycle via p21 and suppression of tumor suppressor genes including CLU [9].

Overall prognosis for neuroblastoma has improved over the last 50 years with 5-year survival rates increasing from 50% in the 1970s to 81% in 2020 [10]. This improvement is attributable to enhanced patient stratification and improved treatment regimens. However, these improvements are not observed equally across the wide range of disease. Improvement in prognosis is attributed mainly to those patients with low to intermediate risk disease, whose 5-year survival is now 98% and 90–95% respectively [11]. Patients classified with high-risk disease still have a 5-year survival of less than 50% [10].

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Current treatments for neuroblastoma

The last decade has seen the adoption of several targeted treatment strategies for neuroblastoma patients. For patients with low-risk neuroblastoma, treatment usually consists of observation or surgical resection and surgery is generally curative for these patients [12]. Recent studies have also shown observation without surgery or chemotherapy may be appropriate for infants with asymptomatic disease and localized tumors, as spontaneous regression will be observed in some patients with low-risk neuroblastoma [5]. For patients with intermediate-risk disease, depending on individual response, 2 to 8 cycles of moderate doses of multiagent chemotherapy are applied as a neoadjuvant treatment before surgical resection. Some of these patients also receive radiotherapy with or without retinoic acid [12,13]. The standard of care for patients with high-risk neuroblastoma comprises three components: induction, consolidation and maintenance therapy [14]. The induction therapy includes chemotherapy which aims to minimize the disease burden pre-operatively, with the aim of clearing metastatic disease, followed by surgical resection of the primary tumor. The consolidation therapy includes different myeloablative chemotherapy regimens (e.g. Busulfan and melphalan) with autologous stem cell rescue, followed by radiotherapy, anti-GD2 immunotherapy and *cis*-retinoic acid (isotretinoin) maintenance therapy (Fig. 1) [15]. Even when patients achieve remission after this intensive multi-model treatment, relapse rates remain high likely due to persistent chemotherapy-resistant minimal residual disease (MRD) [16 17]. The anti-GD2 and isotretinoin maintenance therapy is aimed at eradicating or differentiating any MRD. Isotretinoin is a retinoid that regulates cell proliferation and development and has been demonstrated to induce differentiation and inhibit uncontrolled proliferation of neuroblastoma cells [18]. The combination of anti-GD2 and isotretinoin has shown significant improvements in overall survival and event-free survival [14].

Epigenetic targets in cancer treatment

Unlike some other cancers, neuroblastoma does not have a universal driver genetic mutation, though the underlying causes could be due to MYCN amplification, rare germline mutations, rearrangement, copy number alterations or epigenetic disruption to genes such as ALK, ATRX, TP53, TERT, RAS/MAPK pathway genes [4]. As the imbalance between differentiation and proliferation may lead to malignant transformation, and lack of differentiation is associated with a poorer prognosis, it was proposed 40 years ago by Sidell that neuroblastoma differentiating agents may be used to treat neuroblastoma [19].

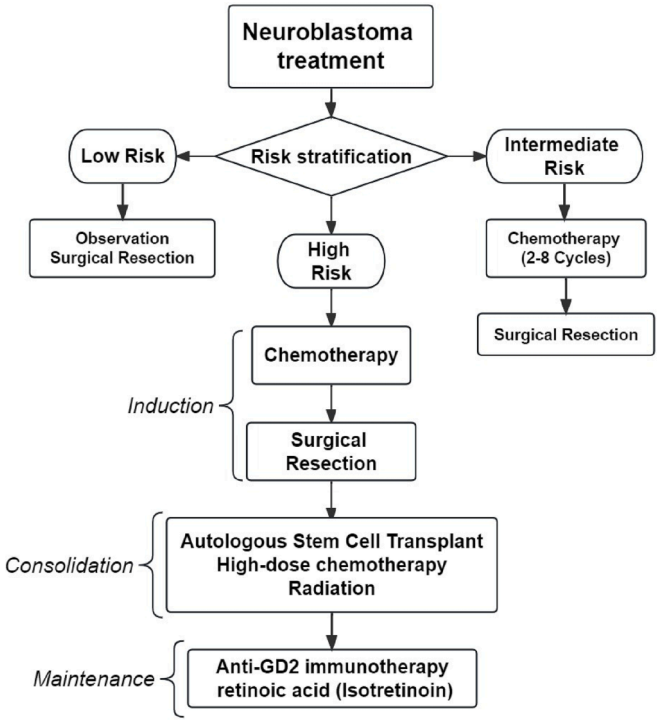

Fig. 1. Treatment classification of neuroblastoma.

Epigenetic factors such as DNA methylation and histone modification, tightly control differentiation of cells by altering gene expression. DNA methyltransferases (DNMTs) are key components of the DNA methylation process which result in transcriptional repression [20]. The first pre-clinical study demonstrated that inhibition of DNMT using 5-aza-deoxycytidine induces neuroblastoma cell differentiation and reduces cell proliferation [21]. Histone modification such as histone methylation, acetylation, phosphorylation and sumoylation also alters the structure of chromatin to allow inactive or active transcription to influence gene expression [22]. Over the past decade, histone modifying enzymes (HMEs) have become popular epigenetic targets for therapeutic intervention. Histone methyltransferases (HMTs) or histone demethylases (HDMs) can either add or remove methyl marks from amino acids of histones [23] and targeting these therapeutically can drastically alter the epigenetic landscape of tumors and result in favorable phenotypes such as cancer cell differentiation and apoptosis [24].

Enhancer of Zeste homolog 2 (EZH2), is a histone methyltransferase, which plays a key role in cell differentiation and cancer progression. Several studies have demonstrated that dysregulation of EZH2 is heavily correlated with poor prognosis of numerous types of cancer including prostate, breast, esophageal, gastric, nasopharyngeal carcinoma and endometrial cancer [25,26]. In this review, we focus on the role of EZH2 in neuroblastoma and the strategies for targeting EZH2 in neuroblastoma treatment.

EZH2 function and epigenetic regulation

Characteristics and mechanism of action of EZH2

EZH2 protein forms the core enzymatic subunit of Polycomb Repressive Complex 2 (PRC2) which is vital in organizing the chromatin into a repressive state, regulating the expression of important transcription factors [27]. PRC2 comprises three other core subunits: Embryonic Ectoderm Development (EED), Suppressor of Zeste 12 (SUZ12), and Retinoblastoma protein Associated protein 46/48 (RbAp46/48) (Fig. 2), which may be associated with enhanced catalytic activity such as recruiting to the chromatin complex [28]. In addition, recent studies have revealed that PRC2 comprises two alternative subtypes, PRC2.1 and PRC2.2. comprising different co-factors in addition to the core subunits. PRC2.1 consists of one of three Polycomb-like (PCL) proteins (PHF1, MTF2 or PHF19, and either Polycomb repressive complex 2-associated protein (EPOP) or PRC2-associated LCOR isoform 1 (PALI1/2). PRC2.2 consists of Jumonji and AT-rich interaction domain 2 (JARID2) and adipocyte enhancer-binding protein 2 (AEBP2) [29]. However, the regulation of PRC2 recruitment and its enzymatic activity is intricately linked to the presence and functionality of these cofactors [30]. Four homologous domains have been discovered in the EZH family: H1 domain, H2 domain, cysteine rich domain and C-terminal SET domain [31]. Notably, the SET domain in EZH2 is required for the activity of histone methyltransferase [31]. Both EZH1 and EZH2 are found in the PRC2 complex, though EZH1 has different functional roles and exhibits low activity of methyltransferase which may be due to EZH1 lacking specific threonine residues [31,32].

EZH2 is responsible for trimethylation of lysine 27 on the tail of histone 3 (H3K27). It transfers a donor methyl group from S-adenosyl-L-methionine (SAM) to H3K27 via its C-terminal SET domain and methylates H3K27 to form H3K27me3 [33]. As a stable repressive chromatin mark, H3K27me3 is recognized and binds to PRC1 subunit CBX family, which induces the mono-ubiquitination of histone 2A lysine 119

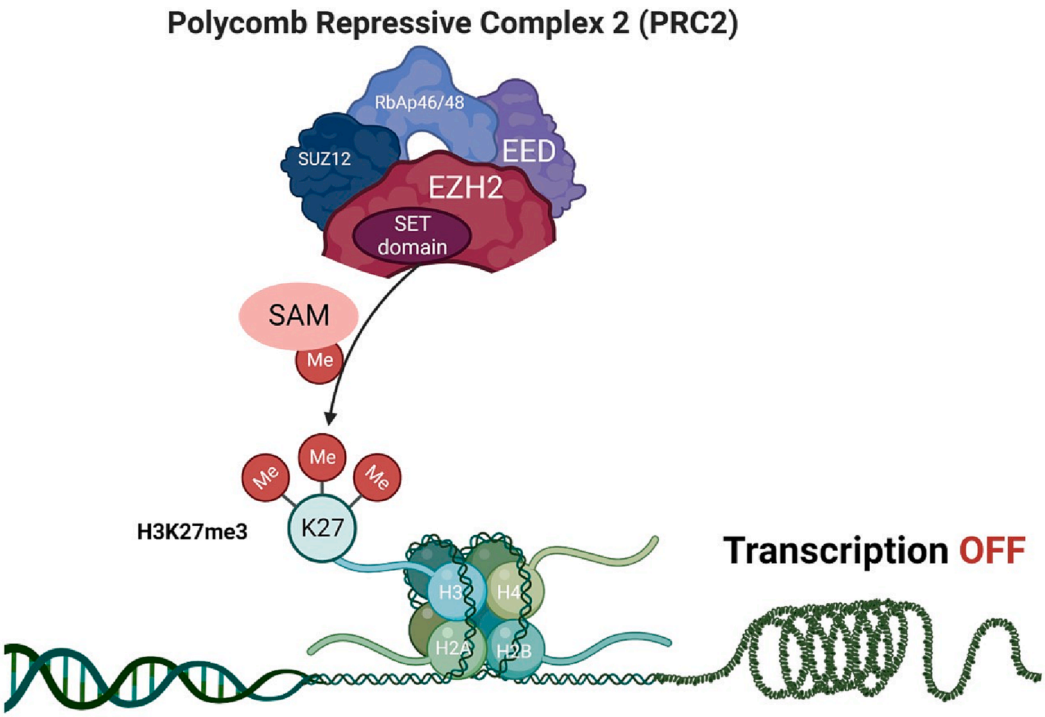

Fig. 2. The enzymatic activity of EZH2 catalyzes trimethylation on H3K27 (H3K27me3), which leads to a conformational change in the structure of chromatin and repression of transcription.

(H2AK119) to prevent RNA polymerase transcriptional elongation and stop gene transcription [34]. EZH2 is also phosphorylated and ubiquitinated while transferring a methyl group to histone H3 tails, which further regulates the activation and stability of EZH2 in different biological processes [33]. Interestingly, Martin et al. demonstrated that recombinant EZH2 did not show the activity of histone methyltransferases activity even though the SET domain was present [35], suggesting the function of EZH2 for histone methylation may require a complex containing EZH2. Subsequent studies demonstrate that EZH2-EED complex binding through the WD40 domain and N-terminal H1 and H2 domains is the response to histone methylation [33]. In addition, EZH2 can also play a non-canonical role in the PRC2-independent pathway as a transcriptional activator through interaction with the estrogen receptor (ER) and b-catenin in ER-positive luminal like MCF-7 cells or activate NFkB genes by forming a ternary complex with RelA and RelB in ER-negative MDA-MB-231 cells [36]. These studies indicate EZH2 functions as either a transcriptional repressor or activator depending on tumor cell type and through a PRC2-dependent or independent pathway.

EZH2 in oncogenic processes

Overexpression of EZH2 has been observed in several cancer types including prostate, breast, esophageal, gastric, nasopharyngeal carcinoma and endometrial cancer [25] and is associated with more aggressive and metastatic disease within these tumor types [37]. The increased expression level of EZH2 was first found to inversely correlate with patient survival in prostate cancer and this was subsequently observed in many other malignancies [38].

Mechanisms behind EZH2 overexpression in oncogenesis include a number of factors such as gene amplification, oncogenic signaling molecules, transcription factors and functional mutations [39]. It was found that a subset of follicular lymphoma with a mutation at amino acid Y641 within the SET domain of EZH2 increased the catalytic function of EZH2 [40]. Similarly, in a study of diffuse large B-cell lymphomas, the mutations of A687V and A677G have also contributed to gain-of-function mutations in EZH2 [41]. In these studies, EZH2 overexpression is thought to repress tumor suppressor genes through its improved enzymatic activity of trimethylation H3K27, promoting oncogenesis. Key tumor suppressor genes identified as being repressed by overexpression of EZH2 include WNT agonist DKK1, angiogenesis factor VASH1 and cell cycle regulator, p16/CDKN2A [42].

However, several studies have also suggested two other mechanisms by which EZH2 may promote of oncogenesis. For example, Richter et al. found that EZH2 promotes oncogenesis and a stem cell like phenotype in Ewing tumors via increased EZH2 expression as a result of transcriptional activation by the EWS/FLI1 fusion protein at the *EZH2* promoter [43]. A number of studies have shown that EZH2 is overexpressed in pediatric rhabdomyosarcoma (RMS), where it plays a role in maintaining the undifferentiated state of these cancer cells [44–48]. In addition, a study in myeloid malignancies suggested inactivation mutations of EZH2 rather than overexpression could promote oncogenesis due to loss of EZH2′s tumor suppressor gene functions [49]. In castration-resistant prostate cancer, the promotion of oncogenesis was due to the non-canonical role of EZH2 in the PRC2-independent pathway as a transcriptional activator, where EZH2 supports transcriptional activation by the androgen receptor [50]. These findings show the complex role of EZH2 in contributing to oncogenesis in different types of cancer.

The role of EZH2 in neuroblastoma

Overexpression of EZH2 in neuroblastoma

EZH2 has been reported to be significantly overexpressed in primary human neuroblastoma. It is possible that overexpression may occur via the common copy number gain of chromosome 7, which harbors the *EZH2* gene [51]. EZH2 overexpression in neuroblastoma is significantly associated with a poorer prognosis. Bate-Eya et al. demonstrated a significant association between higher EZH2 expression with a poor prognosis within a cohort of 88 neuroblastoma patients [51], this finding was mirrored with a public RNAseq data set of 493 neuroblastoma tumors [52]. This demonstrates that EZH2 is important in the

malignant transformation of cells and the development of neuroblastoma. Whether this is a primary event or secondary to other drivers is uncertain, for example, Wassef et al. suggests that EZH2 is overexpressed as a result of cell proliferation and is secondary to other driver events, such as those involving the MYC oncogene [53].

The expression of *EZH2* mRNA and EZH2 protein levels is significantly higher in MYCN amplified neuroblastoma versus MYCN non-amplified neuroblastoma [54,55]. A recent study discovered that EZH2 directly interacts with the oncoproteins MYC and MYCN and stabilizes MYCN independently of its methyltransferase activity. Instead of enzymatic inhibition of EZH2, their findings highlight depletion of EZH2 inducing degradation of MYC(N) and inhibits cell proliferation in MYC(N) driven neuroblastoma as well as small cell lung carcinoma [56]. Tsubota et al. demonstrated that in TH-MYCN mouse neuroblastoma models, MYCN amplification is associated with the enrichment of genes involved in cell proliferation-related pathways as well as PRC2 target genes and that MYCN promotes the transcription of EZH2 [57]. Chen et al. identified by ChIP-quantitative PCR, that MYCN enrichment on the EZH2 gene promoter region, directly activated the transcription of EZH2 [54]. By studying early-stage tumor cells from TH-MYCN mice and gene signature scores, the impact of PRC2 on the transcriptome in early-stage neuroblastoma development is highlighted. It is clear that EZH2 plays an important role in malignant transformation in neuroblastoma and the study of EZH2 inhibition as a treatment for neuroblastoma warrants investigation [57].

EZH2 regulates tumorigenesis and differentiation in neuroblastoma

EZH2 mediated H3K27me3 is an important epigenetic regulatory mark and leads to transcriptional silencing of differentiation genes [58]. *EZH2* gene silencing with lentivirus-mediated short hairpin RNAs (shRNAs), showed that knocking down *EZH2* led to reduced H3K27me3 levels [59]. The expression of a candidate tumor suppressor gene *CASZ1* regulates late-stage neurogenesis, neural fate determination and differentiation, and was found to be increased after *EZH2* knockdown [52]. ChIP analysis identified that EZH2, SUZ12 and EED all bind within the transcription start site of *CASZ1* in neuroblastoma cell lines, coupled with an enrichment of H3K27me3. Demonstrating that *CASZ1* transcription is silenced by EZH2 mediated chromatin modifications [52]. The same study also demonstrated by qRT-PCR that *EZH2* knockdown leads to increased expression of *CLU*, *NGFR* and *RUNX3*, known tumor suppressor genes [52]. This finding is confirmed by Chen et al. who identified EZH2 and H3K27me3 peaks at the NGFR promoter region within neuroblastoma cell lines [54].

In addition, the inverse correlation between EZH2 and RUNX3 gene expression was established in 2008, and reported in numerous other cancers such as lung, breast, pancreatic and colon [60]. Although, evidence is established for RUNX3 as a target of EZH2, PRC2 targets thousands of genes, depending on the cell type, thus effects on the other gene expressions identified in neuroblastoma may not always be mirrored in other types of cancer [61]. However, the *in vitro* effects on gene expression identified within three studies all associated with neuronal cell differentiation and tumor suppression [52,54,55]. Particularly, Tsubota et al. was the first to determine that EZH2 regulates neuroblastoma cell differentiation through NTRK1 epigenetic regulation [55]. Additionally, they showed that *EZH2* knockdown significantly increased the expression level of the neurite extension and neuronal differentiation markers NF68 and GAP43. It was shown by qChIP assay that EZH2 binds directly to a promoter of the region of the *NTRK1* gene in neuroblastoma cells to regulate transcription by H3K27 methylation [55], demonstrating that EZH2 inhibits differentiation pathways in neuroblastoma cell lines. Impaired differentiation and cell development is a hallmark of neuroblastoma, therefore, to reverse the gene silencing that may cause this, could be an effective treatment strategy.

Therapeutic benefit of EZH2 inhibition in neuroblastoma

Development of EZH2 inhibitors

The first generation methyltransferase inhibitor 3-deazaneplanocin A (DZNep) was developed as an inhibitor of S-adenosyl-L-homocysteine hydrolase (SAHase) [62]. However, DZNep is not specific to EZH2 and does not suppress histone methylation through direct inhibition [63]. As EZH2 plays an important role in transferring a donor methyl group from S-adenosyl-L-methionine (SAM), EZH2-specific inhibitors have subsequently been developed which directly target the enzymatic activity of EZH2 via a SAM-competitive pathway [64]. The majority of these inhibitors share a common chemical structure feature 2-pyridone which can occupy the site of SAM and partially block EZH2 from binding [25].(Fig. 3) Direct non-enzymatic inhibitors can disrupt the interaction between EZH2 and EED or SUZ12 in order to break the PRC2 complex [25]. For example, an FDA approved EGFR inhibitor AZD9291 can inhibit the expression of EZH2 by breaking the interaction of EZH2-EED [65]. Table 1 lists current EZH2 inhibitors developed according to different mechanisms of action.

GSK126 is highly selective for targeting EZH2 compared to 20 other histone methyltransferases inhibitors and can inhibit both wild type and mutant EZH2 [39]. Similarly, GSK343 and EPZ005687 were developed as SAM-competitive inhibitors and have been shown to suppress the level of trimethylation of H3K27 in breast and prostate cancer cell lines [25]. However, GSK343 and EPZ005687 are limited to use in laboratory settings due to their substandard pharmacokinetic properties. Phase II clinical trial studies for GSK126 have subsequently been terminated due to insufficient efficacy in patients [66].

Encouragingly, one of the SAM-competitive inhibitors tazemetostat (Tazverik, Epizyme, Inc) was approved by the FDA for treatment of epithelioid sarcoma and follicular lymphoma in 2020 [67]. Tazemetostat has high selectivity and affinity for EZH2 with improved potency and pharmacokinetic characteristics over other SAM-competitive EZH2 inhibitor [66]. Tazemetostat is administered orally, and it is well tolerated [68]. Recently, the Pfizer clinical candidate PF-06821497 has also been developed as an oral-taken SAM-competitive inhibitor, and is currently under investigation in the NCT03460977 clinical trial for relapsed/refractory small cell lung cancer, castration resistant prostate cancer and follicular lymphoma [69].

Effects of EZH2 inhibition in neuroblastoma cell lines

Several studies have demonstrated the validity of targeting EZH2 therapeutically in neuroblastoma [52]. For example, treatment with GSK343 for 24 h, at a concentration of greater than 15 μM significantly reduced cell proliferation in neuroblastoma cell lines and PDX models. Cell viability was significantly reduced in 3 of the 4 cell lines at a concentration of 25 μM [70]. Cell motility after 24 h of treatment was significantly reduced in all four cell lines [70]. This result is mirrored by Mellini et al. whereby GSK126 significantly reduced cell proliferation of the human SK-N-BE cell line at a concentration of 25 μM, and their

Table 1
EZH2 inhibitors based on the different mechanisms of action.

EZH2 inhibitors	Mechanism of action
3-deazaneplanocin A (DNZep) (Non-specific to EZH2)	Inhibits S-adenosyl-L-homocysteine hydrolase and represses histone methylation
GSK126, GSK343, Tazemetostat (EPZ6438), EPZ005687, EPZ011989, DS3201b, JQEZ5, CPI1205 CPI-169, PF06821497, UNC1999, UNC2400	SAM-competitive inhibitors, inhibit EZH2 through occupying the site for SAM in the EZH2's binding pocket
Astemizole, AZD9291, A769662, apomorphine hydrochloride, EED226	Inhibit EZH2 through disrupting the interaction between EZH2 and EED or SUZ12

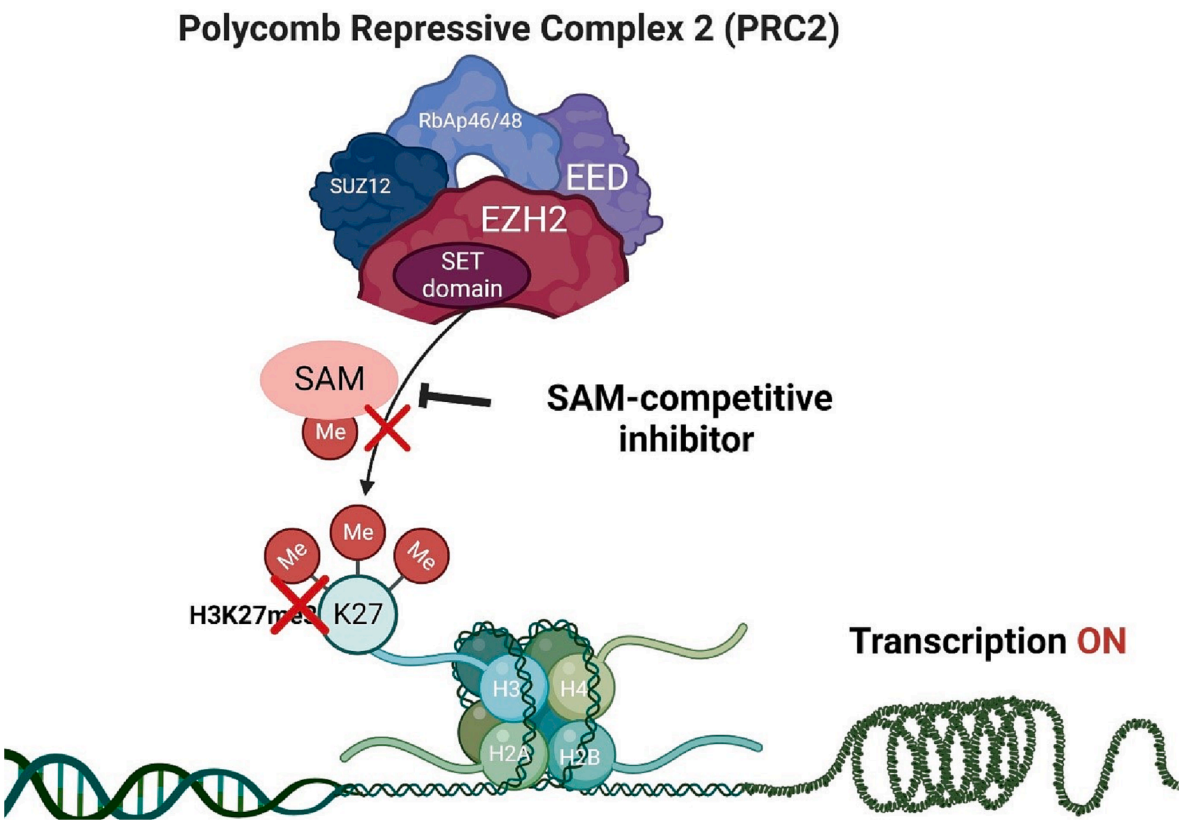

Fig. 3. Mechanism of SAM-competitive EZH2 inhibitor in the regulation of transcription.

experimental EZH2 inhibitor demonstrated significant reduction in proliferation after 5 days at a lower concentration of 1 μM [71]. The effect of GSK126 on cell viability was investigated alongside another EZH2 inhibitor JQEZ5 at increasing concentrations by Chen at al. in 16 human neuroblastoma cell lines [54]. The half maximal inhibitory concentration ranged from 2 to 8 μM with 5 days treatment, which was concomitant with the range at which H3K27me3 was decreased [54]. MYCN-amplified cell lines were significantly more sensitive to EZH2 inhibition than MYCN-non amplified cell lines. Of the 3 MYCN-amplified cell lines tested, both drugs caused an increase in apoptosis after 8–10 days treatment versus control, indicated by a significant increase in annexin-V-positive population and increase in sub-G1 fraction within the cell cycle [54].

A study by Wang et al. concluded that an increase in apoptosis with SAHase inhibition, as demonstrated by a 4-fold increase in the SubG1 phase was partially due to the induction of the caspase-dependent apoptotic pathway, when the cells were treated with DZNep for 96 h [52], as also found by Ren et al. [72]. However, in contrast to these studies, Bate-Eya et al. showed limited impact on cell cycle distribution by EZH2 inhibitors tazemetostat and GSK126 in neuroblastoma cells even after 144 h of exposure, with significant effects on colony-forming ability observed only at high concentrations surpassing those required for inhibiting H3K27 methylation [51].

EZH2 has been shown to exhibit other functions besides methyltransferase activity in the silencing tumor suppressor genes. *EZH2* gene silencing resulted in a strong apoptotic response, and following rescue with the addition of an exogenous *EZH2* mutant (EZH2ΔSET), lacking methyltransferase activity, partial reduced the apoptotic response, further suggesting that EZH2 may have methyltransferase independent activity [51]. In addition, several studies have observed the histology of the cell lines after both RNAi-mediated silencing of *EZH2* and pharmacological treatment with EZH2 inhibitors. Wang et al. observed an increase in neurite like processes [52], as did Chen at al [54]. This is as expected as inhibition of EZH2 leads to increased transcription of NTRK1, thus induction of neurite extension [55]. The increase in neurite extension occurred to a lesser extent with pharmacological inhibition than with genetic silencing, again demonstrating that EZH2 acts via methyltransferase independent pathways [55].

Effects of EZH2 inhibition in neuroblastoma mouse models

Studies testing a variety of EZH2 inhibitors in mouse models of neuroblastoma showed a significant reduction in tumor growth, size or volume when compared to control groups, demonstrating that growth-inhibitory effects EZH2 inhibitors observed *in vitro* were also seen in *in vivo* mouse models. The results are summarized in Table 2 [70 52 57 54].

Notably, ATRX alterations are frequently observed and resulted in the consequent in-frame fusion (IFF) proteins in neuroblastoma. ATRX IFF has been shown to regulate neuronal differentiation. As both EZH2 and transcription factor REST silence neuronal gene programs in ATRX IFF neuroblastoma, Qadeer et al. investigated the inhibition of EZH2 in ATRX IFF neuroblastoma cell line SK-N-MM and CHLA90. The results showed EZH2 inhibitor tazemetostat or UNC1999 significantly leads to ATRX IFF neuroblastoma cell death or decreased tumor growth in ATRX IFF xenograft model respectively. These findings provide support for the use of EZH2 inhibitors as a therapeutic approach for ATRX IFF neuroblastoma [73].

EZH2 inhibition in potential combination therapy for neuroblastoma

Although inhibition of EZH2 has showed some early success in *in vitro* and *in vivo* for neuroblastoma, it has also been reported EZH2 inhibition alone was not curative as tumor regrowth was observed with no extension to overall survival after treatment cessation [54,57]. Therefore, it is highly like that EZH2 inhibition as a treatment strategy would be used as part of combinational therapy.

Table 2
Five different EZH2 inhibitors were investigated in the four studies in a variety of different mouse models, including MYCN-amplified and MYCN-non amplified neuroblastoma cell lines. All experiments showed a significant reduction in either tumor size or volume, after a range of time (12 to 28 days). Chen et al. demonstrated that overall survival of two of the groups were significantly improved with treatment versus no treatment.

Study	EZH2 inhibitor	Mouse Model	Results	Tolerability/Toxicity
Bownes et al. (2021)	GSK343 10 mg/kg/day versus control (sterile PBS) Intraperitoneal injection for 21 days	SK-N-BE(MYCN-amplified) xenograft (14 mice total, 7 mice/group)	Relative tumor growth significantly reduced with GSK343 $p = 0.03$ Fold change in tumor volume – significantly reduced with GSK343 after 16 days treatment $p \leq 0.05$	Well tolerated – constant weight gain, no significant difference between treatment and control group. Data presented.
Wang et al. (2012)	DZNep 2.5 mg/kg twice daily, three days/week for 28 days or control (0.9%sodium chloride)	SMS-KCNR xenograft (14 mice total, 7 mice/group)	Tumor volume (mm^3) significantly reduced versus control after 27 days with DZNep $p < 0.05$	No comment made as to tolerability or toxicity
Tsubota et al. (2017)	Tazemetostat 300 mg/kg/day for 14 days versus vehicle alone	TH-MYCN+/- spontaneous tumor model; 6 mice in control group, 7 mice in treatment group	Tumor weight significantly reduced in EPZ6438 group $p < 0.01$	Stated no observed weight loss or other visible adverse effects – no data presented.
Chen et al. (2017)	JQEZ5 150 mg/kg/day by intraperitoneal injection or vehicle, for 7 days, followed by a 5 day no-drug days Treatment reinitiated at 75 mg/kg/day until sacrifice.	Kelly cell line (MYCN-amplified) in NOD-SCID-IL-2Rγnull (NSG) mice– 10 per group	Tumor volume (mm^3) significantly reduced at 17 days total duration ($p < 0.05$)	After seven days of JG3Z5 – treatment paused due to weight loss, reinitiated at half the dose after 5 days with no intervention. No data presented.
	GSK126 150 mg/kg/day or vehicle by intraperitoneal injection for 21 days.	CHP-212 (MYCN-amplified) xenograft 8 mice/group	Tumor volume (mm^3) significantly reduced at 21 days total duration ($p < 0.01$) for all three xenografts.	No comment made as to tolerability or toxicity. No data presented.
		SK-N-BE(2) (MYCN-amplified) xenograft 7 mice/group SH-SY-5Y (MYCN-non amplified) xenograft 8 mice/group	Significant increase in overall survival $p < 0.01$ in the SK-N-BE(2) and SH-SY-5Y groups.	

EZH2 inhibition has shown potential synergy when combined with other treatment options, especially HDAC-inhibitors or EHMT2-inhibitors in other tumor types [74,75]. Chen et al. demonstrated in an *in vitro* study using neuroblastoma cell line Kelly treated with GSK126 and the histone deacetylase inhibitor Panobinostat, this combination showed the greatest synergy compared to the other combinations tested [54]. A significant combination index for this combination was also observed in further testing on three other cell lines SK-N-BE(2), LAN-1 and CHP-212. Combination therapy led to greater gene expression of tumor suppressor genes than with single treatment [54]. Seier et al. investigated the effect of combining the EZH2 inhibitor EPZ011989 and the histone lysine methyltransferase inhibitor UNC-0638 on neuroblastoma cell lines IMR-32 and SK-N-BE. Results suggested that combination therapy lead to an enhanced effect of IFN-γ on cytokine expression, and thus has the potential to favorably modify the tumor microenvironment [76].

The demonstration that EZH2 inhibition modifies the tumor microenvironment in a favorable way for treatment, indicates combining this with immunotherapy could be a promising area of research. Antibody-dependent cell mediated cytotoxicity, with release of cytotoxic granules and cytokines from natural killer cells is thought to be the key mechanism of action, resulting in tumor cell destruction [77]. It has also been shown that neuroblastoma cells can escape from the innate anti-tumor immune response by downregulating ligands for natural killer cell activating receptors [78]. A recent study demonstrated EZH2 as an essential repressor of natural killer cell activating ligands and inhibition of EZH2 enhances the activity of natural killer cells in hepatocellular carcinoma [79]. Therefore, promoting the antitumor cytotoxicity of natural killer cells through inhibition of EZH2 has the potential to enhance anti-GD2 immunotherapy as anti-GD2 antibodies induce antibody-dependent cell-mediated cytotoxicity (ADCC) via NK cells. Additionally, in Ewing sarcoma, EZH2 inhibition has been shown to enhance the expression of GD2 [80].

Through comprehensive analyses of both bulk and single-cell transcriptomic data, a number of studies have shown that neuroblastoma cell types are defined by expression signatures associated with a more adrenergic or more mesenchymal phenotype [81–83]. Mesenchymal cells exhibited greater resistance to current therapies and were found to be enriched in tumors that appeared after therapy or during relapse [81]. A recent study has suggested adrenergic to mesenchymal transition correlated with reduced GD2 expression which may cause resistance to anti-GD2 immunotherapy [84]. Mabe N.W et al demonstrated that treatment with EZH2 inhibitor tazemetostat in a neuroblastoma cell model reverses the forced adrenergic-to-mesenchymal transition and induces an adrenergic-like state via re-expression of a key GD2 synthase pathway component *ST8SIA1*, thus increasing GD2 expression and enhancing the effectiveness of anti-GD2 immunotherapy [84]. This further suggests that combining EZH2 inhibitor with anti-GD2, which is already used as standard of care immunotherapy in high-risk neuroblastoma, could also lead to enhanced therapeutic responses.

An alternative potential approach is to combine an EZH2 inhibitor with a differentiating agent. Neuroblastoma arises from neuronal crest cells which are unable to develop into mature cells due to the loss of differentiating ability [3]. Particularly, high-risk neuroblastoma has undifferentiated or poorly differentiated histopathological features [7], therefore repairing differentiation becomes a treatment strategy for neuroblastoma patients. Isotretinoin is currently applied as one of the maintenance therapies for neuroblastoma aiming to promote differentiation in remaining tumor cells after conventional treatment. As inhibition of EZH2 can also promote cell differentiation, combining an EZH2 inhibitor with retinoic acid could promote further differentiation and become a promising treatment for high-risk neuroblastoma patients.

Conclusion

Neuroblastoma is an aggressive pediatric tumor arising from the sympathetic nervous system. High-risk neuroblastoma continues to have a poor prognosis despite recent advances in diagnosis and multimodal treatments. It is recognized that undifferentiated or poorly differentiated histology is associated with poorer outcomes, therefore this hallmark provides a potential axis for novel therapeutic strategy. This review has summarized how epigenetic control via EZH2 could represent a new target in the treatment of neuroblastoma. Upregulation of EZH2 activity leads to cell proliferation, cell survival and inhibited differentiation [58]. Evidence from a few studies supports that neuroblastoma has significantly high EZH2 expression which may be involved in the malignant transformation of cells and development of neuroblastoma. Both studies of *in vitro* and *in vivo* models of neuroblastoma have suggested

that genetic and pharmacological disruption of EZH2 inhibits cancer cell or tumor growth. The use of EZH2 inhibitors may be beneficial in combination treatments, EZH2 inhibitor tazemetostat induced reprogramming of the cell state from mesenchymal to adrenergic, which resulted in the upregulation of GD2 and restoring sensitivity to anti-GD2 immunotherapy. Notably, the approval of tazemetostat indicates it may show efficacious and safety in clinical studies for neuroblastoma.

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CRediT authorship contribution statement

Jinhui Gao: Writing – original draft, Writing – review & editing. **Claire Fosbrook:** Writing – original draft, Writing – review & editing. **Jane Gibson:** Writing – review & editing. **Timothy Underwood:** Writing – review & editing. **Juliet Gray:** Writing – review & editing. **Zoë Walters:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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