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Medulloblastoma: Molecular understanding, treatment evolution, and new developments

Xiaohua Liu,[1,2,3] Chunyong Ding,[1,2] Wenfu Tan,[4,*] and Ao Zhang[1,2,3,*]

[1]Research Laboratory of Medicinal Chemical Biology & Frontiers on Drug Discovery (RLMCBFDD), School of Pharmacy, Shanghai Jiao Tong University, Shanghai 200240, China

[2]CAS Key Laboratory of Receptor Research, Shanghai Institute of *Materia Medica* (SIMM), Chinese Academy of Sciences, Shanghai 201203, China

[3]University of Chinese Academy of Sciences, Beijing 100049, China

[4]School of Pharmacy, Fudan University, Shanghai 201203, China

[*]**Correspondence:** wftan@fudan.edu.cn (W. Tan); aozhang@simm.ac.cn (A. Zhang)

Contents

ABSTRACT

Medulloblastoma (MB) is the most common childhood malignant brain tumor, accounting for approximately 20 % of all pediatric central nervous system tumors. Current standard treatments involving surgical interventions followed by craniospinal irradiation and adjuvant chemotherapy have severe motor and cognitive defects. Therefore, individualized treatment regimens with reduced toxicity designed according to the presence of specific oncogenic 'driver' genes are urgently demanded. To this end, recent genetic and epigenetic findings have advanced the classification of MB into the international consensus of four distinct MB molecular subgroups (WNT, SHH, Group 3, and Group 4) based on their respective molecular and histopathological characteristics. More recent studies have indicated that up to seven molecular subgroups exist in childhood MB. Moreover, studies on the inter- and intra-tumoral features of the four subgroups revealed that each subgroup contains variant subtypes. These results have greatly helped risk stratification of MB patients at diagnosis and significantly improved clinical treatment options. Herein, we highlight the recent advances and challenges associated with MB classification, and the development of therapeutic treatments targeting novel subgroup-specific molecular and epigenetic factors, especially those in the SHH-driven MB tumors.

Keywords: medulloblastoma; pediatric brain tumor; oncogenic driver gene; Hedgehog pathway inhibitor, Smoothened receptor antagonist

Abbreviations.

MB, medulloblastoma; WHO, World Health Organization; WNT, wingless; SHH, sonic Hedgehog; PTCH1, patched 1; SMO, Smoothened; GLI, glioma-associated oncogenes; CRD, cystine rich domain; CDK6, cyclin-dependent kinase 6; BCC, basal cellular carcinoma; Hh, Hedgehog; BET, bromodomain and extra C-terminal domain. HDAC, histone deacetylase.

1. Introduction

Medulloblastoma (MB) is a high-grade malignancy that was initially described in 1925 as a distinct series of childhood cerebellum tumors. MB dominantly occurs in infancy (ages less than 3 years) and childhood (ages younger than 16 years) and now represents the most common childhood malignant brain tumor accounting for nearly 20-30% of all pediatric central nervous tumors in children (Pui et al., 2011; Ostrom et al., 2015).

MB generally occurs in the posterior fossa, making its diagnosis and treatment much challenging and often delayed. In the 1930s, surgical intervention was the only treatment option for MB; however, the immediate post-operative mortality rate was found higher than 30% (Millard & De Braganca, 2016). Until the 1950s, craniospinal irradiation was introduced as an adjuvant treatment following surgical resection and showed improved survival rates. Unfortunately, serious motor and cognitive adverse effects, including neurocognitive impairment, secondary malignancies, and endocrine dysfunction were observed in younger patients (Millard & De Braganca, 2016; Paterson & Farr, 1953). Since the 1970s, non-specific cytotoxic chemotherapy was introduced in combination with surgery and/or radiation (Figure 1) (Millard & De Braganca, 2016; Tait et al., 1990). Currently, cytotoxic chemotherapy combined with radiation continues to be used as the standard therapy for MB. However, the long-term use of these therapeutic regimes has shown to induce substantial toxic damages, most notably in developing young patients. The inability of the therapy to recognize both the inter- and intra-tumoral heterogeneity of MB subgroups and the lack of clinical biomarkers to stratify patients, are the major drawbacks underlying the adverse effects (Lannering et al., 2012; Jakacki et al., 2012).

[Figure 1 Here]

2. Molecular and epigenetic classification of MB

2.1 Early classification of MB: from morphological to histological

When MB was first described in the 1930s, it was classified as a large subgroup of malignant invasive embryonal tumors of the cerebellum based on similar morphology with densely packed cells. Metastases of MB barely occur outside the central nervous system, but have been occasionally observed in the bone marrow, lymph nodes, and viscera (Eberhart et al., 2003). In the 1980s the World Health Organization (WHO) classified MB into four main histologic types, namely, desmoplastic/nodular (D/N) MB, MB with extensive nodularity (MBEN), anaplastic MB, and large cell MB, each with variant histologic appearance and different prognosis (Louis et al., 2007). This evolution of MB classification was clinically beneficial since it allowed for the thorough characterization of each tumor prior to commencement of treatment. Unfortunately, differences within similar histological MB variants were overlooked under this classification. Therefore, MB patients were then roughly stratified as being at either average risk or high risk based on clinicopathological variations pertaining to age, extent of resection, stages of metastases, and to some extent the individual genetic alterations (Eberhart et al., 2002; Ellison et al., 2010; Ellison et al., 2011).

2.2 Recent classification of MB: from histological to molecular

The advancement of high-throughput genomic and proteomic techniques has significantly enhanced our understanding of MB. Thus, data obtained through these techniques has contributed to international consensus of MB as the four distinct subgroups by WHO in 2016 at the oncogenic molecular level, namely, wingless (WNT), sonic Hedgehog (SHH), Group 3 and Group 4 (Louis et al., 2016).

The WNT subgroup is the rarest subgroup occurring primarily in children between 4-17 years of age and accounting for approximately 10% of all MB diagnoses. This subgroup is characterized by aberrant activation of the WNT signaling cascade and commonly harbors mutations in exon 3 of *CTNNB1* (encoding β-catenin) and monosomy chromosome 6 (Kool et al., 2012; Ellison et al., 2011). The 5-year survival rate of the WNT subgroup is favorably up to 95%. The SHH subgroup accounts for approximately 30% of all MB diagnoses and is the most common molecular subgroup in both infants (ages < 3 years old) and adults (ages > 17 years old) with few diagnosed during childhood (ages 4-16 years old). The SHH MB is characterized by inactivating germline or somatic mutations in specific SHH pathway genes including *PTCH1* (patched homologue 1), *SMO* (smoothened homologue), and *SUFU* (suppressor of fused homologue), gene amplification of *GLI1*, *GLI2*, and *MYCN*, and mutations in *TP53* (Kool et al., 2012; Ellison et al., 2011; Northcott et al., 2012). The Group 3 MB occurs in both infancy and childhood with few diagnosed in patients older than 18 years old, accounting for 25% of all MB diagnoses. Recurrent *MYC* amplification is a common genetic feature of this subgroup, and the clinical outcome is the worst due to frequent metastases. The MB tumors categorized as Group 4 are the most prevalent accounting for approximately 35% of all MB diagnoses and frequently occurring in teenagers and children with 30% metastatic at diagnosis. This group features aberrations of isochromosome 17q, amplification of *MYCN* and *CDK6* (cyclin-dependent kinase 6), duplication of *SNCAIP* and loss of isochromosome 11q. Group 4 MB are the least biologically characterized with cell of origin remaining unclear (Ramaswamy & Taylor, 2017; Wang et al., 2018; Cavalli et al., 2017).

The molecular characterization of MB ignited further pursuit of clinically

effective genetic alterations specific for MB subgroups, which subsequently promoted more accurate refining of risk stratification and the biomarker-driven clinical diagnosis. It is now agreed that the WNT subgroup and the non-metastatic Group 4 MB tumors with whole chromosome 17 gain or whole chromosome 11 loss are defined as low risk tumors, whereas high risk tumors are those with metastatic or *MYCN* amplified SHH and metastatic Group 4 tumors. The metastatic Group 3 and the SHH with *TP53* mutations are defined as the very high-risk MB (Ramaswamy et al., 2016).

The subgrouping of MB has made the WNT and SHH MB patients readily diagnosed based on their primary transcriptional and methylation profiling. However, the Group 3 and Group 4 subgroups share some molecular and biological similarities that make their discrimination suffering from difficulty. Meanwhile, high heterogeneity is found essentially existing within each subgroups and varies among infants, children and adults.

Recently, Northcott and co-workers studied the genomic landscape across 491 previously untreated MBs, and identified new subgroup-specific driver genes and epigenetic effectors, especially for Group 3 and Group 4. They found that recurrent hotspot insertions targeting *KBTBD4* (Kelch repeat and BTB domain containing 4) are common feature in both Group 3 and Group 4 subtypes, and *PRDM6* (PR/SET domain 6) - a presumed histone methyltransferase was identified as the most prevalent driver alteration in Group 4 (Northcott et al., 2017). Meanwhile, Schwalbe and co-workers assessed 428 samples from patients with childhood MB aging between 0-16 years old at diagnosis, and investigated their biological heterogeneity and survival differences within each subgroup (Schwalbe et al., 2017). This study identified seven robust and reproducible primary molecular subgroups of childhood

MB, among which, the WNT group remains intact, whereas each of the other three subgroups was split into two. The SHH subgroup was split into two age-dependent subgroups, SHH_{infant} (< 4.3 years old) and SHH_{child} (> 4.3 years old); The Group 3 and Group 4 subgroups were each divided into high-risk (Group 3_{HR}, Group 4_{HR}) and low-risk (Group 3_{LR}, Group 4_{LR}) subgroups. Further molecular and clinicopathological features specific for each of the seven subgroups were established to elaborate disease risk-factors, which will be of significance to improve disease risk-stratification and treatment decision on childhood MB patients, who receive craniospinal irradiation as the therapy.

Interestingly, to address the inter-tumoral heterogeneity within MB subgroups, Goldenberg and co-workers recently applied similarity network fusion (SNF) strategy to evaluate the gene expression and DNA methylation across a cohort of 763 primary frozen MB samples and identified 12 different MB subtypes (Cavalli et al.; 2017), including two WNT (α, β), four SHH (α, β, γ, δ), three Group 3 (α, β, γ), and three Group 4 (α, β, γ). The WNTα and WNTβ subtypes differ in age at diagnosis and in frequency of monosomy 6. As such, the WNTα subtype is for young patients with median age of 10 years and with high frequency of monosomy 6, whereas WNTβ is for adults with median age of 20 years. Likewise, the SHHα and SHHδ subtypes are for childhood/adolescent and adults, respectively, whereas the SHHβ and SHHγ correspond to infants with median ages of 1.9 and 1.3 years at diagnosis, respectively. It was found that *TP53* mutations are exclusively prognostic in the SHHα subtype, and not in the other non-SHHα subtypes. Within the Group 3, the 3α subtype is for metastatic infants, whereas 3β is for less metastatic. The 3γ subtype is a high-risk subtype enriched for *MYC*-amplification or *MYC*-activation and has poor clinical outcomes. The 4α, 4β and 4γ subtypes in Group 4 is characteristic of *MYCN*

amplification, *SNCAIP* duplication and *CDK* amplification, respectively. Taken together, these encouraging results on recognition of clinically relevant substructures within each subgroup will not only allow for more precise refinement of risk stratification, and also encourage the identification of biological markers and genetic drivers to discriminate specific subtype. Undoubtedly, this result will also spur new insights for the development of subtype-based molecularly targeted therapy (Bavle & Parsons, 2017; Hovestadt et al. 2020).

2.3 Further classification of MB: from molecular to epigenetic

Posttranscriptional histone modifications significantly impact the structure of chromatin and regulation of gene transcription (Bavle & Parsons, 2017; Jones, Issa, & Baylin, 2016). Recently, multiple genes encoding enzymes associated with histone modifications have been found to be differentially expressed within MB tumors. Moreover, the expression patterns demonstrated specific subgroup bias, thus motivating further investigation into the epigenetic factors associated with different MB subgroups (Batora et al., 2014).

DNA Methylation. By using a restriction landmark genomic scanning approach, Plass and co-workers reported that approximately 1% of all CpG islands in primary MB, and 6% in MB cell lines are abnormally hypermethylated. Some of these hypermethylated sequences have prognostic potential (Fruhwald et al., 2001). Furthermore, using DNA methylation microarrays, the DNA methylation status of 1505 loci in 807 genes were analyzed from 230 MB samples (Schwalbe et al., 2013). It was found that many genes were identified as irregularly methylated in different MB subgroups. For example, partially methylated domains were primarily detected in the WNT and Group 3 subgroups covering up to one-third of the genome. An

oncogene *VAV1* was found hypomethylated with high expression in most human and murine SHH MB. The gene *LIN28B* was found specifically expressing in Group 3 and Group 4 subgroups (Hovestadt et al., 2014; Yi & Wu, 2018).

Histone Modifications. A study that employed single nucleotide polymorphism arrays on 212 MB samples identified a series of epigenetic aberrations targeting histone lysine methyltransferases, histone demethylases, histone acetyltransferases, and members of the polycomb groups of transcriptional repressors (Northcott et al., 2009). Additionally, somatically mutated genes were identified in 310 primary MB tumors in 2012, and the driver genes in each MB subgroup were subsequently classified using a next-generation sequencing strategy (Jones et al., 2012; Pugh et al., 2012; Robinson et al., 2012). The results revealed that a loss-of-function mutation in *MLL2* frequently exists within the WNT and SHH subgroups; whereas the *MLL3* mutation was found to be most common in Group 3 and Group 4. Mutations in *BCOR* (BCL-6 co-repressor) and *LDB1* were also identified with a high occurrence rate of 14 % in the SHH group. Furthermore, *CREBBP* and *ARID1B* mutations were frequently identified in the WNT and SHH subgroups. Importantly, there were also mutations identified exclusively in Group 4 MBs, specifically, in genes encoding *KDM6A*, a MLL2-binding partner, and the Zinc finger MYM-type protein 3 (*ZMYM3*). Moreover, an aberrant H3K27 methylation state was identified specifically in Group 3 and Group 4 MBs. Both subgroups also demonstrate aberrations in somatic copy number and alternations in transcriptional profiles that converged on modifiers of H3K27-methylation (*EZH2*, *KDM6A*, and *KDM6B*) (Northcott et al., 2012; Dubuc et al. 2013).

3. Development of molecular targeted therapies for MB

3.1 Treatment challenges and current available therapies

Since MB tumors are located in the posterior fossa, early diagnosis and treatment are difficult and metastases often occur at diagnosis, which are the leading cause of mortality, especially for those high-risk MB patients. Maximal surgical resection followed by radiation and chemotherapy is the current standard therapy. However, >25% of patients experience cerebella mutism, dysarthria and neurocognitive disorders after surgical operation, and the adjuvant craniospinal radiotherapy and non-specific chemotherapy (vincristine, cisplatin, etoposide, temozolomide, *et. al.*) are reported to cause severe adverse effects and occurrence of secondary tumors (Wang et al., 2018). The classification of MB into four subgroups by WHO in 2016 and the recent identification of more subgroups in childhood MB as well as many subtypes within each subgroup have significantly innovated risk stratification of MB patients at diagnosis and tailored the clinical treatment options, leading to significantly improved survival rates. However, specific treatment for each of the MB subgroups or subtypes are still in the infancy, mostly due to the high heterogeneity within each of the subgroups, as well as the lack of clinically validated specific 'driver' genes for the development of molecularly targeted therapy. Some of the most recent identified oncogenes such as *MYCN*, *MYC*, *TP53*, *CDK6*, *SNCAIPR* and many others may better serve as molecular biomarkers for specific subgroup or subtype, rather than as drug targets. Therefore, corresponding clinically effective inhibitors selectively targeting these genes are either unavailable or have been proven unsuccessful due to their involvements in many other signaling pathways that cause unwanted toxicity (Quinlan & Rizzolo, 2017; Hovestadt et al. 2020). However, since the WNT subgroup of patients are primarily older children and have high prognosis, recent effort is to establish a de-escalated treatment to reduce long-term adverse

sequelae of the standard treatment. Meanwhile, since the Group 3 has the worst prognosis and the Group 4 is least characterized, current attempts on these two subgroup tumors are to optimize combinations of different cytotoxic therapies (*e.g.* gemcitabine and pemetrexed) to balance the efficacy and the toxicity. Relatively, the SHH subgroup is the most studied and well defined MB subtype, representing the majority of infant and adult MB patients. Many small molecule inhibitors targeting the somatic mutations or gene amplifications of the SHH signaling pathway have been designed and evaluated extensively in both preclinical and clinical trials with a few already approved for clinical use. Therefore, the rest of this Review will focus on advances of the development of molecular targeted inhibitors for the treatment of SHH-driven MB tumors (Samkari et al., 2015).

[Figure 2 Here]

3.2 Development of Hedgehog pathway inhibitors by targeting the Smoothened receptor

First Generation Smoothened Receptor Antagonists. The sonic Hedgehog (SHH) MB subgroup is characterized by constitutive activation of the Hedgehog (Hh) signaling pathway. In the absent of Hh ligand (SHH, IHH, DHH) binding, the 12-transmembrane receptor protein Patched 1 (PTCH1) inhibits the Smoothened receptor (SMO), thus acting as a negative regulator of the Hh signaling pathway. This Hh pathway is activated by binding of one of the extracellular Hh proteins (*e.g.* sonic hedgehog, SHH) to PTCH1, which abolishes its inhibition of SMO, another critical receptor in the Hh signaling pathway (Figure 2). SMO is subsequently translocated into the primary cilium where it activates one of the GLI zinc finger transcription factors (GLI1, GLI2 and GLI3). GLI activation consequently initiates transcription of Hh target genes, namely *GLI1*, *PTCH1*, *cyclin D1*, *BCL-2* and *SNAIL*. Aberrant

activations of SHH signaling pathway due to germline or somatic mutations of these target genes, primarily loss of PTCH1, gain-of-function of SMO, and loss of SUFU are the leading causes of SHH MB tumors (Pak & Segal, 2016). Several strategies have been explored to target this pathway and thus to suppress the development of SHH MB, including development of ligands to interfere with the binding of the PTCH1 receptor, designing antagonists to target SMO, or development of direct or indirect modulators of the transcriptional factor GLI (Yin & Esmaeli, 2017; Khatra et al., 2017; Wellbrock et al., 2015). Furthermore, genetically engineered mouse models were established in which MB was induced by loss of one copy of the *Ptch* gene and deletion of the *p53* gene ($Ptch^{+/-}$ $p53^{-/-}$ MB model) (Wetmore et al., 2001; Briggs et al., 2008), or by knock-in of the *SMO* gene bearing an activation point mutation (W535L; SMO A1 model) (Goodrich et al., 1997; Hatton et al., 2008). Numerous Hh inhibitors have been validated as efficient treatment of Hh-dependent cancers, including basal cellular carcinoma (BCC), SHH MB, non-small-cell lung cancer, colon cancer and others (Yin & Esmaeli, 2017; Khatra, Bose, & Sinha, 2017). In addition, the Hh pathway is also implicated in keeping leukemic stem cells dormant thus promoting resistance and disease progression. Therefore, Hh inhibitors are also proposed to enhance the sensitivity of leukemic cells to cytotoxic drugs (Wellbrock et al., 2015).

Cyclopamine. The steroid cyclopamine (compound **1**, Figure 2) is the first Hh pathway inhibitor to be discovered. It functions by directly binding to the hepathelical bundle on the SMO receptor and has an IC_{50} value of 300 nM in solution with the Hh target genes, *PTCH1* and *GLI1*. However, cytotoxicity and poor pharmacokinetic properties of cyclopamine limited its clinical development (Iovine et al., 2016).

Vismodegib and Sonidegib. Vismodegib is an aryl amide (compound **2**, GDC-0449, Figure 2) that interacts with SMO via its

4-chloro-3-(pyridin-2-yl)aniline component. It has IC_{50} values of 13 nM and 2.8 nM in a Hh-responsive GLI luciferase assay and an analogous assay that employs human embryonic palatial mesenchyme cells, respectively (Robarge et al., 2009). In *Ptch*$^{+/-}$-derived MB allograft mice, vismodegib was shown to induce complete tumor regression at doses as low as 12.5 mg/kg when administered twice per day. Moreover, in 2012, this compound became the first Hh pathway inhibitor (Erivedge) to receive the FDA's approval for treatment of locally advanced and metastatic BCC (Gould et al., 2014; Dlugosz, Agrawal, & Kirkpatrick, 2012).

In 2015, an additional SMO antagonist, sonidegib (Erismodegib/LDE-225/Odomzo, compound **3**, Figure 2) was also approved as an alternative treatment for BCC (Pan et al., 2010). This compound can penetrate the blood-brain barrier (BBB), making it potentially effective for treatment of MB. When administered 5 mg/kg/day subcutaneously to the *Ptch*$^{+/-}$-derived MB allograft mice, sonidegib was found to significantly inhibit tumor growth with a treatment/control value of 33%, and tumor regression was achieved when sonidegib was used at higher doses. Unfortunately, the therapeutic effect of this SMO antagonists was determined to be transient, and prolonged drug exposure led to resistance and relapse due to the development of several resistant mutations in SMO residues (Casey et al., 2017; Atwood et al., 2015; Danial et al., 2016).

Glasdegib (Daurismo, PF-04449913). Glasdegib (compound **4**, Figure 2) is a newly approved third-to-market SMO antagonist bearing a benzoimidazole scaffold (Munchhof et al., 2011). This compound is orally available and has demonstrated high potency at a concentration of 5 nM in the GLI-luciferase reporter assay. In 2018, the US FDA approved glasdegib in combination with low doses of cytarabine for treatment of newly diagnosed acute leukemia based on its promising preclinical

results in this disease (Fukushima et al., 2016; Wolska-Washer & Robak, 2019). Unfortunately, there are no reports on both preclinical and clinical studies of this drug in MB patients either alone or in combinations.

Artemisinin Derivatives. Our laboratory has recently designed a series of novel SMO antagonists by combining the basic skeleton of the natural product artemisinin with a SMO-targeting warhead (Liu et al., 2016). Artemisinin has moderate antitumor activity with unknown molecular mechanism, but the new derivatives **5a** and **5b** (Figure 3) demonstrated potent activity against the Hh pathway, with the most potent showing an IC_{50} of 9.53 nM. Moreover, intraperitoneal injection of the compound in *Ptch*$^{+/-}$*p53*$^{-/-}$ MB allograft mice, at concentrations of 25 mg/kg or 50 mg/kg twice per day for 13 consecutive days induced 81% and 91% tumor growth inhibition, respectively. However, these compounds were found invalid to combat the SMO resistant mutants (Liu et al., 2016; Wang et al., 2014).

[Figure 3 Here]

3.3 Hedgehog inhibitors targeting Smoothened receptor mutations

The gene mutations of SMO protein are the primary mechanism for the resistance of SMO antagonists that cause loss of durable response and induce disease progression within several months of treatment. Several point mutations that disrupt the binding of SMO antagonists have been observed clinically, including D473H (mouse D477G), E518, and among others. In addition, inappropriate amplifications or mutations of the Hh downstream components (*e.g.* SUFU, GLI) and activations of other interacting pathways such as PI3K/mTOR/AKT and RAS/RAF/MEK pathways might also contribute to the resistance of SMO antagonists (Samkari et al., 2015). Therefore, new generation Hh inhibitors targeting SMO resistant mutations or the downstream factors are emergently needed to

address the acquired resistance to existing SMO antagonists.

MK-4101. As shown in Figure 3, MK-4101 (compound **6**) is a SMO antagonist structurally distinct from the previous arylamide series of SMO antagonists. This compound has been shown to inhibit the proliferation of MB cells derived from neonatally irradiated *Ptch*$^{+/-}$-derived mice *in vitro* with an IC_{50} of 0.3 μM. It has also demonstrated good bioavailability (> 87 %) with low-to-moderate plasma clearance in mice and rats. Notably, MK-4101 induced tumor regression at a concentration of 80 mg/kg twice per day in *Ptch*$^{+/-}$-derived MB allograft mice, and is currently in phase I clinical trials for treatment of solid tumors (Rimkus et al., 2014; Xin et al., 2018).

TAK441. The pyrrolo[3,2-c]pyridine-4-one derivative (TAK-441, compound **7**, Figure 3) is another SMO antagonist that potently inhibits Hh signal transduction (Ohashi et al., 2012; Ishii et al., 2014). It has exhibited nearly equal binding affinity for the clinically observed SMO D473H resistant mutant (SMO^{D473H}) and the wild-type SMO (SMO^{WT}). Further, TAK-441 significantly inhibited the proliferation of both SMO^{WT}- and SMO^{D473H}-dependent cells with IC_{50} values of 3.2 nM and 79 nM, respectively. In *Ptch*$^{+/-}$-derived MB allograft mice, oral administration of TAK-441 25 mg/kg/day for 14 days resulted in complete growth inhibition. Unfortunately, further development of this compound was suspended for unspecified reasons.

NVP-LEQ506. Structural optimization of a hit phthalazine compound **8** has led to NVP-LEQ506 (compound **9**, Figure 3) as a novel SMO antagonist bearing a pyridazine core (Peukert et al., 2013). This compound exhibited IC_{50} values of 2 nM and 4 nM in solution with human and mouse SMO, respectively; and inhibited the SMO^{D473H} mutant with an IC_{50} of 96 nM. Furthermore, in *Ptch*$^{+/-}$-derived MB

allograft mice, oral administration of NVP-LEQ506 40 mg/kg for 8 days, or 10 mg/kg for 9 days in rats, induced 85% and 98% tumor growth inhibition, respectively.

Bisamide derivatives. Bisamide compound **10** (Figure 3) was reported to be a potent SMO antagonist with robust activity against both SMO^{WT} and SMO^{D473H} with IC_{50} values of 300 nM and 700 nM, respectively. Moreover, the IC_{50} values of compound **10** against endogenous human and mouse SMO in the presence of Hh ligand was 8 nM and 27 nM, respectively. Further, administration of this compound at concentrations of 100 mg/kg/day caused significant tumor growth suppression in mice that had demonstrated resistance to vismodegib due to SMO^{D477G} mutations (Dijkgraaf et al., 2011).

2-Aryl benzimidazoles. *N*-Phenylbenzamide derivative **11** (Figure 3) is a potent Hh inhibitor with an IC_{50} value of 40 nM for SMO (Romer et al., 2004). This compound is nearly equipotent against all SMO alleles by exhibiting 93% inhibition against SMO^{WT}, 90% inhibition against SMO^{D473H}, and 98% inhibition against SMO^{E518} at a concentration of 1 μM. Further, treatment of tumors expressing SMO^{D477G} with compound **11** led to significant tumor growth inhibition. Unfortunately, this compound exhibited moderate-to-high hepatic clearance in rats that limited its further development.

Itraconazole. The antifungal medication itraconazole (compound **12**, Figure 3) has been found to function as a SMO receptor antagonist. However, it exhibits a unique mechanism distinct from other known SMO antagonists (Kim et al., 2010). Itraconazole prevents the ciliary accumulation of SMO, which is normally induced following Hh stimulation. Further, this compound displayed equivalent antiproliferative activity in both the SMO^{WT} (IC_{50}: 55 nM) and the SMO^{D477G} (IC_{50}: 62 nM) dependent MB cells. In vismodegib-resistant tumors, treatment with

single-agent itraconazole resulted in significant tumor growth inhibition (Kim et al., 2013).

ALLO-1 and ALLO-2. ALLO-1 (compound **13**, Figure 3) and ALLO-2 (compound **14**) were found to inhibit both wild-type and mutant SMO. ALLO-1 has IC_{50} values of 489 nM and 1.2 μM in SMO^{WT} and SMO^{D477G} expressing cells, respectively; whereas ALLO-2 has IC_{50} values of 132 nM and 440 nM, respectively in the same cells. Both compounds inhibited the proliferation of mouse MB cells in a dose-dependent manner, with IC_{50} values of 0.47 μM and 0.12 μM, respectively (Kim et al., 2013).

3.4 Cystine rich domain-binding Smoothened receptor modulators

The cystine rich domain (CRD) is required for SMO to adopt a fully active conformation in response to SHH-binding. Endogenous natural molecules oxysterols were found to activate SMO allosterically by binding the CRD of SMO (Huang et al., 2016). The 22(S)-azacholesterol analogue 22-NHC (compound **15**, Figure 3) was found to act as the first CRD-binding inhibitor of SMO, with an IC_{50} value of approximately 3 μM in Hh responsive NIH-3T3 cells (Byrne et al., 2016; Wang et al., 2014; Wang et al., 2013). Meanwhile, reduced binding and activation were observed for the 20(R)-yne (compound **16**, Figure 3) and the 20-keto-yne (compound **17**, Figure 3) in both mouse SMO^{M2} and SMO^{D477H} mutants. Thus, antagonists that engage the oxysterol binding site in the CRD may represent an orthogonal strategy for development of new Hh inhibitors against both SMO wild-type and mutants (Nedelcu et al., 2013).

3.5 BH3 mimetics as alternative Hedgehog pathway inhibitors

BH3-mimetics have been shown to cause apoptosis in MB cells and neural progenitor cells, suggesting their potentials in treatment of MB (Levesley et al., 2011;

Levesley et al., 2018). Recently, BH3 mimetic small molecules were identified to disrupt the BCL-2 protein/SUFU interaction through targeted anti-apoptotic BCL-2 proteins (Cherry et al., 2013; Wu et al., 2017). In C3H10T1/2 cells, the MCL-1 inhibitor MIMX (compound **18**, Figure 4) exhibited IC_{50} values of 0.7-1.2 μM in inhibition of the Hh activation induced by SHH-N, PTCH1 and GLI1 (Souers et al., 2013; Cohen et al., 2012). Compound **18** also demonstrated similar potency against both SMO^{M2} and the resistant SMO^{D477G} mutants with IC_{50} values of 0.9 and 1.0 μM, respectively. Furthermore, the multi-target inhibitor ABT-263 (compound **19**, Figure 4) had IC_{50} values between 1.2-1.8 μM in inhibition of the Hh activation induced by SHH-N, PTCH1, GLI1, SMO^{M2} and SMO^{D477G}. Additionally, the only FDA-approved selective BCL-2 inhibitor, ABT-199 (venetoclax, compound **20**, Figure 4) exhibited similar potency (1.4-2.2 μM) in the same assays (Park et al., 2008). Taken together, these observations confirmed the capacity of BH3 mimetics as suppressants of cancerous cell growth through the disruption of GLI transcription.

[Figure 4 Here]

3.6 Direct inhibitors of the transcriptional factor GLI

The GLI transcription factors (GLI1, GLI2, and GLI3) are the terminal effectors of the SHH-SMO signaling pathway, thus, suppression of GLI is widely considered as a more effective approach both for treatment of Hh-driven cancers and for overcoming drug resistance for SMO antagonists (Infante et al., 2015; Bosco-Clément, 2014).

GANT58/GANT61. GANT58 (compound **21**) and GANT61 (compound **22**, Figure 5) were the earliest identified inhibitors against GLI-mediated transcription with IC_{50} values of approximately 5 μM in SHH light II cell lines that express the luciferase gene under regulation of the GLI promoter element (Agyeman et al., 2014).

It has been proposed that these compounds bind to the groove located between zinc finger-2 and -3 in GLI, without interference with the DNA binding site (Wang et al., 2018). Although GANT61 has been identified as more potent than GANT58, it is less stable at physiological conditions that hinders its further investigations.

Arsenic trioxide. Arsenic trioxide (As_2O_3) is a FDA-approved drug for treatment of acute promyelocytic leukemia. It was found to directly bind to GLI1 with an IC_{50} of approximately 0.7 μM and suppress the transcriptional activity of GLI1. In *Ptch*$^{+/-}$-derived MB allograft mice, As_2O_3 treatment was found to cause nearly complete growth suppression at a concentration of 10 mg/kg. It was also found to inhibit growth of SMO^{D477G} MB cells with similar potency to that of SMO^{WT}-dependent cells (Kim et al., 2013).

3.7 Indirect inhibitors of the transcriptional factor GLI

GPR39 agonists. A series of cyclohexylmethyl aminopyrimidines (CAMPs, structure **23**, Figure 5) were reported to block GLI transcription without direct binding to GLI (Bassilana et al., 2014). The orphan G-protein coupled receptor, GPR39, was identified as the potential target for these compounds. Although further study is needed to confirm this binding profile, ectopic expression or knockdown of GPR39 was found to decrease GLI-activated signaling.

[Figure 5 Here]

DYRK1B inhibitors. The dual-specificity tyrosine-phosphorylation-regulated kinase 1B (DYRK1B) was found as a critical regulator of Hh/GLI signaling (Lauth et al., 2010). Recently, the DYRK1B inhibitor **24** (Figure 5) was found to suppress expression of GLI mRNA at IC_{50} values of 1.16 μM and 1.04 μM, respectively, in SMO-sensitive and SMO-resistant human MB cells (Gruber et al., 2016).

GLI expression suppressors. Recently, our group identified

benzo[b]pyrido[3,4-g][1,5]oxazocin-11-yl)cyclopropanecarboxamide **25** (Figure 5) (Liu et al., 2017) as an inhibitor of Hh signaling, showing an IC_{50} value of 23 nM. Mechanistic studies indicated that, rather than interfering with GLI-DNA binding complex, this compound likely acted on targets downstream of SMO by suppressing expression of the GLI transcription factors.

CK1a allosteric activator. The FDA-approved anti-pinworm drug pyrvinium **26** (Figure 5) was recently identified as an allosteric activator of CK1α kinase. This compound was shown to reduce the stability of GLI factors through direct association. The *in vivo* study showed that this compound at a concentration of 0.8 mg/kg induced nearly complete tumor growth inhibition in *Ptch*$^{+/-}$-derived MB allograft mice (Li et al., 2014).

3.9 New generation of Hedgehog pathway inhibitors by epigenetically targeting GLI

[Figure 6 Here]

Histone deacetylase (HDAC) inhibitors. It has been reported that acetylation of Lys518 on the C-terminus of GLI1 represents a transcriptional inhibitory switch and its subsequent deacetylation by HDAC1 is responsible for transcriptional functioning of GLI (Coni et al., 2013). Gulino *et al.* recently described an integrated circuitry system that is activated by HDAC and deactivated by ubiquitin, thereby highlighting the role of GLI acetylation as a key transcriptional checkpoint in the Hh signaling pathway (Canettieri et al., 20010; Dhanyamraju et al., 2015). Indeed, the *pan*-HDAC inhibitor SAHA (compound **27**, Figure 6) has been reported to induce apoptosis in MB mice. Moreover, the *pan*-class I and class II HDAC inhibitor, tricostatin A (TSA, compound **28**, Figure 6), the class I HDAC1-2 inhibitor, HDiA (compound **29**, Figure 6) and the HDAC1-3 inhibitor, HDiB (compound **30,** Figure 6) were all found to suppress SMO-dependent Hh signaling in a dose- and time-dependent manner;

whereas the class III HDAC inhibitor, sirtinol (compound **31**, Figure 6) and the selective HDAC6 inhibitor, tubacin (compound **32**, Figure 6) were ineffective. Further, HDAC6 was identified as overexpressed in Hh-driven MB cells and as essential for complete activation of the Hh pathway, likely acting downstream of primary cilia. Finally, the HDAC6 selective inhibitor, ACY-1215 (compound **33**, Figure 6) was found to successfully penetrate the BBB and significantly reduce tumor growth in allografts of primary SMO A1 MB cells.

BRD4 inhibitors. Specific bromodomain and extra C-terminal domain proteins (BET), including BRD4, are epigenetic regulatory factors of *MYC* (Beroukhim et al., 2010). The selective BET inhibitor, JQ1 (compound **34**, Figure 6) significantly reduced proliferation of MB cells, and prolonged the survival of $Ptch^{+/-}$-derived allografts of Med1-MB cells. Meanwhile, another BRD4 inhibitor, I-BET151 (**35**) was found to attenuate Hh activity by decreasing the occupancy of BRD4 on the GLI1 locus. *In vivo*, I-BET151 also displayed remarkable tumor growth inhibition in $Ptch^{+/-}$-derived MB mice at a dose of 30 mg/kg (Long et al., 2014).

Further, Cho et al. recently found that BRD4 directly binds to the GLI1 and GLI2 promoters, and that treatment with JQ1 caused a significant decrease in engagement of these sites (Tang et al. 2014). Further, they treated SMO^{WT}- or SMO^{D477G}-$Ptch^{+/-}$-derived MB flank allografts (with JQ1 (50 mg/kg/d) by intraperitoneal injection or orally with the SMO antagonist, vismodegib (100 mg/kg/d), and found marked reduction in the growth of SMO^{D477G}-MB flank allografts in response to JQ1. Together, these studies suggest that BET protein modulation may be an attractive therapeutic strategy for treating Hh-driven tumors that exhibit emerged or priori resistance to SMO antagonists.

3.10 Clinical investigations of Hh pathway inhibitors against MB tumors

Thanks to the extensive efforts devoted to the development of Hh pathway inhibitors, three Hh inhibitors (vismodegib, sonidegib, and glasdegib) acting as SMO antagonists have been launched. However, these inhibitors are not approved as treatment of MB tumors. Both vismodegib and sonidegib are approved respectively in 2012 and 2015 for the treat of metastatic or locally advanced non-resectable BCC, whereas, glasdegib was approved in 2018 for treatment of acute leukemia. Although these drugs showed promising profiles in preclinical trials as treatment of SHH-driven MB tumors, optimal clinical outcomes have not been succeeded yet.

The first phase I clinical trial of Hh pathway inhibitors as molecular targeted therapy of MB tumors was reported in 2009 (Rudin et al., 2009). Based on the promising preclinical study of the SMO antagonist vismodegib, it was applied to treat a 26-year-old man who was diagnosed bearing SHH-driven MB tumors four-year ago and treated with surgical resection followed by the adjuvant craniospinal irradiation as well as a number of chemotherapies, but recurrence and metastasis occurred later. The patient was administered a single oral dose of 540 mg of vismodegib per day for appropriately three months and then discontinued due to disease progression caused by SMO mutation. During the treatment, the patient showed a rapid but transient tumor regression along with symptom reduction without adverse events higher than grade 1. In 2013, Gajjar et al. reported a phase I study of vismodegib in pediatric patients with refractory or recurrent MB to determine the toxicity, pharmacokinetics and the recommended dosage for phase II study (Gajjar et al., 2013)). In this study, 13 eligible childhood (3-21 years old) patients were enrolled on the initial study with 6 receiving 85 mg/m^2 and 7 receiving 170 mg/m^2 vismodegib. Twenty eligible patients were then enrolled on a flat-dosing of 150 mg or 300 mg. Three dose-limiting toxicities were observed with flat-dosing, and the concerned Hh-related skeletal

growth toxicities such as damages on cartilage and bone formation were not observed. Therefore, 150 mg or 300 mg dosage depending on patients' BSA (body surface area) is recommended for phase II trial of vismodegib. In subsequent phase II trials with 31 adult and 12 pediatric MB patients, progression-free survival (PFS) was found longer in those with SHH MB (3 adults and one pediatric) than those non-SHH subgroups. Among the limited patients of SHH-MB, prolonged disease stabilization was 41% (Robinson et al., 2015).

Recently, Li *et al.* performed a systemic review on the current available phase I and II clinical data of Hh inhibitors vismodegib and sonidegib (Li, Song, & Day, 2019). They found that all the MB patients enrolled have good compliance n these trials and show specificity for SHH MB over other non-SHH subgroups. In total, there are 32 SHH over 22 other subgroups of MB patients enrolled for vismodegib, whereas the respective patients for sonidegib are 14 over 60. Overall, the pooled objective response rate (ORR) for vismodegib is 17% for SHH-MB, and 0% for other non-SHH MB subgroups. Likewise, the pooled ORR for sonidegib is 55% for SHH-MB, and 0% for other MB subgroups. In both adult and pediatric MB patients, sonidegib outperforms vismodegib in efficacy, but both inhibitors show similar toxicity profile in the trials. These results confirm that Hh inhibitors have promising antitumor efficacy and well-tolerated in SHH MB patients, therefore further clinical trials with these drugs are promising.

4. Concluding remarks and future perspectives

MB is the most common and yet highly heterogeneous childhood malignant brain tumor. Since MB occurs in the posterior fossa, discrimination is difficult and 70-80% of MB patients are diagnosed before metastatic dissemination survive, thus making treatment of this disease even harder (Quinlan, & Rizzolo, 2017). Recent application

of new genomic and epigenetic profiling technologies has revolutionized the classification of MB from strictly morphology-based to molecular and epigenetic characterization-based, leading to international consensus of four distinct MB molecular subgroups: WNT, SHH, Group and Group 4 (Cavalli et al.; 2017; Bavle, & Parsons, 2017; Ramaswamy et al., 2016). More recent studies have indicated that up to seven molecular subgroups exist in childhood MB. Moreover, studies on the inter- and intra-tumoral features of the four subgroups revealed that each subgroup contains variant subtypes.

The progress on the classification of MB together with identification of specific gene mutations or amplifications have significantly helped risk stratification of MB patients at diagnosis and improved clinical treatment options. However, these biological advance in MB has not led to specific treatment for each of the MB subgroups or subtypes. Maximal surgical resection followed by craniospinal irradiation and adjuvant chemotherapy retains the standard of care for all MB subgroups. Molecular Drugs targeting the identified subgroup- or subtype-specific 'driver' genes such as *MYCN*, *MYC*, *TP53*, *CDK6*, *SNCAIPR* and many others are still immature. Relatively significant progress in the development of Hh pathway inhibitors has been achieved as promising treatment of SHH-driven MB, with a few already in the clinical trials.

Since aberrant activation of the Hh signaling cascade is a hallmark of SHH MB tumors, several somatic mutations (*PTCH1, SMO, GLI*) underlying the pathway activation have been recognized as 'driver' genes for the development of molecular targeted therapies for SHH subgroup tumors (Xie et al., 2019). Currently, three Hh pathway inhibitors that target the SMO receptor (vismodegib, sonidegib, and glasdegib) have been clinically approved for treatment of several SHH-dependent

cancers. But unfortunately, these inhibitors are primarily approved for BCC or acute leukemia, rather for MB. Many clinical trials with vismodegib and/or sonidegib have shown optimal objective responses against pediatric and adult SHH MB, however, the enrollment of limited pediatric SHH-driven MB patients together with transient effects in these trials precludes a solid treatment conclusion.

In addition, several challenges may become additional obstacles for invention of a successful treatment for MB patients. First, the observed limited and transient clinical efficacy of SMO inhibitors in clinical trials might be partially due to their poor ability to cross the blood-brain barrier (BBB). To circumvent this challenge, novel strategies should be explored to promote inhibitors to penetrate the BBB. Second, to address the SMO-mutation driven drug resistance and treatment relapse, novel Hh inhibitors with distinct chemical structures and different modes of action, specifically those that act downstream of SMO such as GLI and BRD4, should be more extensively investigated since these inhibitors have shown effectiveness in mouse models to combat SHH-driven cancers as well as the acquired resistance associated with SMO inhibitors. However, potent and specific GLI inhibitors with optimal druglike properties are lacking, and current available BRD4 inhibitors possess poor safety and pharmacokinetic properties. Therefore, more structural elaborations on these inhibitors are emergently needed.

Moreover, leptomeningeal dissemination, the spread of MB cells through cerebrospinal fluid to the brain and spinal cord, severely affects the prognosis of MB patients. Numerous genes have been identified as involved in this dissemination process (Jenkins et al., 2014). In addition to this metastatic mechanism, it has recently been shown that circulating MB cells driven by the CCL2-CCR2 chemokine axis disseminate through the blood in the leptomeningeal space form leptomeningeal

metastases (Garzia et al., 2018; Simon et al., 2019). Thus, it is imperative to investigate whether therapies designed to combat the metastatic capacity of MB by targeting the Hh signaling pathway is effective.

In conclusion, the recent achievements in classification of MB tumors into more subtypes with distinct subtypes have greatly broadened our landscape on the high heterogeneity of this disease. Future efforts should be focused on identification of biomarkers for early diagnosis, clinically useful genetic or epigenetic factors for more precise patient stratification, and identification of subgroup/subtype-specific driver oncogenes for the development of molecular targeted therapy with reduced toxicity.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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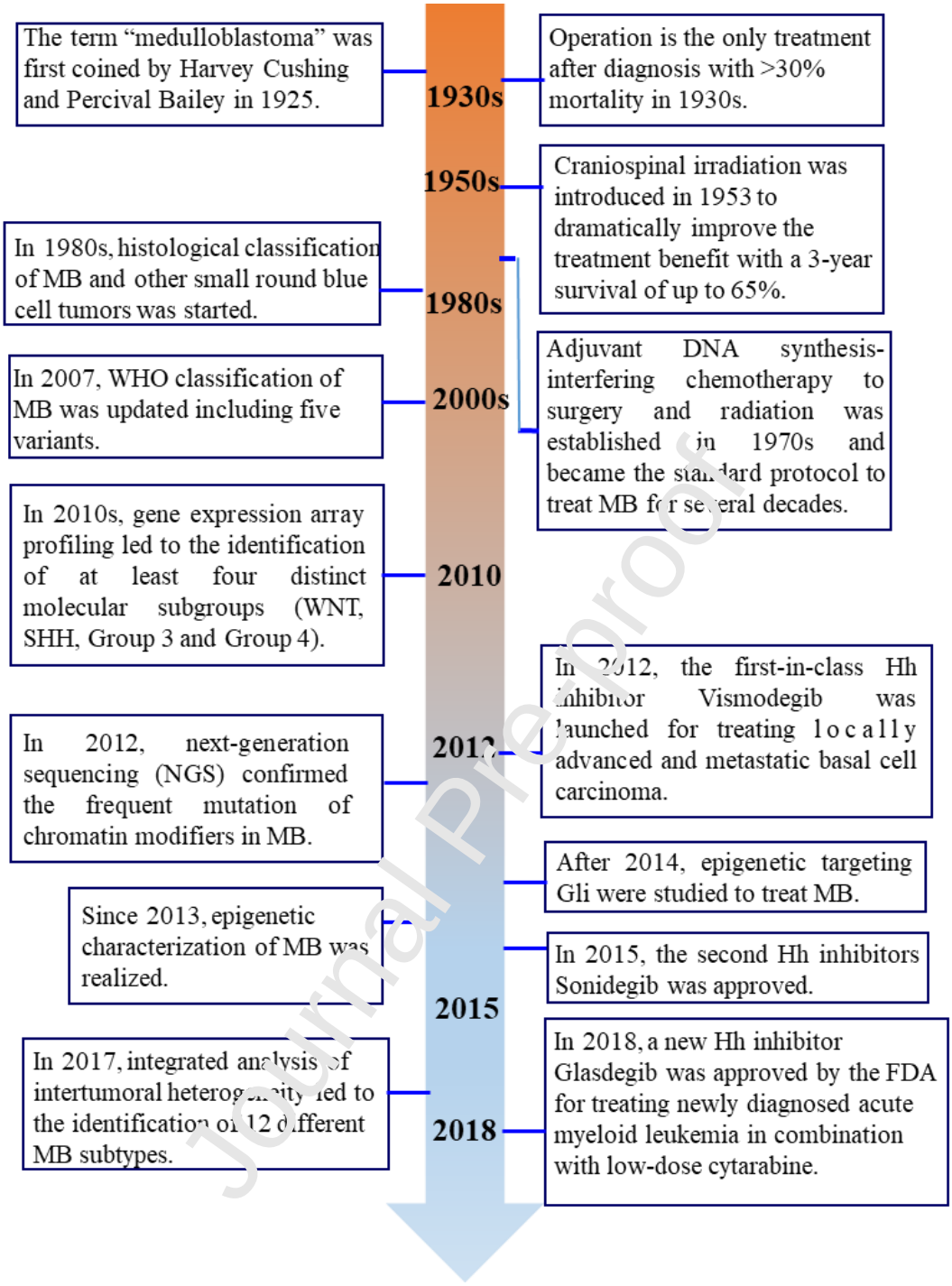

Figure 1. Advances on the pathology, classification and treatment of medulloblastoma. Abbreviations: MB, medulloblastoma.

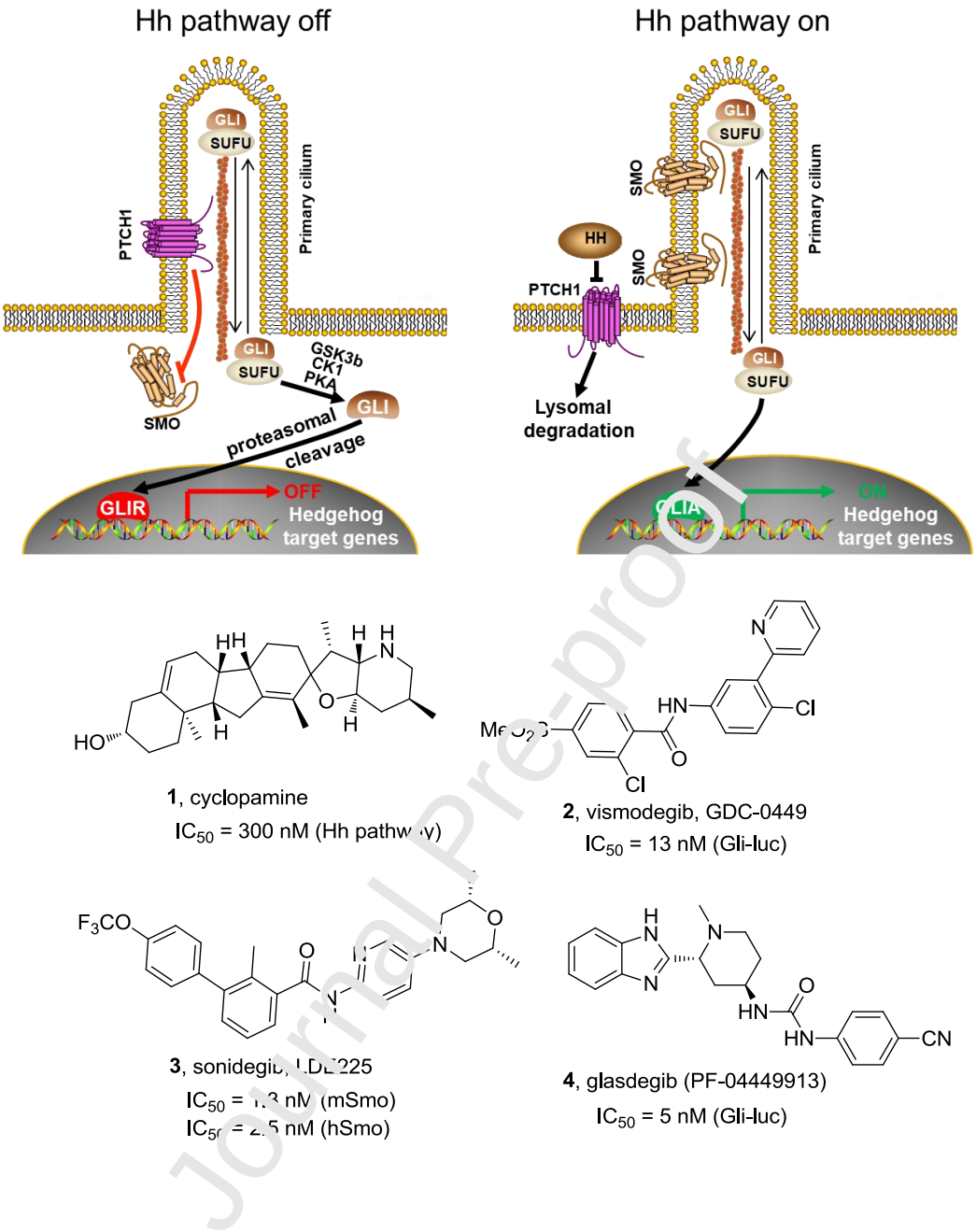

Figure 2. The complex Hedgehog signaling pathway and the first-generation inhibitors. Hh pathway is initiated by binding its 12-pass transmembrane receptor PTCH1 with one of the three ligands (SHH, IHH, and DHH). The membrane protein SMO is then released and accumulated in cilia. The ciliary accumulated SMO can promote activation of the transcriptional factor GLI by inhibition of SUFU and several important protein kinases, leading to target genes transcription. Abbreviations: Hh, Hedgehog; PTCH, patched; SHH, sonic Hedgehog; IHH, Indian Hedgehog; DHH, desert Hedgehog; SMO, Smoothened; GPCRs, G-protein-coupled receptors;

GLI, glioma-associated oncogenes; SUFU, suppressor of fused.

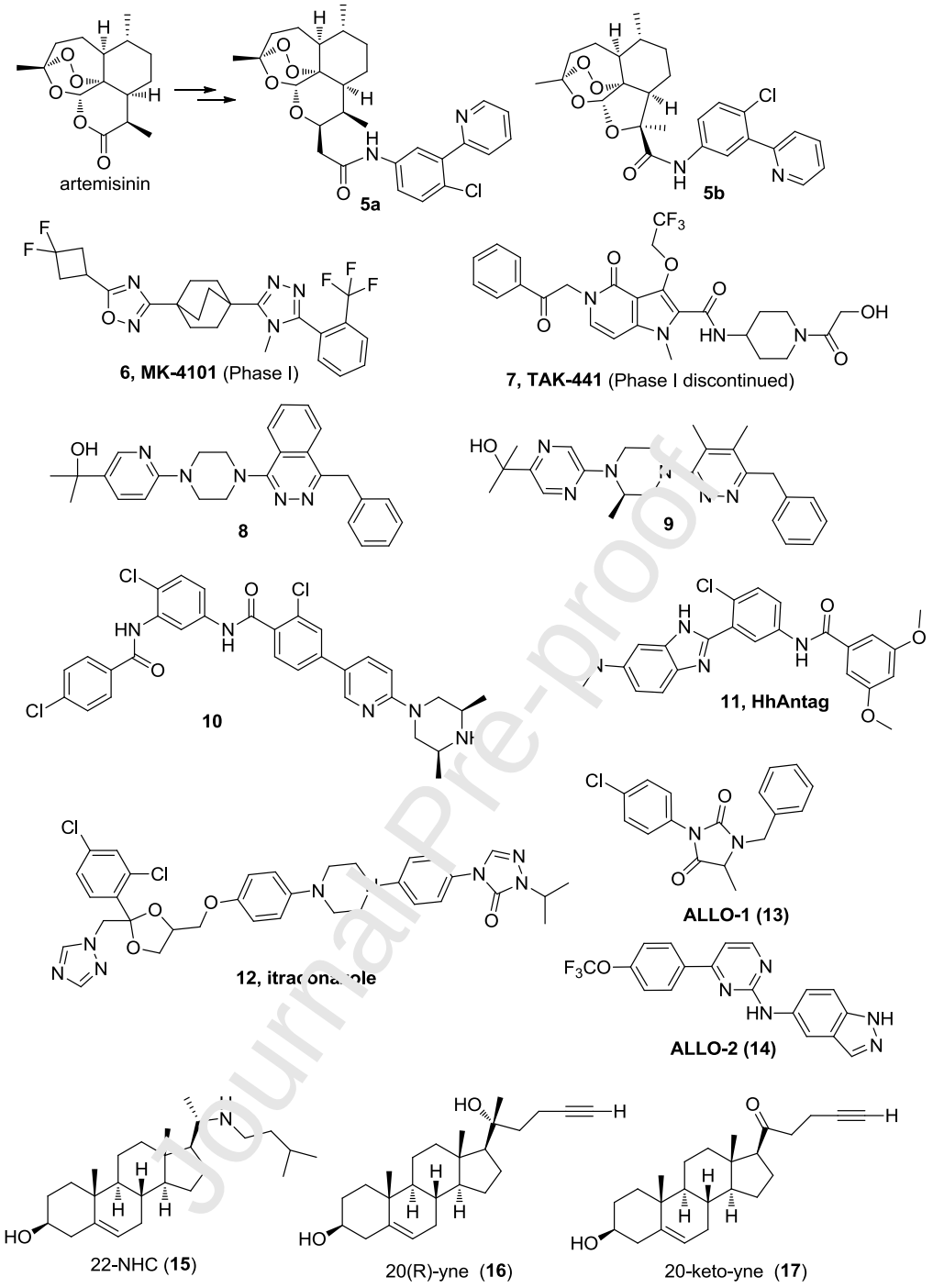

Figure 3. New SMO antagonists **5-9**. Compounds **5a** and **5b** are two artemisinin derivatives binding to SMO. Compounds **6-14** are antagonists of SMO mutants. Compounds **15-17** are three modulators binding with SMO CRD.

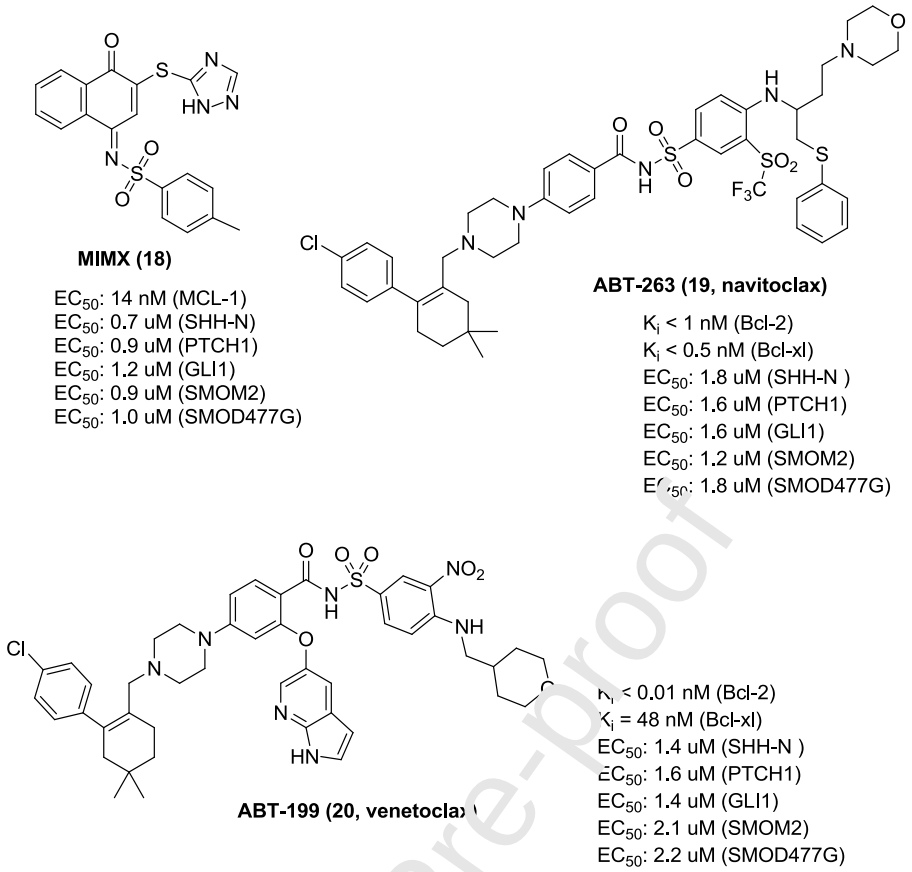

Figure 4. BH3 mimetics as Hh Inhibitors. **MIMX** is a MCL-1 inhibitor. **ABT-263** is a multi-target inhibitor of Bcl-2, Bcl-xl and Bcl-w. The FDA approved **ABT-199** is a selective Bcl-2 inhibitor. Abbreviations: BH3, Bcl-2 homolog 3; MCL-1, myeloid cell leukemia-1 ; Bcl-2, B-cell lymphoma 2; Bcl-xl, B-cell lymphoma-extra large; FDA, Food and Drug Administration.

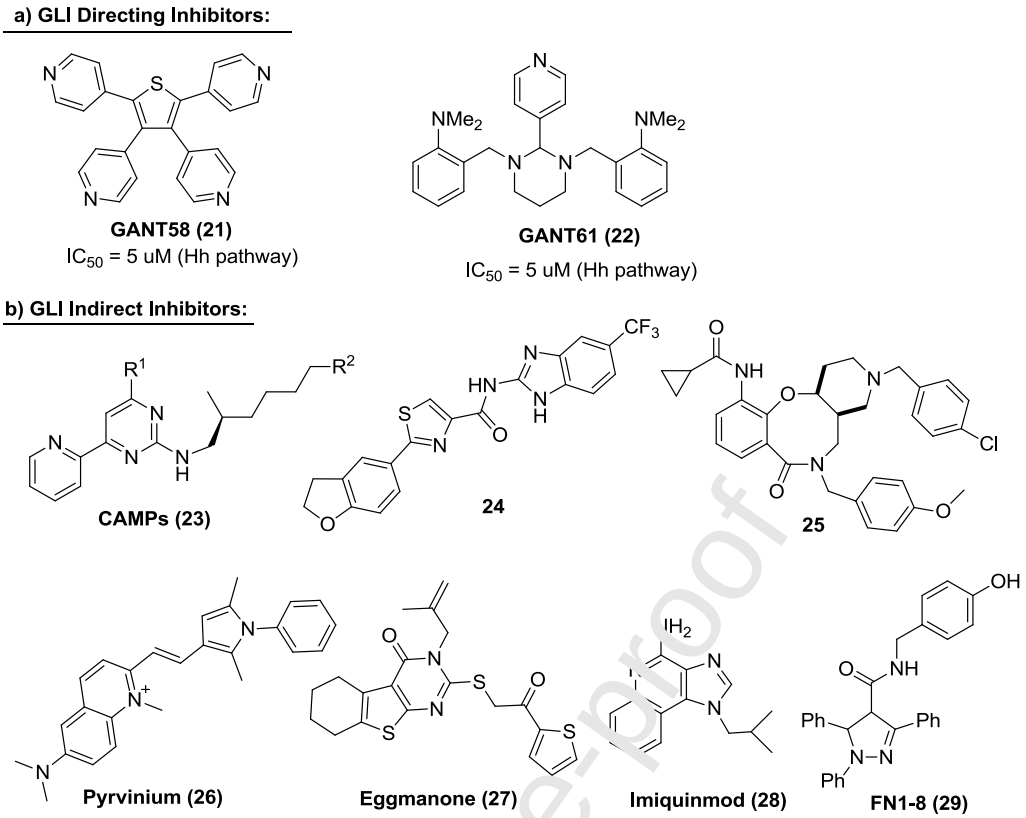

Figure 5. New Hh pathway inhibitors interacting at the level of GLI. Abbreviation: Gli, glioma-associated oncogenes.

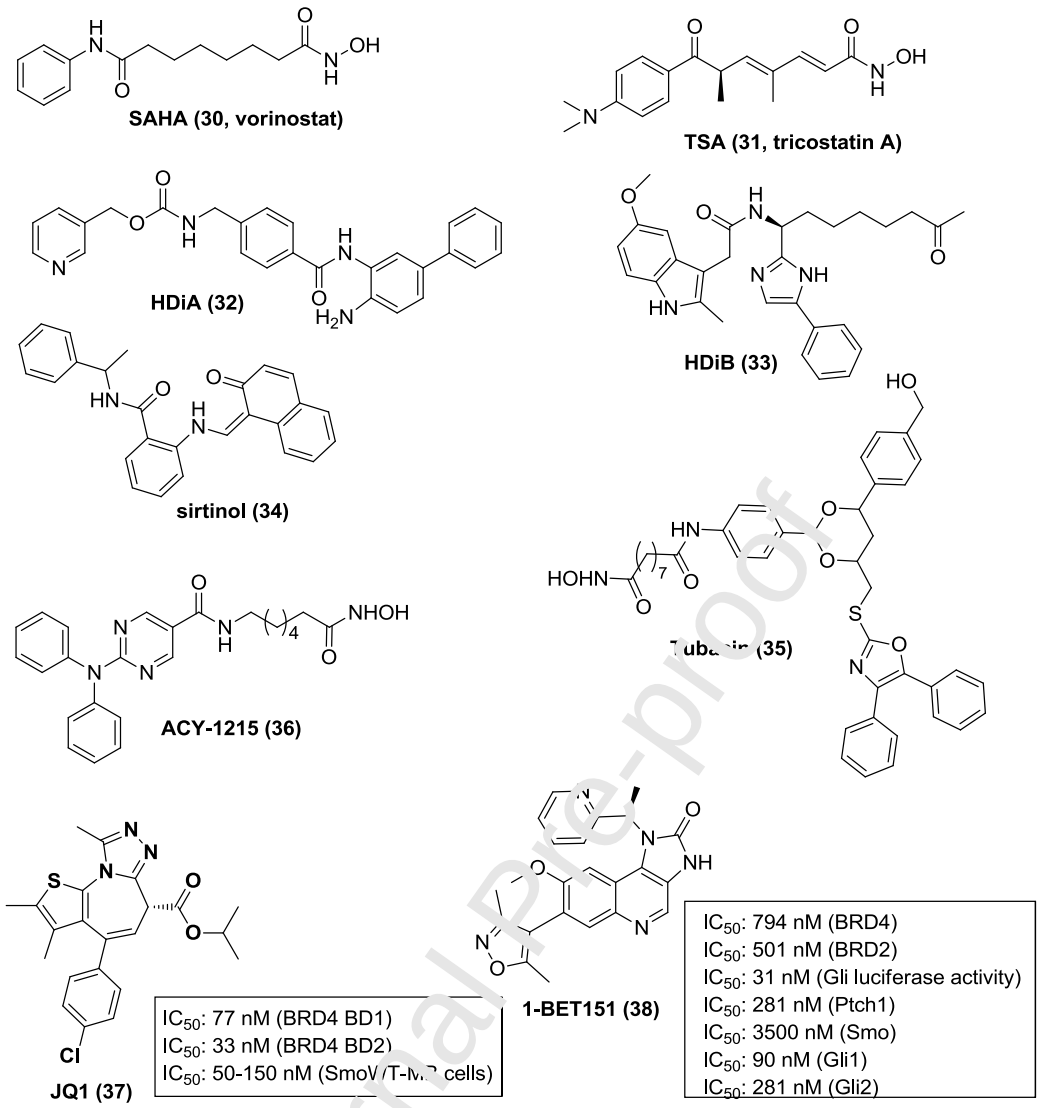

Figure 6. Chemical structures of compounds **30-38.** Compounds **30-36** are HDAC inhibitors. Compounds **37-38** are BET inhibitors. Abbreviations: HDAC, histone deacetyltransferase; BET, The bromodomain and extra C-terminal domain.