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Preclinical therapeutic targets in diffuse midline glioma

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ABSTRACT

Diffuse midline gliomas (DMG) are rapidly fatal tumors of the midbrain in children, characterized by a diffuse growing pattern and high levels of intrinsic resistance to therapy. The location of these tumors, residing behind the blood-brain barrier (BBB), and the limited knowledge about the biology of these tumors, has hindered the development of effective treatment strategies. However, the introduction of diagnostic biopsies and the implementation of autopsy protocols in several large centers world-wide has allowed for a detailed characterization of these rare tumors. This has resulted in the identification of novel therapeutic targets, as well as major advances in understanding the biology of DMG in relation to therapy resistance. We here provide an overview of the cellular pathways and tumor-specific aberrations that have been targeted in preclinical DMG research, and discuss the advantages and limitations of these therapeutic strategies in relation to therapy resistance and BBB-penetration. Therewith, we aim to provide researchers with a framework for successful preclinical therapy development.

Introduction

Diffuse midline gliomas (DMG) are aggressive and incurable pediatric brain tumors, characterized by high levels of intrinsic and acquired resistance to therapy, as well as therapy failure due to an intact blood-brain barrier (BBB), leading to a dismal median survival of ~11 months (Jones et al., 2017; Veldhuijzen van Zanten et al., 2017). In general, chemoresistance to antitumor agents in DMG, brain tumors and various human cancers is a primary hindrance towards curative cancer therapy (Abdul et al., 2018; Saleem et al., 2019; Sharifzad et al., 2019; van Tellingen et al., 2015). Hence, deciphering the molecular mechanisms underlying intrinsic and acquired cancer drug resistance (Mudduluru et al., 2016; Wijdeven et al., 2016; Zhitomirsky and Assaraf, 2016) is a major goal of cancer resistance, in order to develop novel modalities that may overcome multidrug resistance in cancer (Bar-Zeev and Assaraf, 2017; Cui et al., 2018; Levin et al., 2019; Livney and Assaraf, 2013; Mudduluru et al., 2016).

Formerly, DMG tumors were known as diffuse intrinsic pontine glioma (DIPG), or high grade gliomas (HGG) occurring in midbrain structures. The discovery of highly prevalent, oncogenic *Histone 3* mutations in these tumors, prompted the World Health Organization in 2016 to redefine these tumors as diffuse midline gliomas (DMG) carrying H3K27 M mutations (Khuong-Quang et al., 2012; Lewis et al., 2013; Louis et al., 2016; Wu et al., 2012). Until about a decade ago, very little research was performed on this type of cancer. This was mainly due to the lack of biological material, since the majority of children did not undergo biopsies or surgeries due to the presumed risk of operating in such delicate areas, and at the same time the possibility to confirm the diagnosis by magnetic resonance imaging (MRI). Central in DMG biology are the lysine-to-methionine substitutions, at position 27 in *Histone 3* genes (H3K27M), identified in over 80% of cases (Bender et al., 2013; Castel et al., 2015; Chan et al., 2013; Khuong-Quang et al., 2012; Lewis et al., 2013; Wu et al., 2012). The introduction of autopsy and biopsy studies has also led to a significant increase in the availability of cell culture and animal models of DMG, enabling preclinical therapeutic testing (Becher et al., 2010; Grasso et al., 2015; Hennika et al., 2017; Misuraca et al., 2015; Pathania et al., 2017; Plessier et al., 2017; Veringa et al., 2013). Consequently, a variety of therapeutic targets has been discovered, some of which have been the topic of intensive preclinical studies. However, currently there is no therapeutic trial in patients that has reported any convincing survival benefit of pharmacological interventions.

The current review summarizes the druggable targets that have been identified for the treatment of DMG, and provides a critical evaluation of the preclinical studies performed to validate the efficacy of inhibiting or modulating these putative targets. Currently, the main therapeutic targets that have been identified for DMG can be classified as targeting epigenetic modulators, receptor tyrosine kinases and their

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Table 1
Epigenetic regulators as therapeutic targets in diffuse midline glioma.

Target	*In vitro* efficacy	*In vivo* efficacy	Remarks	References
KDM6B	+	+	Synergy with HDAC inhibition	Hashizume et al., 2014; Grasso et al., 2015
PRC2	+	+	Synergy with BET inhibition	Wiese et al., 2016; Mohammad et al., 2017; Piunti et al., 2017; Zhang et al., 2017
HDACs	+	+	Synergy with KDM6B and BET inhibition	Grasso et al., 2015; Hennika et al., 2017; Nagaraja et al., 2017; Pal et al., 2018
CDK7	+	+	Synergy with HDAC inhibition	Nagaraja et al., 2017
BET	+	+	Synergy with EZH2 and HDAC inhibition	Piunti et al., 2017; Zhang et al., 2017
PRC1	+	n/a		Kumar et al., 2017; Filbin et al., 2018

n/a: data not available.

related signal transduction pathways, cell cycle checkpoints, the stem cell phenotype of DMG cells, DNA damage repair systems and targets for immunotherapy. Each of these groups of therapeutic targets will be discussed in detail in the following sections.

Epigenetic modification

With the high prevalence of mutations in genes encoding *Histone 3* in DMG, and their consequences for chromatin remodeling and global transcriptional patterns, epigenetic modification represents a logical and promising therapeutic strategy in these tumors (Table 1 and Fig. 1) (Aihara et al., 2014; Bender et al., 2013; Castel et al., 2015; Chan et al., 2013; Cordero et al., 2017; Fang et al., 2018; Hoffman et al., 2016; Khuong-Quang et al., 2012; Lewis et al., 2013; Nagaraja et al., 2017; Nikbakht et al., 2016; Pathania et al., 2017; Ryall et al., 2016; Stafford et al., 2018; Wu et al., 2012). In this regard, the most obvious strategy would be to restore the trimethylation of H3K27, which is reduced in a transdominant manner as a consequence of heterozygous H3K27 M mutations. As such, the histone demethylase inhibitors GSKJ1 and GSKJ4 (a prodrug of GSKJ1), targeting the histone lysine demethylase 6B (KDM6B/JMJD3), have shown promising activity *in vitro* as well as *in vivo* in patient-derived xenograft models of DMG (Grasso et al., 2015; Hashizume et al., 2014). Recently it was shown that in H3K27 M mutated cells, H3K27 trimethylation is actually increased at certain specific loci in the genome, leading to repression of transcription (Chan et al., 2013; Cordero et al., 2017; Fang et al., 2018; Mohammad et al., 2017; Piunti et al., 2017; Stafford et al., 2018). Based on these findings, inhibition of enhancer of zeste 2 (EZH2), the catalytic subunit of the polycomb repressor complex 2 (PRC2) – which is responsible for trimethylation of H3K27 – has demonstrated preclinical efficacy *in vitro* and *in vivo*, although one study failed to demonstrate such an antitumor effect (Mohammad et al., 2017; Piunti et al., 2017; Wiese et al., 2016; Zhang et al., 2017).

Another consequence of H3K27 M mutations is an increase in acetylation of the remaining H3K27 residues, resulting in an open chromatin structure and superenhancer activation, and subsequent transcriptional activation, at these genomic loci (Fang et al., 2018; Piunti et al., 2017). Inhibition of histone deacetylases (HDACs) by the pan-HDAC inhibitor panobinostat and the dual PI3K/HDAC inhibitor fimepinostat (CUDC-907) further enhanced this consequence of H3K27 M mutations, while at the same time indirectly restoring H3K27 trimethylation (Grasso et al., 2015; Hennika et al., 2017; Nagaraja et al., 2017; Pal et al., 2018). These global chromatin alterations induced by HDAC inhibitors seem specifically cytotoxic to H3K27 M mutated cells and as a result have shown promising preclinical efficacy in treating DMG *in vitro* and *in vivo* (Grasso et al., 2015; Hennika et al., 2017; Pal et al., 2018). Nonetheless, DMG cells rapidly acquire resistance to panobinostat, suggesting that monotherapy with HDAC inhibitors is unlikely to exhibit curative potential (Grasso et al., 2015; Hennika et al., 2017).

A less specific strategy for epigenetic-based therapy, which has proven to be effective in other cancer types with strong transcriptional dysregulation, is the disruption of RNA polymerase II (RNAPII)-mediated transcription. This can be achieved in two ways: either via inhibition of bromodomain and extra-terminal (BET) proteins, or via inhibition of cyclin-dependent kinase 7 (CDK7), which phosphorylates and alters the activity of RNAPII (Nagaraja et al., 2017; Piunti et al., 2017; Zhang et al., 2017). In this regard, preclinical efficacy has been shown for the BET inhibitors JQ1, I-BET151 and OTX-015, and the CDK7 inhibitor THZ1, both *in vitro* and *in vivo*, although resistance to JQ1 eventually developed as well, and cross-resistance between JQ1 and panobinostat has been reported, limiting the potential of this type of combination therapy (Nagaraja et al., 2017; Piunti et al., 2017; Zhang et al., 2017). Furthermore, the polycomb repressor complex 1

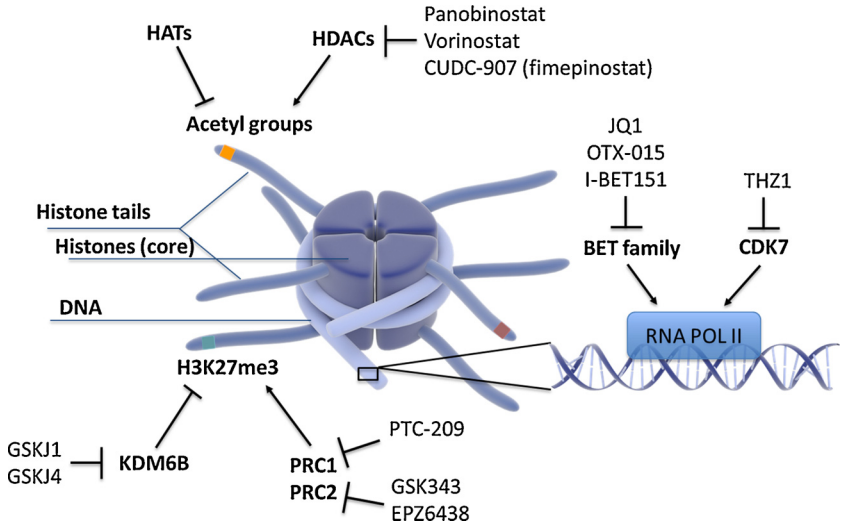

Fig. 1. Schematic illustration of modes of action of commonly used drugs targeting epigenetic processes in diffuse midline glioma. Image generated using the Library of Science and Medical Illustrations package.

Table 2
Growth factor receptors as therapeutic targets in diffuse midline glioma.

Target	*In vitro* efficacy	*In vivo* efficacy	Remarks	References
PDGFRα	+/-	–	Ambiguous results, multikinase inhibition essential	Grasso et al., 2015; Truffaux et al., 2015; Mittapalli et al., 2016; Meel et al., 2017; Filbin et al., 2018
EGFR	+/-	n/a	Ambiguous results, multikinase inhibition essential	Bartels et al., 2014; Grasso et al., 2015; Meel et al., 2017; Fleischhack et al., 2019
IGF1R	+/-	–	Ambiguous results, multikinase inhibition essential	Grasso et al., 2015; Halvorson et al., 2015; Nagaraja et al., 2017; Meel et al., 2017
MET	+/-	–	Ambiguous results, multikinase inhibition essential	Grasso et al., 2015; Halvorson et al., 2015; Truffaux et al., 2015; Meel et al., 2017
KIT	–	n/a	Multikinase inhibition necessary	Grasso et al., 2015
VEGFR2	+/-	n/a	Multikinase inhibition necessary	Meel et al., 2017
FGFR1	+	n/a	Efficacy of inhibition not evaluated	Schramm et al., 2019
ACVR1	+/-	+	In vitro results ambiguous	Taylor et al., 2014,.Meel et al., 2017; Hoeman et al., 2019
Ephrins	+	n/a	Multikinase inhibition likely essential	Truffaux et al., 2015; Nagaraja et al., 2017; Meel et al., 2017
NG2	+	n/a		Yadavilli et al., 2015

n/a: data not available.

(PRC1) subunit BMI1 has been shown to be overexpressed in DMG, possibly as a result of H3K27 M mutations (Kumar et al., 2017). Although the role of BMI1 in this regard has not been extensively investigated yet, its inhibition by CRISPR knockout or the small molecule PTC-209 resulted in partial restoration of H3K27 trimethylation and *in vitro* antitumor efficacy (Filbin et al., 2018; Kumar et al., 2017).

Importantly, combinations of multiple epigenetic modifiers, targeting different components of the chromatin remodeling machinery and transcriptional activation, has shown promising synergistic antitumor effects in several studies (Table 1). Therefore, epigenetic modification represents a highly promising target candidate for the treatment of DMG, although further preclinical and clinical studies are required to fully understand the underlying molecular mechanisms and allow for tumor-specific treatment.

Growth factor receptors

Among the first genetic aberrations and therapeutic targets identified in diffuse midline gliomas were amplifications and overexpression of receptor tyrosine kinases (RTKs, Table 2). Most importantly, the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor α (PDGFRα) genes are frequently amplified and overexpressed in these tumors. At a lower frequency, gene amplification in other RTKs has also been identified, including the insulin-like growth factor 1 receptor (IGF1R), hepatocyte growth factor receptor (MET), stem cell-factor receptor (KIT) and vascular-endothelial growth factor receptor 2 (VEGFR2/KDR) (Buczkowicz et al., 2014a, b; Filbin et al., 2018; Fontebasso et al., 2014; Gilbertson et al., 2003; Grill et al., 2012; Hoffman et al., 2016; Mackay et al., 2017; Paugh et al., 2011, 2013; Vinci et al., 2018; Warren et al., 2012; Zarghooni et al., 2010). Additionally, next generation sequencing (NGS) studies have identified activating mutations in PDGFRα, the activin receptor 1 (ACVR1) and fibroblast growth factor receptor 1 (FGFR1), the latter of which occurs mainly in DMG located in the thalamus (Buczkowicz et al., 2014a, b; Fontebasso et al., 2014; Grill et al., 2012; Hoffman et al., 2016; Mackay et al., 2017; Paugh et al., 2013; Ryall et al., 2016; Taylor et al., 2014; Vinci et al., 2018). Based on these discoveries, inhibitors of these RTKs were tested in a series of preclinical studies (Fig. 2). Although inhibition of PDGFRα and MET effectively inhibited proliferation and migration of DMG cells in an *in vitro* study (Truffaux et al., 2015), the results from other studies were more ambiguous, and only showed relevant antitumor effects of multi-kinase inhibitors such as dasatinib and crizotinib (Grasso et al., 2015; Meel et al., 2017). Interestingly, knocking out PDGFRα in H3K27 M DMG cells by CRISPR/Cas9 displayed a strong impact on cell survival (Filbin et al., 2018). Only one study tested dasatinib *in vivo*, employing a murine DMG model driven by PDGFB overexpression and loss of p53 (Mittapalli et al., 2016). Although some survival benefit of dasatinib was seen in this model, the efficacy was severely limited by multidrug efflux transporters overexpressed on the plasma membrane of tumor cells. Results of preclinical *in vitro* studies evaluating inhibitors targeted at EGFR, IGF1R, KIT and VEGFR2 yielded comparable results, with most efficacy observed with multikinase inhibitors and a global failure of more specific inhibitors (Grasso et al., 2015; Meel et al., 2017). Only one other multi-targeted RTK inhibitor, BMS-754807, has been tested *in vivo*, using the same PDGFB;p53$^{-/-}$ murine DMG model as for dasatinib. Again, treatment with this multikinase inhibitor did not result in a survival benefit for the mice, although inadequate BBB penetration may have played a role here (Halvorson et al., 2015). Parallel to the preclinical studies, two clinical trials have been performed using multitargeted RTK inhibitors for the treatment of children with DMG. In line with the failure of these drugs to achieve relevant antitumor effects *in vivo*, no survival benefit was seen for RTK inhibition in these trials (Broniscer et al., 2013, 2018). One phase 2 trial using nimotuzumab, a monoclonal antibody targeting EGFR, suggested the potential existence of a small subgroup of patients benefiting from this type of therapy, although these results could not be confirmed in a subsequent phase 3 trial (Bartels et al., 2014; Fleischhack et al., 2019). FGFR1 mutations may still represent a valuable therapeutic target in a subset of DMG patients, but to date no preclinical or clinical studies have tested FGFR1 inhibition as a treatment strategy in these tumors. Nonetheless, a recent study identified FGFR signaling as a therapeutic target in DMG *in vitro*, independent of mutations in FGF receptors, emphasizing the need for further research on the potential of FGFR inhibition in DMG (Schramm et al., 2019). Finally, ephrin receptors are often activated in DMG cells, and *in vitro* experiments show antitumor efficacy of inhibitors of these receptors, even in the absence of mutations and gene amplifications (Meel et al., 2017; Nagaraja et al., 2017; Truffaux et al., 2015). Most importantly, ephrins have been shown to be essential for glioma cell migration and invasion, and their blockade inhibits these processes (Nagaraja et al., 2017; Truffaux et al., 2015). Several explanations exist for the overall failure of targeting RTKs for the treatment of DMG. Firstly, recent studies have shown that gene amplification and mutations of PDGFRα are subject to intratumoral and subclonal heterogeneity, which may explain the lack of efficacy of PDGFRα inhibition (Filbin et al., 2018; Hoffman et al., 2016; Vinci et al., 2018). Although not explicitly studied, the same may be true for other RTKs, explaining the failure of therapeutic strategies based on RTK inhibition. Secondly, the presence of an intact BBB, as well as multidrug efflux transporters on the tumor cells, may contribute to the failure of RTK inhibition, simply because adequate drug concentrations are not achieved in tumor cells (Halvorson et al., 2015; Mittapalli et al., 2016; Veringa et al., 2013; Warren, 2018). Thirdly, *in vitro* drug efficacy studies are prone to yield false positive or false negative results, as a consequence of the chosen

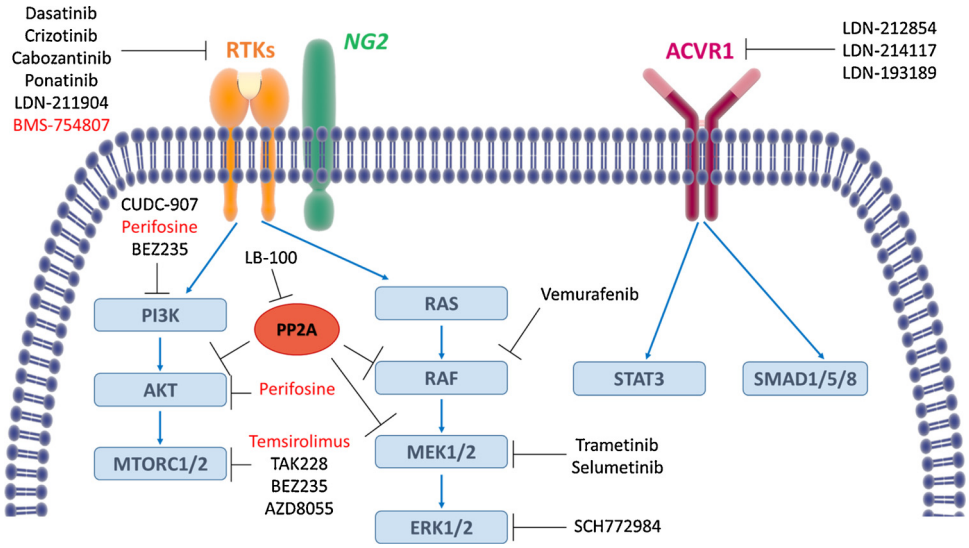

Fig. 2. Growth factor receptors and signal transduction pathways as molecular targets of commonly used drugs in DMG research. Marked in red are drugs that have been shown to be ineffective in (pre-)clinical DMG studies. Image generated using the Library of Science and Medical Illustrations package.

culture methods of tumor cells, and because kinase inhibitors rarely target a single kinase, but are subject to off-target effects, which can skew the balance of efficacy *versus* toxicity in an unfavorable direction (Meel et al., 2017). Finally, tumor cells may rapidly develop escape mechanisms for targeted therapies by switching their dependency from one signaling pathway to another (Tan et al., 2017). To overcome these mechanisms of resistance and treatment failure of RTK inhibitors, one study suggested targeting NG2, a transmembrane protein that stabilizes a variety of RTKs, which is upregulated in DMG and essential for DMG cell survival (Fig. 2) (Yadavilli et al., 2015). However, evidence regarding the feasibility of such a therapeutic approach would have to be generated *in vivo*, which has not been done so far.

The situation may be different for therapeutic strategies targeting ACVR1, which is mutated in a subset of DMG patients, mainly those carrying H3.1 K27M mutations (Buczkowicz et al., 2014a, b; Fontebasso et al., 2014; Mackay et al., 2017; Taylor et al., 2014). The same studies identifying intratumoral and subclonal heterogeneity for PDGFRα show that ACVR1 mutations are generally conserved among all tumor cells (Hoffman et al., 2016; Nikbakht et al., 2016; Vinci et al., 2018). Additionally, ACVR1 mutations are associated with the mesenchymal phenotype in DMG, and their role in the mesenchymal transition may contribute to therapy resistance in these tumors (Castel et al., 2015; Hoeman et al., 2019; Meel et al., 2018b; Puget et al., 2012). Despite limited antitumor efficacy *in vitro* of monotherapy with ACVR1 inhibitors (Meel et al., 2017; Taylor et al., 2014), efficacy could be demonstrated *in vivo* in genetically engineered mice carrying ACVR1 mutant murine DMG tumors (Hoeman et al., 2019). As such, combination therapies incorporating ACVR1 inhibition may be an effective approach in this subset of DMG.

Signal transduction pathways

Like many other types of cancer, DMG is characterized by frequent aberrations involving signal transduction pathways downstream of cell surface receptors. Although no single event is highly prevalent in these tumors, studies have identified mutations in both PIK3CA and PIK3R1, gene amplifications in HRAS and loss of PTEN, all of which resulting in aberrant phosphatidyl-inositol-3 kinase (PI3K) and/or mitogen-activated protein kinase (MAPK) pathway activation (Buczkowicz et al., 2014b; Fontebasso et al., 2014; Grill et al., 2012; Mackay et al., 2017; Nikbakht et al., 2016; Puget et al., 2012; Taylor et al., 2014; Vinci et al., 2018; Warren et al., 2012). Consequently, preclinical studies have investigated the antitumor potential of inhibitors of core components of these pathways (Table 3 and Fig. 2). Based on the identification of activating PI3K mutations, several studies have been conducted to determine the preclinical efficacy of PI3K inhibitors, although all of these studies were performed using combination therapies or compounds with multiple targets. Two studies evaluated the dual PI3K/Akt inhibitor perifosine, and found it to be effective only *in vitro* (Becher et al., 2010) or in combination with the MEK inhibitor trametinib (Wu et al., 2017). In this respect, a phase 1 clinical trial did not demonstrate a survival benefit in DMG patients, although the number of patients included was very low (n = 3) (Becher et al., 2017b). In a functional drug screen, some *in vitro* efficacy was observed with the dual PI3K/mTOR inhibitor dactolisib (BEZ235), but this compound has not yet been evaluated *in vivo* (Grasso et al., 2015). Another recent study demonstrated promising preclinical efficacy of the dual PI3K/HDAC inhibitor fimepinostat (CUDC-907), both *in vitro* and *in vivo*, although the contribution of PI3K inhibition to the treatment efficacy cannot be deduced from this study (Pal et al., 2018).

Activation of the PI3K pathway results in downstream activation of the mammalian target of rapamycin complex (mTORC). As such, mTOR inhibitors have also been investigated for the treatment of DMG. Whereas *in vitro* efficacy of some mTOR inhibitors, especially those targeting both mTORC1 and mTORC2, has been reported by multiple groups (Asby et al., 2018; Flannery et al., 2018; Grasso et al., 2015; Meel et al., 2017; Nagaraja et al., 2017), *in vivo* efficacy has been observed only for the mTORC1/2 inhibitor TAK228 in a murine DIPG model, whereas treatment of human DMG xenograft-bearing mice with the mTORC1 inhibitor temsirolimus was ineffective (Miyahara et al., 2017; Tsoli et al., 2018). The precise reason for this discrepancy may lie in a) the need to inhibit both mTORC1 and mTORC2, b) the differential BBB penetration of temsirolimus and TAK228, or c) an intrinsic difference in sensitivity to mTOR inhibition between the models used in these studies. Based on the lack of antitumor efficacy of temsirolimus in a phase 1 clinical trial (Becher et al., 2017a), further studies are required to assess the potential of mTOR inhibition for the treatment of DMG.

As a result of the activation of RTKs and HRAS gene amplification, the MAPK pathway is frequently activated in DMG as well. Therefore, inhibition of key components of this pathway, such as MEK1/2 and ERK1/2, has received attention in preclinical therapeutic studies in these tumors. Although *in vitro* studies show that DMG cells are, to some extent, sensitive to inhibition of MEK1/2 and ERK1/2, the results are ambiguous, and the observed efficacies may be a result of the tumor cell culture method used (Grasso et al., 2015; Meel et al., 2017; Nagaraja

Table 3
Signal transduction pathways as therapeutic targets in DMG.

Target	*In vitro* efficacy	*In vivo* efficacy	Remarks	References
PI3K	+/-	+/-	Monotherapy PI3K inhibition not tested, multikinase inhibition likely essential	Becher et al., 2010; Grasso et al., 2015; Wu et al., 2017; Pal et al., 2018
AKT	+/-	–	Monotherapy AKT inhibition not tested, multikinase inhibition likely essential	Becher et al., 2010; Hoeman et al., 2019
MTORC1/2	+/-	+/-	Inhibition of both MTORC1 and 2 likely essential	Grasso et al., 2015; Nagaraja et al., 2017; Meel et al., 2017; Miyahara et al., 2017; Asby et al., 2018; Flannery et al., 2018; Tsoli et al., 2018
MEK1/2	+/-	n/a	Ambiguous results, multikinase inhibition likely essential	Becher et al., 2010; Grasso et al., 2015; Meel et al., 2017; Wu et al., 2017
ERK1/2	+/-	n/a	Ambiguous results, multikinase inhibition likely essential	Nagaraja et al., 2017
PP2A	+	n/a	Paradoxical efficacy by overactivation of PI3K and MAPK pathway	Schramm et al., 2019

n/a: data not available.

et al., 2017; Wu et al., 2017). To this day, no studies have been performed that evaluate the antitumor efficacy of MEK or ERK inhibitors *in vivo*, and further research is needed to definitively assess the preclinical potential of these drugs.

In contrast to the traditional therapeutic strategies aimed at inhibiting oncogenic PI3K and MAPK signaling, a recent study identified protein phosphatase 2 (PP2A) as a therapeutic target in DMG *in vitro* (Schramm et al., 2019). PP2A is responsible for the dephosphorylation of AKT, RAF and MEK, among others, with its inhibition resulting in overactivation of these pathways and subsequent apoptosis, as demonstrated for DMG (Schramm et al., 2019). While interesting, the *in vivo* efficacy of such a therapeutic strategy is yet to be demonstrated in DMG, as well as potential combination therapies using other classes of cytotoxic agents.

Cell cycle checkpoints

The deregulation of cell cycle progression is a common feature of many types of cancer; as such, it is not surprising that DMG possesses several aberrations in cell cycle checkpoints which are potential therapeutic targets (Table 4 and Fig. 3). H3K27 M mutations, being the hallmark of DMG, result in epigenetic repression of $p16^{INK4A}$ (CDKN2A), an important regulator of the G1/S checkpoint (Cordero et al., 2017; Mohammad et al., 2017). Furthermore, chromosomal gains of regions containing cyclin-dependent kinases 4 and 6 (CDK4/6) and associated cyclins, all essential at the G1/S checkpoint, have been detected in DMG patient specimens (Paugh et al., 2011). As a result, DMG cells appear to be sensitive to pharmacological inhibition of CDK4/6 *in vitro* (Asby et al., 2018; Grasso et al., 2015). Moreover, one study showed a significantly prolonged survival of genetically engineered mice with DMG, upon treatment with palbociclib (PD-0332991) (Barton et al., 2013). However, the murine DMG model used carries a genomic deletion of the Ink4-ARF locus, which does not occur in DMG patients and may cause susceptibility to CDK4/6 inhibition. Nonetheless, based on the epigenetic repression of $p16^{INK4A}$ in DMG, CDK4/6 inhibition may represent a therapeutic strategy in these tumors that warrants further investigation.

Apart from deregulation of the G1/S checkpoint, DMG was found to overexpress several key players in G2/M progression as well including WEE1, maternal embryonic leucine zipper kinase (MELK), polo-like kinase 1 (PLK1) and aurora kinase B (AURKB), all of which have been studied as potential therapeutic targets (Amani et al., 2016; Buczkowicz et al., 2013; Caretti et al., 2013b; Meel et al., 2018a; Mueller et al., 2014). Inhibition of WEE1 by the small molecule MK1775 (AZD1775) was shown to be effective in treating and radiosensitizing DMG cells. Importantly, treatment of mice carrying patient-derived xenografts of DIPG and other diffuse gliomas, resulted in improved survival, especially when combined with radiotherapy (Caretti et al., 2013a; Mueller et al., 2014). These results are in apparent discordance with the limited BBB penetration of AZD1775, implying that either the BBB is disrupted in the xenograft mouse models, or that very low levels are sufficient for an antitumor effect (de Gooijer et al., 2018). Further studies are therefore required to determine the definitive potential of WEE1 inhibition, especially in light of the development of novel WEE1 inhibitors with superior brain bioavailability (de Gooijer et al., 2018).

Inhibition of another G2/M checkpoint kinase, AURKB, resulted in some antitumor efficacy in DMG cells *in vitro*, but so far no successful *in vivo* trials using specific inhibitors have been performed (Buczkowicz et al., 2013). Only one *in vivo* trial has been performed with a multikinase inhibitor that has aurora kinases among its target, and this failed to show preclinical efficacy, possibly as a result of poor BBB penetration of the compound chosen (BMS-754807) (Halvorson et al., 2015). Strikingly, a functional drug screen failed to show efficacy of AURKB inhibitors in a panel of DMG cultures, whereas inhibition of its paralog AURKA seemed to be effective (Grasso et al., 2015). As such, the role of aurora kinases as therapeutic targets in DMG requires further studies,

Table 4
Cell cycle checkpoints as therapeutic targets for DMG.

Target	*In vitro* efficacy	*In vivo* efficacy	Remarks	References
CDK4/6	+	+/-	Synergy with mTORC1/2 inhibition, *in vivo* data generated in not fully representative DMG model	Barton et al., 2013; Grasso et al., 2015; Asby et al., 2018
WEE1	+	+	Limited BBB penetration of inhibitor used	Caretti et al., 2013a, 2013b Mueller et al., 2014; De Gooijer et al., 2018
AURKB	+/-	–	AURKB *in vivo* only tested with multikinase inhibitor, AURKA inhibition possibly more effective	Buczkowicz et al., 2013; Grasso et al., 2015; Halvorson et al., 2015
PLK1	+	n/a		Amani et al., 2016; Schramm et al., 2019
MELK	+	+	*In vivo* efficacy shown in Mdr1a/b$^{-/-}$;Bcrp1$^{-/-}$ mice, as inhibitor did not cross BBB	Meel et al., 2018a, 2018b

n/a: data not available.

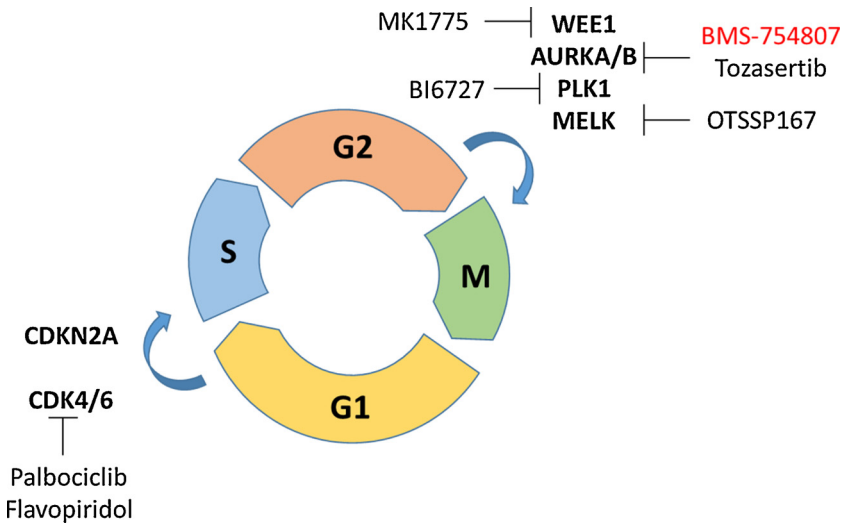

Fig. 3. Cell cycle checkpoints as therapeutic targets of commonly used drugs in preclinical DMG research. Marked in red are drugs that have been shown to be ineffective in (pre-)clinical DMG studies.

also to elucidate the potential translational relevance of aurora kinase inhibitors.

Although extensively studied in other types of cancer as a gatekeeper of the G2/M transition, only one study has described *in vitro* efficacy of direct PLK1 inhibition in DMG (Amani et al., 2016). As *in vivo* data are lacking, it is not possible to draw any conclusions regarding the potential of PLK1 inhibition for the treatment of DMG. As with PI3K and MAPK signaling, enhancing PLK1 activity by inhibiting PP2A has also been suggested as therapeutic strategy, although the contribution of PLK1 stimulation to the antitumor effect of PP2A inhibition is yet to be elucidated (Schramm et al., 2019).

Finally, a recent study by our group has identified MELK as a potential therapeutic target in DMG, showing *in vitro* efficacy of the small molecule OTSSP167, and shRNA targeting MELK, in a panel of primary DMG cultures (Meel et al., 2018a). Although this study did not address the influence of MELK inhibition on the cell cycle progression of DMG cells, MELK has been described as an important regulator of G2/M progression, which likely contributes to the antitumor efficacy of its inhibition. Unfortunately, brain pharmacokinetic studies showed that OTSSP167 does not cross the BBB due to the activity of multidrug efflux transporters. Nevertheless, treatment of Mdr1a/b$^{-/-}$;Bcrp1$^{-/-}$ mice harboring patient-derived DMG xenografts with OTSSP167 resulted in a strong antitumor activity and increased survival, validating MELK as a therapeutic target in these tumors and encouraging the development of novel, MELK inhibitors that penetrate the BBB (Meel et al., 2018a).

Stem cell phenotype

Starting from the very first studies into the biology and oncogenesis of DMG, evidence has arisen that these tumors develop in primitive neural progenitor cells (NPCs) or oligodendrocyte progenitor cells (OPCs) in the fetal brainstem, resulting in stem cell-like tumor cells with high levels of therapy resistance (Anderson et al., 2017; Funato et al., 2014; Larson et al., 2019; Monje et al., 2011; Pathania et al., 2017). Consequently, attempts have been made to target the stem cell phenotype of DMG cells as a therapeutic strategy, as these stem cell characteristics are held responsible for at least part of the therapy resistance of DMG cells (Table 5).

The first such study showed that DMG cells originate from Sonic hedgehog (SHH) expressing OPCs, and retain their dependence on hedgehog signaling, resulting in antitumor efficacy of hedgehog signaling inhibitors *in vitro* (Monje et al., 2011). Another study corroborated these observations by demonstrating overexpression of the SHH receptor Patched 1 (PTCH1) in a subgroup of DMG, and differential methylation of genes involved in hedgehog signaling compared to normal brain tissue (Saratsis et al., 2014). The *in vitro* efficacy of SHH inhibition has since been demonstrated once more, but *in vivo* trials studying the therapeutic potential of these drugs are lacking (Grasso et al., 2015). One study demonstrated overexpression of NOTCH and activated downstream signaling of the Notch pathway in DMG, which may also result in the stem cell phenotype of DMG cells (Taylor et al., 2015). Inhibition of Notch signaling resulted in decreased tumor cell viability and radiosensitization of DMG cells (Taylor et al., 2015). As with SHH inhibition, *in vitro* trials studying the therapeutic effect of Notch inhibitors are lacking, warranting further investigation.

Additionally, our study which identified MELK as a therapeutic target in DMG, further enforced the potential of targeting the stem cell phenotype of DMG cells, as MELK has been shown to be essential for

Table 5
The stem cell phenotype, DNA damage repair pathway and cell surface antigens as (immune)therapeutic targets for diffuse midline glioma.

Target	*In vitro* efficacy	*In vivo* efficacy	Remarks	References
SHH/PTCH1	+			Monje et al., 2011; Grasso et al., 2015
NOTCH	+		Radiosensitization upon NOTCH pathway inhibition	Taylor et al., 2015
PARP	+	+/-	Mouse models used in *in vivo* study not fully representative of DMG, Radiosensitization by PARP inhibition, Possible PARP inhibition-sensitive subclones in DMG	Chornenkyy et al., 2015; Vinci et al., 2018
PPM1D	+	+	Only effective in *PPM1D* mutated, p53 wild-type DMG; Radiosensitization by PPM1D inhibition in DMG cells with that specific genomic profile	Akamandisa et al., 2019
GD2	+	+	CAR T-cell target	Mount et al., 2018
B7-H3	+	+/-	CAR T-cell target, *in vivo* efficacy only in other pediatric brain tumor models	Majzner et al., 2019

cancer stem cell maintenance, and expression patterns of MELK during embryonic development correspond to the putative time of origin of DMG (Meel et al., 2018a). Finally, telomerase maintenance mechanisms, an important hallmark of both normal and malignant stem cells, have been proposed as therapeutic targets in DMG, although inhibition of telomerase and related enzymes has not been pursued for the treatment of DMG to date (Dorris et al., 2014).

DNA damage repair

Like the majority of other cancer types, DMG is characterized by a high prevalence of defects in DNA damage repair systems. Most importantly, p53 function is often compromised by loss-of-function mutations, genomic deletions or indirectly as a result of *PPM1D* mutations (Aihara et al., 2014; Akamandisa et al., 2019; Buczkowicz et al., 2014b; Fontebasso et al., 2014; Grill et al., 2012; Hoffman et al., 2016; P. International Cancer Genome Consortium PedBrain Tumor, 2016; Khuong-Quang et al., 2012; Mackay et al., 2017; Nikbakht et al., 2016; Puget et al., 2012; Saratsis et al., 2014; Taylor et al., 2014; Vinci et al., 2018; Warren et al., 2012). Furthermore, mutations or losses involving ATM or ATRX are found in subsets of DMG patients (Aihara et al., 2014; Buczkowicz et al., 2014b; Fontebasso et al., 2014; Grill et al., 2012; Hoffman et al., 2016; P. International Cancer Genome Consortium PedBrain Tumor, 2016; Khuong-Quang et al., 2012; Mackay et al., 2017; Nikbakht et al., 2016; Saratsis et al., 2014; Taylor et al., 2014; Vinci et al., 2018). Excluding the use of traditional chemotherapeutic agents, a few studies have been performed that attempt to exploit these DNA damage repair deficiencies for the development of therapeutic strategies for DMG (Table 5). Nonetheless, inhibition of poly-ADP-ribose polymerase (PARP), involved in single strand break repair, may be effective in the presence of certain DNA damage repair deficiencies, especially as it is overexpressed in a subset of DMG (Chornenkyy et al., 2015; Zarghooni et al., 2010). As such, PARP inhibition has been studied as a potential therapeutic intervention in DMG, resulting in radiosensitization and antitumor efficacy *in vitro* and *in vivo*, although the mouse models used in this study are not fully representative of DMG (Chornenkyy et al., 2015). Interestingly, a recent study has identified the potential presence of subclones in DMG that are highly responsive to PARP inhibition, warranting further investigation of these compounds in combination treatment strategies (Vinci et al., 2018). More specifically, a recent study has shown that targeting PPM1D effectively treats *PPM1D* mutated DMG *in vitro* and *in vivo*, especially when combined with radiotherapy (Akamandisa et al., 2019). These results indicate that PPM1D inhibitors represent a promising therapeutic strategy for the subset of DMG that carries *PPM1D* mutations in the presence of wild-type p53. As such, exploiting DNA damage repair deficiencies for the treatment of DMG may represent a promising strategy for the treatment of these tumors, despite the limited number of studies on this topic.

Targets for immunotherapy

In recent years, immunotherapy has emerged as a promising new treatment modality for various types of cancer, including adult glioma (Dunn-Pirio and Vlahovic, 2017; Hodges et al., 2016; Polivka et al., 2017). As a result, similar approaches are being explored for the treatment of DMG. However, the lack of immunocompetent mouse models of DMG hampers the study of immunotherapy for these tumors. An exception is formed by chimeric antigen receptor (CAR) T-cell therapy, in which T-cells are genetically engineered *ex vivo* to target surface antigens on tumor cells (Table 5). By using donor T-cells, these therapies can be tested in immunodeficient, xenograft-bearing mice, although the lack of a recipient immune system still imposes a limitation in the interpretation of the results of such trials. Nonetheless, impressive results have been obtained in the treatment of DMG xenograft-bearing mice with CAR T-cells targeted at GD2 or B7-H3 (Majzner et al.,

2019; Mount et al., 2018; Zhou et al., 2013). Although the latter has not been specifically studied in DMG mouse models, the extensive data on using GD2 CAR T-cells in DMG, and B7-H3 *in vitro* and in other pediatric brain tumor models *in vivo*, makes it likely to be effective as well. A definitive assessment of the potential of CAR T-cell therapy and other forms of immunotherapy for the treatment of DMG requires the development of immunocompetent DMG mouse models. The development of these models is also essential to investigate the potential development of resistance to immunotherapy. Until then, caution is warranted when developing clinical trials.

Discussion and future perspectives

Until a decade ago, preclinical research into diffuse midline gliomas was virtually non-existent, and clinical trials were largely based on adult treatment protocols. With the reintroduction of DMG biopsies, and the implementation of autopsy protocols for the collection of biological material, preclinical research is expanding rapidly. As a result, our understanding of the pathobiology, oncogenesis and treatment failure of DMG has greatly improved, especially with the identification of *Histone 3* mutations as driver genetic events. Subsequently, preclinical studies have begun to be performed in an attempt to develop specific, targeted therapies for DMG. Most of these studies have been based on large-scale genomic and transcriptomic studies, which identified a variety of oncogenic programs relevant to the cancer biology of DMG.

Expectedly, these studies are still in their infancy, as only a few years have passed in which researchers had the technology, biological material and relevant preclinical models of DMG available. Nonetheless, from the studies summarized in this review, one can draw some preliminary conclusions as to which research directions seem most promising. Based on the current knowledge, epigenetic modification has yielded highly promising results in multiple models of DMG, and is likely to be selective for *Histone 3* mutated cells, due to the inherent differences in chromatin structure between these and normal cells. The same is true for inhibition of cell cycle checkpoints, which has yielded similar encouraging results in preclinical studies, possibly as a result of deregulation of cell cycle checkpoints by the *Histone 3* mutations, as has been shown for p16. On the other hand, targeting activated signaling pathways and overexpressed growth factors receptors has so far yielded mainly disappointing results, with the exception of ACVR1 inhibitors in *ACVR1* mutated DMG. These results may partially be explained by the lack of specific inhibitors with good BBB penetration, which emphasizes the importance of brain pharmacokinetic studies of promising drugs in preclinical DMG research. Finally, targeting the stem cell phenotype and DNA damage repair systems, as well as exploring immunotherapy, may be effective therapeutic strategies for DMG, however research in these areas is still limited, preventing us from drawing firm conclusions.

An important research direction will be the evaluation of combined therapeutic strategies, especially given that only very few cancers can be treated effectively by targeting a single molecule or cellular process, and that preclinical studies have already identified the development of drug resistance of DMG cells to novel agents that initially appeared effective. Some studies have already effectively combined multiple epigenetic modifiers or multiple kinase inhibitors, but research into combination therapies using compounds from different classes is still lacking, despite the presence of a strong biological and clinical rationale to do so. Importantly, combined therapeutic interventions with radiotherapy should be studied, as this is still the conventional treatment for children suffering from DMG, and as several studies have already demonstrated the potential of radiosensitization in these tumors. In summary, major strides have been made over the past decade towards the development of a curative therapeutic strategies for DMG. Now that biological material and adequate *in vitro* and *in vivo* models are available as well as the knowledge on how to use them effectively along with the identification of the main oncogenic drivers, the possibility for the preclinical development of an effective treatment for these fatal childhood brain tumors in the coming years is at an apparent reach.

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