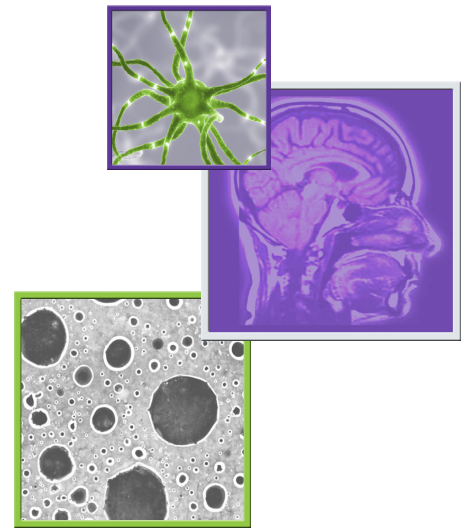
REVIEW



Targeting of histone deacetylases in brain tumors

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Practice Points

- Expression of histone deacetylases (HDACs) is frequently dysregulated in brain tumors compared with normal brain tissue.
- Expression patterns differ depending on histology and tumor grade.
- Mutation rate of HDAC genes in brain tumors is low to nonexistent.
- HDACs have been validated as potential targets for the treatment of brain tumors in preclinical models.
- Small-molecule HDAC inhibitors can induce apoptosis, differentiation and cell-cycle arrest in brain tumor cells.
- Biomarkers for response prediction to HDAC inhibitor treatment are missing.
- HDAC inhibitors might reveal their full anticancer potential in combination therapy approaches.
- HDAC inhibitors currently used in ongoing clinical trials are vorinostat, panobinostat, entinostat and valproic acid.
- Initial results from completed trials do not show efficacy of HDAC inhibitors as monotherapy.
- Individual patients do show responses to HDAC inhibitor treatment.
- Development of biomarkers for response to HDAC inhibition is crucial for the successful translation of the promising preclinical findings into the clinic.
- Currently, HR23B is the most prominent predictive biomarker, and histone acetylation of peripheral blood mononuclear cells the most widely used pharmacodynamic biomarker for HDAC inhibition.
- Critical points for the successful progression of research exploring the targeting of HDACs in brain tumors are elucidation of the function of HDAC isoenzymes, development of inhibitors with a class- or isoenzyme-specific inhibitory profile, development of biomarkers for response prediction and investigation of rational combination therapies.

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SUMMARY Histone deacetylase inhibitors (HDACis) have fascinated researchers in almost all fields of oncology for many years owing to their pleiotropic effects on nearly every aspect of cancer biology. Since the approval of the first HDACi vorinostat for the treatment of cutaneous T-cell leukemia in 2006, more than a hundred clinical trials have been initiated with a HDACi as a single agent or in combination therapy. Although a number of epigenetic and nonepigenetic molecular mechanisms of action have been proposed, biomarkers for response prediction and patient selection are still lacking. One of the inherent problems in the field of HDACis is their 'reverse' history of drug development: these compounds reached clinical application at an early stage, before the biology of their targets, HDAC1–11, was sufficiently understood. This review summarizes the current knowledge on the human family of HDACs as drug targets in pediatric and adult brain tumors, the efficacy and molecular action of HDACis in preclinical models, as well as the current status of the clinical development of these compounds in the field of neuro-oncology.

The human histone deacetylases (HDACs) are divided into the families of 'classical' HDAC1–11 and of sirtuins (also referred to as class III HDACs) [1]. Classical HDAC inhibitors (HDACis) act through complexing the catalytically critical zinc ion of HDAC1–11 at the base of the enzymatic pocket. By contrast, sirtuins do not share this catalytical mechanism, they are NAD^+ dependent and thus are not affected by classical HDACis [1]. This review focuses on the classical HDACs and their small-molecule inhibitors.

The 11 classical HDACs are subdivided into class I, IIa/b and IV according to their homology to yeast orthologs [2]. Class I HDAC1, 2, 3 and 8 are predominantly located in the nucleus and there seems to be a tissue-specific subcellular distribution; however, as has been reported for smooth muscle cells, HDAC8 is mainly found in the cytoplasm [3]. Class IIa HDAC4, 5, 7 and 9 can shuttle between the nucleus and the cytoplasm, while class IIb HDAC6 and 10 are predominantly located in the cytoplasm [4]. Much less is known regarding the single class IV HDAC11. It is now clear from knockout mice experiments that HDACs have nonredundant functions during embryonal development, resulting in distinct phenotypes ranging from early embryonal death to postnatal heart defects, growth plate and endothelial cell dysfunctions, and craniofacial defects [5]. In cancer biology, distinct functions of individual HDAC family members have also been described. For example, class I HDAC1–3 have been found in multiprotein complexes with oncogenic fusion transcripts, such as PML–RARa and AML-1–ETO, driving dedifferentiation of leukemic cells [6,7]. In addition, class I HDAC1–3 are frequently overexpressed in adult solid cancers [8]. In pediatric neuroblastoma, class I HDAC8 is associated with advanced-stage disease and poor clinical outcome and plays a distinct role in differentiation [9]. Much less is known regarding the function of class II HDACs in cancer biology. Class IIa HDAC5 and 9 are overexpressed in subgroups of pediatric medulloblastoma tumors and functional analysis showed that they are involved in proliferation of medulloblastoma cells [10]. Only scarce information on class IV HDAC11 in cancer biology is available. Very recently, HDAC11 was found to be a promising selective drug target in carcinomas [11].

Historically, the first substrates known to be deacetylated by HDACs were histone proteins, hence their name; however, it is now clear that there is a great number of nonhistone nuclear as well as cytoplasmatic substrate proteins that are deacetylated. Thus, many researchers are in favor of the term 'lysine deacetylases' to indicate the fact that HDACs are a more general acting class of enzymes removing acetyl groups from ε-amino-lysine residues of many different proteins [12]. Among the nonhistones substrates of HDACs there are key proteins, such as p53, STAT3, HSP70 and tubulin that are regulated in their biological function by reversible acetylation [1]. In fact, it has been estimated by high-resolution mass spectrometry that more than 3600 acetylation sites in 1750 proteins are regulated after treating a given cell type with a HDACi, giving rise to the term 'acetylome' [13]. It has been proposed that the acetylome, regulated by HDACs and HATs, functions as a regulatory signaling network in cells similar to the phosphoproteome network controlled by kinases and phosphatases [14]. A useful acetylome database covering a wide range of protein acetylation information is the CPLA [201].

HDACs can be inhibited by several small-molecule chemical compounds that fall into five basic structural classes: hydroxamic acids, benzamides,

cyclic tetrapeptides, short-chain fatty acids and electrophilic ketones [15]. The compounds currently in clinical use are unselective, and, therefore, they target several HDACs simultaneously. Vorinostat (suberoylanilide hydroxamic acid) was the first HDACi to be approved for clinical use in cutaneous T-cell lymphoma in 2006, followed by depsipeptide (romidepsin, FK228) in 2009 [16]. Although vorinostat and many of the other hydroxamic acid compounds (i.e., TSA, ITF 2357 and panobinostat) have been described as being pan-HDACis, this view was recently challenged through a chemical phylogenetics approach suggesting that these compounds are in fact class I HDACis [17].

Of great clinical importance in neuro-oncology is the ability of compounds to cross the blood–brain barrier (BBB) (Table 1). The ability of HDACis to penetrate the BBB is not well studied. Some preclinical mouse models and clinical trials suggest that vorinostat is able to pass the BBB [18–20]. Data from PET-based experiments performed by Hooker *et al.* suggest that MS-275 (entinostat) does not penetrate the BBB of nonhuman primates at clinically relevant concentrations [21], although it has been described as a potent brain region selective HDACi in rodents [22]. Valproic acid (a short-chain fatty acid) is an HDACi with extensively clinically proven BBB penetration, as it has long been used for the treatment of epilepsy. Phenylbutyrate, another short-chain fatty acid-type HDACi, has been developed for the treatment of urea cycle disorders. Both compounds are weak HDACis in that they require 1–5 mM concentrations to exert HDAC inhibitory activity *in vitro*. However, these concentrations can hardly be reached in patients (Table 1) and phenylbutyrate must be given as continuous intravenous infusion or ingested in large amounts (several grams) of tablets per day, which still results in submillimolar plasma concentrations (Table 1).

All HDACis currently in clinical use are associated with class-specific toxicities, the most frequent being fatigue, electrolyte disturbances, and gastrointestinal and hematological side effects, which limit the applicable dosages and, therefore, impede the realization of HDACis full anticancer potential. It is currently under discussion which of the HDAC family members are mediating these limiting toxicities. As a consequence, several groups are trying to develop truly isoform-selective inhibitors with a broader therapeutic window [23]. However, this approach requires exact knowledge regarding the molecular function and mechanism of single HDAC isoforms in tumor tissue and normal organs. One of the few current promising examples is the development of HDAC8-selective inhibitors that are at least 200-fold more active against HDAC8 compared with other HDAC family members [24,25] and the identification of HDAC8 as a selective drug target in neuroblastoma [9]. Finally, a recent publication describes novel class IIa selective HDACis [26]; however, true class IIa isoenzyme-selective HDACis are still missing.

Expression of HDACs in brain tumor tissue

Dysregulation of the expression of HDAC genes has been reported for numerous cancer

Table 1. Clinically achievable concentrations of HDAC inhibitors.

Compound	C_{max} (mol/l)	Class	Penetration of BBB	Ref.
VPA	3.41E^{-04}	Short-chain fatty acid	Yes [114]	[115]
Phenylbutyrate	3.00E^{-04}	Short-chain fatty acid	Yes [116]	[117]
Vorinostat (SAHA)	4.49E^{-06}	Hydroxamic acid	Yes [118]	[119]
Panobinostat (LBH589)	4.09E^{-08}	Hydroxamic acid	Yes [120]	[121]
Belinostat (PXD-101)	1.00E^{-07}	Hydroxamic acid	Little in nonhuman primates [122]	[123]
Abexinostat (PCI-24781)	3.39E^{-07}	Hydroxamic acid	N/A	[124]
Dacinostat (LAQ824)	2.17E^{-06}	Hydroxamic acid	N/A	[125]
Givinostat (ITF2357)	4.98E^{-07}	Hydroxamic acid	N/A	[126]
Entinostat (MS-275)	3.90E^{-07}	Benzamide	No/little in nonhuman primates [22]; yes [116]	[127]
Romidepsin (depsipeptide)	8.74E^{-07}	Cyclic tetrapeptide	No/little [101]	[128]

BBB: Blood–brain barrier; C_{max}: Maximum concentration; N/A: Not applicable; SAHA: Suberoylanilide hydroxamic acid; VPA: Valproic acid.

entities [27]. However, literature systematically analyzing the expression of all 11 classical HDAC isoenzymes in cancer cells and the correlation of expression patterns with stage of the disease or patient survival remains sparse. This holds true for the expression of HDACs in brain tumor tissue in particular. Lucio-Eterovic *et al.* demonstrated that class II and IV HDACs are downregulated in glioblastoma multiforme (GBM) in comparison with low-grade astrocytoma and normal brain tissue at the mRNA level [28]. Campos *et al.* restricted their HDAC expression analysis to HDAC1–3 in astrocytic gliomas and found that HDAC1 and 2 are strongly expressed in gliomas independent of tumor grade. HDAC3, however, showed an inverse correlation with grade, with strong HDAC3 expression correlating with increased survival probabilities [29]. Milde *et al.* found a correlation of high HDAC5 and 9 expression and an unfavorable prognosis for patients with medulloblastoma [10].

In order to systematically display HDAC1–11 expression patterns across a wide spectrum of different brain tumor entities of different grade, as well as normal brain tissue, we analyzed publicly available data via the R2 microarray analysis and visualization platform (Figure 1) [202]. For this analysis we used the probesets with the highest average present signal. Other probesets for the same HDAC did not show considerable differences in observed trends in expression levels.

As shown in Figure 1, many brain tumor entities show considerable alterations in their HDAC expression compared with normal brain tissue. In addition, the expression of the same HDAC isoenzyme may differ within the same tumor entity depending on tumor grade or molecular subgroup. The expression data presented in Figure 1 suggests a correlation of increased HDAC1 and 3 expression and tumor grade in astrocytoma (data available for astrocytoma [WHO II° and III°] and GBM [WHO IV°]). Medulloblastomas, in particular, show a diverse HDAC expression pattern. While upregulated in all examined brain tumor entities, HDAC1 is relatively downregulated in only one molecular subgroup of medulloblastoma (group 4). HDAC2, however, showing no alteration of expression in all the other brain tumor entities, is considerably overexpressed in three out of four molecular subgroups in medulloblastoma (groups 3, 4 and SHH, relative to WNT). A similar trend can be observed for HDAC5 and 6. Intriguingly, all brain tumor entities show an upregulation of class I HDAC1 and 3 and a downregulation of class IV HDAC11 compared with normal brain tissue.

In pediatric brain tumors, next-generation sequencing technologies have identified mutations in genes directly or indirectly involved in epigenetic regulation as a common theme [30–35]. However, little information is available on mutations in HDAC genes. *HDAC2* mutations have been discovered in group 4 medulloblastoma [35]; other publications describing the sequencing of large medulloblastoma cohorts, however, did not describe HDAC mutations [32,34]. Therefore, the mutation rate of HDAC genes in medulloblastoma seems to be very low.

While epigenetically effective mutations in the chromatin component H3.3 have been described in GBM [30,31], data on HDAC mutations again is scarce.

We analyzed the publicly available The Cancer Genome Atlas data set on GBM [203] via the open access cBio Cancer Genomics Portal [36,204]. At the time of manuscript submission, data of whole-exome sequencing for 276 GBM tumor samples was available. We found that only very few GBM tumors have mutations in or copy number alterations of HDAC genes (amplification or homozygous deletion) (Table 2). According to MutSig analysis [205], no mutation of HDAC genes in GBM is considered a significantly mutated driver candidate.

Taken together, the mutation frequency of HDACs in all brain tumors currently sequenced seems to be very low to nonexisting. Therefore, it seems likely that the expression or functional status within multiprotein complexes determines the oncogenic potential of HDAC family members.

Functional validation of HDACs & efficacy of HDACis in preclinical models

As mentioned above, HDAC expression is frequently dysregulated in brain tumors compared with normal brain tissue. Considering that HDACs act as important epigenetic, as well as nonepigenetic, regulators of key signaling pathways involved in many, if not, all hallmarks of cancer [15], and since, unlike genetic mutations, epigenetic modifications are thought to be reversible, it is tempting to speculate that targeting of HDACs has the potential to reverse the malignant phenotype of cancer cells. This

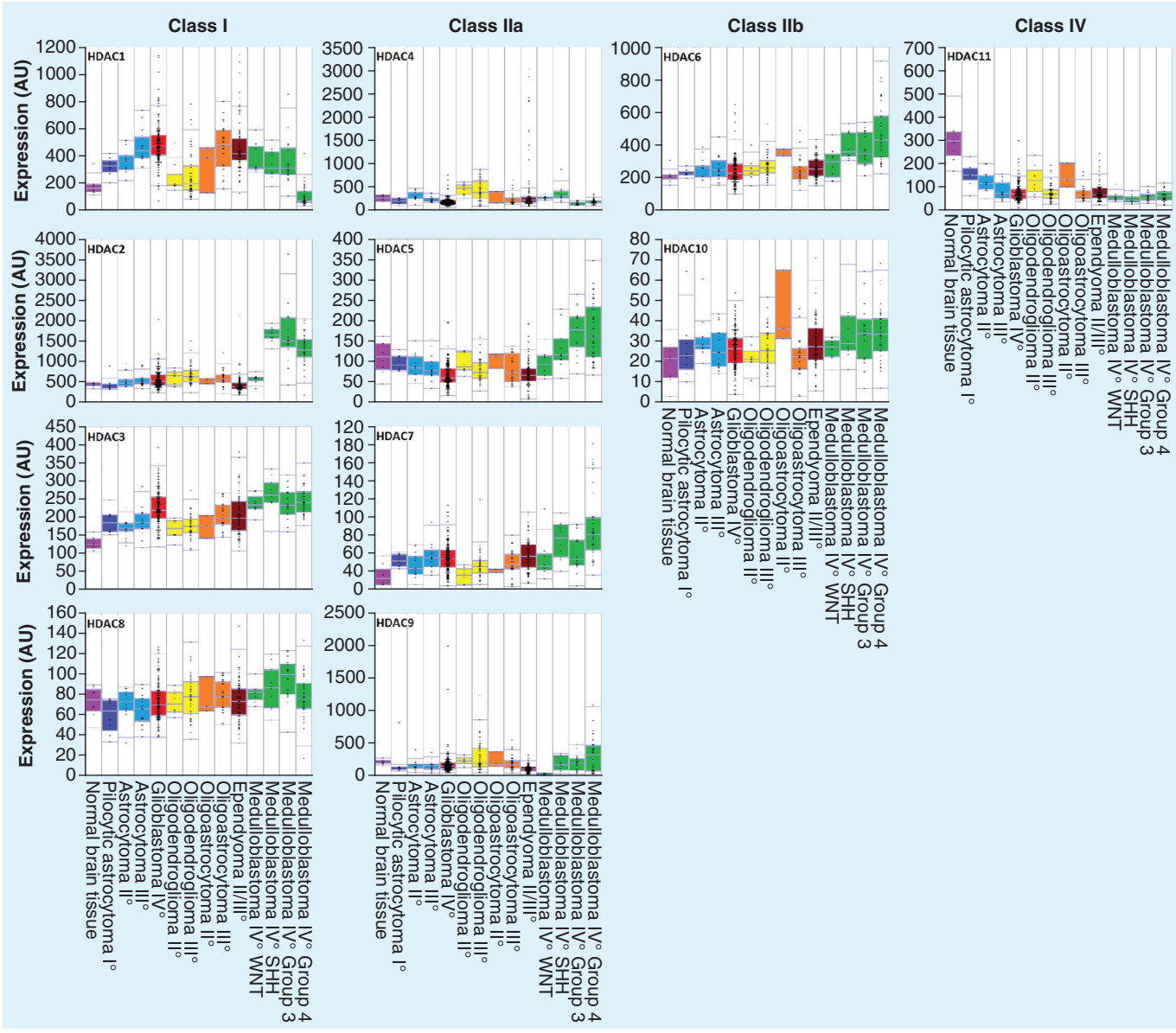

Figure 1. Histone deacetylase mRNA expression in brain tumors, as analyzed by gene-expression microarrays, visualized by R2. Dataset GSE16011 [129]: age at diagnosis: 11–81 years; normal brain tissue: n = 8; pilocytic astrocytoma WHO I°: n = 8; astrocytoma WHO II°: n = 13; astrocytoma WHO III°: n = 16; glioblastoma multiforme WHO IV°: n = 159; oligodendroglioma WHO II°: n = 8; oligodendroglioma WHO III°: n = 44; oligoastrocytoma WHO II°: n = 3; and oligoastrocytoma WHO III°: n = 25. Dataset GSE21687 [130]: age: 0–59 years; ependymoma WHO II° and III°: n = 83. Dataset GSE37418 [131]: age: 3–16 years; medulloblastoma WHO IV° WNT: n = 8; medulloblastoma WHO IV° SHH: n = 10; medulloblastoma WHO IV° group 3: n = 16; and medulloblastoma WHO IV° group 4: n = 39. Probesets: HDAC1: 201209_at; HDAC2: 201833_at; HDAC3: 216326_s_at; HDAC4: 204225_at; HDAC5: 202455_at; HDAC6: 206846_s_at; HDAC7: 217937_s_at; HDAC8: 223345_at; HDAC9: 205659_at; HDAC10: 226672_s_at; and HDAC11: 227679_at.

renders HDACs highly interesting targets for anticancer therapy.

In preclinical investigations much effort has been made to evaluate the functional relevance of HDACs in brain tumor cells. Currently, the most frequent experimental approaches of modulating HDAC activity include interference with HDACs either by si/shRNA-mediated knockdown or by enzymatic inhibition with small-molecule inhibitors. While knockdown experiments have the advantage of exclusively targeting single HDAC isoenzymes individually, established HDACis usually target a variety of HDAC family members, impeding the

Table 2. Rate of mutation rates and copy number alterations in glioblastoma.

Gene	Somatic mutations		Copy number alterations	
	Cases (n)	%	Cases (n)	%
HDAC9	4	1.4	7	2.5
HDAC2	3	1.1	4	1.4
HDAC5	3	1.1	0	0.0
HDAC6	3	1.1	0	0.0
HDAC8	2	0.7	0	0.0
HDAC1	1	0.4	1	0.4
HDAC3	1	0.4	0	0.0
HDAC4	1	0.4	2	0.7
HDAC10	0	0.0	4	1.4
HDAC11	0	0.0	1	0.4
HDAC7	0	0.0	1	0.4

As analyzed by whole-exome sequencing of the The Cancer Genome Atlas data set *'Glioblastoma Multiforme'* retrieved via the cBio Cancer Genomics Portal [39,203,204].

attribution of an observed effect to a specific enzyme. However, knockdown experiments are technically much more challenging, more difficult to control and the pharmacology of si/shRNA-mediated knockdown in *in vivo* models remains complex [37], while experiments with HDACis promise a quick translation of interesting findings from bench to bedside, if applied in clinically relevant concentrations. Finally, since HDACs usually function within multiprotein complexes, knockdown of a particular HDAC can cause disruption of the function of a whole protein complex. Thus, a given biological effect cannot necessarily be attributed to enzymatic function, and, therefore, be translatable into a strategy involving HDACis, but could also be explained by HDACs functioning as bromodomain proteins, that is, readers of epigenetic marks acting independently from their catalytic activity.

As reviewed below, HDACis have a broad range of effects on brain tumor cells. Most of the research has focused on the two most frequent devastating malignancies in neuro-oncology: GBM in adults and medulloblastoma in pediatrics. It is important to note that in all investigations, the observed effect of HDAC inhibition was dose dependent and relevant anti-tumoral effects were only observed above a distinct threshold level. In many investigations, final inhibitor concentrations are far from clinically achievable dosages, which renders these findings untranslatable into clinical applications. This review only comments on investigations working with clinically achievable concentrations of inhibitors as summarized in Table 3. Two reasons for applying inhibitors at higher concentrations were considered valid to make exceptions:

- The clinically achievable concentration of the applied inhibitor is unknown, but the investigators demonstrated in control experiments that the inhibitor does not affect normal brain tissue or cells at the highest concentration used;
- High concentrations of the inhibitor are required to be able to investigate molecular mechanisms involved in functional consequences of HDAC inhibition. Elucidation of these mechanisms might offer rationales for intelligent combination therapies that might allow lowering the dosage of the inhibitor.

It appears that, in preclinical models of all investigated brain tumor entities, small-molecule HDACis of different chemical classes were able to considerably change the cellular phenotype. Although very promising, these findings are far from satisfying. The constantly observed response of brain tumor cells to HDAC inhibition, especially in contrast with the missing response of normal brain tissue [38], suggests a certain degree of tumor addiction to the catalytic activity of HDACs. Tumor addiction to a targetable enzyme, opens up a therapeutic window. However, cell lines of the same entity show big differences in their response to the same HDACi: some go into apoptosis while others experience only a cell-cycle arrest, half maximal inhibitory concentration values for induction of apoptosis differ dramatically from cell line to cell line across all entities. Why are some tumor cells more susceptible to HDACi treatment than others? This is a key question to be answered as it could ultimately result in the development of biomarkers for response prediction in the clinical setting. This is of particular clinical interest since a limited number of individual patients show a dramatic response, while for the majority of patients no benefit of HDACi treatment could be demonstrated.

As HDACs have a broad variety of substrates apart from histones, such as transcription factors p53 and HSP90, HDAC inhibition will ultimately result in a rather difficult-to-survey global change in acetylation patterns of cellular proteins. The challenge of current HDAC research is to identify those HDAC-dependent acetylation patterns and to elucidate molecular

mechanisms of tumor addiction to functional consequences of the acetylation status of different proteins.

When it comes to histones, the most prominent target of HDACs, the functional consequences of the catalytic activity of HDACs are anything but easy to predict. Mechanistically speaking, it is known that an altered histone acetylation pattern will change the availability of DNA for transcription. Functionally speaking, this will ultimately result in a change of gene expression. However, to functionally link the action of a specific HDAC isoenzyme to the altered expression of a specific (set of) gene(s) is much more difficult. Unlike transcription factors, HDACs do not interact directly with DNA. This renders them incapable of directing their regulatory effect on transcription towards a defined region of the DNA. Therefore, HDACs are dependent on other proteins to recruit them to specific regions of the DNA where they will then effectuate their histone deacetylating activity. HDAC expression alone is, therefore, not sufficient to predict a cancer cell's susceptibility to HDACi treatment. The given set of recruitment proteins (e.g., transcription factors and repressive complexes), which will vary considerably from cancer cell to cancer cell, determines the gene expression-modifying effect of HDACs on the histone level. However, it appears reasonable that, if both HDAC isoenzyme and the appropriate DNA recruitment protein(s) are present in a cancer cell, the HDAC can be targeted towards a region of the DNA encoding for tumor suppressor pathways and/or inhibition of oncogenic pathways. This could partially explain tumor addiction to HDACs and has been shown for several tumor suppressor genes in different brain

Table 3. Ongoing clinical trials using histone deacetylase inhibitors in brain tumors.

HDAC inhibitor	Combination treatment	Histology	Phase	Age (years)	Clinicaltrials identifier/ status
Panobinostat	Stereotactic radiation	Recurrent glioma, high-grade meningioma, brain metastasis	I	>18	NCT01324635; recruiting
Valproic acid	Radiation therapy, temozolomide	High-grade glioma	II	18–90	NCT00302159; recruiting
Vorinostat	Stereotactic radiation	Anaplastic astrocytoma, anaplastic oligodendroglioma, glioblastoma, gliosarcoma, mixed glioma	I	>18	NCT01378481; recruiting
Vorinostat	Bevacizumab	Recurrent glioblastoma multiforme, malignant glioma	II	>18	NCT01738646; recruiting
Vorinostat	Bevacizumab, temozolomide	Recurrent malignant glioma	I/II	>18	NCT00939991; active, not recruiting
Vorinostat	Erlotinib, temozolomide	Recurrent glioblastoma multiforme	I/II	>18	NCT01110876; recruiting
Vorinostat	Bevacizumab	Recurrent glioblastoma	I/II	>18	NCT01266031; active, not recruiting
Vorinostat	Temozolomide	Relapsed primary brain tumors and spinal chord tumors, gliomas, AT/RT, germ cell tumors, MB, PNET, meningioma	I	1–21	NCT01076530; completed
Vorinostat	Temozolomide	Malignant glioma	I	>18	NCT00268385; active, not recruiting
Vorinostat	Temozolomide, bevacizumab, radiation therapy	Newly diagnosed high-grade glioma	Randomized Phase II/III trial	3–21	NCT01236560; recruiting
Vorinostat	Radiation	Newly diagnosed pontine glioma	I/II	3–21	NCT01189266; recruiting
Valproic acid	Etoposide	Neuronal tumors and brain metastases	I	ND	NCT00513162; completed
Vorinostat	Bevacizumab, irinotecan	Recurrent glioblastoma	I	>18	NCT00762255; active, not recruiting
Phenylbutyrate	–	Relapsed brain tumors	II	2–21	NCT00006450; completed

Details of each trial can be found on www.clinicaltrials.gov and by searching for the relevant trial number.
AT/RT: Atypical teratoid rhabdoid tumor; HDAC: Histone deacetylase; MB: Medulloblastoma; ND: Not defined; PNET: Primitive neuroectodermal tumor.

tumor entities (see the 'Modulation of tumor suppressor pathways' section).

Inhibition of HDACs results in a change of expression in up to 20% of the genes of the total genome in a given cell [39]. Depending on the genetic and epigenetic make-up of the cell and HDACi concentration applied, the following mechanisms of action have been implicated in the response of brain tumor cells to HDAC inhibition:

- Modulation of tumor suppressor pathways and/or of oncogenic pathways;
- Inhibition of cell cycle and induction of differentiation;
- Induction of cell death mechanisms;
- Sensitization towards irradiation and other chemotherapeutic agents;
- Interference with cell–matrix interaction: invasion and angiogenesis;
- Other mechanisms: telomerase activity and an increase in genomic instability;
- Crosstalk between regulative acetylation and methylation mechanisms.

Modulation of tumor suppressor pathways

As HDAC activity is linked to increased chromatin condensation and subsequent silencing of gene expression, a tempting strategy of exploiting molecular alterations in cancer involves the reactivation of tumor suppressor genes by inhibition of HDAC activity. Indeed, several groups have shown that HDACis might be of use when it comes to reinducing tumor suppressor genes in brain tumors. Schmidt *et al.* identified the Ras-related protein on chromosome 22 (*RRP22*) as an important tumor suppressor gene in gliomas [40]. *RRP22* expression is negatively correlated with tumor grade and overall survival. This group demonstrated that upon treatment with TSA, *RRP22* is re-expressed. Gao *et al.* described *NECL1* as a frequently lost tumor suppressor gene in GBMs. *NECL1* expression could be reinduced by TSA treatment [41]. Vibhakar *et al.* showed that *DKK1,* a Wnt antagonist and tumor suppressor gene in medulloblastoma, is frequently downregulated compared with normal tissue of the cerebellum and could be reinduced by TSA treatment [42]. Tamannai *et al.* identified *ING1* as a tumor suppressor gene in GBM, and described the reinduction of ING1 expression through TSA treatment [39]. Spiller *et al.* have found that treatment with retinoic acid can induce the transcription of *BMP2*, leading to apoptosis, and concurrent treatment with vorinostat potentiated the effect of retinoic acid by facilitating accessibility for transcription, resulting in a dramatic increase in apoptosis [43].

Inhibition of cell cycle & differentiation

Cell cycle

For uncontrolled proliferation, cancer cells have to find a way to escape their intrinsic control machinery during the cell cycle. Mutations and dysregulations of proteins essential for controlling check points of the cell cycle are, therefore, constitutively found in cancer cells [44]. A frequently observed effect of HDAC inhibition in brain tumor cells, is the induction of cell-cycle arrest. Accumulation of cells in either the G_1 or G_2/M phase of the cell cycle after HDAC inhibition has been reported, and several mechanistic explanations for the HDACi-mediated cell-cycle arrest have been proposed.

Concerning G_1 arrest, the majority of published data point to the involvement of p21. Treatment of GBM cells with vorinostat has been shown to increase the acetylation of the histones in the promoter region of *CDKN1A*, inducing the expression of p21 [45], leading to G_1 cell-cycle arrest. Yin *et al.* also observed an increase in expression of p27, and decreased expression of *CDK2*, *CDK4*, *CCND1* and *CCND2* upon treatment with vorinostat [45]. An upregulation of p21 associated with G_1 cell-cycle arrest was also found in GBM cells after treatment with TSA [46], MS-275 [47], LAQ824 and sodium butyrate [48]. TSA also induced G_1 cell-cycle arrest by upregulation of p21 in a medulloblastoma cell line [49]. However, it has been shown that both 4-PB and TSA can induce G_1 cell-cycle arrest in GBM cells independently of p21 [49,50]. With regard to G_2/M arrest, it has been demonstrated that vorinostat in clinically relevant concentrations leads to G_2/M arrest in GBM cells [45,51,52] and TSA causes G_2/M arrest in medulloblastoma cells [49,53].

The role of p53 in HDACi-mediated cell-cycle arrest remains unclear. While Jin *et al.* demonstrated that HDACi-induced cell-cycle arrest is dependent on p53 in GBM cells [54], other investigations came to the conclusion that cell-cycle arrest in GBM cells was independent from p53 [45,51]. Further clarification of these observations is of great interest since p53 is frequently mutated in GBM.

Differentiation

While HDACis such as TSA and 4-PB suppress differentiation of neural stem cells into astrocytes and oligodendrocytes in the physiological setting [55], several groups report differentiation effects in brain tumor cells after treatment with HDACi. For both medulloblastoma [56] and GBM [57,58], induction of differentiation has been reported after treatment with HDACis. Of note, the differentiated phenotype became irreversible when HDACis were applied for long periods of time, for example, 28 days [56,58]. Milde *et al.* demonstrated that treatment with vorinostat induced neuronal differentiation with loss of stem cell properties in an ependymoma stem cell model, including inhibition of self-renewal as shown by a reduction of neurosphere formation [59]. Several groups propose that HDAC inhibition leads to the activation of transcription factors that control differentiation programs. Morita *et al.* suggest that in glioma cells HDACis lead to an elevation of *BDNF* gene expression [57], induced by an increase of neuroactive 5α-reduced steroid metabolites. Her *et al.* demonstrated that this increase is caused by a TSA-mediated increase in 5α-R expression [60]. Taylor *et al.* showed that HDAC1 and 2 are complexed with REST in medulloblastoma, and HDACi treatment ultimately results in expression of the REST target gene *SYN1*, leading to neuronal differentiation [61]. In a very interesting approach, Wei *et al.* demonstrated in a rat glioma model that metabolites, such as alanine, lactate, inositol, *N*-acetylaspartate and creatinine, which usually show a diverse picture in tumor tissue, are restored to the levels of normal brain tissue after treatment with vorinostat, indicating differentiation [62].

■ Induction of cell death mechanisms

Apoptosis

The overwhelming body of published data creates the impression that the application of an HDACi can induce apoptosis in all investigated brain cancer entities. However, many published experiments did not use clinically achievable concentrations and applying HDACi at relevant concentrations provides a more diverse picture when it comes to the induction of apoptosis. Premkumar *et al.* have found that in several different GBM cell lines clinically achievable concentrations of vorinostat monotherapy, while inhibiting cell proliferation, do not induce apoptosis [52]. Others have shown a slight increase in apoptotic GBM cells after treatment with relatively low doses of TSA or 4-PB [39,48,58]. In medulloblastoma, vorinostat, TSA and MS-275 have been demonstrated to have a proapototic effect as single agents [43,51,63,64]. However, while TSA induced apoptosis in ependymoma cells [65], clinically achievable concentrations of vorinostat did not lead to increased cell death but to differentiation of ependymoma cells [66].

HDACis can disturb the equilibrium of pro- and anti-apoptotic proteins present in the cell, resulting in apoptosis. For example, Sawa *et al.* showed that romidepsin induces apoptosis in several GBM cell lines partially by the increased expression of the proapoptotic protein Bad and reduced transcription of antiapoptotic proteins Bcl-xL and Bcl-2 [67]. HDAC inhibition was also found to increase the transcription and acetylation of p53, while simultaneously negatively regulating the IKK–NF-κB pathway and survivin expression, promoting the mitochondrial apoptosis pathway in GBM [54,68]. In a GBM *in vivo* model, Ugur *et al.* showed that vorinostat increased Caspase 3 expression [69]. Rahman *et al.* also identified Caspase 3 as essential for TSA-induced apoptosis in medulloblastoma, primitive neuroectodermal tumor (PNET) and ependymoma [49]. Häcker *et al.* found that HDAC inhibition with MS-275 leads to acetylation of Ku70 in medulloblastoma, which leads to its dissociation from the proapoptotic Bax protein, the latter being activated by simultaneously upregulated p53 [70]. Upregulation of Caspase 8 upon HDACi treatment has been reported for medulloblastoma [71], atypical teratoid rhabdoid tumor [63], as well as GBM cells [46], while Sonnemann *et al.* found an activation of Caspase 9 and 3 upon HDACi treatment in medulloblastoma cells [64].

Sensitiziation to extrinsic & intrinsic apoptotic pathways

Disturbing the equilibrium of pro- and anti-apoptotic proteins with HDACis, even if not necessarily directly inducing apoptosis, appears to reliably sensitize tumor cells to extrinsic and intrinsic apoptotic pathways. Several groups report that HDACi pretreatment of medulloblastoma [71] and GBM cells *in vitro* [64], as well as GBM cells in an *in vivo* model [71], sensitizes towards TRAIL-induced apoptosis. Bangert *et al.* showed that treatment of GBM cells with MS-275, leads to the downregulation of cFLIPL and cFLIPS, partially by MS-275-induced

upregulation of cMYC, which mediates the increased sensitivity towards TRAIL-induced apoptosis [72]. Sharma *et al.* found that the observed induction of apoptosis in glioma cells after treatment with scriptaid was concurrent with a dramatic increase in Ras activity, while apoptosis induction could be prevented by inhibiting JNK [73].

■ Sensitization towards oxidative stress, irradiation & other chemotherapeutic agents

Oxidative stress and DNA damage are known to induce apoptosis via the intrinsic (mitochondrial) pathway. As HDACis have been reported to result in reactive oxygen species generation [74], and to induce and sensitize cancer cells towards DNA damage [75,76], combining HDACis with proteasome inhibitors, irradiation or DNA-damaging chemotherapeutics is a rational approach to try to increase the induction of apoptosis in cancer cells.

Proteasome inhibition

Proteasomes are an important part of the survival machinery of cancer cells. Yu *et al.* have shown that the combination of the proteasome inhibitor bortezomib with different HDACis (TSA, LBH589 and LAQ824) results in dramatic induction of apoptosis in GBM cells [77]. Both agents synergistically lead to mitochondrial injury with loss of mitochondrial membrane potential and consecutive release of cyctochrome C, an increase in the generation of reactive oxygen species and an increasing level of proapoptotic Noxa and MCL-1 cleavage [52].

HDACis & radiation therapy

Heterochromatization of DNA protects DNA from radiation-induced DNA damage [78]. Leading to the loosening of the chromatin structure, HDACis bear the potential to facilitate radiation-induced DNA double-strand breaks (DSB) in cancer therapy. For both GBM [51,75,79,80] and medulloblastoma [64,81], increases in radiation-induced apoptosis upon concurrent HDACi treatment has been described. Shabason *et al.* found that the radiation-sensitizing effect of HDACis is most pronounced when the HDACis are applied both before and after irradiation [79]. This may be due to the fact that HDACis not only promote radiation-induced DSB by euchromatization of the genome, but also negatively regulate the expression of DNA repair proteins, such as Rad51 and DNA-PK, which are crucial to nonhomologous end joining and homologous recombination [52,82]. However, data from Manova *et al.* showed that chromatin acetylation itself does not affect DSB repair by nonhomologous end joining in GBM cells [83].

DNA-damaging chemotherapeutic agents

Several groups have tested whether HDACis can increase apoptosis induced by clinically applied DNA-damaging chemotherapeutic agents. It has been reported, that MS-275 and vorinostat enhance apoptosis in both GBM [47] and medulloblastoma [64] induced by doxorubicin, etoposid and cisplatin. An interesting finding by Kitange *et al.* demonstrates the synergistic effect of combining vorinostat with temozolomide on induction of cell apoptosis in a GBM mouse model despite upregulation of MGMT, which is considered a mechanism of acquired temozolomide resistance [76].

■ Interference with cell–matrix interaction: invasion & angiogenesis

HDACis reveal great potential in influencing the interaction of brain tumor tissue with its environment. Osuka *et al.* showed in *in vitro* experiments that treatment with the HDACi valproic acid significantly reduced the secretion of the VEGF in several GBM cell lines under normoxic conditions [84]. A reduction of angiogenesis upon treatment with vorinostat was also observed in *in vivo* models of GBM [62,63].

With increasing degrees of malignancy, brain tumors present a more invasive phenotype. Invasiveness and malignancy show a positive correlation with expression of the matrix metalloproteases MMP-2 and -9 in GBM [85]. Chen *et al.* found that the tumor suppressor gene *RECK*, which is downregulated in high-grade gliomas and negatively regulates MMP-2 and -9, can be reinduced in glioma cells by treatment with the HDACi valproic acid [86]. In addition, Papi *et al.* demonstrated that HDAC inhibition in GBM cells leads to the upregulation of the protein level of TIMP [87], while Konduri *et al.* showed that HDAC inhibition reinduces the expression of TFPI-2, a broad-range proteinase inhibitor in glioma cells [88]. An *et al.* found that the observed reduction of invasiveness in glioma cells upon treatment with vorinostat was not related to the activity of MMPs, but to an increase in cell–cell adhesion [89]. Together these findings suggest that HDACis block the invasiveness of GBM cells.

■ Other mechanisms: telomerase activity & an increase in genomic instability

Telomerase

Telomerase activity (or an alternative lengthening of telomeres) is crucial for the immortalization of cells [90]. While generally repressed in somatic cells, the telomerase reverse transcriptase *hTERT*, encoding the catalytic domain of the human telomerase, is reactivated in approximately 85% of all malignancies, including malignant brain tumors [91]. Although the repression of *hTERT* in the physiological setting is at least partially mediated by HDAC1 and 2 [91], it has been shown that HDAC inhibition by TSA decreases *hTERT* expression at the mRNA level in medulloblastoma and GBM cell lines [53], as well as in CNS PNET and ependymoma cell lines [49]. Sharma *et al.* showed that scriptaid decreases telomerase activity in glioma cells [73], which could lead to telomere damage and consecutively contribute to induction of apoptosis [92].

Increase in genomic instability

While most research in the field of HDACs has been focusing on the change of gene expression after HDAC inhibition for many years, several groups recently have shown that part of the cytotoxicity of HDACis can be attributed to an increase in genomic instability following increased histone acetylation [53,82]. DNA DSBs were evidenced by H2AX activation after treatment with TSA in high-grade childhood CNS PNET, medulloblastoma and ependymoma cell lines, suggesting an activated DNA damage response [49]. As a consequence, HDACis were combined with other DNA-damaging chemotherapeutics. Combining vorinostat with the topoisomerase inhibitor SN38 in GBM cell lines was shown to have a synergistic effect on DNA damage [93]. MS-275 potentiated the DSB-inducing effect of doxorubicine in GBM cell lines and triggered the acetylation of the DNA repair protein Ku70, attenuating its damage response activity [70].

■ Crosstalk between regulative acetylation & methylation mechanisms

The tight coordination of histone acetylation with other regulative epigenetic mechanisms, including histone and DNA methylation, has been described in several cancer entities [55]. While hypermethylation of CpG islands, mediated by DNMT, already leads to transcriptional repression of genes in cancer cells [63], proteins with a binding domain for methylated DNA can ultimately serve as recruitment proteins for HDACs. Concurrent histone deacetylation and DNA hypermethylation results in long-term silencing of these genes [47]. Espada *et al.* could show that loss of the DNMT enzyme leads to an alteration of the histone acetylation patterns [94]. On the histone level, Singh *et al.* could show that treating GBM cells with the HDACi PCI-24781 induces hypermethylation of histones [39]. The methylation status of histones (e.g., dimethylation of lysine 4 on histone H3, leading to transcriptional activation) is generally coordinated by the enzyme LSD1, residing in a complex with HDAC1 and 2 [95]. Simultaneously inhibiting LSD1 with tranylcypromine and HDACs with PCI-24781 strongly induced apoptosis in GBM cells [38]. Finally, Ecke *et al.* demonstrated that combination therapy of the DNMT inhibitor 5-aza-dC and the HDACi valproic acid effectively diminishes tumor formation in a *ptch* medulloblastoma *in vivo* model [96].

Clinical trials of HDACis in neuro-oncology

Early case reports applying HDACis in individual patients with brain tumors suggested activity in clinical neuro-oncology [97,98]. Owing to its good safety profile and easy clinical availability, valproic acid was applied as the first HDACi in maintenance therapy for pediatric patients with high-grade glioma [99]. Recently, a retrospective analysis of 544 adult GBM patients receiving antiepileptic drug treatment during radiation therapy suggests improved overall survival of patients receiving valproic acid [100]. Phenylbutyrate is another weak short-chain fatty acid HDACi that has been developed for the treatment of urea cycle disorders with limited clinical experience (Table 3) due to the short half-life and high doses required. The first prospective clinical trial applying a hydroxamic acid-type HDACi in neuro-oncology was reported in 2009 [20]. In this Phase II study, vorinostat was given as a single agent to 66 patients with recurrent GBM. The trial met the prospectively defined primary efficacy end point, with nine of the first 52 patients being progression free at 6 months, suggesting modest activity. Biopsy in pre- and post-treated GBMs showed increased histone acetylation in immunohistochemical staining in tumor samples, suggesting BBB penetration of vorinostat and on-target effects in GBM tissue. However, owing to the small number of samples and technical limitations of quantification of

immunohistochemistry, this observation has to be interpreted with caution. A Phase I/II trial of single-agent romidepsin included 35 patients with recurrent GBM. No objective response was seen in the Phase II trial and the authors concluded that single-agent romidepsin in its standard dose was not effective. Besides low antiglioma activity of romidepsin as a single agent these disappointing results might partially be due to insufficient BBB penetration of the compound or ABCB1-mediated increase of drug efflux in the tumor tissue [101].

Since HDACis show antiangiogenic, chemotherapy- and radiation-sensitizing activity in preclinical models, current trials combine HDACis with bevacizumab, chemotherapy and radiation therapy. The HDACis applied include the hydroxamic acids vorinostat and panobinostat, the benzamide MS-275 (entinostat) and the short-chain fatty acid valproic acid.

Drappatz *et al.* reported a Phase I study of panobinostat in combination with bevacizumab, for recurrent high-grade glioma. The trial determined a dose recommendation of panobinostat 30 mg three-times per week, every other week, in combination with bevacizumab 10 mg/kg every other week. There were some promising signals of efficacy with three patients showing partial responses and seven stable diseases, and a Phase II trial of this combination is underway [102]. In another Phase I trial, vorinostat was combined with bevacizumab and irinotecan in patients with recurrent GBM [103]. The authors report a high toxicity of this regimen and the need for frequent dose reductions of irinotecan. Of interest, the authors noted that high-dose vorinostat was associated with an improved progression-free and overall survival when compared with low-dose vorinostat. In a plasma proteomic-based antibody array platform, the authors identified low IGFBP-5 pretreatment levels as significant predictors of progression-free survival and decreasing PDGF-AA plasma levels as predictors of tumor recurrence.

Another rational combination partner of HDACis is the proteasome inhibitor bortezomib, since both compounds target critical components of the proteasomal machinery. In a Phase II trial, Friday *et al.* assessed the combination of vorinostat and bortezomib in patients with recurrent GBM. However, the trial was closed at the predetermined interim analysis with none out of 34 patients being progression-free at 6 months, suggesting that this combination is not effective in recurrent GBM [104].

In addition, several early clinical trials are currently recruiting patients with brain tumors, mainly patients in relapse, but also upfront (Table 3). In these trials, the HDACis are combined with standard therapy, including radiation and temozolomide, but also with other targeted compounds, such as bevacizumab, erlotinib and bortezomib (Table 3). Of note, a Phase II/III randomized trial is currently testing vorinostat plus radiation versus temozolomide plus radiation versus bevacizumab plus radiation as first-line treatment in young patients (3–21 years) with high-grade glioma (NCT01236560) (Table 3).

At this point, it is too early to make a firm statement on the use of HDACis in the treatment of brain tumors. The first Phase I/II completed trials indicate that single-agent HDACis have no or only modest efficacy. However, some trials indicate that combination treatment might be more effective and there seems to be a dose-dependent effect of HDACis. Since single patients do show responses, the greatest challenge still is to identify reliable biomarkers for patient selection in future clinical trials.

Predictive & pharmacodynamic biomarkers for HDACis in clinical trials

As with all molecularly defined therapeutic strategies, development of predictive markers allowing for stratification of patients in clinical trials will be crucial to the success of HDACis in the clinic. Data on predictive markers, however, are sparse, the most prominent marker today being HR23B, a protein involved in nucleotide excision repair, as well as the ubiquitin-mediated proteolytic pathway. First described by Khan *et al.* in cutaneous T-cell lymphoma, it has been shown that HR23B protein expression serves as a predictive marker for responsiveness to vorinostat, with strong HR23B protein expression predicting a good response to vorinostat [105]. Moreover, HR23B can easily be assessed in formalin-fixed paraffin-embedded samples, making it ideally suited for routine clinical application. HR23B has since been validated as a predictive marker for response to belinostat in hepatocellular carcinoma (HCC) by Yeo *et al.*, indicating that this marker could possibly be valid for more than single tumor entities [106]. However, it remains to be shown if HR23B can serve as a predictive marker in the field of neuro-oncology. Other biomarkers predictive of poor response to HDAC inhibition include nuclear accumulation of STAT1 and high levels of nuclear pSTAT3 in malignant cells in skin samples of cutaneous

T-cell lymphoma patients [107], and overexpression of 17 antioxidant genes in acute myeloid leukemia patient's peripheral blood cells [108].

Preclinical data from HCC *in vivo* studies indicate that the methylation status of a set of tumor suppressor genes is a predictive marker for HDAC class I inhibition, in general, and HDAC1 inhibition, in particular, with strong methylation predicting poor response [109]. Moreover, low *ID2* gene expression has been proposed as a predictive marker in HCC [110]. Almost no data on HDAC expression as predictive markers exist. In brain tumor cell lines, the expression pattern of HDACs was shown not to be predictive for the responsiveness of these cell lines to HDACis [111].

Histone acetylation of peripheral blood mononuclear cells (PBMCs) has been proposed as a pharmocodynamic biomarker in the clinical setting since PBMCs can be collected easily and repetitively in patients during clinical trials. Indeed, histone H4 acetylation has been shown to be a strong positive predictor of response in a clinical trial applying vorinostat in breast cancer [112]. However, determination of histone acetylation in PBMCs strongly depends on the type of acetylation assay used, as well as the time point of PBMC collection, and is associated with a large variability. Pretreatment HDAC2 protein expression levels in PBMCs have been proposed as predictive markers for vorinostat response, since they correlated with the extent of the histone H4 acetylation change in a Phase I study [113].

Further studies on predictive and pharmacodynamic biomarkers in brain tumors and their molecular subgroups are, therefore, urgently required, if HDACis are to be successful in the clinic.

Conclusion & future perspective

In summary, the success of HDACis in the treatment of brain tumors will be largely dependent on:

- Elucidation of the molecular function of single HDAC isoenzymes;
- Development of class- or (possibly more desirably) isoenzyme-specific HDACis;
- Patient selection by application of valid predictive markers and/or entity-specific isoenzyme inhibition;
- Development of synergistic combination therapies.

Although preclinical and some clinical data suggest a promising action of unselective HDACis in brain tumors, we are still lacking a thorough understanding of individual HDAC isoenzyme function(s) in brain tumors and normal tissues. However, the understanding of molecular functions of HDAC isoenzymes is crucial if we are to improve cancer therapy by offering drugs that are more effective with a broad therapeutic window. This can be achieved with HDACis that have either higher specificity against cancer cells or higher selectivity for single HDAC isoenzymes. The basis for this is the development of class- and/or isoenzyme-selective HDACis; although very promising first steps have been undertaken, the panel of inhibitors applicable in the clinical setting needs to be significantly expanded.

As has been reported in numerous publications, expression of HDAC isoenzymes is highly variable, and is dependent on the tumor entity, and clinical and/or molecular subgroup within one entity. Not surprisingly, HDAC isoenzyme expression is often correlated with clinical course. Conversely, our knowledge on HDAC isoenzyme expression profiles and their predictive value for response to HDACis remains largely unexplored. Success and failure of clinical trials testing HDACis will, however, be highly dependent on selecting the right patient population that is clearly defined by molecular markers. Further efforts are required in the development of predictive and pharmacodynamic biomarkers, be it based on HDAC isoenzymes themselves, their downstream targets, or functionally linked or surrogate markers.

Since HDACi monotherapy will probably not be successful in the treatment of cancer patients, rational and synergistic combination therapies need to be fully characterized. This will require employment of adequate preclinical *in vitro* and *in vivo* models for the corresponding tumor entities and their molecular subgroups.

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