

Epigenetics in Brain Tumors: HDACs Take Center Stage

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Abstract: Primary tumors of the brain account for 2 % of all cancers with malignant gliomas taking the lion's share at 70 %. Malignant gliomas (high grade gliomas WHO° III and °IV) belong to one of the most threatening tumor entities known for their disappointingly short median survival time of just 14 months despite maximum therapy according to current gold standards. Malignant gliomas manifest various factors, through which they adapt and manipulate the tumor microenvironment to their advantage. Epigenetic mechanisms operate on the tumor microenvironment by de- and methylation processes and imbalances between the histone deacetylases (HDAC) and histone acetylases (HAT). Many compounds have been discovered modulating epigenetically controlled signals. Recent studies indicate that xCT (system xc-, SLC7a11) and CD44 (H-CAM, ECM-III, HUTCH-1) functions as a bridge between these epigenetic regulatory mechanisms and malignant glioma progression. The question that ensues is the extent to which therapeutic intervention on these signaling pathways would exert influence on the treatment of malignant gliomas as well as the extent to which manipulation of HDAC activity can sensitize tumor cells for chemotherapeutics through 'epigenetic priming'. In light of considering the current stagnation in the development of therapeutic options, the need for new strategies in the treatment of gliomas has never been so pressing. In this context the possibility of pharmacological intervention on tumor-associated genes by epigenetic priming opens a novel path in the treatment of primary brain tumors.

Keywords: Glutamate, HDAC inhibitors, malignant gliomas, supra-complete resection, tumor zone model, xCT.

EPIGENETIC REGULATORY MECHANISMS IN ONCOLOGY

The acetylation and deacetylation of histones represent an important epigenetic regulatory mechanism of gene expression [1]. Histone acetylation leads to relaxation of the chromatin structure through cancellation of electrostatic affinity between histones and DNA. In consequence, this leads to stimulation of gene transcription, whereas histone deacetylation is associated with chromatin condensation and mediates suppression of gene activity [2]. This is primarily achieved through addition or removal of negatively charged lysine residues from histones facilitated by histone acetyltransferases (HAT) on the one hand, and through histone deacetylases (HDAC) as counterparts of HATs on the other. The HATs are classified into the three groups termed GNAT, MYST and p300/CBP. The family of HDACs is so far formed of at least four subclasses: zinc-dependent class I, II and IV, and the NAD-dependent class III [3-5] (Fig. 1).

Balanced activity between the corresponding HATs and HDACs is critical to physiological cell differentiation and commensurate cell metabolism. Disturbance in this fine

equilibrium is associated with oncogenic transformation [6]. This primarily concerns the HATs, as evidenced by their structural destruction through deletion or inactivation of mutations in numerous malignant tumor entities [7, 8]. Inhibition of HATs results in a dominance of HDAC-activity, leading in turn to the inactivation of several tumor suppressor and cell-cycle regulatory genes [2, 9]. A further mechanism behind oncogenic transformation lies in HDAC-associated activation of the transcription factor c-myc, leading to stimulation of cell proliferation and inhibition of apoptosis through repression of the tumor suppressive miRNA miR-29 [10-12]. In this manner, oncogenic transformation is facilitated by inactivation of tumor suppressing genes as well as activation of oncogenic signaling pathways as a consequence of dominance in HDAC-activity [13]. The option of blocking the activity of HDACs through specific inhibitors opens up a completely new treatment front with the possibility of influencing gene transcription through drug-induced epigenetic regulation, thereby permitting counteraction of the previously described oncogenic imbalance. Various promising HDAC inhibitors with cytostatic properties have been developed, with those characterized by primary action on HDACs of the zinc-dependent class I, IIa, IIb and IV currently in the clinical test phase [14] (Fig. 1).

Recent studies have further shown that class III NAD-dependent HDACs also decisively control oncogenic processes [15, 16]. It is anticipated to be able to induce additional signaling pathways for differentiation and/or cell

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Class	Typ	Localization	VPA	But	SAHA	LBH	PXD	LAQ	SK1	SK2	SB	Tub	MS	Dep	Api	EX527	Sir	Sal	Thio	GW	Ref
I	HDAC1	nucleus	x	x	x	x	x	x	x	x			x	x	x						Bhalla 2005, Bolden 2006
	HDAC2	nucleus	x	x	x	x	x	x	x	x			x	x							Bhalla 2005, Bolden 2006
	HDAC3	nucleus/cytoplasm	x	x	x	x	x	x					x	x	x						Bolden 2006
	HDAC8	nucleus/cytoplasm	x	x	x	x	x	x			x		(x)	x							Bolden 2006
IIa	HDAC4	nucleus/cytoplasm	x	x	x	x	x	x													Bolden 2006
	HDAC5	nucleus/cytoplasm	x	x	x	x	x	x													Bolden 2006
	HDAC7	nucleus/cytoplasm	x	x	x	x	x	x													Bolden 2006
	HDAC9	nucleus	x	x	x	x	x	x													Bolden 2006
IIb	HDAC6	cytoplasm			x	x	x	x				x									Bolden 2006
	HDAC10	nucleus/cytoplasm			x	x	x	x				x									Bolden 2006
III	SIRT1	nucleus													x		x	x			Napper 2005, Lara 2009, Jung-Hynes 2010
	SIRT2	cytoplasm															x	x	x		Grozinger 2001, Lara 2009, Jung-Hynes 2010
	SIRT3	mitochondria																	x		Jung-Hynes 2010, Disch 2013
	SIRT4	mitochondria																			Jung-Hynes 2010
	SIRT5	mitochondria																		x	Jung-Hynes 2010, Suenkel 2013
	SIRT6	nucleus																			Jung-Hynes 2010
	SIRT7	nucleus																			Jung-Hynes 2010
IV	HDAC11	nucleus			x	x	x	x													Bolden 2006

Fig. (1). Overview of the HDAC classes and corresponding HDAC-inhibitors. HDACs are divided into four different classes with localization in various cell compartments. Abbreviations used: Api = Apicidine, But = Butyrate, Dep = Depsipeptide, GW = GW5074, LAQ = LAQ824, LBH = LBH589, MS = MS-275, PXD = PXD101, Sal = Salemid, SB = SB-379872-A, Sir = Sirtinol, SK1 = SK-7041, SK2 = SK-7068, Thio = Thieno[3,2-d]pyrimidine-6-carboxamide, Tub = Tubacin, VPA = valproic acid.

death mechanisms through the implementation of HDAC inhibitors beyond direct cytotoxicity towards tumor cells [17, 18]. This process is designated as ‘epigenetic priming’, describing sensitizing tumor cells in order to render them more susceptible to cytostatic drugs [19, 20]. In this manner it has been shown that individual HDAC-inhibitors are in a position to induce the expression of death receptors such as death receptor DR5, leading to subsequent activation of caspases in epigenetically primed cells [21-24]. These special properties render HDAC-inhibitors highly relevant to neuro-oncology, especially in the case of patients with high grade gliomas (WHO°III and WHO°IV) where they would constitute a new treatment concept to the backdrop of the currently severely limited therapy options.

THE 3 TUMOR ZONES (TZ) MODEL IN NEURO-ONCOLOGY AND HDAC INHIBITION

Belonging to the group of primary malignant brain tumors which account for a staggering 70 % of the most commonly occurring primary brain processes, glioblastomas (GBM, WHO grade IV gliomas) can be counted amongst the most malignant of all tumor entities with a median survival time of just 14 months [25]. The poor prognosis of this tumor entity is significantly facilitated by heightened proliferation, diffuse invasive growth, increased angiogenesis, and the ability to manipulate the tumor microenvironment to their own advantage [26-28]. These characteristics imply that clinicians are continually faced with imminent tumor recurrence bordering on certainty in glioma patients. Due to the lack of a current cure, this represents a fulcrum for

treatment with HDAC inhibitors, which are in a position to exert specific and crucial influence on tumor cells (Fig. 2) [11, 29, 30].

A peculiarity of malignant gliomas lies in their ability to reorganize their microenvironment according to their needs [31]. These pathophysiological features are governed according to the so-called tumor zone model comprising 3 zones which encompass all clinical properties and pathophysiological traits of malignant gliomas [32]. Tumor zone I corresponds to the contrast-agent enhancing area in MRI scans, comprising the primary tumor bulk, and consists of the so-called “core cells”. Tumor zone II, also known as the peritumoral zone, corresponds to the zone of perifocal edema in MRI scans and surrounds the tumor zone I. Tumor cells in this area are known as “transitory cells” since they exhibit many but not necessarily all histological and molecular hallmarks of the “core cells” (Fig. 2). Clinical and experimental investigations conducted point to an increased accumulation of microglia, reactively altered astrocytomas and endothelial cells. It is assumed that this area represents the biologically active area of the tumor. Although tumor zone III appears to be pathophysiological inactive due to its macroscopically unremarkable appearance and consists of healthy brain parenchyma per se, it still harbors solitary tumor cells corresponding to precursor or stem cells collectively designated as “partisan cells” according to the model presented here. Despite belonging to the group of malignant glioma cells, they appear not to be same in character and differ in their molecular profile from the remainder of the tumor cell population. The heterogeneity of intra-glioma cell

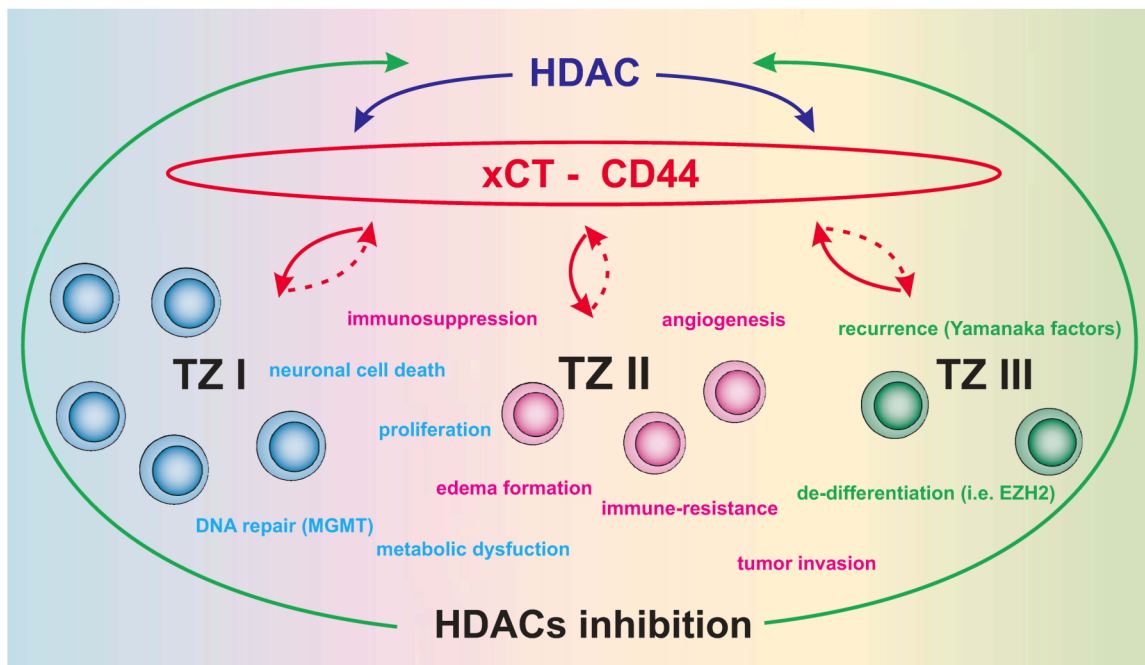


Fig. (2). Conceptual scheme of the tumor zone model (TZI-III) and epigenetic regulation. The diagram depicts the known aspects of interaction between HDACs and the xCT system. Hallmarks of malignant glioma cells are color-coded in blue, while green and pink correspond to the various tumor zones (TZI-III). HDACs regulate various properties of malignant gliomas *via* the xCT (SLC7a11/ system Xc⁻). Since the glycoprotein CD44 (H-CAM/Hyaluronate receptor) has been previously shown to regulate and stabilize xCT, we suggest this complex as a therapeutic target for HDAC inhibitors. Inhibition of HDAC activity has been shown to render glioma cells vulnerable by diminishing the potency of their destructive properties. The xCT-CD44 complex could represent a crucial starting point for targeting xCT function. Furthermore, transcription factors critical for inducing pluripotent stem cells (Yamanaka factors) could be another vital target of HDAC inhibitors.

architecture is reflected by the polyclonal properties of this tumor entity [33]. Thus, HDAC inhibition in such a heterogeneous tumor microenvironment may affect tumor biology at various levels, *i.e.* from simple cytostatic/cytotoxic growth arrest to inhibition of their pluripotent nature (Fig. 2).

VISUALIZATION OF THE 3 TUMOR ZONES (TZ) IN NEURO-ONCOLOGY

Tumor visualization is limited due to a very diffuse and infiltrative growth pattern. For example, it is possible to identify the tumor zone I well both intraoperatively as well as in MRI scans [34]. 5-ALA is an amino acid from the group of ketocarboxylic acids, which is a precursor of Hem in porphyrin synthesis and normally metabolized through protoporphyrin IX into Hem. This transformation is not completed in tumor cells however, leading to intracellular accumulation of intermediary protoporphyrin IX. This leads to fluorescent excitation following exposure to a blue light source and enables direct intraoperative visualization of tumor cells. 5-ALA guided surgery of brain tumors therefore represents a direct biochemical approach enabling differentiation between tumor and healthy tissue. Differentiation here between auto-fluorescence and a vague fluorescence signal from solitary tumor cells in transition zones represents a technical challenge for the neurosurgeon [35]. An alternative method here is indirect visualization involving the property of increased angiogenesis in malignant gliomas. The identification of zones with a tumor

cell density below a threshold permitting visualization of the fluorescing metabolites tumor-occupied zones is possible through visualization of hyper-vascularized areas, the so-called “angiogenic hotspots” [36]. This would correspond most closely to the tumor zone II according to the above described tumor zone model. In contrast, visualization of tumor cells in the tumor zone III is still difficult even following histopathological evaluation due to low numbers and growth activity [35]. This zone is in all probability therefore the most difficult area to tackle from the neuro-oncological perspective, since current therapy options target tumor cells in the individual tumor zones either poorly or so effectively that healthy brain parenchyma would be collaterally damaged.

HDAC INHIBITORS AS AN OPTION IN THE TREATMENT OF MALIGNANT GLIOMAS

HDAC inhibitors are characterized by the singular property of being able to interact specifically with tumor cells [37]. As opposed to conventional chemotherapeutic agents, they selectively act upon transformed cells instead of globally on brain parenchyma [1, 38]. Although a mechanism fundamental to this selectiveness is known to lie in influencing the balance between thioredoxin and reactive oxygen species [37], this phenomenon is yet to be fully explained [39]. The specific and tremendous advantage this tumor selectiveness awards to neuro-oncology lies in the fact that HDAC inhibitors can modulate gene expression of

exclusively glioma cells in all three tumor zones without noticeable effect on healthy brain parenchyma. A possible growth inhibiting mechanism in glioblastomas lies in the specific up regulation of the cell cycle protein p21/WAF [40]. Through this, tumor cells are pushed into a G0-G1 cell-cycle arrest and consequently into apoptotic cell death [41]. Malignant gliomas are hence characterized by the following properties besides high proliferative activity:

1. gliomas are characterized by high angiogenic potential [27],
2. gliomas are accompanied by perifocal edema [31],
3. gliomas are characterized by high neurotoxic potential and induce neuronal cell death [42, 43], and
4. gliomas act in an immune-suppressive manner on immune-competent cells [44, 45].

These properties are primarily traced back to the synthesis and secretion of factors in the three tumor zones which manipulate the microenvironment according to the needs and advantage of the tumor [32]. One of these factors is the amino-acid glutamate. It is secreted by tumor cells *via* the channel protein xCT in exchange for cysteine [46]. Glutamate can potentially increase tumor cell proliferation through the activation of AMPA receptors *via* an autocrine loop [47]. In malignant gliomas tumor-derived glutamate additionally takes center stage in the induction of perifocal edema, thereby forming the tumor zone II [31]. Glutamate further stimulates microglial activity and leads to their accumulation [48, 49], which can additively trigger neuronal cell death through dendritic retraction [50]. The cytokine *macrophage migration inhibitory factor* (MIF) is then secreted by glioma cells in tumor zones I and II, leading to paralysis of microglial activity and absence of an immune response to the neoplastic process [44, 51]. In addition, the secreted glutamate concentration is toxic and leads to neuronal cell death, through which room for uninhibited tumor expansion is created [42]. This provides the base for modulatory influence on this process through HDAC inhibitors (Fig. 2). The expression of the glutamate transporter xCT can be specifically modulated through inhibition of HDAC activity in malignant gliomas, which leads to a reduction in tumor associated glutamate release in all three tumor zones [52]. In this manner, it is conceivable to normalize the tumor microenvironment:

1. microglial activity is normalized and an immune reaction takes place,
2. astrocyte swelling is reduced,
3. angiogenesis is impeded and
4. glutamate associated peritumoral neuronal cell death is considerably reduced

The cell-surface glycoprotein molecule CD44 (formerly termed HCAM, Pgp-11/phagocytic glycoprotein-1, Hermes antigen, lymphocyte homing receptor, ECM-III, HUTCH-1) is important in this respect as it stabilizes and controls the xCT-complex [53]. It is to be noted, that a selective removal of the tumor-specific variants of the CD44 molecule from

the complex, *i.e.* Cd44v6-12, leads to a loss of the xCT transporter from the tumor cell surface with subsequent suppression of tumor growth. Additionally, an increase in the expression of the cell-cycle inhibitor p21/WAF occurs [53] – similar to the phenomenon of treatment with HDAC inhibitors: xCT expression is reduced and the generation of p21/WF increased. The extent to which normalization of the tumor microenvironment also encompasses the partisan cells in tumor zone III as well is yet to be investigated. Since however glioblastomas recur with a penetrance of almost 100 % despite resection with surgical margin (*i.e.* the 100 + approach) followed by adjuvant radio-chemotherapy, it can be assumed that partisan cells from the tumor zone III may account for this, the sensitivity of which to HDAC inhibitors needs to be investigated in the future. Additionally, CD44 is involved in the formation of resistance to radio-chemotherapy through regulating anti-oxidation and detoxification pathways [54].

It is known with respect to adult stem cells that individual substances such as SAHA or MS-275 induce a differentiation with a shift in the direction of neurons [55]. The so-called “Yamanaka-factors“ are of clinical interest in this context as just the activity of these four factors or a combination of at least two of these, *i.e.* Oct3/4, Sox2, c-Myc and Klf4 alone are sufficient to initiate mechanisms which generate embryonic-like stem cells from any cell type including adult differentiated cells [56]. It is meanwhile possible to initiate these mechanisms by utilizing “small-molecule compounds” [57]. The clinical phenomenon of glioma recurrences could be explained through this “gain-of-function” activity of individual tumor stem cells. Although the role of enhancer of zeste homologue 2 (EZH2) which belongs to the group of polycomb proteins has not yet been defined, EZH2 is over-expressed in glioblastomas and could potentially take part in the self-renewal of tumor cells [58]. Tumor cells are maintained in an undifferentiated state through the increased expression of EZH2. Inhibition of EZH2 expression through the specific inhibitor 3-deazaneplanocin A (DZNep) releases these cells into a differentiated state similar to following treatment with individual HDAC inhibitors such as SAHA. Although these results suggest that partisan cells probably react well to HDAC inhibitors too, this is yet to be demonstrated.

Individual HDAC-inhibitors influence another epigenetic signaling pathway too, *i.e.* the MGMT promotor methylation status, although it was assumed that DNA methylation suppresses gene transcription independent of HDAC [59]. This observation is important from the clinical point of view. The established standard adjuvant treatment of glioblastomas according to current studies includes temozolomide (Temodal®), a DNA alkylating agent [60, 61]. Patients with hypermethylated MGMT status profit most from temozolomide treatment [60]. Generally, tumor cells can repair temozolomide-induced DNA damage through the DNA repair enzyme O6-Methylguanine-DNA-Methyltransferase (MGMT). Thus, patients with a non-methylated MGMT promotor status profit little from treatment with DNA alkylating agents such as temozolomide [60]. This group is designated as temozolomide non-responder and comprises about 65 % of all glioblastoma patients [62, 63]. This

underlines the importance of being able to offer these patients an alternative therapy option. In this context it is to be examined in future whether the MGMT promotor status can be changed through treatment with HDAC inhibitors, thereby achieving “epigenetic priming” for subsequent treatment with temozolomide. These findings are furthermore especially important in order to arrive at a better understanding of the xCT/CD44/glutamate system. xCT expression is nowadays being considered as a predictive factor similar to MGMT promotor methylation status [64]. Furthermore, CD44 is controlled through methylation processes [65]. It has also been shown that the glutamate-receptor mGlu3 controls the expression of the MGMT-enzyme and can therefore confer resistance against chemotherapy agents [66]. Inhibition of the mGlu3 receptor here leads to sensitization of tumor cells towards temozolomide. xCT control of the MGMT system through secreted glutamate therefore represents a realistic overlapping of two systems considered to be independent of each other according to current knowledge. This means that normalization of the xCT system could lead to a change in MGMT promotor methylation status.

Recent data suggest that a modulation or intervention in the balanced HAT and HDAC signaling in malignant gliomas could be an effective treatment route. A decisive factor here is to define the particular functions of the 11 HDACs and 7 SIRT1s. This is especially important since current HDAC inhibitors in use show certain cross-specificity with several HDACs at the same time.

Hence, HDAC inhibitors are accompanied by numerous side effects as reported in initial clinical studies ranging from weight loss to severe changes in the blood count. The identification of ‘glioma-specific’ HDACs and tumor zone-specific HDACs (TZ-specific HDACs) would be relevant to the development of new inhibitors to target particular glioma functions.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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