Histone deacetylase 6 inhibition enhances oncolytic viral replication in glioma

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Oncolytic viral (OV) therapy, which uses genetically engineered tumor-targeting viruses, is being increasingly used in cancer clinical trials due to the direct cytolytic effects of this treatment that appear to provoke a robust immune response against the tumor. As OVJs enter tumor cells, intrinsic host defenses have the potential to hinder viral replication and spread within the tumor mass. In this report, we show that histone deacetylase 6 (HDAC6) in tumor cells appears to alter the trafficking of post-entry OVJs from the nucleus toward lysosomes. In glioma cell lines and glioma-stem–like cells, HDAC6 inhibition (HDAC6i) by either pharmacologic or genetic means substantially increased replication of oncolytic herpes simplex virus type 1 (oHSV). Moreover, HDAC6i increased shutting of post-entry oHSV to the nucleus. In addition, electron microscopic analysis revealed that post-entry oHSVs are preferentially taken up into glioma cells through the endosomal pathway rather than via fusion at the cell surface. Together, these findings illustrate a mechanism of glioma cell defense against an incoming infection by oHSV and identify possible approaches to enhance oHSV replication and subsequent lysis of tumor cells.

Introduction

Malignant gliomas (such as glioblastoma [GBM]) remain formidable cancers based on their poor prognosis, with a median survivorship of 15 months or less, extensive neurologic morbidity, and cost of care (1, 2). Surgical, radiation-based, and pharmacologic therapies have extended patients’ lives by a few months, but the extensive and complex genetic heterogeneity of these tumors renders therapeutic targeting of a few aberrant signaling networks unlikely to succeed (3, 4). Various immunotherapies have recently been approved by the FDA for the treatment of some cancers and are now also being tested in GBM (5). The theoretical advantage of some immune-based treatments relates to immune cell recognition of any aberrant tumor-associated pathway/molecule and possible immune cell adaptability to the anatomic and temporal heterogeneous nature of the GBM. One form of immunotherapy uses genetically engineered tumor-selective pathogens, such as oncolytic viruses (OVs), to replicate in and kill tumor cells, thereby increasing immune cell recognition of tumor and viral antigens exposed in the lysed tumor “debris” field (6–8).

As OVJs are administered into tumors, entry of the agent into the cell, viral replication, cell lysis/death, and release of progeny virions to infect surrounding tumor cells are critical processes that should occur efficiently in order to obtain sufficient tumor cell death to provoke an effective antitumor immune response, resulting in clearance of the neoplasm. Yet, these initial stages of OV action against tumors can still be impeded by a variety of tumor and host factors that limit efficient entry, replication, and intratumoral spread (9, 10). Recognition and identification of these host factors can thus be utilized to try and improve these critical initial phases of OV therapy. One type of OV that has been tested, even in phase III clinical trials (11), is based on genetically engineered herpes simplex virus type 1 (HSV-1). HSV-1 is thought to primarily enter infected cells by fusion of its viral envelope with the cellular membrane and release of the viral capsid into the cell cytosol, after which it travels to the nucleus using the microtubular (MT) apparatus (12, 13). Recently, though, HSV-1 has also been shown to enter some cells through endocytic vesicles that are subsequently fused with viral envelopes to release capsids into the cytosol, suggesting an alternative mechanism of post-entry trafficking of virus capsids from the plasma membrane (PM) into the nucleus (14). Through this alternative mechanism that is widely used by other viruses, such as adenoviruses, incoming viral capsids would need to exit endosomes before these fuse with lysosomes in order to shuttle viral capsids to the nucleus. Sensing of viral infection and recognition of viral nucleic acids also occurs within endosomes (15). Endocytic materials and cytoplasmic proteins mainly are transported on MT networks, and posttranslational modifications of tubulin control MT function (16, 17). Of particular interest, histone deacetylase 6 (HDAC6) (18), a member of the class IIb histone deacetylases (HDACs), has been characterized as a deacetylase of tubulin and of other cytoplasmic proteins (HSP90 and cortactin) (19) responsible for homeostasis of the cellular MT apparatus (20). In addition, HDAC6 has been...
shown to be required for selective autophagic processes involving autophagic vesicle fusion with lysosomes, and it is also involved in the process of cellular endocytic uptake (21–23). As a pathogenic defense mechanism, HDAC6 activity has been reported to selectively upregulate type I IFN (24) and prevent HIV-1 entry.

(e) HDAC6i also led to improved oHSV replication in a panel of post-entry oHSV to the nucleus rather than to the lysosome; and (d) HDAC6i led to increased shuttling of HDAC6 in the antiviral effect is linked to the acetylation status of oHSV, while augmentation of HDAC6 reduced it; (b) the role of HDAC6 was transiently overexpressed in U251 glioma cells, HSV-1 capsids traffic to the nucleus to deliver viral DNA via the cellular MT apparatus and that one of HDAC6’s activities is to deacetylate tubulin, we then asked whether the increased acetylation of type 1 IFNs; (d) HDAC6i led to increased shuttling of post-entry oHSV to the nucleus rather than to the lysosome; and (e) HDAC6i also led to improved oHSV replication in a panel of patient-derived spheroid-grown glioma cells. These results thus establish that HDAC6 can be an intrinsic cell mechanism against oHSV and oncolytic tumor therapy.

Table 1. VPA effect on replication efficacy of OVs

<table>
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<tr>
<th>Cell line</th>
<th>Input (PFU)</th>
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<th>rHSVQ1</th>
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<td>n.d.</td>
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<tr>
<td>GBM42</td>
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<td>6.00</td>
<td>14.40</td>
</tr>
</tbody>
</table>

Total PFU at 3 days after infection were measured using the indicated cells with rQNestin34.5 or rHSVQ1 infection. We used 10,000 PFU as the input virus amount upon VPA treatment (+VPA; 30 mM) or mock treatment (–VPA) for 15 hours before infection. Average titer values after VPA treatment were represented as fold increase (fold), compared with mock treatment (mean of 3 biological replicates). P < 0.01, P < 0.05, P < 0.001, compared with mock, 1-way ANOVA test. n.d., nondetectable level.

enhanced oHSV replication in both established and primary gliomas, confirming previously published observations (28–31).

Specific HDAC6i enhances oHSV infection and replication. The finding that pan-HDAC inhibition led to increased oHSV replication suggests that pan-HDAC activity inhibited it (29). We sought to determine whether any one particular HDAC was responsible for this finding and focused on HDAC6, based on the rationale described in the Introduction. We used tubacin, a relatively specific deacetylase inhibitor of HDAC6 (32), to determine whether it affected HSV-1-mediated transgene expression and oHSV replication in U251 human glioma cells. As shown in Figure 1, tubacin did indeed increase luciferase-mediated bioluminescence delivered by an HSV-1 amplicon vector, either in the absence or in the presence of exogenous IFN-β (Figure 1A), and also improved the kinetics of oHSV replication between 24 and 48 hours after infection (Figure 1B).

In fact, transient overexpression of human MEC17 (33), an α-tubulin acetyltransferase (also called TAT1), significantly enhanced HSV-1–mediated transgene expression and HSV-mediated gene expression. When exogenous human HDAC6 was transiently overexpressed in U251 glioma cells, HSV-1 amplicon–mediated luciferase gene expression was significantly reduced (Figure 2, C and D). With the knowledge that post-entry HSV-1 capsids traffic to the nucleus to deliver viral DNA via the cellular MT apparatus and that one of HDAC6’s activities is to deacetylate tubulin, we then asked whether the increased acetylation level of α-tubulin changed HSV-mediated gene expression. In fact, transient overexpression of human MEC17 (33), an α-tubulin acetyltransferase (also called TAT1), significantly enhanced the bioluminescence mediated by an HSV-1 amplicon (Figure 2, E and F), thus providing further evidence that HSV-mediated gene expression is enhanced by tubulin acetylation (i.e., counteracting...
HDAC6 activity). To further ensure that the effect of HDAC6 against HSV-1 infectivity was specifically due to HDAC6 mod-
ulation of tubulin acetylation, we used two α-tubulin mutants: a hyperacetylation (K40Q) and an acetylation-resistant (K40R)
tubulin mimic. Stable expression of either the K40Q or the K40R mutant resulted in the expected increased or decreased replica-
tion of oHSV, respectively (Figure 3). This indicated that HDAC6 activity (i.e., causing tubulin deacetylation) was the specific func-
tion that decreased oHSV replication, rather than an “off-target”
effect of shRNA or pharmacologic agents.

**Tubacin alters post-entry trafficking of HSV capsid proteins.**

To determine how HDAC6i improved HSV-1–mediated gene expression (Figure 1A) and replication kinetics (Figure 1B), we
asked whether HDAC6i altered the intracellular trafficking of post-entry capsids toward the nucleus for viral genomic release
or toward the lysosomes for digestion of large multimolecular
particles. We used HSV-1 virions with a GFP-tag located within
the VP26 capsid protein, designated K26GFP (34), to infect U251
cells expressing red fluorescent protein–tagged (RFP-tagged)
LAMP1, a lysosome membrane–bound protein, in order to enable live monitoring and quantification of HSV-1 capsid trafficking.
At 90 minutes after infection, in untreated cells, the majority of green fluorescence localized primarily at the edge of DAPI-
stained nuclei (average percentage GFP colocalization at nuclei,
57.27% ± 21.56%) rather than in association with RFP lysosomes
(25.41% ± 13.27%) (Figure 4). Tubacin treatment led to a signifi-
cant increase in GFP-VP26 capsids localizing to the perinucleus
(68.45% ± 14.98%) rather than to lysosomes (17.60% ± 8.39%).
Conversely, IFN-β treatment led to the opposite effect, with a sig-
nificant increase in the number of capsids localizing to lysosomes
(39.19% ± 18.25%) compared with the number localizing to nuclei
(28.07% ± 18.52%). Tubacin treatment reversed this effect (lys-
osome, 27.17% ± 14.39%, versus perinucleus, 49.42% ± 15.95%).
Together, these data indicated that tubacin preferentially routed
post-entry HSV-1 capsids to nuclei rather than to lysosomes at
early time points after infection, even in the presence of IFN-β.

**Figure 1. The HDAC6-specific inhibitor, tubacin, improves HSV-1–mediated gene expression and oHSV replication.** (A) Bioluminescence (measured as RLU) assay was performed 24 hours after infection with a replication-defective HSV-1 encoding a Fluc cDNA of U251 cells (MOI of 3). (B) Replication of rQNestin34.5 (MOI of 0.1) in tubacin-treated (dashed line) and control U251 cells (solid line). The input dose was given at 0 hours. (C) Titration of oHSV-infected (rQNestin34.5-infected) U251 cells (MOI of 0.03) in the presence of IFN-β, with and without VPA or tubacin, 3 days after infection. Doses of tubacin and IFN-β were 5 μM and 1,000 units/ml, respectively. (D) LDH cytotoxicity assay 3 days after infection of U251 cells by rQNestin34.5 in the presence of tubacin (0, 1, 2, and 5 μM; starting at 14 hours before infection). (E) LDH cytotoxicity assay 5 days after infection of U251 cells by rQNestin34.5 in the presence of CI994 (0, 1, and 3 μM; starting at 14 hours before infection). (F) LDH cytotoxicity assay of U251 cells in the presence of tubacin for 5 days. *P < 0.05, **P < 0.01, ***P < 0.001 by 1-way ANOVA test in A–C and F and 1-way ANOVA with Turkey’s multiple comparisons tests in D and E. Error bars correspond to mean ± SD (n = 3; in A–C and n = 4; in F). Horizontal bars represent the average in A–C. Bars represent the average (n = 4; in D–F). (See also Supplemental Figure 1.).
with our aforementioned findings. Therefore, we tried to evaluate whether in gliomas entry or uptake of oHSV occurs via the endocytic pathway or via direct fusion of the viral envelope to the plasma membrane. To analyze the localization of oHSV capsids in two independent GSCs (GBM326 and GBM83) and U251 cells upon oHSV infection. Transmission electron microscopy (TEM) analysis showed virion uptake occurring via endocytosis (Figure 6, A and B), with occasional fusion of the viral envelope to the plasma membrane (Figure 6C). In addition, virions could be visualized sequestered in endosomal vesicles within the cytosol, with some vesicles showing fusion with virions, suggestive of capsid release from vesicles (Figure 6D). Quantitative analyses further confirmed that the majority of oHSV uptake in GSCs occurred via endocytosis (Figure 6E). These experiments thus provided further evidence that post-entry oHSV capsids are encapsulated in intracellular vesicles (and thus more likely to be targeted by lysosomes) rather than being released into the cytosol upon infection in glioma cells.

In vivo effect of HDAC6i in glioma models. We next sought to determine whether oHSV replication was also enhanced in vivo in a animal model of glioma. We used human GBM30 cells, a GSC that grows aggressively in the brains of athymic mice. We also used tubastatin A (TA), another highly specific HDAC6 inhibitor (38), because tubacin biodistribution in the brain is limited by the brain-blood barrier. After establishing GBM30 gliomas in the brains of athymic mice and treating them with systemic TA or vehicle, two different types of oHSVs were tested by intratumoral administration. Four days later, brains and tumors were harvested, and oHSVs were titrated. As shown in Figure 7A, TA-mediated inhibition of HDAC6 did indeed lead to a significant increase in the in vivo titers of two different oHSVs. To determine whether this translated into a significant antitumor effect, survival of mice with orthotopic GBM30 gliomas was followed after oHSV intratumoral injection, as a function of TA administration. There was a trend toward improved survival for mice treated with TA and oHSV compared with that for mice treated with oHSV alone (Figure 7B). In lieu of detailed pharmacodynamic studies of TA, we analyzed the target of TA action (i.e., HDAC6i hyperacetylated tumors, leading to increased tubulin acetylation in tumors). As shown in Supplemental Figure 3, TA administration led to increased tubulin acetylation in orthotopic tumors in mouse brains. The sum of these results thus suggested that in vivo HDAC6i improved intratumoral replication of oHSV in highly aggressive GSC tumors.

Variability of the HDAC6i effect on oHSV replication in a panel of well-characterized GSCs. To further study the HDAC6i effects in patient-derived samples, we repeated oHSV replication assays on multiple GSCs that were very well characterized in terms of their molecular profiles (37). As shown in Table 2, there was variability not only in the HDAC6i effect, but also in the VPA-mediated pan-HDACi effect on oHSV replication when

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Figure 2. Functional assays of HSV-1 infection in glioma cells with altered deacetylase or acetyltransferase functions. (B) Stable HDAC6 gene knockdown mediated by two different HDAC6 targeting shRNAs (referred to as #1 and #2) and (D) transient overexpression of the HDAC6 gene or (F) myc-tagged human MEC17 (an α-tubulin acetyltransferase) in U251 glioma cells were confirmed by Western blots using antibodies against HDAC6, myc, and acetylated total α-tubulin on parallel gels. Infectivity of replication-defective HSV-1 encoding the Fire gene at a (A and C) MOI of 3 or (E) other indicated MOIs was measured by luciferase enzymatic activities. (A) Empty vector (Vec) was used as a control and was set as 100 percent for RLU. (C and E) HDAC6 and myc-tagged MEC17 were transiently overexpressed in U251 cells for 48 hours prior to the infection. Horizontal bars and error bars in the plots correspond to average values and mean ± SD, respectively (n = 3; *P < 0.05, **P < 0.01 by 1-way ANOVA test).

Visibly faster. In addition, tubacin-treated cells exhibited a visibly increased number of GFP-positive punctae in nuclei (Figure 5, B and F, 92% at 20 h.p.i.) when compared with that in nontreated cells. In agreement with the previous observation (Figure 4), IFN-β treatment delayed the temporal kinetics of both GFP expression and perinuclear accumulation of GFP-positive punctae, which were also decreased in number when compared with control cells (Figure 5, C and G, 54% at 20 h.p.i.), but with an increase in GFP signal within RFP-expressing aggregates, indicative of lysosomal accumulation and possible lysosomal degradation, as observed above (Figure 4). Tubacin was able to reverse the effects of IFN-β treatment (Figure 5, D and H, 75% at 20 h.p.i.). Taken together, these results suggested that HDAC6i altered post-entry trafficking of HSV-1 capsids, increasing it toward nuclei rather than lysosomes and reversing the effect of IFN-β that instead routed capsids toward lysosomes rather than nuclei.

oHSV capsids can be found in endocytic vesicles in GSCs. Endosomes can fuse with lysosomes to degrade the uptake of pathogenic particles, and our data presented above showed that HSV-1 capsids colocalized with lysosomes. This would imply that HSV-1 capsids were trafficked into cells via endosomes rather than being trafficked freely into the cytosol immediately upon the viral uptake. HSV-1 entry has not been classically associated with uptake via endosomes, and, in a number of cells, such as EBV-LPD primary cells (35) and Vero cells (ref. 36 and data not shown), oHSVs are taken up by direct fusion of the viral envelope to the cell membrane, releasing capsids into the cytosol. This trafficking pathway thus would not require HDAC6, in contrast to the cell membrane, releasing capsids into the cytosol. This was shown (48) to be due to direct fusion of the viral envelope PM (Figure 6C). In addition, virions could be visualized sequestered in endosomal vesicles within the cytosol, with some vesicles showing fusion with virions, suggestive of capsid release from the vesicle (Figure 6D). Quantitative analyses further confirmed that the majority of oHSV uptake in GSCs occurred via endocytosis (Figure 6E). These experiments thus provided further evidence that post-entry oHSV capsids are encapsulated in intracellular vesicles (and thus more likely to be targeted by lysosomes) rather than being released into the cytosol upon infection in glioma cells.

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assayed on this GSC panel. In fact, in 2 of 6 GSCs (GBM157 and GBM326), either tubacin- or TA-mediated HDAC6i significantly enhanced oHSV replication, a finding that did not occur in 3 GSCs (GBM83, GBM528, and GBM1123); in contrast, in GBM19 cells, HDAC6i reduced oHSV replication. Interestingly, the pan-HDACi effect of VPA was also variable, with significant enhancement in 2 of 6 GSCs (GBM528 and GBM83). Tubacin also significantly augmented oHSV cytotoxicity for GBM326 cells to the same magnitude as pan-HDACi with VPA (data not shown). However, this did not occur with GBM19 cells, in agreement with the viral replication data (Table 2), and did not significantly occur with GBM528 cells (data not shown).

To determine whether HDAC6 levels correlated with responses, we evaluated HDAC6 mRNA levels in the tested GSCs. As shown in Figure 8A, all 6 GSCs expressed HDAC6 mRNA, but there was no correlation between HDAC6i sensitivity and HDAC6 mRNA levels: for instance, GBM528 cells expressed the highest levels of HDAC6 mRNA, but HDAC6i had no effect on these cells, while GBM326 cells expressed low to intermediate levels of HDAC6 mRNA and yet were the most sensitive to HDAC6i of all tested GSCs. We also determined that human GBMs expressed HDAC6 mRNA, as shown in Figure 8B. These data thus indicated that there was variability in enhancement of oHSV replication and cytotoxicity by HDAC6i against different GSCs.

Discussion
Eukaryotic cells possess intrinsic defense mechanisms against infections by viruses that directly restrict viral replication and assembly upon infection via ubiquitously existing proteins. Over the last two decades, a variety of genetically engineered viruses have been tested as cytotoxic agents against tumor cells, but little is known about epigenetic mechanisms of the intrinsic defenses that tumors may have against different OVs. We and others have previously shown that pan-HDAC inhibitors lead to enhanced infectivity and replication of different OVs in tumors (28–30, 39). Although it is still possible that the epigenetic effects of the nuclear class of HDACs were responsible for this effect, we have found that the nonnuclear cytosolic HDAC6 can provide antiviral effects in tumor cells exposed to an oHSV. Specifically, we have been able to show that (a) pharmacologic and genetic inhibition of HDAC6 led to enhanced replication of oHSV, while augmentation of HDAC6 function reduced oHSV replication; (b) HDAC6’s role in the antiviral effect is linked to the acetylation status of MTs; (c) HDAC6i counteracted the antiviral effect of type 1 IFNs; (d) HDAC6i led to increased shuttling of post-entry oHSV to the nucleus rather than to the lysosome; and (e) HDAC6i also led to improved oHSV replication in a panel of patient-derived spheroid-grown glioma cells, albeit with some variability. Coupled with the knowledge that HDAC6 is ubiquitously expressed in human GBMs (see Figure 8B), these findings thus indicate that HDAC6 can provide antiviral functions in tumor cells exposed to OVs.

Multiple HDACs with diverse functions exist in cells. In fact, at least 18 HDACs have been discovered that not only control deacetylation of histones, thus modifying gene expression, but that also deacetylate other proteins involved in cellular homeostatic functions (40, 41). We and others have previously published results that indicate that inhibition of HDACs with pharmacologic agents, such as VPA, trichostatin A, or SAHA, significantly enhances the OV anticancer activity in vitro and in vivo (28, 31, 39, 42, 43). However, it was not clear whether the effect of pan-HDAC inhibitors was due to inhibition of a specific HDAC protein or multiple HDAC proteins. This study showed that tubacin, a relatively specific HDAC6 inhibitor unlike others that target intranuclear substrates such as histones (32), enhanced HSV-1 vector-mediated transgene expression and improved the kinetics of oHSV replication in glioma cells. It also reversed the antiviral effects of IFN-β (24). We demonstrated that overexpression of tubulin deacetylase HDAC6 and tubulin acetyltransferase MEC17 led to
Table 2. HDACi effect on rQNestin34.5 replication efficacy

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<th>Cell name</th>
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<th>Tubacin (fold)</th>
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Total PFU were measured using the indicated patient GBMs with rQNestin34.5 infection (72 hours) at 5,000 PFU (Input) after treatment with VPA (1 mM), tubacin (100 nM), or TA (50 nM) or mock treatment (oHSV) starting at 15 hours before infection. Average titer values after drug treatment are represented as fold increase (fold) compared with mock treatment (PFU, mean of 3 biological replicates). *P < 0.01, **P < 0.05, 1-way ANOVA test, subsequent to pairwise comparison test of groups with Bonferroni-adjusted P values, compared with the mock (oHSV alone).

Figure 4. Percentage colocalization of GFP-VP26 intracellular capsids in RFP-LAMP1–expressing U251 cells. At 90 minutes after infection, in cells under the indicated treatment conditions (IFN-β, 1,000 units/ml; tubacin, 5 μM), colocalization of GFP fluorescence either (A) with RFP fluorescence (RFP-LAMP1) or (B) with DAPI fluorescence (perinucleus) was counted in each cell after they were fixed with 4% paraformaldehyde and stained with DAPI. The horizontal bars and error bars represent average and mean ± SD, respectively (n = 22–27; *P < 0.05, **P < 0.01, ***P < 0.001 by 1-way ANOVA test). Numbers of cells used for counting GFP dots are as follows: -tubacin/-IFN-β, n = 27; +tubacin/-IFN-β, n = 26; -tubacin/+IFN-β, n = 22; and +tubacin/+IFN-β; n = 23.
The aggresome pathway was recently shown to promote the process of capsid uncoating of influenza A virus (IAV), allowing for release of viral ribonucleoprotein (vRNP) into the nucleus (56): the HDAC6 B uz domain was shown to play an important role in this process. During HSV-1 infection, the UL36-encoding tegument proteins VP1-2, also known as viral DUB, are proteolytically cleaved at the nuclear pore, triggering a conformational change of the capsid, an essential step for release of the HSV-1 genome (57, 58). Therefore, the nuclear release of IAV vRNP and that of HSV-1 DNA are mechanistically different, and this difference may be a reason for the different outcome that HDAC6 activity has in facilitating versus inhibiting IAV versus HSV-1 replication. However, there may also be other reasons for HDAC6’s different role in IAV and HSV-1 replication kinetics that relate to the differences in IAV versus HSV-1 entry and transport to the nucleus. In fact, HDAC6i seems to enhance trafficking and budding of progeny IAVs (56, 59) as well as trafficking of transfected naked DNA (60, 61).

A possible clinical translation of our findings would be to combine specific pharmacologic HDAC6i with oHSV administration. The rationale for this is that pan-HDAC inhibition may be more toxic due to pleiotropic effects on multiple HDACs. We found that the effect of HDAC6i on a panel of GSCs was variable. Although the reasons for this variability remain to be answered, this should not be too surprising, since it is likely that the degree and magnitude of the HDAC6 effect could depend on the level of intact IFN signaling or the ability to use HDAC6 in endosome/autophagosome fusion or the variability of the trafficking of post-entry oHSVs. These possibilities will require additional experimentation to determine their importance. Nevertheless, the variability of responses does not detract from the finding of a mechanistic link in some tumor cells between HDAC6, IFN signaling, and handling of post-entry oHSV trafficking.

We observed a trend of improved survival in mice with an intracranial GSC treated with oHSV and HDAC6i compared with that of mice treated with oHSV alone, although the difference in survival between these treatment groups did not reach significance, if an α level of 0.05 is used as the cutoff. The P value of 0.0852 shows a trend to significance that would probably be achieved if a larger sample size was used (e.g., a sample size of 24 animals per group will achieve 80% power at a 0.05 significance level to detect a hazard ratio of 0.4 when the proportion surviving in the oHSV treatment group is 0.40 at 30 days, using the log-rank test and 2-sided test). In addition, extensive experimentation may be required to determine if the dose and schedule of TA in vivo were optimal for the intended outcome. Yet, the finding that the tested dose of TA led to significantly increased yields of two different oHSVs in mice with tumors 4 days after injection may indicate that more prolonged administration of the drug would be required to visualize a more significant effect. It is also possible though that HDAC6i by itself may not lead to a sufficient increase in oHSV replication to translate into a significant difference and that additional HDACs would need to be inhibited. In fact, when we used...
Figure 6. TEM analysis of oHSV uptake events in GBM83 and GBM326 GSCs and U251 glioma cells. (A) GBM326 cells at 20 minutes after infection (p.i.) with rQNestin34.5. The inset shows an enveloped-virion–containing vesicle on the PM (arrowhead). N, nucleus. (B) GBM326 cells. The inset shows macropinocytosis of a virion on the PM. (C) GBM383 cells. The inset shows fusions with HSV-1 virions (arrowheads) and the PM. (D) GBM326 cells. The inset shows endocytic vesicles containing virions in the cytoplasm. Arrowheads indicate fusions of virions with vesicle membrane. (E) Summary of TEM analyses in GBM83, GBM326, and U251 cells at 2, 10, and 20 minutes after infection with rQNestin34.5 virus. PM, oHSV on PM; cytosol (vesicle), virions in the cytosolic endocytic vesicle; cytosol (naked), virions in cytosol without vesicles. Scale bar: 500 nm.

Methods

Reagents. Human IFN-β–1a was obtained from PBL Assay Science; VPA was obtained from Sigma-Aldrich; tubacin used in Figure 1, A–C, Figure 4, and Figure 5 was a gift from Stuart Schreiber (Broad Institute of MIT and Harvard, Boston, Massachusetts, USA) and tubacin used in Figure 1, D–F, and Table 2 was obtained from MedKoo Biosciences; TÂ was obtained from BioVision Inc.; and CÎ994 was obtained from Selleck Chemicals. Human IgG was obtained from Talecris Biotherapeutics Inc.

Human specimens. For each patient, samples of both tumor and brain devoid of gross tumor were resected, aliquoted, and processed for either extraction of total RNA (TRIzol, Invitrogen) or isolation and establishment of patient-derived GSCs (62).

Cell culture. African green monkey Vero kidney cells (and the derivative 7b cell line) (63), human U251 glioma cells (28) and their derivative cell lines, human Gib36 glioma cells and their derivative cell lines (G16–9, Gib36AEGFR), and U87AEGFR and U373 cells were cultured on adhesive culture dishes containing DMEM (Invitrogen) supplemented with 2% or 10% FBS (Sigma-Aldrich), 100 μg/ml penicillin/streptomycin (Invitrogen), and 10 mM HEPES (Invitrogen) at 37°C in a humidified incubator at 5% CO₂. For passage, trypsin (Invitrogen) was used as a dissociation reagent. Primary GSCs were maintained as nonadhesive spheroids in flasks containing neurobasal medium supplemented with B27 (Invitrogen), 100 μg/ml penicillin/streptomycin, GlutaMAX (Invitrogen), and 50 μg/ml of both human EGF and FGF-2 (both from R&D Systems). Spheres were dissociated using TrypLE (Invitrogen).

DNA constructs. Human MEC17 cDNA (BC047303) was obtained from a mammalian gene collection cDNA library and amplified by PCR (forward: 5′-AGATCTGTCGACATGTGGTT-GACCTGGCCTTTCTG-3′; reverse: 5′-GGATCCGCGGCCGCT-CACCAAGGCCTGGTGCTGCGACGTT-3′) in the pCR4Blunt-TOPO vector (Life Technologies), followed by engineering a site to join the BglII/NotI fragment to the SalI site of pCMV-Myc (Sigma-Aldrich) with a Myc-epitope tag at the N-terminus (called pCMV-myc–human MEC17). HDAC6 flag (plasmid #13823), pmCherry_a_tubulin_IRES_puro2 (plasmid#21043), and LAMP1-RFP (plasmid #1817) were obtained from Addgene. The K40Q and K40R mutants of α-tubulin were generated using the QuikChange XL Site-Directed mutagenesis Kit (Agilent Technologies), following the manufacturer’s protocol. The correct identity of all mutant constructs was verified by DNA sequencing.

Gene transfer and knockdown. Gene transfer was performed using Lipofectamine (Life Technologies), following the manufacturer’s protocol. To isolate stably expressing transfectants, cells were treated with G418/ geneticin (500 μg/ml, Life technologies), and individual G418-resistant clones (for LAMP1-RFP and mCherry–α-tubulin cells) were selected, followed by analysis using fluorescence microscopy (Nikon). To knockdown the HDAC6 gene in U251 cells, we used the pGIPZ vector containing HDAC6-targeting sequences (V2LHS_71187 for clone 1 and V2LHS_71188 for clone 2 in Figure 2A, OpenBiosystems). Lentiviral vectors (including control shRNA vectors) were packaged in 293FT cells. Infected cells were cultured in the presence of puromycin (2 μg/ml, Sigma-Aldrich) prior to use.

In summary, these findings indicate that HDAC6 activity is a factor in the intrinsic intracellular defense of some tumor cells against virotherapy. In light of multiple clinical trials of OVs against cancers, this may be highly relevant to improve treatment efficacy.
HSV-1 viruses and titration. K26GFP virus was obtained from P. Desai (Johns Hopkins University School of Medicine, Baltimore, Maryland, USA) (34). rQNestin34.5, rHSVQI, and KNE viruses used in this study have been reported previously (26, 64). In brief, rQNestin34.5 recombinant virus contains a deletion of the UL39 gene (encoding ICP6) and both copies of the γ34.5 genes, with the addition of one copy of γ34.5 gene under the control of nestin promoter inserted into the deleted UL39 locus. KNE virus contains a mutated form of glycoprotein D, with residues 2 to 24 deleted and a single amino acid change at residue 38 (Y38C) in the KOS strain that eliminates entry via HVEM and nectin-1, with insertion of a single-chain antibody (scFv) directed against EGFR in the deleted position. Titration assays followed a standard plaque formation assay with minor modifications. Briefly, the virus was serially diluted 1:3 (25 μl into 50 μl DMEM containing 2% FBS) across the 96-well plate toward the end columns, with changing of pipette tips between each well. Vero cells in 100 μl medium were then added at a final density of 20,000 cells in each well, and the plates were incubated at 5% CO₂, 34°C, overnight for 20 hours before adding 50 μl of 0.4% human IgG solution diluted in DMEM containing 2% FBS for the next 3 days.

oHSV purification. All oHSV viral stocks were prepared using the 7b cell line (63) in 5- or 10-layer Nunc Cell Factories (Thermo Fisher Scientific) at a MOI of 0.005 to 0.01 at 37°C in serum-free media (Life Technologies). After the 2-hour adsorption period, media with fetal bovine serum (Sigma-Aldrich) were added to a final concentration of 10%, aliquoted into cryovials containing 2% FBS for the next 3 days. Luciferase assay. The engineering and packaging of the pHGCagY-Luc vector, which expresses the firefly luciferase (Fluc) under the control of a CAG promoter and the EGFP reporter gene under the control of a HSV-1 IE4/5 promoter, was previously described (66). Briefly, pHGCagY-Luc plasmid DNA was cotransfected with fHSVΔpac in Vero 2-2 cells using Lipofectamine (Life Technologies). After 3 days, transfected cells were scraped into the medium, the suspension was frozen and thawed 3 times, and the cell debris was removed by centrifugation (10 minutes, 3,000 g). The virus, the supernatant was added to the layer of 25% sucrose in HBSS in a centrifuge tube, followed by ultracentrifugation using SW-41 rotor (2.5 hours, 106,000 g). The pellet was dissolved in HBSS, and the solution was frozen by dry ice/ethanol and stored at –80°C until use. Titration was assayed in G16-9 human glioma cells and calculated as GFP transduction unit per ml (TU/ml). Luciferase assays were performed using the Luciferase Assay Kit (Promega), and luciferase activities in each well were measured using a microplate reader (POLARStar Omega, BMG Labtech) to obtain RLU.

Viral infection assay. Human U251 glioma cells and derivative clones were plated at 500,000 cells per 6-well plate containing DMEM supplemented with 2% FBS (DMEM/2F) the day before infection. In some experiments, cells were treated with VPA, tubacin, or TA for 15 hours prior to the infection, and IFN-β (1,000 units per ml) was added in cultured media for 14 hours before the infection. VPA was removed from media prior to infection because prolonged exposure generated cytotoxicity, while the HDAC6 inhibitors (tubacin and TA) were replenished.
GBM157, and GBM528), poly-l-lysine (Sigma-Aldrich, 2 g/ml for GBM19, μ ethanol and thawing in a 37°C water bath for virus stock titration. 15-ml conical tubes, followed by 3 repeated cycles of freezing in dry ice–
ethanol and thawing in a 37°C water bath for virus stock titration.

After 3 days, intracellular and extracellular lactate dehydrogenase (LDH) levels were measured using a Pierce LDH Cytotoxicity Assay
ment 96-well plate and then transferred to the plates with tumor cells. Serially diluted aliquots of oHSV were prepared in a ultralow attach-
ment 96-well plate and then transferred to the plates with tumor cells. After 3 days, intracellular and extracellular lactate dehydrogenase (LDH) levels were measured using a Pierce LDH Cytotoxicity Assay Kit (Life Technologies), following the manufacture’s protocol. Cyto-
toxicity was calculated with following formula: percentage cytotoxic-
ity = ([LDH of uninfected cells] - [intracellular + extracellular LDH of
sample cells])/[LDH of uninfected cells] × 100.

Virus uptake through TEM analysis. Cells were incubated with rQnestin34.5 at a high MOI (MOI = 50) on ice for 1 hour to facilitate attachment of numerous viruses to single cells for TEM. The temperature of the cultures was then shifted to 37°C for up to 30 minutes to trigger virus uptake. Cell pellets were collected, washed 3 times with PBS, and then fixed in 2.5% glutaraldehyde (catalog 16220, Electron Microscopy Sciences) in cacodylate buffer for 4 hours at 4°C. Cells were then washed 3 times with cacodylate buffer followed by post-

Detection of HRp was performed by incubating blots with ECL Plus reagent (GE Healthcare) and subsequently exposing them to film.

Fluorescence microscopy analysis. To observe living cells under confocal microscopy (LSM-510META or LSM-710, Zeiss), cells were seeded onto glass-bottom dishes (In Vitro Scientific) containing DMEM/2F, the day before analysis. Infection of K26GFP was per-
formed on ice or in a 4°C refrigerator for 1 hour, and unattached virus particles were washed out using D-PBS, followed by the addition of
DMEM warmed to 37°C containing 2% FBS. The dish was then set up
in the 5% CO2 chamber on the stage of the enclosure microscope at
37°C. The z-axis stack images were taken every 15 minutes and assem-
ded by ImageJ 1.48 or an earlier version of the ImageJ software (NIH)
with the function of Z project (standard deviation type); we started
using time stamps after the temperature shift to 37°C. To analyze the colocalization of GFP-VP26 with either LAMP1-RFP or the periphery region of DAPI-stained nuclei, cells were seeded on the 12-well plate containing a German glass round coverslip (#1 thickness) the day before K26GFP infection. At 1.5 h.p.i. (temperature shift from 4°C to
37°C), cells were fixed, incubated with 4% paraformaldehyde (Sigma-Aldrich) in PBS (pH 7.4) at room temperature, and excess paraformaldehyde was removed using 50 mM NH4Cl for 5 minutes at room temperature. After permeabilization with 50 μg/ml digitonin (EMD), cellular nuclei were stained with DAPI before the coverslips were mounted with Vec-
taShield (Vector Laboratories) on glass slides. Fluorescent images were acquired with a Zeiss LSM510 META or LSM710 confocal microscopy system and processed using ImageJ. For quantitative analysis of colo-
calizing GFP/RFP/DAPI signals, we fixed the cells at 90 minutes after virus-cell fusion and quantified the number of individual GFP-tagged
VP26 capsids present in RFP-LAMP1 lysosomes versus nuclei/perinu-
clei as a function of IFN-β and/or tubacin treatment. GFP dots were
manually counted using each individual z-stack sectioned image, and
toal colocalized GFP dot numbers in a single cell were summed from
whole z-stack images and plotted in Figure 5.

Virus uptake through TEM analysis. Cells were incubated with rQnestin34.5 at a high MOI (MOI = 50) on ice for 1 hour to facilitate attachment of numerous viruses to single cells for TEM. The temperature of the cultures was then shifted to 37°C for up to 30 minutes to trigger virus uptake. Cell pellets were collected, washed 3 times with PBS, and then fixed in 2.5% glutaraldehyde (catalog 16220, Electron Microscopy Sciences) in cacodylate buffer for 4 hours at 4°C. Cells were then washed 3 times with cacodylate buffer followed by post-

HDAC6 (sc-28386; Santa Cruz), α-tubulin (T6074; Sigma-Aldrich), acetylated α-tubu-
lin (T6793; Sigma-Aldrich), FLAG M2 (F3165; Sigma-Aldrich), and myc 9E10 (11667149001;
Roche). ECL anti-mouse or anti-rabbit IgG and HRP-linked whole antibodies (NA931V and
NA934V, respectively; GE Healthcare) were used for the detection of primary antibodies.
fixation in 1% OSO₄ (catalog 0972A, Polysciences) in cacodylate buffer for 1 hour at 4°C. After 3 cacodylate buffer washes, the pellets were then dehydrated through graded alcohol, embedded in Epon/Araldite, and polymerized at 60°C overnight. Blocks were then sectioned at 70 to 80 nm and collected on the grids. The grids were first stained with 1% aqueous uranyl acetate (catalog NC0740462, Fisher Scientific) for 12 minutes, followed by a ddH₂O rinse. The grids were then stained with Reynolds lead citrate (catalog 17900, Electron Microscopy Sciences) for 5 minutes in a moisture-free chamber. After the rinse with ddH₂O, the grids were set to dry before TEM analysis via Hitachi H-7650 SEM/TEM Hitachi S-4800.

**Animal experiments.** Female athymic nu/nu mice at 6 to 8 weeks olds were purchased from Charles River or Harlen Laboratories. GBM30 cells (100,000 cells) in 5 μl HBSS buffer were injected intra-cranially to establish the xenograft tumor in the mouse brain. After 14 days (in Figure 7A) or 5 days (in Figure 7B), 3 μl of oHSV in HBSS buffer was injected intratumorally. TA or vehicle diluted in DMSO was administrated at a dose of 1.3 mg per kg body weight. oHSV types, their administration doses, and TA administration conditions are described in the legend of Figure 7.

**Statistics.** One-way ANOVA and pair-wise comparisons with adjusted P-values were used for statistical analyses, using R Studio software run with the R language (R Core Team; http://www.R-project.org) and R package “stats” version 2.15.3. Kaplan-Meier survival curves were compared using the log-rank test, using JMP Pro version 9 (SAS Institute Inc.). P-value of less than 0.05 were considered significant.

**Study approval.** The protocols for collection of human specimens and use of microbe agents were approved by the IRBs of The Ohio State University and Brigham and Women’s Hospital. Informed consent was acquired from participants who provided specimens. All animal studies were reviewed and approved by the IACUC of The Ohio State University and Harvard Medical School.

**Author contributions.**

HN and EAC developed the concept, designed experiments, and wrote the manuscript. EAC oversaw and supervised all analyses and strategies. HN and TN performed in vitro experiments using cell lines, and JKK and KO performed in vitro experiments using primary gliomas. TN, MCS, and HN conducted an in vivo experiment. PYW performed TEM and flow cytometry analyses, and TPC supervised these analyses and interpretation. AO performed an experiment using HDAC1 knockdown. HN and TN constructed the DNA used in this study. SF supervised statistical data analyses. IN supervised analysis and preparation of primary gliomas. WFG performed analyses of oHSV products. PG and JCG supervised oHSV vectors. KK and SL provided the critical review and technical expertise.

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