DNA Demethylating Agents Synergize with Oncolytic HSV1 against Malignant Gliomas

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Abstract

**Purpose:** Oncolytic viruses (OV) based on herpes simplex virus type 1 (HSV1) are being used in clinical trials for a variety of cancers. The OV, rQNestin34.5, uses a nestin promoter/enhancer to selectively drive robust viral replication in malignant glioma cells. We have discovered that this promoter becomes extensively methylated in infected glioma cells, reducing OV efficacy.

**Experimental Design:** We used demethylating drugs [5-azacytidine (5-Aza)], decitabine, or valproic acid (VPA) in both in vitro and in vivo malignant glioma models to determine if they improved the efficacy of rQNestin34.5 therapy.

**Results:** The use of demethylating agents, such as 5-Aza, improved OV replication and tumor cell lysis in vitro and, in fact, synergized pharmacologically on Chou–Talalay analysis. In vivo, the combination of the demethylating agents, 5-Aza or decitabine, with rQNestin34.5 significantly prolonged the survivorship of athymic mice harboring intracranial human glioma xenografts over single agent alone.

**Conclusion:** These results, thus, provide further justification for the exploration of demethylating agents when combined with the OV, rQNestin34.5, in preclinical therapeutics and, possibly, clinical trials for malignant glioma.

Introduction

Oncolytic viruses (OV) are either natural mutants or genetically engineered strains of viruses that replicate and lyse tumor cells in a relatively selective fashion (1). Although there are several types of OV’s, those based on herpes simplex virus type 1 (HSV1) have been widely studied in animal models and in human clinical trials (2, 3). Subjects have been reported to tolerate the treatment well, although evidence for efficacy awaits testing in phase 3 trials. Efficacy is predicated on sufficient replication and OV biodistribution to permit direct cytotoxicity and/or antitumor immune responses to occur. In this context, evidence from clinical trials seems to show that replication needs to be improved for a sufficient therapeutic effect to occur (4, 5).

To improve replication, efforts to understand how the OV and the host interact are needed. One of the intracellular mechanisms of defense against a viral infection lies in epigenetic silencing of viral genes. Although for some viruses, such as EBV, methylation of CpG islands in genes and promoters is one mechanism for gene silencing (6), for HSV1 the evidence for CpG methylation playing a major role has been controversial, with recent data showing that the HSV1 genome is not extensively methylated at CpG sites (7–9). Notwithstanding this, it is not known if oncolytic HSV1s (oHSV1), engineered with heterologous (such as tumor-specific) promoters, would be different. This is a significant question to explore because effective demethylating agents exist that could be used in a clinical trial with oHSV.

rQNestin34.5 is a genetically engineered oHSV1 where a heterologous cellular Nestin promoter–enhancer element drives expression of a single copy of the late viral gene, ICP34.5, whose function results in increasing the ability of HSV1 to replicate (10, 11). This oHSV1 is under preclinical development for an eventual clinical trial (E.A. Chiocca; unpublished data). We hypothesized that this promoter element could be a potential site for silencing of ICP34.5 gene expression by methylation. In this report, we show that infected glioma cells extensively methylate the Nestin promoter and demethylating agents, such as 5-azacytidine (5-Aza) or decitabine, synergize with rQNestin34.5 replication and glioma cytotoxicity in vitro as well as leading to...
Enhanced therapeutic effects in vivo. Therefore, demethylating agents could be combined with rQNestin34.5 in the treatment of gliomas.

Materials and Methods

Reagents

Dulbecco’s modified Eagle’s medium (DMEM), Neurobasal medium, Hank’s Balanced Salt Solution (HBSS), penicillin and streptomycin, GlutaMax, B27 supplement were purchased from Invitrogen. Human basic fibroblast growth factor (hFGF) and hEGF were purchased from R&D Systems Inc. 5-Aza, and decitabine (5-aza-2′-deoxycytidine) were purchased from Sigma Aldrich. The Cell Proliferation Kit I (MTT) was purchased from Roche.

Cells and medium

Human U87AEGR glioma cell lines were maintained in DMEM supplemented with 2% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Human primary OG02 and G169 gliomas were established from surgical specimens resected from patients with glioblastoma and were grown as tumor spheres in medium consisting of Neurobasal medium with x1 GlutaMax, with B27 supplement (1A

Virus replication assay

The oHSV, rQNestin34.5, was previously described (10), and the replication assay was conducted as described in publications (12). Briefly, after incubation with valproic acid (VPA) for 20 hours or 5-Aza for 2 days, cells were seeded onto 24-well plates at 2 × 10⁴ cells/well in 500 μL of media and infected with oHSV at a multiplicity of infection (MOI) of 0.05. GFP expression was used as an indicator for the presence of oHSV. After 3 days, cells were harvested with supernatants at indicated times in triplicate. After three freeze–thaw cycles and sonication, titers of infectious progeny virus were determined by plaque assay on Vero cells.

Methylation status analysis

Genomic DNA and virus DNA from rQNestin34.5-infected cells were obtained using the PureLink Genomic DNA Mini Kit (Invitrogen). The DNA was treated using bisulfite modification with an EZ DNA Methylation Kit purchased from the Zymo Research Corp. The Nestin promoter–enhancer region (10) of rQNestin34.5 was amplified using the following methylation-specific and nonmethylation-specific primer pairs: methylation-specific F agatgcgggaaagag and R tatttactggattga; non–methylation-specific F gggggttgggagag and R tctcaaaacaaccaact. Polymerase chain reaction (PCR) products were extracted and cloned using the TOPO TA Cloning Kit (Invitrogen). Each clone was subjected to sequence analysis using the James Comprehensive Cancer Center Nucleic Acid Shared Resource.

Quantitative real-time PCR

Total RNA was isolated using the Quick-RNA Miniprep Kit (Zymo Reseach Inc.) and reverse transcribed using the ImProm-II Reverse Transcriptase (Promega). Quantitative real-time PCR (RT-PCR) was performed using a ReplEx2 Master Cycler (Eppendorf) and Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). The following sequences of PCR primers were used for the analysis: gC: F ccttgcgtttctggaga and R gtgggttcggggtctgct; γ, 34.5: F acagtcgcggaaccctca and R agtcgcggaactgcgtgct; ICP4: F cgacccgatcaccacgcc and R atgacgcggtgtcgtgct; CTUS1: F ggggtacctgcgtcc and R cattgccgtaacac; CTRS1/2: F caacagctctgcaacc and R gacgggtctgcaac; CTRS3: F caacagcatcggagcc and R caccaagccgtgatcc; ICPO7: F accacagcggattc and R acacacttggatgt; GAPDH: F ggaatcgcggtgcg and R ggaatcgcggattgcgagc.

Cell viability assay by MTT exclusion assay

Cells were treated with 5-Aza for 2 days before infection with rQNestin34.5. Three days after the infection, cell viability was determined by MTT assay as previously described. Triplicate wells were counted each time. The results were computed and the combination index for pharmacologic synergy analysis was used with the software program CompuSyn (ComboSyn, Inc., Paramus, NJ).

Mouse in vivo survival experiments

Female athymic mice (6–8 weeks of age) were obtained from the National Cancer Institute (NCI). To generate intracerebral xenograft models, 2.5 × 10⁵ G169 cells in 4 μL or 2 × 10⁵ human U87AEGR established glioma cells were stereotactically implanted into the brains of athymic mice. After implantation, mice were randomly assigned to each experimental group. On day 7, mice were treated by intratumoral injection of 1 × 10⁵ PFU of rQNestin34.5 alone, 1 × 10⁵ PFU of rQNestin34.5 in 4 μL of 10 μmol/L...
5-Aza, 10 μmol/L 5-Aza alone, or HBSS alone. Mice were then observed until they became moribund, at which point they were sacrificed, and the presence of intracranial tumors was confirmed. All in vivo procedures were approved by the Subcommittee on Research Animal Care at Ohio State University Medical Center.

Statistical analyses

The log-rank tests were used to compare Kaplan–Meier survival curves and were followed by the procedures of the Holm–Sidak method for multiple comparisons. For the experiment shown in Fig. 4, due to crossing survival curves, the Fleming and Harrington weight function with a P value of 0.5 and q value of 2 was used to place greater weight on events that occurred at later time points (13).

Results

The oHSV, rQNestin34.5, possesses a CpG rich island in the Nestin promoter–enhancer region upstream of ICP34.5

rQNestin34.5 (10, 11) is an oHSV that is currently in preclinical development for a possible clinical trial in humans with malignant glioma (E.A. Chiocca; unpublished data). The engineered virus encompasses a Nestin promoter–enhancer sequence upstream of one copy of the γ1 34.5 gene that encodes for ICP34.5. This allows for robust replication and lysis in Nestin-expressing cells. Malignant gliomas express high levels of Nestin, likely in the glioma "stem like" cell subpopulation (14–17). rQNestin34.5 possesses other deletions: both endogenous copies of γ1 34.5 have been removed and the viral gene, encoding for ICP6, has also been partially deleted and its remaining sequences have been fused with an active copy of GFP. This defect in ICP6 restricts virus replication to cells with p16 defects (i.e., tumor cells; ref. 4). When we analyzed this Nestin promoter sequence, we realized that several CpG rich islands were present (Supplementary Fig. S1). This information, thus, may mean that expression of γ1 34.5 could be limited by methylation of its promoter shortly after infection.

Demethylation of rQNestin34.5’s ICP34.5 promoter

Based on these earlier findings, we hypothesized that this promoter would be methylated upon tumor cell infection, and that treatment with demethylating agents could increase oHSV gene expression and replication. In fact, analysis of this promoter region after infection confirmed that it is hypermethylated (Fig. 1A). There were 45 methylated cytidines (Supplementary Fig. S1). Treatment with the demethylating agent 5-Aza led to promoter demethylation, as expected (Fig. 1A). Treatment of infected cells with VPA, an histone deacetylase (HDAC) inhibitor reported to lead to DNA demethylation (18–22), also led to Nestin promoter demethylation (Fig. 1A), and treatment with both VPA and 5-Aza resulted in demethylation of almost all previously methylated cytidines (Fig. 1B). Therefore, demethylation of the Nestin promoter in infected cells was possible with either 5-Aza or VPA.

Demethylating agents enhance oHSV gene expression and replication

We then proceeded to determine if 5-Aza or VPA enhanced rQNestin34.5 gene expression and viral replication. Figure 2A shows that treatment of rQNestin34.5-infected cells with either drug led to a significant several-fold
increase in the expression of the viral genes \( g1 \), ICP4, CTUS1, gC, CTRS1/2, CTRS3, and ICP27. In addition, \( rQNestin34.5 \) expresses a GFP transgene. To test if 5-Aza also increased virus replication, we visualized GFP fluorescence from oHSV in glioma cells. There was a visible increase in the number and size of GFP–positive-infected glioma cells in both U87\(D\) and primary GBM-derived OG02 neurosphere-cultured spheroids. In OG02 primary glioma neurospheres, 5-Aza doses were increased up to 6.2 \( \mu \)mol/L (Fig. 2B). At higher doses, 5-Aza led to cell death and reduced viral plaque formation. Similarly, there was a visible increase in the number and size of GFP-positive plaques, as 5-Aza doses were increased to 2.1 \( \mu \)mol/L in the established human \( U87\DeltaEGFR \) glioma cell line (Fig. 2C), with reduction at higher doses due to 5-Aza-mediated cell death. Quantification of viral titers confirmed a significant increase in viral replication mediated by 5-Aza in both OG02 (Fig. 2D) and \( U87\DeltaEGFR \) glioma cells (Fig. 2E). Therefore, these results show that demethylating agents lead to an increase in oHSV gene expression and enhanced replication in established and primary glioma cells.

**rQNestin34.5 and 5-Aza act synergistically in glioma cell killing**

We sought to determine if there was evidence of pharmacologic synergy between oHSV and 5-Aza in glioma cell killing. First, we determined dose–effect combinations of 5-Aza and oHSV on two primary glioma cells, G169 and OG02. Figure 3A shows that oHSV alone led to 50% cell killing at a dose of 0.05 plaque-forming unit (pfu) in G169 and 0.5 pfus in OG02 primary glioma cells. 5-Aza alone led to a median effect on cell survival at a dose of approximately 4 \( \mu \)mol/L for either cell (not shown). Chou–Talalay analyses were then performed (23, 24). Figure 3B shows that there was very strong synergy for the majority of affected
fractions after combination treatment with 5-Aza and oHSV. These results, thus, provided confirmation that oHSV and 5-Aza act in a synergistic fashion in mediating cytotoxic effects against glioma cells.

rQNestin34.5 and demethylating agents lead to significantly increased survival in mice bearing orthotopic human gliomas

Based on the aforementioned findings, we sought to determine if the combination of oHSV and 5-Aza led to a significant increase in survival in an animal model of glioma. The rationale for carrying out the combination experiments was based on the in vitro data from previous figures showing that 5-Aza demethylated the nestin promoter–enhancer element, leading to improved rQNestin34.5 replication. After orthotopic implantation of human G169 primary glioma cells in athymic nude mice, animals were treated on day 7 with either HBSS, 5-Aza (10 μmol/L), oHSV (1 × 10^5 pfu/mouse), or a combination of 5-Aza (10 μmol/L) and oHSV (1 × 10^5 pfu/mouse). The survival experiments include a heat-inactivated oHSV control but not a replication-defective HSV vector, because we have previously shown that this vector alone lacks significant anticancer effects (25). Figure 4A shows that there was a significant increase in the survival of animals treated with the combination of 5-Aza and oHSV, compared with controls. Decitabine is being tested in clinical trials of humans suffering from a variety of cancers (www.clinicaltrials.gov). We, thus, asked whether the combination of decitabine and oHSV was also beneficial. Figure 4B shows that, indeed, the combination of decitabine (10 μmol/L) and oHSV (1 × 10^5 pfu/mouse) led to a statistically significant increase in the survival of animals with orthotopic G169 gliomas. We then attempted to determine whether the combination of the oHSV and decitabine was effective in a second human glioma model. After implanting human U87 D_EGFR glioma cells in the brains of athymic mice, we treated them with vehicle, oHSV alone, or oHSV and demethylating agent. Figure 4C shows that there was an overall significant increase in survivorship in treated animals when compared with controls.

Figure 3. Pharmacologic synergy between 5-Aza and rQNestin34.5. A, cell viability (measured by MTT) of primary G169 and OG02 glioma cells was assayed 2 days after infection with rQNestin34.5 at different MOIs (0–5) in the presence of different doses of 5-Aza and oHSV. Data shown represent the mean ± SD of three replicates for each sample. B, the combination indexes of 5-Aza and oHSV were calculated using Chou–Talalay analyses. An IC50 was selected to determine outcome. Each ratio group included MOIs of 0.05, 0.5, 1.0, 2.5, and 5.0 for oHSV. Ratio group 1 included 0.05, 0.5, 1.0, 2.5, or 5.0 μmol/L of 5-Aza combined with each of these abovementioned MOIs for oHSV. Ratio group 2 included 0.1, 1.0, 2.0, 5.0, or 10 μmol/L of 5-Aza with each MOI. Ratio group 5 included 0.25, 2.5, 5.0, 12.5, or 25 μmol/L of 5-Aza with each MOI. Confidence intervals (CI) were plotted against the affected fraction (Fa), that is, the percentage of cell death resulting from combination therapy. CI of less than 0.9 indicates synergy, CI between 0.9 and 1.1 is additive; and CI of more than 1.1 indicates antagonism.
Experimental attempts to improve the replication efficiency of OV in order to lyse more tumor cells require a detailed understanding of how the infecting virus and its host cell interact. In the context of the oncolytic HSV (oHSV), rQNestin34.5, where a heterologous promoter–enhancer is used to drive expression of a viral gene needed for robust replication, it is not clear how the host cell would attempt to modify incoming viral DNA. In fact, in this report, we show that CpG islands in the nestin promoter–enhancer element become extensively methylated in glioma cells. In vitro, the addition of demethylating agents leads to demethylation of this promoter with improved replication of the virus and enhanced cytotoxicity. In fact, demethylating agents and rQNestin34.5 pharmacologically synergize in tumor lysis. In vivo, this leads to significantly improved survival of mice harboring orthotopic gliomas derived from a freshly explanted human tumor and a trend toward significantly improved survivorship in mice harboring glioma from a commonly established human glioma line. Therefore, the addition of demethylating agents to rQNestin34.5 virotherapy provides a beneficial therapeutic effect in a preclinical experimental setting.

In vitro, we also showed that the HDAC, VPA, led to demethylation of the nestin promoter. Previously, we had shown that VPA significantly improved OV replication and survival of mice with orthopic glioma xenografts (10, 12). The mechanism for this effect was reported by us to occur through VPA’s action on reducing STAT1’s activation of interferon signaling. In addition, we have recently shown that VPA also enhances rQNestin34.5 replication and anticancer effects in vivo, by inhibiting antiviral immune responses such as the generation of IFNγ by NK cells that rapidly infiltrate virally infected gliomas (26, 27). The finding of reducing the hypermethylation of the oHSV’s heterologous promoter uncovers another mechanism that plays a role in VPA’s improvement of rQNestin34.5 replication.

Recently, VPA has been reported to decrease DNA methyltransferases (DNMT) protein levels and reduce DNMT enzymatic activity (28). The role of epigenetics and the significance of aberrant gene regulation in the etiology of cancer is a well-established phenomenon. The hallmark of cancer epigenetics is aberrant DNA methylation—a hallmark of cancer epigenetics is aberrant DNA methylation consisting of global hypomethylation and regional hypermethylation of tumor suppressor genes (TSG) by DNMTs (29, 30). Overexpression of DNMT1 and DNMT3B in gliomas leads to hypermethylation of various tumor suppressor genes, resulting in a lack of cellgrowth regulation and higher genomic instability. This hyperactivation of DNMTs may, thus, lead to reduced efficacy of oHSVs. In fact, there have been reports that the expression of transgenes in tumors after viral-mediated delivery decreased, but that 5-Aza and/or trichostatin A reversed this, implying gene methylation as a silencing mechanism for oncolytic viruses in tumors (31). However, the role of methylation in silencing HSV genes is controversial.

Discussion

Experimental attempts to improve the replication efficiency of OV in order to lyse more tumor cells require a detailed understanding of how the infecting virus and its host cell interact. In the context of the oncolytic HSV (oHSV), rQNestin34.5, where a heterologous promoter–enhancer is used to drive expression of a viral gene needed for robust replication, it is not clear how the host cell would attempt to modify incoming viral DNA. In fact, in this report, we show that CpG islands in the nestin promoter–enhancer element become extensively methylated in glioma cells. In vitro, the addition of demethylating agents leads to demethylation of this promoter with improved replication of the virus and enhanced cytotoxicity. In fact, demethylating agents and rQNestin34.5 pharmacologically synergize in tumor lysis. In vivo, this leads to significantly improved survival of mice harboring orthotopic gliomas derived from a freshly explanted human tumor and a trend toward significantly improved survivorship in mice harboring glioma from a commonly established human glioma line. Therefore, the addition of demethylating agents to rQNestin34.5 virotherapy provides a beneficial therapeutic effect in a preclinical experimental setting.

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Kubat and colleagues reported that the HSV1 genome was not hypermethylated after infection of rabbit skin cells. Our findings showed that the nestin promoter CpG islands were extensively methylated. Although this could be explained because it is heterologous, Fig. 2 shows that the transcription of several oHSV genes was also enhanced by 5-Aza or VPA. One explanation may be that the enhanced transcription of the nestin promoter-driven γ34.5 gene also led to increased transcription of other viral genes. The alternative explanation is that these epigenetic reagents demethylated CpG islands of endogenous HSV1 promoters, leading to the observed increase in transcription. In fact, in support of the latter explanation, the methylation status of the endogenous ICP4 promoter in oHSV-infected glioma cells also showed extensive methylation with expected demethylation by 5-Aza (data not shown).

Furthermore, demethylating agents could affect OV replication by reversing the hypermethylation status of promoter–enhancers of IFN and IFN response genes. However, this is unlikely to be operative in our model because we have observed that 5-Aza enhances rQNestin34.5 replication even in the human U87 glioma cell line that is IFN-insensitive (data not shown), because of deletion of chromosomal loci where such genes reside and not because of IFN gene promoter hypermethylation (32, 33). Instead, VSV action in breast cancer cells has been reported to be enhanced by DNA demethylating agents not because they demethylated the IRF5 and IRF7 promoters, but rather through another unknown mechanism (34). To determine in vivo effects of the combination therapy on human glioma animal models, we had to use athymic mice. Possible effects of demethylating agents on T cells in immunocompetent animal models might require further experimentation, although we have minimized for this in our model by intratumoral, local administration of drug. In fact, to minimize possible systemic effects of demethylating agents and maximize effects on the oHSV itself, we also administered the two together intratumorally instead of administering the drug systemically. Local administration of agents in humans with malignant gliomas is possible via polymers, endovascular catheterization, or convection-enhanced delivery. Nevertheless, although the in vivo data confirms that the combination of oHSV and demethylating agent therapy was beneficial, it is possible that the mechanism for the observed in vivo benefit may be different than direct enhancement of rQNestin34.5 replication by demethylation of the Nestin promoter–enhancer.

The discovery of significant in vitro pharmacologic synergism in glioma cells when rQNestin34.5 was combined with 5-Aza extended the finding of a significant survival effect in mice with intracranial gliomas treated with the combination. When the affected cell fraction was low (<0.3), there was some evidence for antagonism. This could indicate a situation where low doses of demethylating agent may be stimulating glioma cell proliferation (i.e., by demethylation of oncogenes) while the replicative kinetics of the low initial dose of oHSV have not had sufficient time to generate sufficient numbers of viral progeny to “catch up” with the proliferating tumor cells.

Decitabine is being widely used in clinical trials for subjects afflicted with a variety of cancers, and our findings may provide justification for further exploration of the combination of decitabine with rQNestin34.5. We are in the process of filing an investigational new drug (IND) use for rQNestin34.5 for a phase 1 clinical trial in patients with recurrent glioma. The findings from this report indicate that further biodistribution and toxicity data in animals should be carried out to evaluate if decitabine can be justifiably added to rQNestin34.5 in subsequent clinical trials, particularly because demethylating agents could reactivate latent viruses. In addition, the schedule of demethylating agent treatment with oHSV will need further detailed analysis.

There have now been several instances/reports of drugs that can be used to pharmacologically enhance viral oncolysis (35). The mechanism of action of such pharmacologic enhancement is dependent on the drugs’ mode of action. For instance, some will modulate the immune system to reduce immune cell–mediated clearance of injected OVs or OV-infected tumor cells. Other drugs will upregulate tumor cell pathways that can be also used by the OV for improved replication. Still others will modulate the tumor microenvironment to facilitate OV distribution or survival. In this context, the findings in this report represent the first example of a drug that acts directly on the viral genome to improve its antitumor effect. As the field of epigenetic therapy progresses, it will be interesting to determine if other agents that act on other aspects of the epigenome will also modulate OV DNA to improve its replicative ability.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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