Cidofovir: A Novel Antitumor Agent for Glioblastoma

Piotr Hadaczek1, Tomoko Ozawa2, Liliana Soroceanu1, Yasuyuki Yoshida2, Lisa Matlaf1, Eric Singer1, Estefania Fiallos1, C. David James2, and Charles S. Cobbs1,2

Abstract

**Purpose:** Cidofovir (CDV) is an U.S. Food and Drug Administration (FDA)-approved nucleoside antiviral agent used to treat severe human cytomegalovirus (HCMV) infection. Until now, no clear therapeutic effects of CDV have been reported outside of the setting of viral infection, including a potential role for CDV as an antineoplastic agent for the treatment of brain tumors.

**Experimental Design:** We investigated the cytotoxicity of CDV against the glioblastoma cells, U87MG and primary SF7796, both in vitro and in vivo, using an intracranial xenograft model. Standard techniques for cell culturing, immunohistochemistry, Western blotting, and real-time PCR were employed. The survival of athymic mice (n = 8–10 per group) bearing glioblastoma tumors, treated with CDV alone or in combination with radiation, was analyzed by the Kaplan–Meier method and evaluated with a two-sided log-rank test.

**Results:** CDV possesses potent antineoplastic activity against HCMV-infected glioblastoma cells. This activity is associated with the inhibition of HCMV gene expression and with activation of cellular apoptosis. Surprisingly, we also determined that CDV induces glioblastoma cell death in the absence of HCMV infection. CDV is incorporated into tumor cell DNA, which promotes double-stranded DNA breaks and induces apoptosis. In the setting of ionizing radiotherapy, the standard of care for glioblastoma in humans, CDV augments radiation-induced DNA damage and, further, promotes tumor cell death. Combination therapy with CDV and radiotherapy significantly extended the survival of mice bearing intracranial glioblastoma tumors.

**Conclusion:** We have identified a novel antiglioma property of the FDA-approved drug CDV, which heightens the cytotoxic effect of radiotherapy, the standard of care therapy for glioblastoma. Clin Cancer Res; 19(23); 6473–83. ©2013 AACR.

Introduction

Glioblastoma is the most common primary brain tumor in adults, and despite advances in our understanding of glioblastoma biology and the use of these molecular insights to test innovative approaches for treating glioblastoma, the mean patient survival in glioblastoma remains 12 to 15 months (1). We hypothesized that persistent viral infection plays a role in the pathogenesis of glioblastoma, given the extensive results from our laboratory and others demonstrating that human cytomegalovirus (HCMV) infection is present in a majority of human glioblastomas (2). Several HCMV gene products that are expressed in human glioblastomas are thought to contribute to tumor pathogenesis, including the expression of the HCMV IE1 protein, which has been associated with length of patient survival (3–7). Furthermore, improved survival was recently reported for patients with glioblastoma who were treated with the oral antiviral drug valganciclovir, when used in combination with the standard of care treatment for this cancer, radiation and temozolomide, suggesting that anti-HCMV therapy might provide clinical benefit to patients of glioblastoma (8).

To determine whether the inhibition of HCMV gene expression impacts the biology of glioblastoma, we used the antiviral agent cidofovir (CDV), an acyclic nucleoside phosphonate analog with a broad-spectrum antiviral DNA synthesis activity that has been approved for the treatment of CMV retinitis in patients with AIDS (9, 10). In addition, CDV has proved effective for the treatment of other DNA virus–related diseases including the central nervous system disease progressive multifocal leukoencephalopathy (PML; ref. 11). The antiviral activity of CDV is thought to result from the selective inhibition of viral DNA polymerase.
Radiation (28). “Radio-sensitiser” role for CDV in the setting of ionizing radiation-damaged DNA may activate tumor cell apoptosis (26, 27). CDVpp incorporation into secondary rounds of DNA synthesis (24, 25). Because of and gemcitabine) block tumor growth by inducing DNA synthesis and inhibit tumor cell proliferation independent of endothelial cell apoptosis (13, 21). This effect was associated with S-phase arrest, PARP activation, p53 upregulation, and caspase activation (22). The latter study results, therefore, suggest that CDV can inhibit cellular DNA synthesis and inhibit tumor cell proliferation independent of endogenous viral presence, potentially by incorporation into cellular DNA as a substitute for dCTP, as demonstrated in the case of HPV-infected human keratinocytes (23). In the setting of virus-driven oncogenesis, CDV has been shown to inhibit cancer growth in various in vivo models, including those involving human tumor xenografts (Epstein–Barr virus-associated nasopharyngeal carcinoma and human papillomavirus (HPV)–induced cervical carcinoma) and polyomavirus-induced rodent tumors (hemangiomatas in rats as well as hemangiosarcoma development in mice; refs. 16–20). In addition, hemangiomatas tumors are inhibited by CDV, independent of viral infection, by induction of endothelial cell apoptosis (13, 21). This effect was associated with S-phase arrest, PARP activation, p53 upregulation, and caspase activation (22). The latter study results, therefore, suggest that CDV can inhibit cellular DNA synthesis and inhibit tumor cell proliferation independent of endogenous viral presence, potentially by incorporation into cellular DNA as a substitute for dCTP, as demonstrated in the case of HPV-infected human keratinocytes (23). Although most cytosine nucleoside analogs (e.g., ara-C and gemcitabine) block tumor growth by inducing DNA chain termination, CDV appears to act by inhibiting secondary rounds of DNA synthesis (24, 25). Because of this, CDV may have unique potential as a radiation-sensitizing agent (26, 27). CDVpp incorporation into radiation-damaged DNA may activate tumor cell apoptotic response mechanisms. Indeed, the literature supports a “radio-sensitiser” role for CDV in the setting of ionizing radiation (28).

In order to determine whether CDV inhibits glioblastoma cell growth, as well as to address viral dependency of observed antitumor effects, we conducted experiments to evaluate CDV effects on glioblastoma cell lines and primary explant cultures, both in the presence or absence of HCMV infection. To determine how growth-inhibitory effects of CDV interact with ionizing radiotherapy, we extended our experiments to include in vivo analysis of combination therapy with CDV + radiotherapy, using orthotopic glioblastoma xenograft models. Our data indicate a previously unrecognized potent anti-glioma effect of CDV, which acts in combination with radiotherapy for increased tumor cell kill through increased apoptotic response.

Materials and Methods

Primary glioblastoma samples and neurosphere growth assays

Tissue samples were obtained during surgery from patients diagnosed with glioblastoma using an Institutional Review Board (IRB)-approved protocol. Fresh tumor tissues were subjected to enzymatic digest, mechanically dissociated, and cultured as neurospheres as previously described by our group (29). For growth assays, cells were cultured in neurosphere media (Neural Basal Media + EGF/FGF2) at 1,000 cells/well in 96-well plates. Media was replaced every other day, with or without CDV (100 μmol/L, where indicated) and tumor cells were monitored using an inverted microscope fitted with a camera. At 96 to 120 hours following initial culturing, spheres were photographed and counted. All sample incubations were in quadruplicate. Experiments were repeated twice for each primary culture tested.

Apoptosis Antibody Arrays

Proteins were extracted from glioblastoma cultures, treated with or without CDV for 48 hours, and assayed with the Human Apoptosis Array Kit (R&D Systems) according to the manufacturer’s instructions.

Protein extraction for Western blot and Western blot analysis are described in the Supplementary Materials.

Cell culture, infection, treatment, and viability assays are described in the Supplementary Materials.

RNA extraction and real-time PCR are described in the Supplementary Materials.

Incorporation of 14C-CDV into cellular DNA

U87MG cells and MRC-5 fibroblasts were grown in Minimum Essential Medium (MEM) supplemented with 10% FBS and penicillin/streptomycin. Cells were seeded into T-25 flasks and allowed to reach approximately 50% confluence. One variant with MRC-5 was carried with the cells at 100% confluence (contact inhibition–no mitoses). Cells were incubated for 24, 48, and 72 hours with 2 nCi/5 ml of 14C-labeled CDV. In addition, three log-phase cultures of U87MG were treated with 5 Gy using a cesium-137 source (I.L. Shepherd & Associates) at a dose rate of 3.2 Gy/min. Immediately following irradiation, the medium was exchanged with fresh medium and 14C-labeled CDV.

Translational Relevance

CDV is an FDA-approved nucleoside analog used to treat severe human cytomegalovirus (HCMV) infection. Until now, no clear antineoplastic effects of CDV have been reported outside of the setting of viral infection, and no prior evidence has shown a role for CDV as an antineoplastic agent for glioblastoma. Our preclinical evaluation of CDV revealed its potent antitumor activity against glioblastoma, independently of the status of HCMV infection. CDV is incorporated into tumor cell DNA, thereby promoting double-stranded DNA breaks and triggering downstream apoptotic signaling. In vivo data with human glioblastoma intracranial xenografts showed that, when treatment with CDV was combined with radiation therapy, it significantly increased the survival of tumor-bearing mice. We believe that this finding may represent a new therapeutic avenue for treatment of glioblastoma.
Irradiated cells with corresponding control cultures were harvested after 2, 6, and 24 hours. Cultures were harvested at each time point and, after three washes, frozen cell pellets were shipped to Accium Biosciences (Seattle, WA) where DNA was isolated using a Qiagen PureGene Tissue Kit to remove unbound 14C-CDV. DNAs were dissolved in 100 μL of hydration buffer, and incorporation of 14C into cellular DNA was quantified by ultrasensitive accelerator mass spectrometry (AMS; Accium BioSciences, Seattle, WA).

Glioblastoma xenografts

U87MG cells were cultured as described earlier. Human glioblastoma primary tissue SF7796 was established and maintained as serially passaged subcutaneous xenografts in athymic mice. Both U87MG and SF7796 were modified by lentiviral infection for stable expression of firefly luciferase to enable in vivo bioluminescence imaging, as previously described (30). To prepare tumor cells from subcutaneous xenografts for intracranial injection, previously described protocols were used (31). Both SF7796 and U87MG cells were resuspended in Dulbecco’s modified Eagle medium (DMEM) at a concentration of 1 × 10^8 cells/mL.

Intracranial tumor establishment in athymic mice

Five- to 6-week-old female athymic mice (nu/nu, homozygous: Simonsen laboratories, Gilroy, CA) were housed under aseptic conditions, and received intracerebral tumor cell injection as previously described (31). Briefly, mice were injected with 300,000 cells (3 μL) into the right striatum using a 26-gauge needle. Animals were monitored daily and imaged 1 to 2 times weekly until they were euthanized when exhibiting significant neurologic deficit, or showed greater than 15% reduction from their initial body weight. There were 8 to 10 animals in each treatment group. CDV treatments were carried out by intraperitoneal (i.p.) injection (100 mg/kg), 3 times per week until the animals required euthanasia, as indicated by animal subject body condition. The vehicle used was sterile saline.

Bioluminescence monitoring of intracerebral tumor growth

In preparation for bioluminescence imaging (BLI), mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), then administered 150 mg/kg of luciferin (n-luciferin potassium salt; Gold Biotechnology) via i.p. injection. Ten minutes after luciferin injection, mice were examined for tumor bioluminescence using an IVIS Lumina imaging station (Caliper Life Sciences). Regions of interest, defined using Living Image software (Caliper Life Sciences), were recorded as photons per second per steradian per square centimeter (31, 32).

Mouse irradiation

Mice were anesthetized via inhalation of 2.5% isoflurane with 1 liter of oxygen per minute for 5 minutes before being positioned on an irradiation platform located 16.3 cm from a cesium-137 source (J. L. Shepherd & Associates). Their eyes, respiratory tracts, and bodies were protected with lead shielding. Mice received whole-brain irradiation (32) at a dose rate of 2.47 Gy/min until 2 Gy radiation for U87MG or 1 Gy radiation for SF7796 had been delivered. After irradiation, animals were monitored until recovery. Radiation treatment was initiated when tumors were in a log-phase growth, as determined by bioluminescence monitoring, with mice irradiated once daily for 5 consecutive days.

Tissue processing, immunohistochemistry, and quantification of staining

Within each treatment group of mice, 3 animals were euthanized under deep general anesthesia (sodium pentobarbital, 90 mg/kg i.p.) and brains were harvested for immunohistochemical (IHC) analysis. Detailed procedures are described in the Supplementary Materials.

Statistical analysis

PRISM 5, Version 5.03 (GraphPad Software) was used to conduct all statistical analyses (EC50 values, log-rank Mantel–Cox test for survival analysis, and the Student t test for tumor bioluminescence). The EC50 values with corresponding 95% confidence limits were compared using the unpaired Student t test. All data are presented as a mean ± SD. We considered P values of less than 0.05 as statistically significant.

Results

To investigate whether CDV inhibits HCMV-infected glioblastoma tumor cell growth, we treated primary patient-derived glioblastoma cells (passage zero) with CDV (20 μmol/L) for 72 hours following initial establishment of neurosphere cultures. Cells treated with CDV (Fig. 1A and B) displayed phenotypic changes suggestive of cell apoptosis. To investigate the mechanism underlying this response, we used an apoptosis antibody array to profile cell lysates from control and CDV-treated samples, with results indicating at least two apoptotic pathways being activated in the presence of CDV: p53 and Fas/TNFa-Trail (Fig. 1C). Results from the antibody array were quantified (Fig. 1D) and validated using Western blot analysis (Fig. 1E). Furthermore, the expression of HCMV genes (e.g., UL44) in this glioblastoma sample was inhibited by CDV treatment (Fig. 1E). We similarly examined the CD133+ (stem-like) cellular fraction from another glioblastoma sample, which was also positive for endogenous HCMV. CDV treatment inhibited tumor sphere formation for these CD133+ cells (Fig. 1B, white bars) as well as the expression levels of various viral genes (e.g., pp71) and cellular proteins that are important contributors to the pathogenesis of glioblastoma (e.g., Sox2, SCF). These latter data (Supplementary Fig. S1) suggest that the use of antiviral drug CDV inhibits the expansion of primary glioblastoma spheres by multiple mechanisms, including the activation of proapoptotic pathways, and the inhibition of self-renewal and mitogenic pathways.
To further investigate HCMV as a determinant of the glioblastoma cell response to treatment with CDV, we next utilized an isogenic model in which U87MG cells, with or without HCMV infection, were examined. The viability of infected cells was more affected by CDV than was that of the uninfected U87MG (Fig. 2A, \( P = 0.0059 \)). Importantly, however, the viability of uninfected cells was also reduced by CDV treatment (but not HCMV alone; Supplementary Fig. S2), suggesting that additional mechanisms, independent of CDV suppression of HCMV gene expression (which is demonstrated in Supplementary Fig. S3), underlie the proapoptotic effects of CDV. Cidofovir treatment of HCMV-infected U87MG and primary glioma stem cell culture (GSC) 387 resulted in significant inhibition of expression of several viral genes, including UL84, UL55 (gB), UL83 (pp65), and UL82 (pp71; Supplementary Fig. S3). Primary-derived glioblastoma culture SF7796 was found to be endogenously infected with HCMV (Supplementary Fig. S4), and was used for subsequent testing of CDV antitumor effects, as described further. In vitro superinfection of SF7796 with the HCMV TR strain (MOI = 0.5) further sensitized these cells to CDV (Fig. 2B, \( P = 0.034 \)). Thus, the results of the viability assay, from both an established glioma cell line (U87MG) and a patient-derived glioblastoma primary culture, demonstrate that CDV inhibits glioblastoma cell expansion, regardless of the presence or extent of HCMV infection. In the dose–response curve, the EC\(_{50}\) was significantly higher (635.3 \( \mu \)mol/L) in normal MRC-5 fibroblasts treated with CDV (Fig. 2A) than in both the glioblastoma cell lines tested (371.1 \( \mu \)mol/L for U87MG and 375.2 \( \mu \)mol/L for SF7796). Interestingly, when the fibroblasts were grown at confluence (no mitoses due to contact inhibition), the EC\(_{50}\) was dramatically increased (Supplementary Fig. S5A).

Because CDV is a nucleoside analog, we hypothesized that it might induce apoptosis by becoming incorporated into cellular DNA, leading to DNA injury and subsequent activation of programmed cell death. Further, we hypothesized that the combination of CDV with ionizing radiotherapy, which is the standard of care for glioblastoma, might act in concert to promote further tumor cell apoptosis. To test these hypotheses, we examined proliferating U87MG cells for the incorporation of \(^{14}\)C-labeled CDV into newly synthesized DNA, both in the presence and absence of 1.5 Gy irradiation. Cells were harvested at 2, 6, and 24 hours posttreatment, and DNA was extracted and analyzed using ultrasensitive AMS (Accium BioSciences). After a 2-hour incubation, \(^{14}\)C-labeled CDV incorporates into genomic DNA at a frequency of \(10^{-3}\) nucleotide per DNA base pair in both “CDV only” and “CDV + radiotherapy”-treated cultures. Analysis of additional time points revealed that the extent of CDV incorporation was time-dependent, but was
not significantly affected by radiotherapy (Supplementary Fig. S5B).

To test the effects of CDV on normal cells, MRC-5 fibroblasts were exposed to the same doses of 14C-labeled CDV as U87MG (without irradiation). The results showed that the glioblastoma cell line incorporates the drug into DNA at a much higher rate (Fig. 2C) than does MRC-5. In addition, when cellular divisions were halted by contact inhibition, the incorporation was minimal. This would indicate that CDV treatment primarily affects rapidly dividing cells.

To test the hypothesis that CDV causes cellular DNA damage, and that the combination of CDV plus...
radiotherapy promotes more robust DNA damage than either agent alone, we performed Western blot analysis with antibodies specific to proteins involved in DNA repair, using cell lysates from U87MG cells exposed to CDV, irradiation, or the two in combination. Analysis of cellular phospho-histone H2A.X, a sensitive indicator of double-stranded DNA breaks (33), showed that both CDV and radiotherapy induce H2A.X phosphorylation. This effect was dramatically enhanced when CDV was combined with radiotherapy (27.6- or 21-fold increase when compared with treatment with only radiation or CDV, respectively; Fig. 2D). Interestingly, when normal fibroblasts were treated with CDV, no induction of H2A.X phosphorylation was observed (Fig. 2E and F). However, when fibroblasts were previously infected with HCMV, exposure to CDV caused significant upregulation of activated H2A.X (sensitizing effect). HCMV infection alone (without CDV) caused only slight induction of H2A.X (Fig. 2E and F), in concordance with previous studies which demonstrated that HCMV can induce chromosomal and DNA damage (34).

In order to determine if the antitumor effects of CDV observed in cultured glioblastoma cells would translate to in vivo conditions, we used a mouse intracranial glioblastoma xenograft model. Our initial in vivo analysis was aimed at addressing the antitumor activity of CDV, as administered systemically, via intraperitoneal injection, to athymic mice in which human glioblastoma xenografts had been established by intracranial injection of tumor cells infected with HCMV, and/or that express HCMV gene products (Supplementary Fig. S4). As anticipated based on published work showing that virus-associated subcutaneous xenograft tumors responded to CDV treatment (13, 17), 100 mg/kg CDV administered to mice, 3 times weekly, either delayed or prevented intracranial tumor development, compared with mice treated with vehicle only (Fig. 3A and B). Importantly, CDV treatment significantly extended animal subject survival for each of the two xenograft models we tested (Fig. 3C and D).

No significant differences in cell proliferation were identified in the control versus CDV-treated SF7796 primary xenograft glioblastoma tumors after 2 weeks of treatment: Ki-67 proliferation index for mice receiving CDV was 28.9 ± 7.1%, versus untreated control tumors showing an average of 22.4 ± 6.0% cells positive for Ki-67 (Fig. 4A and B, P = 0.13). In contrast, IHC analysis of tumors from mice receiving treatment with CDV showed extensive apoptotic response of tumor cells, as indicated by DNA fragmentation revealed by TUNEL staining (Fig. 4A): 4.9 ± 0.5% for CDV-treated tumors versus 0.54 ±
Figure 4. Effect of CDV on human SF7796 xenografts in vivo. A, ex vivo immunohistochemical staining for Ki-67, TUNEL, and cleaved caspase-3 in xenografts treated with CDV. Results shown for Ki-67 are at 2 weeks, although similar results were obtained at other time points. Representative photomicrographs (control and week 2) of TUNEL and cleaved caspase-3 immunohistochemical staining similarly show increased numbers of positive cells in the tumors of mice treated with CDV (magnification × 20). The third column of photographs shows lower magnification (× 10) of the margin between invasive tumor and surrounding non-tumor tissues. Positive cells (red arrows) were detected only in tumor areas of CDV-treated mice. Size bars = 200 μm. B, statistical analysis of staining for Ki-67, TUNEL, and cleaved caspase-3 between treated (black columns) and control animals (white columns) is shown as means ± SD. *, P < 0.001. C, Western blot analysis of extracts from SF7796 xenografts treated with CDV. CDV treatment results in increased phosphorylation of the histone H2A.X at Ser139.
0.06% in controls (Fig. 4B, \( P < 0.0001 \)). Detection of apoptotic cells in treated tumors was also confirmed by staining for cleaved caspase-3 (Fig. 4A; 2.68 ± 0.25% vs. 0.17 ± 0.03% in untreated mice: \( P < 0.0001 \); Fig. 4B). Interestingly, the positive cells for TUNEL and cleaved caspase-3 were detected only within the tumor areas of CDV-treated mice (Fig. 4A, third column). All nontumor areas of treated and control animals were negative for both markers. In addition, CDV treatment inhibited expression of the late HCMV antigen in these endogenously infected primary glioblastoma cells (Supplementary Fig. S6). Furthermore, phospho-histone H2A.X was detected in the tumor lysates from mice treated with CDV, but not in tumors from untreated control mice (Fig. 4C).

Taken together, these data indicate that CDV induces apoptosis in proliferating glioblastoma cells by promoting DNA damage and, as a result, CDV treatment decreases tumor growth and extends animal subject survival. To address the importance of anti-HCMV effects of CDV in terms of tumoricidal activity, we repeated the experiment with U87MG cells, but this time without HCMV infection of U87MG prior to implantation. Consistent with our in vitro results, substantial antitumor effect from CDV treatment was evident (Fig. 5A and B), suggesting that CDV antitumor activity is not dependent on, and is not necessarily affected by presence of virus.

Our next in vivo experiment was conducted to evaluate the antitumor effect of CDV when administered in...
combination with radiotherapy. For U87MG-derived tumors without HCMV infection, comparison of group bioluminescence values revealed substantial antitumor effect from radiotherapy or CDV treatment alone. Although combination therapy further suppressed tumor growth (Fig. 6A) and increased animal survival, as indicated by comparison of group mean values and in relation to corresponding monotherapies, the effects of combination therapy were not significantly different relative to the effects of monotherapy (Fig. 6B).

A repeat of the combination therapy experiment with SF7796-derived tumors (primary-derived glioblastoma, endogenously positive for HCMV expression; Supplementary Figs. S4 and S6) showed that combined treatment significantly extended survival compared with either monotherapy (Fig. 6C and D). Therefore, based on the results of two distinct intracranial xenograft models, we conclude that combination CDV and radiotherapy has the most substantial antitumor effect.

Discussion

Here, we show for the first time that CDV, a U.S. Food and Drug Administration (FDA)-approved drug for the treatment of HCMV infection, possesses potent antitumor properties against glioblastoma. We found that CDV inhibits HCMV viral gene expression in HCMV-infected primary glioblastoma tumors derived from patient biopsy specimens, but that viral gene expression was not required for CDV antitumor activity. Our data indicate that CDV becomes incorporated into the DNA of proliferating tumor cells, which likely initiates DNA double-stranded breaks and/or stalling of the DNA replication fork, leading to tumor cell apoptosis. When CDV treatment was combined with ionizing radiation, we observed a dramatic increase in phosphorylation of histone H2AX, a sensitive indicator of DNA double-stranded breaks, thereby showing that the DNA-damaging effects of radiotherapy are exacerbated by CDV.

The current paradigm for the mechanism of action of CDV in treating various types of virus-associated diseases suggests that this nucleoside analog becomes preferentially incorporated into viral DNA (35). The interpretation of preferential antiviral effect is based on results indicating that the viral DNA polymerase, rather than human DNA polymerase, is selectively inhibited. In the case of the antiviral drug ganciclovir, a thymidine nucleoside analog, there is clearly a greater specificity for inhibition of HCMV DNA synthesis, rather than cellular DNA synthesis, because the activity of ganciclovir depends on viral thymidine kinase for its conversion to the active metabolite that is incorporated into viral DNA as a chain terminator (36). In contrast, CDV does not depend upon a viral gene product to become phosphorylated, but rather is phosphorylated by the human cellular cytidine kinase enzyme. Thus, there is no virus-specific activity required for conversion of CDV to an active state. An important issue is the safety of CDV treatment to normal cells of a patient. Our data strongly suggest that CDV primarily affect rapidly dividing cells, sparing cells that replicate their DNA at a lower rate. The incorporation of CDV molecules into newly synthesized DNA is, therefore, dependent on the mitotic rate (23). No apoptotic cells were detected outside the tumor tissue of treated mice, and normal fibroblasts grown in contact inhibition did not incorporate toxic levels of CDV.

The current literature suggests that, as opposed to other cytosine analog antineoplastic agents such as ganciclovir, CDV does not cause direct DNA-chain termination upon incorporation into cellular DNA, but likely promotes stalling of the DNA replication fork (37). DNA fork stalling could lead to ‘futile cycling’ of the replication fork or of DNA double-strand break repair. In either case, if the DNA replication process is not sustained, affected cells may undergo programmed cell death.

By the addition of further DNA insult, such as ionizing radiation, we hypothesized that the cellular apoptotic response would increase. In vitro data revealed a dramatic increase (>21-fold) of a phosphorylated histone H2AX, indicating DNA injury/instability after exposure to both CDV and ionizing radiation, and that this effect resulted in reduced tumor growth in vivo, which, in turn, results in extending animal subject survival.

Further investigation is needed to address the extent to which the antitumor effect of CDV is due to a direct anti-HCMV effect versus an effect on cellular DNA synthesis, repair, and apoptotic signaling. HCMV infection seems to sensitize cells (both tumor and normal) to CDV treatment. The underlying mechanism is not fully understood; however, this and other studies show that during infection, host cells activate DNA damage signaling pathways (phosphorylation of H2AX; refs. 38–40). Using HCMV-infected glioblastoma cells (U87MG and SF7796), we determined enhanced in vitro tumor cell CDV sensitivity with the presence of the virus. The impact of HCMV gene expression may play a significant role in the pathogenesis of glioblastomas (3, 6, 7, 41) and medulloblastomas in vivo (42). Carro and colleagues, for example, have demonstrated that activation of the STAT3 signaling pathway is a critical event in the progression of proneural glioblastoma to the highly aggressive mesenchymal subtype of glioblastoma (43). We as well as others, have shown that the expression of HCMV IE1 and US28 gene products promote STAT3 signaling (3, 4, 6). Additional studies using endogenously infected glioblastoma tissues, measuring the effects of CDV and related compounds on HCMV-induced growth-promoting signaling are underway in our laboratory.

Regardless of the issue concerning the roles and effects of HCMV in glioblastoma oncogenesis, our results show that CDV is an effective drug for interfering with DNA synthesis, both in the presence and absence of HCMV. Its incorporation into cellular DNA activates DNA damage-response pathways due to increased DNA breaks, which prompts elevated tumor cell apoptotic response. Given the mode of cell killing by CDV, its use in combination treatments with radiation represents a promising and practical strategy to
improve treatment outcomes for patients of glioblastoma. Enhanced radiation sensitivity in HPV-positive head and neck cancer has been recently shown (44). Studies with analogs of CDV having more favorable pharmacokinetics are underway.

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

Authors' Contributions
Conception and design: P. Hadaczek, T. Ozawa, L. Soroceanu, C.D. James, C.S. Cobbs
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Hadaczek, L. Soroceanu, Y. Yoshida, L. Matlaf, E. Singer, E. Fiallos, C.D. James, C.S. Cobbs
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Hadaczek, T. Ozawa, L. Soroceanu, Y. Yoshida, E. Singer, E. Fiallos, C.D. James, C.S. Cobbs
Writing, review, and/or revision of the manuscript: P. Hadaczek, L. Soroceanu, C.D. James, C.S. Cobbs

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Hadaczek, C.D. James
Study supervision: P. Hadaczek, T. Ozawa, L. Soroceanu, C.D. James, C.S. Cobbs

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