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METHODS: ORIGINAL ARTICLE

Toxicology and Biodistribution Studies for MGH2.1, an Oncolytic Virus that Expresses Two Prodrug-activating Genes, in Combination with Prodrugs

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MGH2.1 is a herpes simplex virus type 1 (HSV1) oncolytic virus that expresses two prodrug-activating transgenes: the cyclophosphamide (CPA)-activating cytochrome P4502B1 (CYP2B1) and the CPT11-activating secreted human intestinal carboxylesterase (shiCE). Toxicology and biodistribution of MGH2.1 in the presence/absence of prodrugs was evaluated in mice. MGH2.1 ± prodrugs was cytotoxic to human glioma cells, but not to normal cells. Pharmacokinetically, intracranial inoculation of MGH2.1 ± prodrugs was cytotoxic to human glioma cells, but not to normal cells. MGH2.1 DNA was detected in brains of mice inoculated with 108 pfus for up to 60 days. However, only one animal showed evidence of viral gene expression at this time. Expression of virally encoded genes was restricted to brain. Intracranial inoculation of MGH2.1 did not induce lethality at 108 pfus in the absence of prodrugs and at 109 pfus in the presence of prodrugs. This study provides safety and toxicology data justifying a possible clinical trial of intratumoral injection of MGH2.1 with peripheral administration of CPA and/or CPT11 prodrugs in humans with malignant gliomas.

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Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive type of brain tumor with the median survival of 14.6 months for newly diagnosed patients. Considering that the median age of onset is 64 years, it is a sobering that the 5-year survival rate is reported to be under 5% among patients of 65 years of age or older. The current standard therapy for newly diagnosed glioma patients consists of maximum safe surgical resection of tumor mass followed by concomitant or sequential radiation and chemotherapy with temozolomide. Despite this aggressive multimodal treatment, the prognosis of most GBM patients remains poor and recurrence is almost inevitable with median survival of 5.0 months. Treating recurrent GBM is even more difficult than the newly diagnosed cases since the second surgical resection is not always feasible and in many cases, the tumor develops resistance to radiation and chemotherapy.

GBM is a disease caused by the accumulation of genetic mutations resulting in uncontrollable proliferation of cells, which are also highly heterogeneous. Recent advances in genomics, proteomics, and bioinformatics provide a vast amount of information underlying molecular mechanisms of GBM initiation and progression, leading to development of gene-targeted therapies. The three critical pathways involved in GBM are the RTK/RAS/PI3K pathway, the p53 pathway, and the CDK/cyclin/CDK inhibitor/RB pathway. These have been found to be disrupted in more than 75% of GBM samples. Considering the complex and compensatory nature of the signaling, therapeutic targeting of a single molecule, or even of an entire pathway, is not likely to achieve clinically significant or meaningful anticancer effects. In this context, oncolytic virus-based gene therapy emerges as a potentially attractive platform by combining the viral replicative lytic capacity for tumors with additional transfer of genes that mediate anticancer effects. For GBM therapy, herpes simplex virus (HSV), adenovirus, measles, and reovirus have been extensively studied and their safety and efficacy have been evaluated in Phase 1 clinical trials. HSV type 1 (HSV1) oncolytic viruses possess several advantages for brain tumor treatment, namely, its capacity to accommodate several transgenes, its natural neurotropism, its high infectivity for and rapid lysis of cancer cells, the relative ease of genetic manipulation, and the availability of antiviral drugs to prevent unintentional viral spread. Very attenuated oncolytic HSVs (oHSV) have been well tolerated in Phase I.
clinical trials for GBM. However, clinically significant efficacy remains elusive, likely because the current clinical generation of oHSV is attenuated and compromised in immune evasion ability and in the degree of replicative cytotoxicity.\textsuperscript{13,15,16}

Newer generations of viral vectors have been developed to improve efficacy without compromising safety. MGH2 was originally engineered from wild HSV1 virus F strain:\textsuperscript{17} in addition to a deletion of the viral UL39 transgene that provides replicative selectivity for cells with p16 tumor suppressor defects\textsuperscript{18} and to deletions of both copies of the ICP34.5 neurovirulence genes \textit{(i.e.,} thereby rendering it genetically similar to G207, an oHSV tested in several clinical trials, refs. 19,20), MGH2 also contains two transgene transcriptional units encoding two different prodrug-activating genes: rat cytochrome P4502B1 (CYP2B1)\textsuperscript{21} and secreted human intestinal carboxylesterase (shiCE).\textsuperscript{22,23} CYP2B1 converts cyclophosphamide (CPA) into the active anticancer DNA-alkylating metabolite, phosphoramide mustard (PM), whereas shiCE converts irinotecan...
(CPT11) into the active topoisomerase I inhibitor, SN-38. MGH2 exhibited significant activity against human glioma cells both in vitro and in vivo that was enhanced by the addition of CPA and CPT11. CPA is an alkylating agent used in cancer treatment with dose-dependent biological activity as a cytotoxic and immunosuppressive agent at high dose and antiangiogenic and immunostimulatory agent at low dose.\textsuperscript{24} CYP2B1 encodes hepatic CYP2B1, an extensively studied prodrug-activating enzyme, which converts CPA to its anticancer metabolite PM.\textsuperscript{25} PM acts as a DNA cross-linking agent,\textsuperscript{26} altering DNA structure, and resulting in apoptotic cell death. CPA can also function as an immunomodulator that enhances HSV replication through inhibition of antiviral natural killer cell and mononuclear cell responses.\textsuperscript{27–29} Irinotecan is also widely used in cancer treatment and activated by carboxylesterase (CE) into SN-38, a potent DNA topoisomerase I inhibitor.\textsuperscript{30} The efficacy of irinotecan has been reported to be enhanced when combined with other anticancer drugs in patients with glioma.\textsuperscript{31} The human intestinal form of CE expresses a truncated carboxyl terminus to enable the extracellular secretion of the drug on the surrounding noninfected cells. (P. Potter, unpublished results). MGH2.1 in combination with CPA/CPT11 exerts its anticancer effects through four distinct modes of action: (i) immunomodulation by CPA improves HSV replication; (ii) transgene-mediated activation of CPA and CPT11; (iii) direct HSV replication and cytotoxicity; and (iv) bystander effect of cytotoxic metabolites released from infected/lysed cells. We have previously shown that oncolytic virus-mediated activation of the prodrugs, CPA and/or CPT11, produced more in vitro cytotoxicity against glioma cells and led to significantly increased survivability of mice harboring brain glioma xenografts, when compared with treatment with prodrugs alone.\textsuperscript{17} In order to provide data related to this strategy’s toxicology, safety, and biodistribution, we report experiments designed to show that mice tolerate the combination of HSV and two prodrugs well. As part of the effort to proceed into clinical trials, MGH2 was genetically modified to MGH2.1 by removing a green fluorescent protein (GFP) expression cassette from its genome, as described in the Materials and Methods section. These data, thus, justify a possible clinical trial of MGH2.1 in combination with CPA and CPT11 in patients with malignant glioma.

**Results**

**Effects of MGH2.1 with and without CPA/CPT11 toward human glioma and normal cells**

We first sought to establish the in vitro cytotoxicity of MGH2.1, CPA, and CPT11 at various dose levels in human astrocytes and three human glioma cell lines (Gli36, U87, and U251). MGH2.1 alone reduced the survival of all three glioma cell lines in a dose-dependent manner, but not that of human astrocytes, even at a multiplicity of infection (MOI) of 10 (Figure 1a). Each of the two prodrugs, CPA and CPT11, also reduced the survival of glioma cell lines but not that of human astrocytes (Figure 1b,c, respectively), in spite of their “prodrug” status perhaps because of incubation at 39.8 °C, when compared to controls. Because there was selective glioma cell cytotoxicity from the prodrugs alone at this high temperature, we next sought to determine if expression of the MGH2.1-encoded transgenes, CYP2B1 and shiCE, respectively, converted the prodrugs CPA and CPT11 in glioma and normal cells to provide additional cytotoxicity (Figure 1d). For glioma cells, doses of MGH2.1, CPA, and CPT11 were selected at MOI of 0.1, 250 µmol/l and 0.05 µmol/l, respectively. For human astrocytes, doses of reagents were increased to MOI = 10, 1,000 µmol/l of CPA, and 0.2 µmol/l of CPT11. In order to study the effect of prodrug conversion without the confounding variable of MGH2.1 replicative cytotoxicity, the next set of experiments were conducted utilizing...
the temperature shift method,\textsuperscript{38} where 4 hours after infection of glioma cells with MGH2.1, viral replication is stopped by raising the temperature from 37 to 39.8 °C, in the presence or absence of prodrugs. Five days later, cells were counted. In spite of the temperature-mediated viral replicative block, the 4-hour replicative infection of MGH2.1 at MOI = 0.1 still reduced the survival of the three established glioma cell lines compared with control by 20–30% (Figure 1d). The combination of CPA and CPT11 also reduced the survival of all three glioma cells. Finally, the combination of initial infection with MGH2.1 that was replicatively blocked and of CPA and CPT11 significantly reduced survival when compared with CPA and CPT11 alone for Gli36 and U251 but not U87 glioma cells (Figure 1d). In order to minimize unconverted prodrug cytotoxicity and to maximize the degree of infectivity of cells, we repeated the experiment by reducing the dose of prodrugs by half and increasing the dose of MGH2.1 to a MOI of 1 in the Gli36 and U251 glioma cells only. The data of Figure 1e show that the increased MOI of MGH2.1 also increased the relative cytotoxicity in spite of the temperature-mediated replicative block, when added alone. Again, we detected a significant increase (of about 70%) in cytotoxicity when replicatively blocked MGH2.1 was combined with both prodrugs when compared with prodrugs alone (Figure 1e). In agreement with our previously published findings, this provided evidence that MGH2.1 did lead to additional conversion of the two prodrugs in infected cells.\textsuperscript{17} We then tested the cytotoxicity of MGH2.1 (MOI = 1) ± CPA and/or CPT11 against five primary glioma cells. While the temperature shift-mediated replicative block of MGH2.1 infection at MOI = 1 did not significantly reduce the cell number of primary glioma cells other than an approximate 10% decrease in X12, its combination with either or both prodrugs significantly reduced cell numbers of all glioma cells ($P < 0.001$) (Figure 1f). We next sought to determine if MGH2.1 replication in the presence or absence of prodrugs was cytotoxic to normal cells. These experiments were conducted at 37 °C to allow for viral replication. Infection of MGH2.1 at MOI = 10 in the presence of the both prodrugs led to a very small reduction in survival rates in some (human fibroblasts and skeletal muscle cells) but not all human primary normal cells (astrocytes, hepatic, human umbilical venous endothelial cell, renal, and smooth muscle) and also did not affect mouse neurons (Figure 1g). These data thus showed that MGH2.1 in combination with prodrugs was well tolerated by normal cells.

**Prodrug metabolism**

CPA is metabolized by the hepatic cytochrome 450 system,\textsuperscript{25,39–42} whereas CPT11 is metabolized by serum CE\textsubscript{S}.\textsuperscript{23,43}

To determine if there were alterations in the normal metabolism of both prodrugs when administered systemically in the presence of intracranial MGH2.1, prodrug metabolite levels, SN-38 (CPT11 metabolite) and PM (CPA metabolite), were assayed in plasma, brain, and liver of Balb/C mice intracranially

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**Figure 2 Prodrug metabolite assay.** Concentrations of the prodrug, (a) CPA, and its metabolite, (b) PM, and of the prodrug, (c) CPT11, and its metabolite, (d) SN-38, were measured in plasma, brain, and liver of mice injected with MGH2.1 or mock in the presence of systemic CPA and CPT11. Viable MGH2.1 (1 × 10\textsuperscript{6} pfus) or heat-inactivated mock was inoculated into mouse brains, stereotactically. The next day, 2 mg of CPA and 2 mg of CPT11 was injected intraperitoneally, and blood, brain, and liver were collected at 10 minutes and 30 minutes, 1, 2, 4 and 6 hours after dosing. The concentrations of analytes are shown as ng/ml for the plasma samples and ng/g for brain or liver samples. Each plot represents the average of duplicate samples and standard deviations were indicated by error bars. Student’s t test was used for comparison between viable MGH2.1 and mock groups. CPA, cyclophosphamide; PM, phosphoramid mustard.
injected with 10^6 pfus of MGH2.1 or mock (heat-inactivated MGH2.1). The day after virus injection, both CPA and CPT11 were administered intraperitoneally (i.p.) at a dose of 2 mg in 100 µl phosphate-buffered saline (PBS). Animals were killed at 10 minutes, 30 minutes, 1, 2, 4, and 6 hours after prodrug injection, and blood, brain, and liver were immediately harvested and processed for measurements of metabolites by mass spectrometry (detailed procedure in Supplementary Materials and Methods). As expected, there were higher concentrations of both prodrugs and their metabolites in liver compared with plasma and brain (Figure 2). No significant difference in the levels of CPA, CPT11, PM, and SN-38 in plasma, brain, and livers, except for SN-38 levels in plasma (P = 0.0213, two-tailed paired t test) was observed between MGH2.1 and mock infection groups. Taken together, these results indicated that intracranial injection of MGH2.1 did not significantly alter the metabolism of systemic CPA and CPT11.

Detection of viral DNA and transcripts in brain and trigeminal ganglia

We then sought to determine presence of viral DNA and transcripts in brains and trigeminal ganglia (TG). Seven days after virus inoculation at a dose of 5 × 10^7 pfus, viral DNA polymerase was detected in all brain samples regardless of prodrugs. Interestingly, viral genomic DNA was also detected in TG in two of three animals treated with MGH2.1 + CPA, one of three animals treated with MGH2.1 + CPT11, and none of the animals treated with MGH2.1 alone (Figure 3a).

We then analyzed for presence of MGH2.1 at 60 days. After injection of MGH2.1 (1 × 10^7 or 1 × 10^8 pfus), viral DNA polymerase was still present in five out of five brains inoculated with 1 × 10^6 pfus of MGH2.1 and in three out of five brains inoculated with 1 × 10^8 pfus of MGH2.1 (Figure 3b). However, no viral DNA was detected in TG. Expression of viral latency-associated transcript (LAT) and of the two encoded transgenes (rat CYP2B1 and human shiCE) was evaluated by reverse transcription-PCR (RT-PCR) of brain and TG obtained from the mice, 60 days after MGH2.1 intracranial injection. Expression of LAT was detected in none of the five brains and one of five TGs from the lower MGH2.1 dose group, and one of five brains and two of five TG samples from the higher dose group (Figure 3c). The transgene shiCE transcript was expressed in one of the TGs that also expressed LAT in the higher dose group. However, no CYP2B1 transgene expression was observed in any group.

Systemic biodistribution of virally encoded transcripts and viral LAT after MGH2.1 intracerebral injection

We next proceeded to assay for the presence of MGH2.1-encoded transcripts in multiple organs after MGH2.1 inoculation in mice brains. Brains, liver, lung, heart, intestines, testis, spleen, lymph nodes, and TG of Balb/C mice intracranially injected with MGH2.1 (5 × 10^7 pfus) in the presence or absence of systemic prodrugs were harvested and RT-PCR for CYP2B1, shiCE, and LAT was carried out, 7 days after MGH2.1 injection (Figure 4). Since CYP2B1 expression is under control of the HSV1 IE 4/5 promoter
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Supplementary Figure S1a), its expression would indicate persistent viral infection in a tissue, unlike LAT which may indicate only latent virus. RNA extracted from U251 cells infected with MGH2.1 or wild-type F strain was utilized as positive and negative control, respectively. Distribution of transgenes (rat CYP2B1 and human shiCE), for the viral LAT gene and, for mouse GAPDH as a control was carried out in brains, liver, lung, heart, intestines, testis, spleen, lymph nodes, and TG. RNA from U251 infected with MGH2.1 or wild-type F strain was used as control. RNA after reverse-transcription was amplified for 35 cycles, and amplified products were separated by agarose gel electrophoresis. CPA, cyclophosphamide; CYP2B1, cytochrome P4502B1; LAT, latency-associated transcript; LN, lymph node; shiCE, secreted human intestinal carboxylesterase; TG, trigeminal ganglia.

Figure 4 Biodistribution of intracerebrally injected MGH2.1. MGH2.1 (5 × 10^7 pfus) ± CPA (intraperitoneally (i.p.) at 1, 3, 5, and 7 days) or CPT11 (i.p. at 1 day) was intracerebrally injected in mice and viral mRNA was isolated after 7 days. Reverse transcription-PCR for the two encoded transgenes (rat CYP2B1 and human shiCE), for the viral LAT gene and, for mouse GAPDH as a control was carried out in brains, liver, lung, heart, intestines, testis, spleen, lymph nodes, and TG. RNA from U251 infected with MGH2.1 or wild-type F strain was used as control. RNA after reverse-transcription was amplified for 35 cycles, and amplified products were separated by agarose gel electrophoresis. CPA, cyclophosphamide; CYP2B1, cytochrome P4502B1; LAT, latency-associated transcript; LN, lymph node; shiCE, secreted human intestinal carboxylesterase; TG, trigeminal ganglia.

Table 1 Serum chemokine levels in Balb/C after MGH2.1 intracerebral inoculation

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Reliable range (pg/ml)</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 60</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>120–3,850</td>
<td>87.44 (±201.39)</td>
<td>94.80 (±87.39)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>100.12 (±162.76)</td>
</tr>
<tr>
<td>IL-6</td>
<td>0–250</td>
<td>2.64 (±1.47)</td>
<td>13.52 (±5.33)</td>
<td>7.51  (±1.48)</td>
<td>4.82  (±8.04)</td>
<td>15.93 (±5.15)</td>
</tr>
<tr>
<td>IFN-α</td>
<td>30–500</td>
<td>18.32 (±1.28)</td>
<td>25.37 (±5.77)</td>
<td>34.34 (±18.61)</td>
<td>18.15 (±2.22)</td>
<td>28.47 (±25.27)</td>
</tr>
<tr>
<td>IFN-β</td>
<td>15–1000</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>15–250</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

Serum cytokines associated with acute inflammatory was analyzed by ELISA before and 3, 7, 14, and 60 days after MGH2.1 (5 × 10^7 pfus) inoculation into Balb/C mouse brain. Reliable ranges (pg/ml) were defined by standard curve (P < 0.05).

Lethality of MGH2.1 with and without prodrugs after intracranial injection

To further determine if there was evidence of severe clinical toxicity, a survival study was conducted. Intracerebral toxicity of MGH2.1 was compared with that of wild-type F strain in young (aged 8 weeks) Balb/C mice. Different doses of viruses (5 µl volume) were stereotactically injected into the white matter of the right hemisphere of mice. The body weight of animals was recorded weekly and the survival was followed for 9 weeks. MGH2.1 did not cause lethality at the highest dose tested (10^8 pfus), whereas lethality was evident with F strain at a dose as low as 10^4 pfus (Table 2). In fact, the calculated LD_{50} for F strain was 6.8 × 10^3 pfus, in agreement with published studies. There was some initial weight loss in mice inoculated with MGH2.1 at the higher inoculated doses, but weight returned to baseline within a few weeks after viral inoculation with weight gain ensuing thereafter.
was thus not calculable, that in combination with CPT11 was $1.24 \times 10^{10}$ pfus, while that in combination with both prodrugs was $3.53 \times 10^{10}$ pfus.

**Discussion**

We have previously shown that oncolytic virus-mediated activation of the prodrugs, CPA and/or CPT11, produced more *in vitro* cytotoxicity against glioma cells and led to significantly increased survivorship of mice harboring brain glioma xenografts, when compared with treatment with prodrugs alone.17 In this report, we show that the combination of MGH2.1, CPA, and CPT11 was cytotoxic to human glioma cells, including glioma “stem-like” cells and did not significantly affect the viability of a panel of normal human and mouse cells. Importantly, the intracerebral administration of MGH2.1, expressing the two prodrug-activating transgenes, also did not alter the endogenous metabolism of CPA and CPT11. MGH2.1 DNA and transcripts were largely confined to the brain after intracranial injection and did not end up in organs outside the central nervous system even in the presence of CPA and/or CPT11. MGH2.1 intracerebral injection alone was not associated with lethality at doses up to $10^6$ pfus, while the combination of MGH2.1 (intracerebral) and systemic CPA + CPT11 was not associated with lethality at a dose of $10^6$ pfus. Therefore, these studies establish an animal toxicology and biodistribution set of findings that can be useful for further design of a clinical trial of MGH2.1 with and without CPA and/or CPT11 in humans with malignant glioma.

MGH2.1 provides multimodal therapeutics in the context of a single bioagent for a cancer, such as glioblastoma, where multiple genes are mutated and multiple signaling pathways are deregulated. Therefore, the multiple modes of tumor killing may potentially circumvent the tumor cell’s ability to escape from anyone single treatment. In fact, tumor cell toxicity arises from: (i) the direct cytotoxic action of MGH2.1 viral infection and lysis of tumors; (ii) the alkylation of tumor cell DNA by CPA’s activated metabolites that can also diffuse out and kill other tumor cells, even if MGH2.1 did not infect them;16 (iii) the inhibition of tumor cell topoisomerase I activity by CPT11’s activated metabolite that can also kill other noninfected tumor cells, by diffusion of the metabolite or of the prodrug-activating enzyme44; and (iv) the enhancement of MGH2.1 replication by the immunomodulatory effects of CPA.45 Results from the *in vitro* cytotoxicity studies show that MGH2.1, in combination with prodrugs, was more toxic to human glioma cell lines and primary glioma cells than MGH2.1 alone, providing evidence for the effectiveness of multiple mechanisms of tumor cell kill. It should be noted that the experiments shown in Figure 1d–f measure cytotoxicity mediated by only a 4-hour infection and replicative cycle of MGH2.1 due to the 37 to 39.8 °C temperature shift46 combined with virus-encoded transgene–mediated conversion of CPA and/or CPT11, whereas the experiments shown in Figure 1g measure cytotoxicity mediated by the fully replicative oncolytic virus with and without prodrugs. In this context, when multiple mechanisms of tumor cell toxicity are operative, one concern is that they can also lead to increased toxicity against normal cells. At least *in vitro*, this was not the case, as shown in Table 2, where no statistically significant differences in body weights between mice injected with $1 \times 10^6$ pfus of MGH2.1 versus those injected with mock.
case: even combined at their highest tested dose (MOI = 10 for MGH2.1, 1000 μmol/l for CPA, and 0.2 μmol/l for CPT11), there was no significant effect on human astrocyte viability or on the viability of a panel of normal cells. Therefore, in vitro the combination of MGH2.1 and its two prodrugs increases glioma cell cytotoxicity without affecting the viability of normal cells.

Both CPA and CPT11 are metabolized by endogenous enzymes. CPA is primarily metabolized by the hepatic cytochrome P450 system with rat CYP2B1 being the most active catalytic isoform for this reaction.25 CPA is first metabolized into the unstable intermediate 4-hydroxy CPA which spontaneously degrades into PM and acrolein.39 PM is the active anticancer agent that alkylates DNA and, thus, leads to DNA damage and cell death.40 CPT11 is metabolized by serum CE into its active anticancer metabolite, SN-38, that inhibits topoisomerase I, thus leading to DNA damage.36 In addition, a number of intracellular CEs can also catalyze this reaction and shiCE is such an isozyme.22,23,40 We had previously shown that the active metabolites of CPA (4-hydroxy CPA and its metabolites)45 and that of CPT11 (SN-38) were cytotoxic to glioma cells.17 In addition, expression of CE and CYP2B1 in the context of the oHSV1, MGH2 (i.e., MGH2.1 with a GFP transgene) was highly effective against intracranial gliomas in mice when combined with systemic CPA and CPT11.17 The rationale for this is that, in spite of endogenous bioconversion of the two prodrugs, there was still sufficient prodrug not being converted that could be activated locally in tumor by MGH2. Evidence for this mechanism was shown previously where local conversion of CPA by a replicating HSV expressing CYP2B1 was shown.55 However, one concern may be that the local intratumoral bioconversion of the two prodrugs could alter or change systemic endogenous pharmacokinetics. The data in this paper show that intracranial inoculation of MGH2.1 or mock followed by i.p. injection of CPA and CPT11 into immunocompetent Balb/C mice did not alter the pharmacokinetics of prodrugs and their metabolites in brain, serum, and liver. This suggests that MGH2.1 replication/infection is minimal in naive brains and the observed expression of shiCE and CYP2B1 (Figure 4) would not be meaningful enough to alter the endogenous metabolism of the two prodrugs. Although the same amount of CPA and CPT11 were injected, the concentrations of CPA and its metabolite PM were higher than those of CPT11 and SN-38 by an order of magnitude in brain, which likely reflect the differences in regard to half-life of the prodrugs and metabolites, ability of prodrugs to cross brain–blood barrier, and enzyme activity. Therefore, the endogenous kinetics of prodrug metabolism were not altered by the injection of virus in brains.

In terms of immune reactions, intracranial injection of MGH2.1 in immunocompetent Balb/C did not elicit induction of serum cytokines (TNF-α, IFN-α, IFN-β, or IFN-γ), and only a marginal increase of IL-6 was detected at the various time points. We do anticipate that MGH2.1 administration may induce anticancer immune response through viral replication followed by cell lysis in tumor-bearing animals. Modulating host immunity in viral cancer therapy is tricky. Antiviral immune response should be suppressed until infection and replication is established in target tumor cells. At this juncture, antitumor immune response should be induced by viral molecules produced upon successful viral replication, such as dsRNA, and/or processed antigens specific to virally infected cells. CPA, an alkylating agent widely used in various cancer treatments, has also immunosuppressive effect and has been shown to improve replication of oHSV in tumor cells both in vitro and in vivo.9,27–29,31–33,46–47 We observed that there was an expected CPA effect on enhancement of in vitro cytotoxicity of MGH2.1 in the present study, as well.

Seven days after intracranial virus injection, viral genomic DNA was observed in all brain samples regardless of prodrug administration. In some of prodrug-treated animals, TG also exhibited viral DNA (Figure 3a). This shows that prodrugs (likely CPA) may enhance the ability of virus to travel to TG, where the virus likes to harbor in a latent state. By 60 days after virus injection without prodrug treatment, brains, but not TG, still harbored viral genomic DNA (Figure 3b). HSV1 is thought to establish latency in TG in both human and mouse. Intracerebrally injected HSV was observed to spread via retrograde transport to neurons,45–48 RNA and DNA of LAT and viral genes were detected in TG of mice latently infected HSV1 at 60 days after infection, although there was no conclusive data regarding their replication.51 Systemic dissemination of intracranially administered MGH2.1 was assayed in naive immunocompetent Balb/C mice by Q-PCR. Various organs were harvested from animals killed 7 days after virus injection. All brain samples expressed transgenes and LAT genes regardless of prodrug treatment. Other than brain tissue, only one TG harvested from CPA-treated animal exhibited a LAT transcript. Significantly, there was no evidence of viral gene expression in other organs, even in CPA-treated animals. The lack of transcript expression in organs other than the brain (and TG) and the lack of the CYP2B1 transcript that is under control of the HSV IE4/5 promoter in the brain at 60 days (Figure 3c) argues that MGH2.1 transcripts would be unlikely to be found in organs such as liver at 60 days. The lack of oHSV dissemination in the presence of CPA is consistent with a previous result that pretreatment of CPA did not cause systemic distribution of oHSV1.32,33,46,52 In these studies, we did not observe viral gene expression even in CPA-treated TG. However, samples were harvested only 12 hours after virus injection into the intracranial tumor mass, suggesting that time may have been too short for the virus to travel to TG. Taken together, our study along with previously cited reports confirms that oHSV injected in brains of mice remains localized to the central nervous system even in the presence of prodrugs that are activated by the oHSV.

The primary objective of this study was to show the safety profile of MGH2.1 with/without its prodrugs upon intracerebral injection. Previously, we have shown its efficacy in animal models of glioma.17 The LD₅₀ for MGH2.1 in combination with CPA was not reached, and the LD₅₀ for the virus in combination with CPT11 was calculated as 1.24 × 10⁴ pfus, which is not achievable. MGH2.1 intracerebral administration by itself was not lethal to mice at the highest tested dose (10⁸ pfus), but we did establish an LD₅₀ when it was combined with systemic administration with prodrugs at a dose of 3.53 × 10⁸ pfus. However, we were able to find a dose (10⁶ pfus) that was not associated with animal death. In our previously published study,17 this dose was effective in an animal model of glioma and, thus 10⁵ pfus is the No Observed Adverse
Event Level dose in mice. This, thus, provides a starting dose in an eventual Phase I clinical trial for human subjects with malignant gliomas taking into account differences in brain weight. There was some dose-dependent weight loss in mice intracranially injected with MGH2.1 at the highest dose and systemic produgs exacerbated this adverse effect, but this was a transient effect with animal weight gain returning to the original level by a few weeks after surgery. Since the PBS-injected control group also showed some weight loss, albeit to a lesser degree, a portion of this adverse effect can be attributed to the anesthesia and surgery.

In summary, these studies provide feasibility data related to the toxilology and biodistribution of this novel oHSV in combination with its produgs in preparation for an eventual clinical trial.

Materials and methods

Engineering of MGH2.1. MGH1 is a double-mutant oHSV1 derived from wild-type F strain containing deletions of both copies of ICP34.5 and an insertion in UL39.13 To enhance its anticancer therapeutic efficacy, two transgene cassettes encoding the prodrug-activating enzymes, rat CYP2B1 and shiCE, were integrated into MGH1 and designated as MGH2. CYP2B1 and shiCE convert CPA and irinotecan (CPT11) into their active metabolites, PM and SN-38, respectively. For potential clinical trials, the GFP transgene cassette was removed from the MGH2 sequence due to its possible immunogenicity (Supplementary Figure S1a). Details of the engineering are described in Supplementary Materials and Methods. This new oHSV was designated as MGH2.1 and three different isolates (1-1-5, s1-2-6, and 1-2-8) were initially plaque-purified. Of the three MGH2.1 isolates, 1-2-6 was selected because it showed the highest CE activity, the retention of the transgene cassettes, and the deletion of the GFP expression cassette. To confirm the structural stability of transgenes in its genome, MGH2.1 isolate, 1-2-6, was plaque-purified on Master Cell Bank cells B7 and N23 (obtained from William B Goins, University of Pittsburgh, Pittsburgh, PA) for 20 passages, viral DNA was extracted every five passages, and gross genomic structure was verified by Southern blot analysis by following published procedures (Supplementary Figure S1b). CYP2B1 and shiCE sequences were excised from the transfer plasmid pT-onIS-IE4/5-CMV-hiCEC and used as probes. A parental strain rHSVQ1 (rH) which lacks the transgenes CYP2B1 and shiCE, oriS-IE4/5-CMV-hiCEC and used as probes. A parental strain with glycine saline solution (10 mmol/l glycine, 137 mmol/l NaCl, 24.1 mmol/l KCl, 0.49 mmol/l MgCl2, and 0.68 mmol/l CaCl2, pH 3) followed by PBS to remove unattached viruses and fresh medium was then added. Cells were incubated at 37 °C in an atmosphere containing 5% CO2. In vitro virus cytotoxicity assay. Cytotoxicity was evaluated and reported as the fraction of cells surviving after oncolytic virus infection compared with those treated with vehicle (fractional cell ratio). The day before infection, 5×103 cells were plated onto 96-well plates in medium prepared following manufacturer’s instructions for normal primary cells, brain tumor stem cell medium consisting of Neurobasal medium (Invitrogen) supplemented with GlutaMAX (Invitrogen), B27 supplements (Invitrogen), 20 ng/ml of human recombinant basic FGF (Peprotech, Rocky Hill, NJ), and 20 ng/ml of human recombinant EGF (Peprotech). The day before infection, primary glioma sphere cells were trypsinized and seeded onto plates coated with poly-β-lysine (Invitrogen) containing brain tumor stem cell medium with reduced concentration of fibroblast growth factor and epidermal growth factor (5 ng/ml for each) to form monolayers. Primary human cells (astrocyte, pulmonary fibroblast, hepatocyte, renal epithelial cells, skeletal muscle cells, smooth muscle cells, and human umbilical venous endothelial cell: umbilical vein smooth muscle cells) and mouse neural cells were purchased from ScienCell Research Laboratories (Carlsbad, CA), and cultured in the corresponding medium following the manufacturer’s instructions. The primary normal cells used in this study were passaged no more than five times, and the aliquots were stored in Bambanker serum-free cell freezing medium (Wako Chemicals USA, Richmond, VA) at –80 °C until use. All cells were grown at 37 °C in an atmosphere containing 5% CO2.
lactate dehydrogenase released upon cell lysis with CytoTox96 Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI). For prodrug functional assays, cells were prepared in the same manner as described above. After 1-hour incubation in viral solution at 37 °C, fresh medium containing CPT11 (APP Pharmaceuticals, Schaumburg, IL) and/or CPA (Baxter, Deerfield, IL) at indicated concentrations was added. Viral replication was then stopped 3 hours later by transferring the plates to a 39.8 °C incubator. Cytotoxicity was measured 4 days later with CytoTox96 Non-Radioactive Cytotoxicity Assay kit. For some cell lines, cytotoxicity was measured by enumeration through a Coulter counter. The day before infection, 5 × 10^4 cells were seeded onto 12-well plates. Infection with the virus was measured by the CytoTox96 Non-Radioactive Cytotoxicity Assay kit. For some cell lines, cytotoxicity was measured by enumeration through a Coulter counter (Beckman Coulter, Indianapolis, IN). The day before infection, 5 × 10^4 cells were seeded onto 12-well plates. Infection, drug treatment, and incubation were performed in the same manner as described above. Cells were incubated for 5 days for dose–response assays and 4 days for prodrug functional assays and surviving cells were counted with a Coulter counter.

**Animal studies.** All animal studies were performed in accordance with guidelines issued by The Ohio State University Institutional Animal Care and Use Committee, utilizing an approved animal protocol. Viral inoculation and care of animals harboring virus were conducted in approved BSL2 laboratory rooms. BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA) or the National Cancer Institute (Frederick, MD). For neurotoxicity experiments, 8-week-old mice were anesthetized by i.p. administration of ketamine (100 mg/kg) and xylazine (20 mg/kg). Oncolytic virus was then stereotactically injected into the right frontal lobe of brain (2 mm lateral and 1 mm anterior to the bregma at a depth of 3 mm). PBS and ultraviolet-radiated MGH2.1 were used as negative and mock control, respectively. The survival time for each group was monitored for 60 days after virus injection and body weights were measured weekly. CPA (2 mg in 100 µl PBS) was administered i.p. at 1, 3, 5, and 7 days after virus injection, and CPT11 (2 mg in 100 µl PBS) was injected i.p. 1 day after virus injection.

**Metabolite assays.** CPA, CPT11, and their active metabolites, PM and SN-38, were measured with the API-3000 triple quadrupole mass spectrometer (ABSciex, Framingham, MA) in mouse plasma, brain, and liver tissue. Thirty-six Balb/C mice were divided into two groups which were injected intracranially with either mock or 10^6 pfus of MGH2.1. The next day, mice were treated with i.p. injections of CPA (2 mg) and/or CPT11 (2 mg). Blood, brain, and liver tissues were collected 10 minutes and 30 minutes, 1, 2, 4 and 6 hours after drug administration. Blood was collected into heparin tubes placed on ice, followed by 10 minutes centrifugation (6,000 rpm) at 4 °C. A 200-µl plasma aliquot was transferred into an Eppendorf tube with 20 µl of 2 mol/l semicarbazide in 50 mmol/l potassium phosphate buffer (pH 7.4) and frozen in dry ice. All samples were stored in −80 °C before analysis. Refer to Supplementary Materials and Methods for detailed descriptions of measurement conditions.

**PCR and RT-PCR.** Brain and organs were harvested from animals euthanized at 7 or 60 days after virus inoculation at the dose of 5 × 10^7 pfus (7 days) and 10^8 or 10^9 pfus (60 days). CPA or CPT11 was injected i.p. at 1, 3, 5, and 7 days (CPA) or 1 day (CPT11) after virus inoculation. A small piece of tissue excised from each organ was immediately placed into RNA later tissue storage reagent (QIAGEN, Germantown, MD) and stored at 4 °C until use, for a period up to 4 weeks. Viral genomic DNA was extracted from the tissues using QIAamp DNA Mini Kit (QIAGEN) following the manufacturer's protocol. Total mRNA extraction and first-strand cDNA synthesis was conducted using OneStep RT-PCR system (QIAGEN) following the manufacturer's protocol. Primer sequences and annealing temperatures are listed in Supplementary Table S1. The cDNA products of the reverse transcription reaction were denatured at 95 °C for 15 minutes followed by a 35-cycle PCR reaction (94 °C for 30 seconds, 56 °C or 63 °C for 30 seconds, and 72 °C for 2 minutes). PCR products were separated by agarose gel electrophoresis to confirm the size of products.

**ELISA assay.** Blood was collected from three Balb/C mice per each time point before and 3, 7, 14 and 60 days after MGH2.1 (5 × 10^7 pfus) inoculation into Balb/C mouse brain. Serum was separated by brief centrifugation and used for ELISA assays to quantify the concentration of TNF-α (Hycult Biotechnology, Uden, Netherlands), IL-6 (Cell Science, Canton, MA), IFN-α (Cell Science), IFN-β (Cell Science), and IFN-γ (Cell Science), following manufacturer's instructions.

**Statistical analyses.** For MGH2.1 dose effect analyses, a square root transformation was used to stabilize the variance. Dunnet's method was used to adjust for multiple comparisons. For dose effect of CPA or CPT11, linear models (analysis of variance) were used to compare cytotoxicity among groups and days. Bonferroni was used to control for type I error. For primary glioma cells and normal cells, t test (one-tailed, unequal variance) was performed.

**Supplementary material**

**Figure S1.** Engineering of MGH2.1.

**Figure S2.** Effect of intracerebrally injected MGH2.1 ± CPA and ±CPT11 on body weights of Balb/C mice.

**Figure S3.** In vitro functional assays of transgenes (shiCE and CYP2B1) in MGH2.1.

**Table S1.** List of primers utilized in study.

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