Brain Tumor Oncolysis with Replication-Conditional Herpes Simplex Virus Type 1 Expressing the Prodrug-Activating Genes, CYP2B1 and Secreted Human Intestinal Carboxylesterase, in Combination with Cyclophosphamide and Irinotecan

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Abstract

The treatment of malignant glioma is currently ineffective. Oncolytic viruses are being explored as a means to selectively lyse tumor cells in the brain. We have engineered a mutant herpes simplex virus type 1 with deletions in the viral UL39 and γ1,34.5 genes and an insertion of the two prodrug activating genes, CYP2B1 and secreted human intestinal carboxylesterase. Each of these can convert the inactive prodrugs, cyclophosphamide and irinotecan (CPT-11), into their active metabolites, respectively. This new oncolytic virus (MGI2) displays increased antitumor efficacy against human glioma cells both in vitro and in vivo when combined with cyclophosphamide and CPT-11. Importantly, cyclophosphamide, CPT-11, or the combination of cyclophosphamide and CPT-11 does not significantly affect oncolytic virus replication. Therefore, MGI2 provides effective multimodal therapy for gliomas in preclinical models when combined with these chemotherapy agents. (Cancer Res 2005; 65(15): 6850-7)

Introduction

The treatment of malignant glioma continues to be problematic with 5-year survival rates still <5% (1). However, a variety of experimental approaches have been tested both preclinically and clinically (2), and one such method involves the use of viruses with mutations that allow for relatively selective replication in tumor cells (3). These viruses have been termed oncolytic, replication-conditional, replication-compromised, or tumor-selective viruses. In some cases, it seems that viral replication may be targeted to molecular aberrancies found in tumor cells. In this respect, several herpes simplex virus type 1 (HSV-1) mutants have been shown in a variety of preclinical studies to replicate and lyse tumor cells with relative specificity. In fact, human phase I clinical trials have shown that an oncolytic HSV-1 with deletions of both copies of the viral UL39 and γ1,34.5 genes and the viral UL39 gene, encoding for ICP6, was not associated with serious adverse events in humans when injected into brain tumors at doses up to 3 × 10⁷ plaque-forming units (pfu; ref. 4).

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Materials and Methods

Engineering of recombinant viruses. To insert both CYP2B1 and secreted human intestinal Carboxylesterase (shICE) transgenes into the UL39; γ1,34.5 mutant HSV-1 background, we employed the HSVquik methodology. Briefly, this allowed for site-specific recombination

Single-modality therapy for malignant glioma is not likely to be very effective. We have shown previously that addition of prodrug-activating genes to the genome of an oncolytic HSV-1 allows for enhanced antitumor effects on addition of the prodrug (5). In a rat 9L and human U87EGFR glioma model, ganciclovir/Hs-TK and cyclophosphamide/CYP2B1 gene therapy in the context of replication HSV-1 provided the most effective anticancer effect compared with all other combinations (6). However, one problem with ganciclovir/Hs-TK gene therapy is that the active metabolite will abolish viral replication; therefore, ganciclovir treatment has to be carefully scheduled such that drug levels and viral replication are maximized to achieve the greatest possible antitumor efficacy (7–9).

Because of this limitation, we have been seeking prodrug activation strategies that will not significantly limit viral replication. Cyclophosphamide/CYP2B1 represents one such strategy: its lack of significant inhibition of viral replication may be due to a combination of the drug’s mode of action affecting human DNA replication more than viral DNA replication (5) and of its immunosuppressive properties allowing for decreased host antiviral responses (10).

Recently, it has been shown that topoisomerase I inhibitors, such as irinotecan (CPT-11), can be synergistic in their anticancer effects with alkylating agents (11–14), a drug class that includes cyclophosphamide. CPT-11 is an inactive prodrug that is metabolized by carboxylesterases into its active metabolite, SN-38, and prodrug-activating gene therapies using CPT-11/carboxylesterase have been described (15–20). We thus sought to determine if CPT-11/carboxylesterase significantly inhibits HSV-1 replication and, if it does not, if a carboxylesterase transgene can be combined into an oncolytic viral genome together with the CYP2B1 gene to provide tumor cell chemosensitivity to both cyclophosphamide and CPT-11. Herein, we report that the combinations of viral oncolysis and chemosensation imparted by CPT-11/carboxylesterase and cyclophosphamide/CYP2B1 gene transfer resulted in more efficacious tumor treatment compared with controls, without significant effects on viral replication.
of a IE4/5 promoter-CYP2B1-cytomegalovirus (CMV) promoter-shiCE cassette into the U39 locus of a UL39;γ34.5-mutant HSV-1 cloned into a bacterial artificial chromosome (BAC). This is followed by Cre-loxP-mediated excision of unwanted BAC sequences from the recombinant in mammalian Vero cells. Details of this methodology are currently in preparation. Several plaques designated MGH2 were obtained, and two were selected for Southern analysis by restricting viral DNA from each isolate with HindIII or Kpnl to confirm genetic identity.

After electrophoretic separation on a 0.5% agarose gel, the products were transferred to a Hybond-N+ membrane (Amersham, Piscataway, NJ), and after UV light cross-linking, the presence of the carboxylesterase cDNA and of the CYP2B1 sequences within the putative MGH2 constructs was confirmed.

Cell lines and drugs. Human Gli36EGFR, U87EGFR, U251, and T98G glioma cells were propagated in DMEM (Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 100 units/mL penicillin, and 100 µg/mL streptomycin (Life Technologies) at 37°C in 5% CO2. Serum was sterilized by filtration through 0.2 µm and medium was checked for sterility per routine. CPT-11 (CAMTOSAR injection, CPT-11 hydrochloride injection; Pfizer, New York, NY) was supplied as a sterile aqueous solution (pH 3.5). Vials (5 mL) contained 100 mg CPT-11 hydrochloride. It was stored at room temperature while protected from light. For growth inhibition assays, the drug was diluted in medium immediately before use. SN-38 was a gift from Drs. Susan Ludeman and O. Michael Colvin (Duke University, Durham, NC). Cyclophosphamide was purchased from Mead Johnson Oncology (Princeton, NJ) as a sterile powder. It was diluted in DMEM (10 mL) to provide a stock of 35.8 mmol/L before use. D-Luciferin was purchased from Molecular Probes (Eugene, OR). It was diluted into a stock solution of 50 mmol/L sodium phosphate buffer (pH 7.3).

Cell proliferation and viral replication assays. For studies detailing survival of human U87EGFR glioma cells compared with survival of oncolytic virus, hrB3, as a function of SN-38 concentration, U87EGFR glioma cells (6 x 10^6/10 cm dish) were plated in the presence of varying concentrations of the drug(0, 1 nmol/L, 5 nmol/L, 10 nmol/L, 100 nmol/L, and 1 µmol/L) in triplicate. Cell survival was enumerated by Coulter counting 5 days later. In parallel, Vero cells at 90% confluency were infected with hrB3 (50 pfu/plate) at varying concentrations of drug (0-1 µmol/L) in triplicate. After overlaying with 0.5% agarose, plaques were enumerated by Xgal staining 10 days later.

For dose-response assays, Gli36 cells (3 x 10^5) were plated into wells of six-well plates in DMEM/2% FBS and allowed to adhere overnight. The next day, cells were washed with HBSS and MGH2 virus was added at multiplicities of infection (MOI) of 0.1 or 0.5. The plates were shaken every 15 minutes for 1 hour at 37°C after which fresh medium (2 mL) containing CPT-11 or cyclophosphamide at indicated concentrations was added. Viral replication was attenuated 3 hours later by transferring the plates to a 39.8°C incubator. Surviving cells were counted 4 days later using a Coulter Z2 counter (Beckman Coulter, Hialeah, FL). Statistical analyses were done using Student’s t test.

Carboxylesterase activity and irinotecan conversion assays. Carboxylesterase activity was determined in sonicated cell extracts or culture medium by a spectrophotometric assay using o-nitrophenyl acetate as a substrate. Samples were incubated with 3 mmol/L o-nitrophenyl acetate in 50 mmol/L HEPES (pH 7.4), and the conversion to o-nitrophenyl was determined spectrophotometrically as a change in absorbance at 405 nm with an extinction coefficient of 0.1 (1.34 x 10^-5 mol/L^-1 cm^-1). A minimum of four assays were done for each sample, and values for the cells extracts were corrected for the protein concentration. Activity is expressed as nmol o-nitrophenyl produced/min/mg protein or produced/min/mL culture medium. Conversion of CPT-11 to SN-38 was monitored by incubating either cell extracts or medium samples with 5 or 25 µmol/L CPT-11 for 18 hours in 50 mmol/L HEPES (pH 7.4) at 37°C. Reactions were terminated by addition of an equal volume of acidic methanol, and particulate matter was removed by centrifugation at 100,000 x g for 30 minutes at 4°C. Concentration of both drugs in the supernatant was determined by high-performance liquid chromatography.

In vivo bioluminescence. To quantitatively measure tumor responses to therapy, human Gli36EGFR cells were transplanted with a firefly luciferase cDNA. A stable transfectant, which expressed high levels of luciferase, was subcloned and passaged in G418. Gli36EGFR-luc cells reliably formed tumors in the brains of athymic mice. Athymic mice with such glioma xenografts were treated with (a) saline (n = 3), (b) a combination of i.p. CPT-11 (i.p. injection of 2 mg in 100 µL volume on day 1) and cyclophosphamide (i.p. injection of 2 mg in 100 µL volume on days 1, 3, 5, and 7; n = 3), (c) stereotactic inoculation of MGH2 alone (intratumoral injection of 1 x 10⁶ pfu in 10 µL volume on day 0; n = 4), (d) stereotactic inoculation of MGH2 (on day 0) and i.p. injection of CPT-11 (on day 1; n = 3), (e) stereotactic inoculation of MGH2 (on day 0) and i.p. injection of cyclophosphamide (on days 1, 3, 5, and 7; n = 7), and (f) stereotactic inoculation of MGH2 (on day 0) and i.p. injection of cyclophosphamide (on days 1, 3, 5, and 7) and CPT-11 (on day 1; n = 5). Luciferase activity was measured in animal brains by bioluminescence imaging over a 10-day time span after i.p. administration of D-luciferin. D-Luciferin was inoculated 5 minutes before each imaging procedure as an i.p. inoculation (126 mg/kg). Images were acquired 10 minutes after the substrate administration following an initial optimization study. A light image of the animal was also taken in the chamber using dim illumination. The spatial distribution of luciferase activity within the tumors was then measured by recording photon counts in the CCD with no illumination. Following data acquisition, postprocessing and visualization were done using the Berthold WinLight 32. The mean, SD, and sum of the photon counts in these regions were then calculated. For visualization purposes, bioluminescence images were fused with the corresponding white light surface images by using color overlay mode of the software, permitting correlation of areas of bioluminescent activity with anatomy. The comparison of groups based on in vivo bioluminescence was made using a random effects model (Proc. Mixed in SAS), allowing random intercept and slope effects for the log-transformed bioluminescence. Group comparisons were made using contrasts in the linear(slope) effects.

Survival studies. Human Gli36ΔEGFR glioma cells were implanted on day 0; then, MGH2 (1.4 x 10⁶ pfu) was stereotactically inoculated on day 6 followed by CPT-11 administration as one dose (2 mg/100 µL) at day 7 and a smaller dose (1 mg/50 µL) at day 21, whereas cyclophosphamide was i.p. given on days 7 to 35 every other day at a dose of 2 mg/100 µL. Survival comparisons were displayed using Kaplan-Meier curves and compared using the log-rank test.

Results

SN-38, the active metabolite of irinotecan, does not significantly inhibit herpes simplex virus type 1 replication. We sought to determine if the active metabolite of CPT-11, SN-38, inhibited viral replication. Dose-response curves were generated to compare the concentration of drug that inhibited the proliferation of human U87EGFR glioma cells to that which inhibited the formation of plaques by an oncolytic HSV-1 mutant. Figure 1 shows that glioma cell viability was reduced by 50% at a concentration of SN-38 of 4.5 nmol/L, whereas viral replication was reduced by 50% at a concentration of SN-38 of ~300 nmol/L. These results thus indicated that SN-38 possessed antiproliferative action at doses that were almost 2 orders of magnitude lower than doses required to significantly inhibit viral replication.

Engineering of MGH2 virus mutant. The final expected recombinant virus, designated as MGH2 and initially selected as two different isolates, possessed the IE4/5 promoter-driving expression of CYP2B1 followed by a CMV promoter-regulating expression of shiCE (Fig. 2A). This cassette was inserted in the UL39 locus downstream of an ICP6-GFP fusion sequence and flanked by

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one residual loxP and frt sites. Deleted sequences of the c 134.5 genes were not affected. Southern blot analysis of two plaque picks verified the general accuracy of the genetic structure (Fig. 2B). By using a carboxylesterase probe, restriction enzyme analysis with HindIII revealed expected fragments of 12 and 1 kb (or 4 kb), respectively, for two individual isolates of MGH2 (Fig. 2B). Again using a CYP2B1 probe with the same enzymes, expected signals of 1.6 and 1 kb (or 1.2 kb) were observed. Therefore, one of the MGH2 isolates was selected for further studies.

**In vitro dose-effect studies.** The in vitro effect of both prodrugs was tested by infecting cells with MGH2 (MOI = 0.5) and then temperature shifting to 39.8°C to attenuate viral replication (6). This is done because in vitro viral oncolysis occurs more rapidly than anticancer effects mediated by activated metabolites and it would be difficult to study the latter effects without attenuating viral replication. The temperature shift thus allows one to measure the effect of prodrug activation in the presence of diminished viral oncolysis. Figure 3A shows that, after MGH2 infection, addition of CPT-11 at 0.05, 0.1, or 0.2 μmol/L and cyclophosphamide at 250, 500, or 1,000 μmol/L, respectively, produced significantly increased antiproliferative effect compared with addition of either prodrug alone. This indicated that combining CPT-11 with cyclophosphamide activation resulted in more tumor cell killing than activating only one prodrug.

**In vitro studies against other glioma cells.** The antiproliferative effect of MGH2 was further studied against two other human glioma cell lines (U251 and T98G). Figure 3B shows that CPT-11 and cyclophosphamide activation in the context of an attenuated (temperature shifted) MGH2 infection produced an increased antiproliferative effect compared with activation of only one prodrug. This further confirmed that activation of both CPT-11 and cyclophosphamide resulted in more tumor cell killing compared with activation of only one or the other prodrug.

**Prodrug activation does not inhibit MGH2 replication.** We next sought to show that activation of both prodrugs in tumor cells did not inhibit replication of MGH2. Human Gli36ΔEGFR cells were thus infected with MGH2 in the presence or absence of both prodrugs, singly or in combination. Because MGH2 maintains an active Hs-TK gene, infection in the presence of ganciclovir was carried out as a positive control. Figure 3C

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**Figure 1.** Differential effects of SN-38 on U87ΔEGFR (purple squares) cell survival or hrR3 (blue diamonds) oncolytic virus plaque formation. Human U87ΔEGFR glioma cells (squares) were cultured in the presence of increasing concentrations of SN-38 (the active metabolite of CPT-11). Cell viability was assayed 5 days later by enumerating cells attached onto a dish with a Coulter counter. The viability of the oncolytic virus, hrR3 (diamonds), was assayed by determining plaque formation in agarose gels in the presence of increasing concentrations of SN-38.

**Figure 2.** Genetic structure of MGH2. A, top, structure of the backbone parental virus with deletions in c 34.5 genes, inserted ICP6-GFP fusion, and deleted UL39 gene. MGH2 was generated and it contains both CYP2B1 and hiCE, inserted into the UL39 locus. Arrows, HindIII restriction sites and expected fragment sizes. B, Southern blot analyses of digested recombinant viral DNA are shown after hybridization with a shiCE (left) or a CYP2B1 (right) probe. DNA from parental virus (rHSV-Q1, lane 1) and two different MGH2 isolates (lanes 2 and 3) were restricted with HindIII.
shows that addition of CPT-11 and cyclophosphamide, singly or in combination, did not inhibit MGH2 replication compared with infections carried out in the absence of prodrugs. In contrast, infections carried out in the presence of ganciclovir reduced viral titers by 4 orders of magnitude. This indicated that activation of CPT-11 and cyclophosphamide alone or in combination in the context of MGH2 infection did not inhibit viral replicative capacity.

**Prodrug activation.** In previous studies, we have shown that CYP2B1-mediated activation of cyclophosphamide resulted in expression of the enzyme in infected tumor cells and the generation of a diffusible metabolite in both cells and in medium (5, 21). However, expression of shiCE in the context of an oncolytic viral infection and generation of the metabolite, SN-38, has not been shown previously. We thus infected human Gli36 D EGFR glioma cells with MGH2 and proceeded to harvest medium and cell extracts at different time points. Figure 4A and B shows that, between 20 and 120 hours after infection, there was a time-dependent increase in SN-38 produced (reaching a maximum of 4.9 pmol/h/mg at 120 hours) and in carboxylesterase activity.

Figure 3. In vitro studies. A, human Gli36 D EGFR cells were plated on dishes in the presence of varying concentrations of MGH2, cyclophosphamide (CPA), and CPT-11 as indicated in the figure. Approximately 4 hours after MGH2 infection, the temperature was raised to 39.8 °C to block MGH2 replication (5, 21, 32). Five days later, the number of surviving cells was enumerated. B, human U251 and T98G glioma cells were exposed to MGH2 at the indicated MOI and cyclophosphamide and CPT-11 at the indicated doses. Approximately 4 hours after MGH2 infection, the temperature was raised to 39.8 °C to block MGH2 replication (5, 21, 32). Five days later, the number of surviving cells was enumerated. C, human Gli36 D EGFR cells were infected with MGH2 at a MOI of 1. One hour later, CPT-11 (0.2 μmol/L), cyclophosphamide (250 μmol/L), or both (CPT-11 at 0.2 μmol/L and cyclophosphamide at 250 μmol/L) were added. Twenty four hours later, MGH2 was harvested and titers were determined by plaque assays. As a control, ganciclovir (GCV) was added at 100 ng/mL. *, P < 0.05; **, P < 0.01; ***, P < 0.005 (Student’s t test).
tumor response. Athymic mice with brain glioma xenografts expressing the firefly luciferase gene was employed to quantitate \( D \) of glioma in which a human Gli36 shiCE transgenes and the prodrugs, cyclophosphamide and CPT-11. Luciferase activity was measured in animal brains by bioluminescence imaging over a 10-day time span. Figure 5A shows that the combined use of MGH2, cyclophosphamide, and CPT-11 was more effective than all other tested treatments in decreasing luciferase activity, a correlative measure of decreased cell viability. A representative image of this effect is shown in Fig. 5B, where much less bioluminescence is measured in the brain of a mouse treated with MGH2 plus cyclophosphamide plus CPT-11 compared with that of a mouse treated with MGH2 alone. Therefore, these results indicate that viral oncolysis combined with activation of both prodrugs was more effective at decreasing i.c. glioma viability than all the other combinations for the 10-day time course of treated treatment. We then sought to confirm the above findings by determining the survival time of treated mice. We employed the same schedule of virus and prodrug administration used for the \textit{in vivo} bioluminescence experiments. Mice with intracerebral glioma xenografts were treated with saline, MGH2, or MGH2 + CPA + CPT11. There was a statistically significant increase in the survival time of mice treated with MGH2 + CPA + CPT11 compared to others (median survival time of MGH2 + CPA + CPT11 = 42 days, MST of MGH2 alone = 30 days, MST of saline alone = 21 days, \( P < 0.004 \), two-sided Kruskal-Wallis test). The data thus indicate that the combination of MGH2 plus cyclophosphamide plus CPT-11 provided an effective \textit{in vivo} anti-glioma effect.

\textbf{Survival effects of MGH2 plus cyclophosphamide plus irinotecan.} We then sought to determine if anticancer effects were improved when the schedule and dosing of the prodrugs was altered. A different dose and schedule for the prodrugs was used from the previous experiment. Human Gli36ΔEGFR glioma cells were implanted on day 0; then, MGH2 (1.4 \times 10^6} \text{pfu}) was stereotactically inoculated on day 6 followed by CPT-11 administration as one dose (2 mg/100 \( \mu \)L) at day 7 and a smaller dose (1 mg/50 \( \mu \)L) at day 21, whereas cyclophosphamide was i.p. given on days 7 to 35 every other day at a dose of 2 mg/100 \( \mu \)L. Figure 6 shows that there was a statistically significant increase in the survival of animals treated with MGH2 plus cyclophosphamide plus CPT-11 compared with controls. These results indicate that the combined effect of MGH2 and both prodrugs provided an effective therapeutic effect for this tumor model.

**Discussion**

Experimental treatments for malignant glioma have to be investigated due to the current inability to provide effective treatment. The objective of this report was to show that one such experimental treatment ("oncolytic viral therapy") could be optimized through the addition of genes that activate the prodrugs, cyclophosphamide and CPT-11. For the first time, we were able to show that (a) \textit{in vitro}, the combination of MGH2 (the herpes simplex viral oncolytic mutant that delivers the\textit{CYP2B1} and \textit{shiCE} transgenes) and the prodrugs, cyclophosphamide and CPT-11, was more effective in cytolysis than other combinations; (b) \textit{in vitro}, activation of these two prodrugs did not inhibit MGH2 replication; and (c) \textit{in vivo}, the combination (reaching a maximum of 35.6 nmol/min/mg of activity at 120 hours). This is consistent with release of the diffusible metabolite and secreted enzyme in the medium. In cell extracts, enzyme activity was initially higher than that found in supernatants up to the 96-hour time point but then became approximately equal. This was consistent with cellular production of the enzyme before its secretion. However, SN-38 levels in cell extracts were consistently higher at all time points in cell extracts (between 9.1 and 10.9 pmol/h/mg). This suggests that intracellular activation of CPT-11, due to the increased concentration of carboxylesterase within the cells, likely contributed to the production of SN-38. Another explanation may be that the SN-38 present in the medium at the earlier time points was derived from diffusion from the cell rather than from direct extracellular activation.

**Quantitative measurements of \textit{in vivo} anticancer effects of MGH2 plus cyclophosphamide plus irinotecan.** We first tried to quantify antitumor efficacy in an orthotopic xenograft model of glioma in which a human Gli36ΔEGFR glioma cell line stably expressing the firefly luciferase gene was employed to quantitate tumor response. Athymic mice with brain glioma xenografts were thus treated with saline with combinations of i.p. CPT-11 and/or cyclophosphamide with stereotactic inoculations of MGH2. A variety of combinations of virus and drug were used, including MGH2 with i.p. CPT-11, MGH2 with i.p. cyclophosphamide, or MGH2 with i.p. cyclophosphamide and CPT-11. Luciferase activity was measured in animal brains by bioluminescence imaging over a 10-day time span. Figure 5A shows that the combined use of MGH2, cyclophosphamide, and CPT-11 was more effective than all other tested treatments in decreasing luciferase activity, a correlative measure of decreased cell viability. A representative image of this effect is shown in Fig. 5B, where much less bioluminescence is measured in the brain of a mouse treated with MGH2 plus cyclophosphamide plus CPT-11 compared with that of a mouse treated with MGH2 alone. Therefore, these results indicate that viral oncolysis combined with activation of both prodrugs was more effective at decreasing i.c. glioma viability than all the other combinations for the 10-day time course of treated treatment. We then sought to confirm the above findings by determining the survival time of treated mice. We employed the same schedule of virus and prodrug administration used for the \textit{in vivo} bioluminescence experiments. Mice with intracerebral glioma xenografts were treated with saline, MGH2, or MGH2 + CPA + CPT11. There was a statistically significant increase in the survival time of mice treated with MGH2 + CPA + CPT11 compared to others (median survival time of MGH2 + CPA + CPT11 = 42 days, MST of MGH2 alone = 30 days, MST of saline alone = 21 days, \( P < 0.004 \), two-sided Kruskal-Wallis test). The data thus indicate that the combination of MGH2 plus cyclophosphamide plus CPT-11 provided an effective \textit{in vivo} anti-glioma effect.

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of MGH2 plus cyclophosphamide plus CPT-11 was more effective than other combinations as measured by both a quantitative bioluminescence assay and an animal survival study. This evidence thus indicates that addition of the oncolytic virus genome of genes that activate prodrugs with anticancer (but minimal antiviral) action provides enhanced therapeutic efficacy.

The selection of transgenes to include within an oncolytic virus genome is critical: use of a gene that possesses significant antiviral action is likely to antagonize antiviral oncology and viral spread, ultimately limiting effective delivery of the transgenes. For instance, the Hs-TK gene used to activate ganciclovir can limit viral replication in some instances and thus may not be an appropriate choice in this paradigm (6–9). This report shows that the active metabolite of CPT-11, SN-38, does not significantly inhibit viral replication at concentrations required for cytotoxicity. Therefore, the use of a carboxylesterase that can efficiently activate CPT-11 is a desirable characteristic for use in the enzyme/prodrug strategy described here. This is confirmed by our in vitro and in vivo experiments that show that enhanced viral oncolysis was observed without inhibiting viral replication.

It is interesting to speculate why SN-38 does not significantly affect HSV-1 replication. The metabolite acts as a topoisomerase I inhibitor that affects mammalian DNA replication by blocking the necessary step of nicking one DNA strand for proper unwinding. HSV-1 DNA replication depends on seven viral proteins and in vitro replication models on a eukaryotic cell-free system (22–24). It is not clear if such relaxing enzymatic activity requires both the action of topoisomerase II and I or if only one of these could provide all of the relaxing function. It has been reported that inhibition of topoisomerase II will inhibit HSV-1 replication (25, 26). Our results suggest that inhibition of topoisomerase I does not significantly affect HSV-1 replication at least at doses of SN-38 required to cause cytotoxicity. Clearly, additional experimentation will be required to define the mechanism of selective cellular versus viral toxicity for SN-38.

There are multiple carboxylesterase enzymes that have been described (27). We selected a human intestinal form that was further modified by deleting six COOH-terminal amino acids to allow the protein to be secreted. This would allow for both intracellular and extracellular conversion of CPT-11. Our results indicate that, in the context of delivery by an oncolytic HSV, there was a time-dependent increase in carboxylesterase activity in the supernatant of cells. This could have been due to both secretion of the enzyme and release in the medium of enzyme from cells lysed

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**Figure 5.** In vivo bioluminescence assays of MGH2 + prodrugs anticancer effects. A, athymic mice (n = 5) with orthotopic human glioma (MGH2) were treated with a stereotactic injection of MGH2 (1 × 10^6 pfu in 10 μL volume) on day 0 followed by an i.p. injection of CPT-11 on day 1 and cyclophosphamide on days 1, 3, 5, and 7. Control mice received injections of saline alone (yellow diamonds; n = 3); a combination of i.p. CPT-11 (on day 1) and cyclophosphamide (on days 1, 3, 5, and 7; gray diamonds; n = 3); with stereotactic inoculation of MGH2 alone (on day 0; black triangles; n = 4); with stereotactic inoculation of MGH2 (on day 0) and i.p. injection of CPT-11 (on day 1; green triangles; n = 3); or with stereotactic inoculation of MGH2 (on day 0) and i.p. injection of cyclophosphamide (on days 1, 3, 5, and 7; purple squares; n = 7). Luciferase activity was measured every other day by anesthetizing mice and imaging with a cooled CCD device and values were initially expressed as photon counts per 30 seconds. The plotted values represent the average ratio between the photon counts per second measured that day and the photon counts measured on day 0 for each animal. There was significant variability in measured photon counts per second between animals in each group and animals in different groups at day 0 due to small differences in tumor growth pattern and tumor position within the brain. The absolute average photon counts per 30 seconds for each group were as follows: saline alone, day 0: 3,484, day 1: 17,004, day 3: 39,848, day 5: 44,760, and day 7: 47,180; cyclophosphamide + CPT-11, day 0: 300,000, day 1: 900,000, day 3: >10^6, and day 5: >10^6; MGH2 alone, day 0: 100,000, day 1: 200,000, day 3: 300,000, day 5: 78,959, day 7: 100,000, and day 10: 300,000; MGH2 + cyclophosphamide, day 0: 100,000, day 1: 96,332, day 3: 100,000, day 5: 68,992, day 7: 54,132, and day 10: 76,941; MGH2 + CPT-11, day 0: 300,000, day 1: 106, day 3: 2 × 10^5, day 5: 3 × 10^5, day 7: 36,573, and day 10: 200,000; and MGH2 + cyclophosphamide + CPT-11, day 0: 100,000, day 1: 200,000, day 3: 100,000, day 5: 21,039, day 7: 524, and day 10: 3,041. There was a statistically significant difference (P < 0.05) among the groups using a random effects model (Proc. Mixed in SAS), allowing random intercept and slope effects for the log-transformed bioluminescence. Group comparisons were made using contrasts in the linear(slope) effects. B, representative pictures of bioluminescence emission from the brains of mice treated with MGH2 + cyclophosphamide + CPT-11 (left mouse) or MGH2 alone (right mouse). Luciferase activity is color coded, with red indicating the highest (~300,000 photon counts per 30 seconds) and blue the lowest (~1,000 photon counts per 30 seconds).
by viral oncolysis. The amount of SN-38 in the supernatant also increased with time. This could have been due to both extracellular conversion of the prodrug, CPT-11, and diffusion of the metabolite, SN-38, from the intracellular to the extracellular compartment. In previous studies, we have measured the conversion of cyclophosphamide to its active metabolite(s) after infection with an oncolytic virus. The amount of SN-38 in the supernatant also increased with time. This could have been due to both extracellular metabolites generated by activation of the delivered transgene are fairly low in concentration, also rendering it difficult to study the effect of added therapies, whereas at low MOIs (<0.01) metabolites generated by activation of the delivered transgene are fairly low in concentration, also rendering it difficult to study the effect of added therapies. In vivo experiments. In vivo experiments. In vivo experiments.

In vivo, the combined use of MGH2 replication-induced cell lysis and activation of both prodrugs, cyclophosphamide and CPT-11, produced an anticancer effect that was superior to the other combinations. In contrast, a significant reduction in cell viability was observed for the MGH2 and other ICP6-defective oncolytic viruses in the in vivo temperature shift assay.7

In vivo, cyclophosphamide activation also inactivates early antiviral host responses that limit viral infection of and propagation within tumors (10, 28–30). It is not known if CPT-11 activation produces similar responses.

The temperature shift assay measures the effect of prodrug activation in infected and surrounding cells in the absence of oncolytic virus replication. It depends on the finding that HSV-1 with defective ICP6 function are restricted in replication at a temperature of ≥39.8°C (5, 6). We have found that, at this temperature, infected cells will express transgenes delivered by the oncolytic virus, but instead of a propagating infection, infected cells appear to apoptose.9 We have been employing this experimental modality because viral replication/propagation and tumor cell killing in vitro is rapid and fairly effective at high MOIs (>0.1), rendering it difficult to study the effect of added therapies, whereas at low MOIs (<0.01) metabolites generated by activation of the delivered transgene are fairly low in concentration, also rendering it difficult to study the effect of combined treatment. In vivo, this temperature sensitivity may affect the oncolytic viral activity in animal models and/or treated humans, thus rendering the use of added therapies even more relevant. However, several literature sources indicate that mouse core body temperatures vary between 36°C and 38.5°C based on activity (31, 32). The upper value is at least 1°C less than that required to effectively shut off MGH2 and other ICP6-defective oncolytic viruses in the in vivo temperature shift assay.7

In vitro, the combined use of MGH2 replication-induced cell lysis and activation of both prodrugs, cyclophosphamide and CPT-11, produced an anticancer effect that was superior to the other combinations. In fact, quantitative measurements of tumor cell viability using bioluminescence imaging indicated a rapid effect for this modality, with significant differences observed even 4 days after MGH2 inoculation, 3 days after one dose of i.p. CPT-11, and 1 and 3 days after dosing with i.p. cyclophosphamide. This effect was maintained for the remaining 6 days of the experiment. In contrast, a significant reduction in cell viability was observed for the MGH2 plus cyclophosphamide alone or MGH2 plus CPT-11 alone compared with MGH2 alone only at the 10-day time point. The reason for this enhancement may be due to (a) three differential modes of cytotoxicity on tumor cells (viral lysis, DNA alkylation, and topoisomerase I inhibition), (b) augmentation of viral oncolysis by cyclophosphamide metabolite–mediated suppression of host immune responses, and (c) synergistic pharmacologic effects displayed by topoisomerase I inhibitors and alkylating agents. Further experimentation will be needed to characterize if all or any of these mechanisms are operative in this strategy.

The animal survival studies also confirmed that MGH2 plus cyclophosphamide plus CPT-11 was superior in antitumor efficacy than MGH2 alone. Some apparent discrepancies were apparent among the in vitro bioluminescence experiment and the survival experiments. In vivo bioluminescence provides a rapid and extremely sensitive assay of luciferase activity, which depends on the availability of ATP and oxygen within cells, availabilities found only in live cells. Animal survival is an overall indication of therapeutic success that depends on many more variables (not only tumor lysis but also edema, toxicity/inflammation, animal health, etc.)
than bioluminescence. Using one schedule, the experiments of Fig. 5A and as described in Results show that the difference in the decrease in tumor viability caused by MGH2 plus both prodrugs versus MGH2 alone versus saline alone as measured by bioluminescence preceded the apparent significant difference in increase in animal survival produced by MGH2 plus both prodrugs versus MGH2 alone versus saline alone by ~20 to 40 days. Therefore, this would be in agreement with a model, where bioluminescence measurements reflect very early changes in tumor metabolic status, which may correlate with the overall end point of survival. We stated may because when the schedule/dose of prodruk administration was altered (Fig. 6) the correlation between bioluminescence and long-term survival between saline versus MGH2 alone and saline versus MGH2 plus prodrugs effect was lost. The reason for this loss is not known, but it may be due to alterations in drug schedule/dosing. It is likely that additional optimization of dosing and schedules will have to be carried out to achieve even more significant effects.

The mutations in the genome of MGH2 are similar to those found in G207, an oncolytic HSV-1 that has already been tested in phase I clinical trials without serious adverse events (4). However, it is not known if the addition of cyclophosphamide and CPT-11 will produce an increase in overall toxicity. Our findings justify the performance of preclinical toxicity data in appropriate nonhuman primate animal models to determine appropriate dosing and schedule for this triple combination (33).

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References

Brain Tumor Oncolyis with Replication-Conditional Herpes Simplex Virus Type 1 Expressing the Prodrug-Activating Genes, CYP2B1 and Secreted Human Intestinal Carboxylesterase, in Combination with Cyclophosphamide and Irinotecan
