

ORIGINAL ARTICLE

Oncolytic Herpes simplex virus expressing yeast cytosine deaminase: relationship between viral replication, transgene expression, prodrug bioactivation

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Yeast cytosine deaminase (yCD) is a well-characterized prodrug/enzyme system that converts 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU), and has been combined with oncolytic viruses. However, *in vivo* studies of the interactions between 5-FC bioactivation and viral replication have not been previously reported, nor have the kinetics of transgene expression and the pharmacokinetics of 5-FC and 5-FU. We constructed a replication-conditional Herpes simplex virus 1 (HSV-1) expressing yCD and examined cytotoxicity when 5-FC was initiated at different times after viral infection, and observed that earlier 5-FC administration led to greater cytotoxicity than later 5-FC administration *in vitro* and *in vivo*. In animal models, 12 days of 5-FC administration was superior to 6 days, but dosing beyond 12 days did not further enhance efficacy. Consistent with the dosing-schedule results, both viral genomic DNA copy number and viral titers were observed to peak on Day 3 after viral injection and gradually decrease thereafter. The virus is replication-conditional and was detected in tumors for as long as 2 weeks after viral injection. The maximum relative extent of yCD conversion of 5-FC to 5-FU in tumors was observed on Day 6 after viral injection and it decreased progressively thereafter. The observation that 5-FU generation within tumors did not lead to appreciable levels of systemic 5-FU ($<10 \text{ ng ml}^{-1}$) is important and has not been previously reported. The approaches used in these studies of the relationship between the viral replication kinetics, transgene expression, prodrug administration and anti-tumor efficacy are useful in the design of clinical trials of armed, oncolytic viruses.

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Introduction

Recent advances in the molecular biology of cancer have led to the development of novel therapeutic agents, including viruses that destroy cancer cells by lytic viral replication. This approach has been termed viral oncolysis, and viruses studied for this purpose include adenovirus, Herpes simplex virus 1 (HSV-1), vaccinia virus, reovirus, Newcastle disease virus, Seneca Valley virus and measles virus.^{1,2} Several clinical trials with oncolytic viral vectors have been reported and overall the results demonstrate safety of administration.^{3,4} Some of the

clinical trials have demonstrated anti-tumor activity of oncolytic viruses.^{5–7} Nonetheless, efficacy of these viruses has been limited in clinical studies reported to date.

Combination of viral oncolysis with specific anti-cancer drugs may augment anti-tumor activity.^{8,9} Prodrug/enzyme cancer therapy mediated by armed, oncolytic HSV vectors has been actively investigated as one promising approach.^{10–12} In particular, yeast cytosine deaminase (yCD) is a well-characterized system that converts non-toxic anti-fungal agent 5-fluorocytosine (5-FC) to cytotoxic chemotherapeutic agent 5-fluorouracil (5-FU).¹³ In addition, converted 5-FU can freely diffuse from yCD-expressing cells to neighboring cells (that is, does not require gap junctions), thereby eliciting a so-called bystander effect.¹⁴ One limitation of 5-FU as a systemically delivered agent is the associated toxicity to normal tissues. Prodrug conversion strategies increase 5-FU concentration preferentially within tumors, thereby potentially decreasing systemic side effects and increasing

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therapeutic efficacy. In addition, not only is 5-FU an effective anti-cancer drug, but is also a potent radiosensitizer and may allow for more efficacious combinations that include radiotherapy. In our previous report, we demonstrated that administration of 5-FC along with this armed HSV-1 vector-expressing yCD enhances viral oncolysis.¹³ The mechanism of action appeared to be local intratumoral generation of 5-FU; however, intratumoral and systemic 5-FU levels were not assessed.

Complex interactions between the viral oncolysis and the bioactivated chemotherapeutic agent can occur in the context of viral replication.^{15,16} For example, a bioactivated prodrug which inhibits DNA synthesis may interfere with viral DNA synthesis and limit viral replication, and consequently attenuate lytic viral replication and oncolysis.¹⁷ In our previous study, we reported that 5-FC bioactivation is associated with only a minimal decrease in HSV-1 replication.¹³ Other studies demonstrate inhibition of viral replication associated with 5-FC bioactivation,^{18–20} thereby suggesting that virus strain, cancer cell type and drug concentration influence the balance of the interaction. In the current study, we examine the hypotheses that tumors infected by oncolytic HSV-1 expressing yCD produce intratumoral levels of 5-FU without generating appreciable systemic levels of 5-FU; and the efficacy of armed, oncolytic viral therapy is dependent on the timing of administration of the 5-FC prodrug relative to the HSV-1 oncolytic virus. We constructed an HSV-1 mutant that expresses yCD and assessed the impact of the timing of 5-FC relative to HSV-1 administration by determining the kinetics of viral replication and transgene expression. During these studies we measured intratumoral conversion of 5-FC to 5-FU and systemic levels of 5-FU.

Materials and methods

Cell lines and viruses

African green monkey kidney cells (Vero) and human colon carcinoma cells (HT29) were obtained from the American Type Culture Collection (Rockville, MD). Mouse colon carcinoma cells (MC26) were obtained from the National Cancer Institute Tumor Repository (Frederick, MD). All the cell lines were propagated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (both from MediaTech CellGro, Manassas, VA), supplemented with 100 units ml⁻¹ penicillin and 100 mg ml⁻¹ streptomycin (Invitrogen, Carlsbad, CA). Early passage cells were used for all experiments and were not reauthenticated. Parent herpes simplex virus type 1 (HSV-1) strain Patton was kindly provided by E. Antonio Chiocca (Ohio State University, Columbus, OH).

Plasmid and recombinant virus construction

We used an HSV vector cloning system termed Flip-Flop HSV-BAC (bacterial artificial chromosome) system for the generation of new recombinant HSV vectors²¹ (kindly provided by Dr Roberta Martuza, MGH). Briefly, two site-specific recombinases, Cre and FLPe, were used

sequentially to integrate desired sequences and to excise the BAC sequences, respectively, and as the size of the HSV-BAC-insert genome exceeds the packaging limit of HSV, only correctly recombined virus grow efficiently. Utilizing this system, we developed a HSV-1 mutant expressing the codon-optimized yCD-uracil phosphoribosyltransferase (UPRT) gene (Invivogen, San Diego, CA) with a myc-tag in the ICP6 locus under the control of the CMV-IE promoter in the HSV strain Patton, termed BMP6-CMVFcumB. A yCD-UPRT fusion gene was used as UPRT has been reported to enhance the yCD rate-limiting enzymatic conversion of 5-FC to 5-FU.^{22–24} β -galactosidase (LacZ) gene expression is driven by the ICP6 promoter.

Viral cytotoxicity assay

Cells were plated in triplicate at a density of 4×10^4 ml⁻¹ in a 24-well plate. After 24 h, cells were infected with virus at the indicated multiplicity of infection (MOI) and then media containing 5-FC at the indicated concentrations was added. After 120 h, the effect of virus with or without 5-FC on the growth of cell lines was measured by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, MO) assay as previously described.¹³ The experiment was repeated twice for each cell line.

Detection of HSV-1 gene and transgene expression

Detection of HSV-1 gene and transgene expression by western blot was performed as previously described.²⁵ The following antibodies were used for the detection: 4A6 anti-Myc monoclonal antibody (Millipore, Billerica, MA, USA), anti-HSV-ICP4 monoclonal antibody (US Biological, Swampscott, MA) and β -actin (Abcam, Cambridge, MA).

In vivo studies

Male BALB/c mice (6–8-week-old) (Charles River Laboratories, Shrewsbury, MA) were maintained in accordance with the institutional guidelines of the Massachusetts General Hospital animal care facility. MC26 cells were implanted subcutaneously into the flank area of mice (1×10^7 cells $100 \mu\text{l}^{-1}$ of DMEM) and BMP6-CMVFcumB virus (1×10^7 plaque-forming units per 50 μl of PBS with 10% glycerol) was injected intratumorally when tumor volumes reached 100 mm³. Mice were stratified by tumor volume and then randomly assigned to each treatment group ($n = 5$). 5-FC (750 mg kg^{-1}) was administered intraperitoneally. Tumor sizes were measured every 2 days by external caliper and the volume calculated as length \times width \times depth $\times 0.52$. Each blood sample was drawn 24 h after last 5-FC was injected intraperitoneally. These blood samples were centrifuged at 2000 r.p.m. for 10 min and the plasma was used for the subsequent assay. Each tumor specimen was also collected 24 h after last 5-FC was injected. These tumor specimens were minced by a sterilized scalpel and put into tubes with PBS and garnet beads (MO BIO Laboratories, Carlsbad, CA), and then vortexed for 15 min at 4 °C. The samples were stored at -80 °C. The experiment was repeated in duplicate to ensure reproducible results.

Quantitative real-time PCR

Mouse flank tumor samples were harvested and homogenized at designated time points by ATL buffer (Qiagen, Germantown, MD). DNA was isolated from each sample according to the manufacturer's protocol (QIAamp DNA Mini Kit; Qiagen). HSV-1 DNA, MacIntyre strain (Advanced Biotechnologies Inc., Columbia, MD) was used to create a quantitative standard control. Viral genomic DNA copy number was analyzed by quantitative real-time PCR (LightCycler; Roche Diagnostics Corporation, Indianapolis, IN). Primer sequences to ICP6 were as follows: forward 5'-TCTGGAGTCTGTCGAATTTCA -3' and reverse 3'-ATACATGCTGCGCTTAAAGTG-5'. All reactions were done in duplicate and the experiment was repeated to ensure reproducible results.

Plaque assay

Monolayer cultures of Vero cells grown in a six-well plate were infected with serial dilutions of virus from tumor homogenates as indicated. After removal of virus inoculum, the cells were incubated in DMEM supplemented with 1% inactivated fetal bovine serum and 0.1% pooled human immunoglobulin (Talecris Biotherapeutics, Research Triangle Park, NC) at 37°C for 3 days until plaques were visible. The cells were stained by 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal; Sigma) histochemistry and β -galactosidase-positive plaques were counted.

LacZ staining

Frozen tumor sections of 5 mm thickness in OCT mounted on microscope slides were fixed in 0.5% glutaraldehyde. β -galactosidase activity was measured by incubating the slides in X-gal solution at 37°C overnight. X-gal solution was prepared as follows: X-gal at 20 mg ml⁻¹ in dimethyl formamide, 0.1 mol l⁻¹ potassium ferric cyanide, 0.1 mol l⁻¹ potassium ferrocyanide and 0.1 mol l⁻¹ MgCl₂ (all from Sigma). Sections were coverslipped before examination under a light microscope.

Determination of 5-FC and 5-FU in plasma and tumor homogenate

The concentration of 5-FC and 5-FU in plasma and tumor tissue homogenate was measured by liquid chromatography–tandem mass spectrometry (LC–MS/MS). The procedure used to prepare samples for analysis, the chromatographic conditions and tandem mass spectrometric detection were adapted from several analytical methods that have been previously reported.^{26,27} Analytical reference samples of 5-FC, 5-FU and 5-bromouracil (5-BU), the latter of which was used as the internal standard (IS), were purchased from Sigma-Aldrich (St Louis, MO). Stock solutions with concentrations of approximately 1.0 mg ml⁻¹, based on the actual amount weighed to an accuracy of 0.001 mg using a Cahn microbalance (Analytical Technology, Inc, Boston, MA), were made in water for 5-FC, and methanol for 5-FU and 5-BU. A single analyte working solution containing both 5-FC and 5-FU at a concentration of 0.100 mg ml⁻¹ was made by diluting the stock solutions

with acetonitrile:water (1:1, v/v). Calibration standards were made by serially diluting the analyte stock solution with mouse plasma (Innovative Research, Novi, MI) to provide eight solutions with concentrations ranging from 10 to 1000 ng ml⁻¹. Quality control samples were similarly prepared with concentrations of 30, 300 and 900 ng ml⁻¹. The IS working solution was made by quantitatively diluting the stock solution to 1.50 μ g ml⁻¹ with acetonitrile:water (1:1, v/v).

Frozen plasma samples and tissue homogenates were allowed to thaw over ice and mixed on a vortex stirrer. In a polypropylene microcentrifuge tube, 25 μ l of each sample was vigorously mixed with 5 μ l of IS working solution and 75 μ l of chilled acetonitrile by vortexing for 30 s. The mixture was centrifuged (12 000 g, 5 min) to sediment precipitated protein and 80 μ l of the clear supernatant was transferred into another microcentrifuge tube and evaporated using a centrifugal vacuum concentrator at 40°C (Labconco, Kansas City, MO). The extract was reconstituted in 50 μ l of ammonium acetate buffer (10 mM, pH 3.76) assisted by vortexing and sonicating. An autosampler was used to inject 25 μ l of this solution onto a Synergi 4 μ m Hydro-RP 150 \times 4.6 mm column (Phenomenex, Torrance, CA) preceded by a Phenomenex AQ C18 4.0 \times 3.0 mm guard cartridge (Phenomenex) and 0.5 μ m inline filter. The column was eluted at 0.5 ml min⁻¹ with an isocratic mobile phase composed of 25% methanol and 75% ammonium acetate buffer (10 mM, pH 3.76), delivered using a quaternary pump. At the end of the 6.5-min run, the flow rate was increased to 1.0 ml min⁻¹ to flush the column with 95% methanol for 3 min and re-equilibrate it with the initial composition of the mobile phase for 3 min before starting the next run. Flow from the analytical column was directed into the spray chamber of an Agilent 6410 Triple Quad LC/MS system equipped with an API electrospray source (Agilent Technologies, Inc., Santa Clara, CA) from 0 to 6.5 min during the run and to waste at all other times. Nitrogen was used as the nebulizing gas at 60 p.s.i. and as the drying gas at a flow rate of 13 l min⁻¹ and a temperature of 350°C. The capillary voltage was 1500 V during positive ion detection and -1200 V for negative ion detection. MS/MS detection was performed using high purity nitrogen as the collision gas and operating the instrument in the multiple reaction-monitoring mode. The polarity of the mass spectrometer was positive for monitoring 5-FC, and negative for monitoring 5-FU and 5-BU. The individualized operating parameters that provided maximum response for each compound were as follows; 5-FC: transition, m/z 130.0 \rightarrow 113.0; fragmentor voltage, 110 V; collision energy, 20 V; 5-FU: transition, m/z 129.0 \rightarrow 42.0; fragmentor voltage, -80 V; collision energy, -16 V; 5-BU: transition, m/z 189.0 \rightarrow 42.0; fragmentor voltage, -85 V; collision energy, -16 V. The dwell time was 70 ms for each transition and the quadrupoles were maintained at unit resolution. Quantitation was based on integrating the extracted ion chromatograms for each transition.

The analytical method was thoroughly validated according to current recommendations.²⁸ Average \pm s.d.

retention times were 3.67 ± 0.02 min for 5-FC, 4.03 ± 0.01 min for 5-FU and 5.69 ± 0.02 min for 5-BU. Peaks that interfered with detection of either compound were not evident in chromatograms of pretreatment mouse plasma or tumor homogenate. Study samples were prepared for analysis together with a complete set of calibration standards, drug-free plasma with and without addition of the IS, a set of quality control samples and run in a computer-generated randomized order. The relationship between the 5-FC/IS and 5-FU/IS peak area ratios (y) and known concentration of the analyte in each calibration standard (x) were fit to the equation for a straight line ($y = ax + b$) or an exponential equation ($y = a + b \cdot x^c$), respectively, using WinNonlin Professional 5.0 software (Pharsight Corp., Cary, NC). Values of the parameters for the best-fit equations were used to calculate the analyte concentrations in study samples. Concentrations of 5-FC and 5-FU in tumor tissue, expressed as μg per g tissue weight, were calculated by multiplying their measured concentrations in the homogenate by the combined volume of tumor tissue and PBS added to prepare the homogenate and by dividing the product by the tumor tissue volume, assuming that the density of 1 g ml^{-1} for tumor tissue. The geometric mean concentration of 5-FC and 5-FU was calculated from the observed concentrations for the group of animals sampled at each time point.

The average correlation coefficient for the calibration curves was 0.999 for both 5-FC and 5-FU. Interday accuracy for assaying both compounds in the quality control samples ranged from 95.0 to 103.9% of the known concentrations with a precision of 6.5–10.0%. 5-FC and

5-FU were measured at their 10 ng ml^{-1} lower limits of quantitation with an accuracy $< 108\%$ and precision $< 8\%$. The limits of detection were 2 ng ml^{-1} for 5-FC and 1 ng ml^{-1} for 5-FU.

Statistical analysis

Statistical analyses were performed with a Student's *t*-test. $P < 0.05$ was considered to be significant.

Results

Construction of a replication-conditional HSV-1 expressing the yCD-UPRT gene

HSV-1 mutant expressing the myc-tagged yCD-UPRT gene in the ICP6 locus under the control of the CMV-IE promoter was constructed from HSV-1 strain Patton, using the Flip-Flop HSV-BAC system (Figure 1a).²¹ After three rounds of limiting dilution for purification, viral isolates were selected, with the correct genetic structure for each confirmed. One viral isolate termed bMP6-CMVFcumB was selected for subsequent experiments.

To confirm the protein expression of the yCD-UPRT gene, HT29 cells were infected with bMP6-CMVFcumB at an MOI of 2. Cell extracts after virus infection for 20 h showed protein expression of yCD-UPRT as assessed by western blot for the myc-tag (Figure 1b).

Addition of 5-FC enhances cytotoxicity against cancer cell line

We evaluated cytotoxicity in HT29 and MC26 cells infected with bMP6-CMVFcumB at a constant MOI of 0.01 or

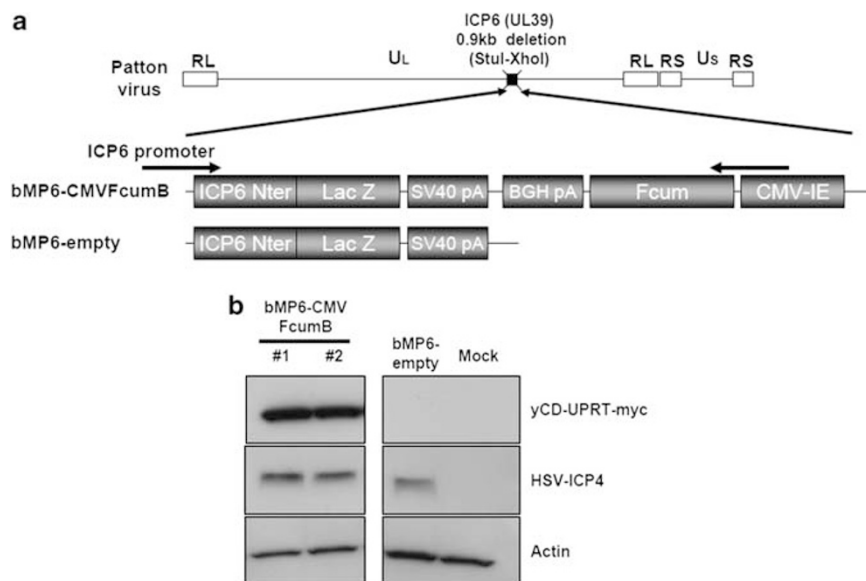


Figure 1 Construction of an armed HSV-1 expressing the yCD-UPRT gene. **(a)** Schematic diagram of HSV-1 expressing yCD-UPRT gene. **(b)** HT29 cells were infected with bMP6-CMVFcumB virus at MOI of 2 for 20 h and western blot was used to detect the expression of yCD-UPRT by the myc-tag. Lane #1 and lane #2 represent two different viral isolates. ICP4 is expressed by both bMP6-CMVFcumB and bMP6-empty. BGH pA, bovine growth hormone polyadenylation; CMV-IE, cytomegalovirus-immediate early; Fcum, codon-optimized myc-tagged yCD-UPRT; HSV-1, Herpes simplex virus 1; ICP4, infected cell protein 4; LacZ, β -galactosidase; MOI, multiplicity of infection; Nter, *N*-terminal; pA, polyadenylation; RL, repeat long; RS, repeat short; SV40, simian virus 40; UL, unique long; US, unique short; yCD, yeast cytosine deaminase.

0.185, respectively, in the presence of increasing concentrations of the prodrug 5-FC. Cell killing at 5 days after viral infection was determined by MTT assay. Compared with the control virus bMP6-empty, bMP6-CMVFcumB produced increased cytotoxicity with increasing 5-FC concentrations, with a 50% cytotoxicity observed at approximately $50 \mu\text{g ml}^{-1}$ for HT29 and $10 \mu\text{g ml}^{-1}$ for MC26 (Figure 2a and b). We then explored the cytotoxicity against HT29 and MC26 cells infected with bMP6-CMVFcumB at increasing MOI ranging from 0.0001 to 1.6 in the presence or absence of a constant concentration of 5-FC ($50 \mu\text{g ml}^{-1}$). 5-FC conferred increased cytotoxicity to HT29 cells at MOI values ranging from 0.01 to 0.1 (Figure 2c) and to MC26 cells at MOI values ranging from 0.02 to 1.6 (Figure 2d). When both of these cell lines were infected with bMP6-empty virus at same MOI in the presence or absence of 5-FC, as expected the addition of 5-FC did not enhance cell killing (Figure 2e and f).

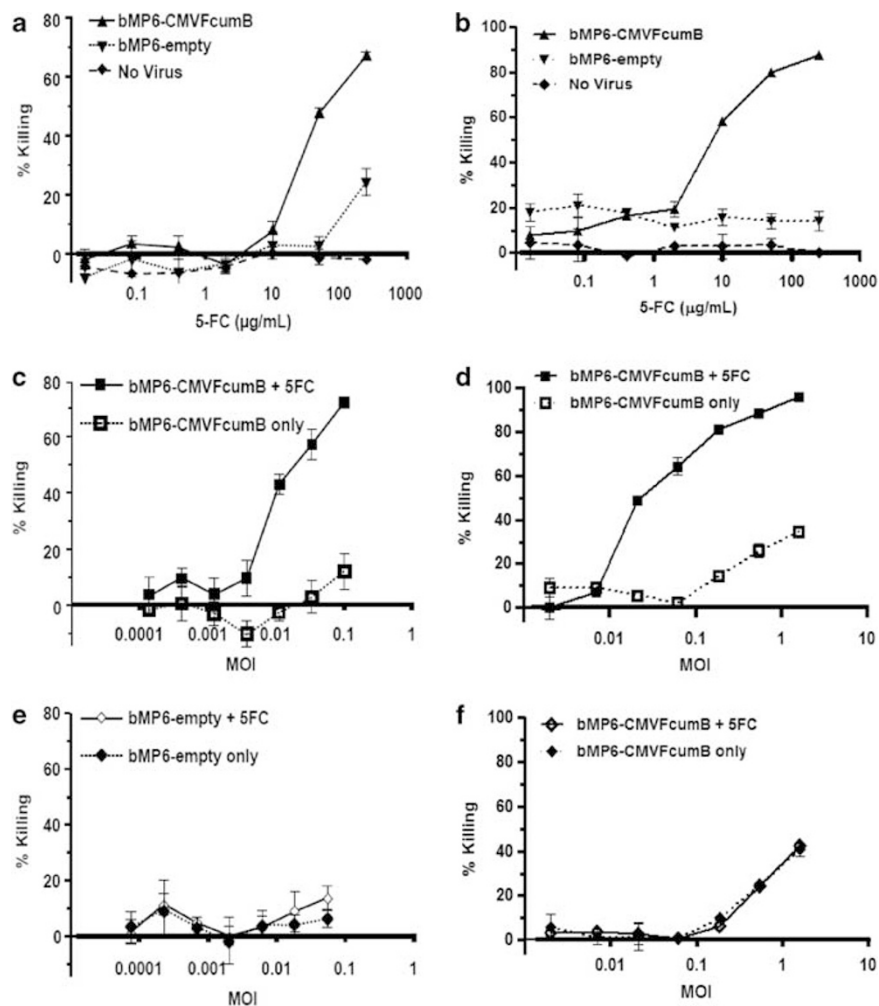


Figure 2 5-FC administration enhances the efficacy of the armed, oncolytic virus, bMP6-CMVFcumB. (a) HT29 and (b) MC26 cells were infected with bMP6-CMVFcumB at a MOI of 0.01 or 0.185, respectively, in the presence or absence of increasing concentrations of 5-FC and cytotoxicity was determined by MTT assay. (c) HT29 and (d) MC26 cells were infected with bMP6-CMVFcumB at increasing MOIs in the presence or absence of 5-FC at a concentration of $50 \mu\text{g ml}^{-1}$ and cytotoxicity was determined by MTT assay. (e) HT29 and (f) MC26 cells were infected with the control virus bMP6-empty at increasing MOIs in the presence or absence of 5-FC at a concentration of $50 \mu\text{g ml}^{-1}$ and cytotoxicity was determined by MTT assay. 5-FC, 5-fluorocytosine; MOI, multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

This strategy of yCD–UPRT expression in combination with 5-FC has the theoretical potential to interfere with viral replication and thereby reduce overall anti-tumor efficacy.^{13,29} Accordingly, we examined cytotoxicity when 5-FC initiation was varied after bMP6-CMVFcumB infection of HT29 cells, as well as infection of murine MC26 cells. Bioactivation of 5-FC enhances cytotoxicity beyond that seen by lytic replication alone. Early 5-FC initiation was more effective than late initiation in HT29 cells and this difference was more apparent at relatively high MOI (Figure 3a). Similar results were seen with MC26 cells (Figure 3b).

Optimal dosing schedule of 5-FC in mouse xenograft model

In order to examine the influence of timing of 5-FC initiation *in vivo*, BALB/c mice with MC26 flank tumors were treated by intratumoral injection of bMP6-CMVFcumB and 5-FC

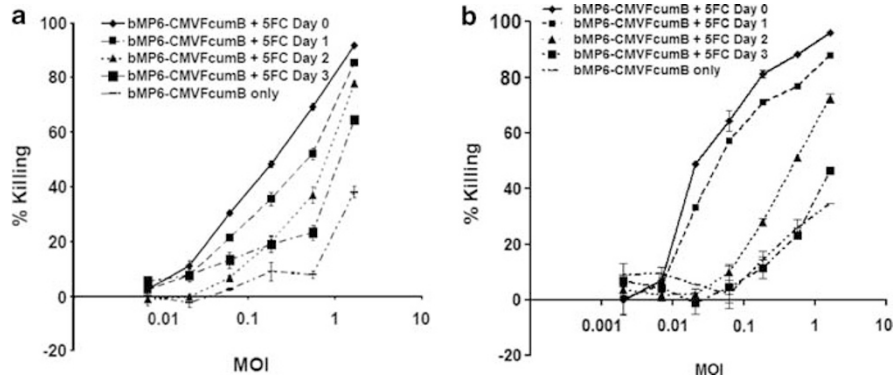


Figure 3 Early 5-FC initiation after viral infection is more effective than late administration. (a) HT29 cells and (b) MC26 cells were infected with bMP6-CMVFcumB at increasing MOIs and cytotoxicity was determined by MTT assay after 5-FC at a concentration of $50 \mu\text{g ml}^{-1}$ was initiated at several time points after viral infection. 5-FC, 5-fluorocytosine; MOI, multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.

was administered intraperitoneally. The initiation date for 5-FC was varied, while the duration of 5-FC was held constant (6 days). As shown in Figure 4a, Group 4 received 5-FC on days 0 through 6; Group 5 received 5-FC on days 3 through 9 and Group 6 received 5-FC on days 6 through 12. A single intratumoral injection of bMP6-CMVFcumB virus alone (no 5-FC administered) resulted in inhibition of tumor growth compared with controls (no treatment or 5-FC alone) (Group 1 vs. Group 3; $P=0.0022$, Group 2 vs. Group 3; $P=0.0062$). Also, the addition of 5-FC on Day 0 or Day 3 enhanced the anti-tumor activity of bMP6-CMVFcumB significantly (Group 3 vs. Group 4; $P=0.011$; Group 3 vs. Group 5; $P<0.001$). Importantly, 5-FC initiation on Day 3 showed greater inhibition of tumor growth compared with late 5-FC initiation (Day 6) (Group 5 vs. Group 6; $P=0.036$), and there was also a trend whereby initiation on Day 3 was more effective than initiation on Day 0 but this did not reach significance. The difference between initiation on Day 0 and initiation on Day 6 was not statistically significant (data not shown).

The optimal duration of 5-FC administration was assessed in the same mouse model. We investigated different durations of 5-FC administration after initiation on Day 3 after viral injection. Virus monotherapy resulted in inhibition of tumor growth compared with 5-FC alone (Group 1 vs. Group 2; $P=0.004$), and treatment with virus and 5-FC administration also resulted in significant inhibition compared with virus monotherapy (Group 1 vs. Group 3; $P=0.022$). Importantly, 5-FC administration for 12 days showed further inhibition of tumor growth than 5-FC administration for 6 days, however, 5-FC beyond 12 days provided no additional benefit (Figure 4b). These results indicated that optimal duration of 5-FC administration in this model was for 12 days and dosing for more than 12 days does not enhance tumor inhibition.

Viral replication and transgene expression in tumor cells
In order to better understand these results, we explored the kinetics of viral replication and transgene expression

in this flank tumor model. bMP6-CMVFcumB virus was administered intratumorally into MC26 tumors on flanks of BALB/c mice, and the tumors were subsequently harvested at different time points between Day 0 and Day 21 following virus injection. Tumor homogenates were analyzed by quantitative real-time PCR to assess genomic HSV-DNA, viral plaque assay to assess replicative virions and LacZ staining to assess transgene expression. Both viral genomic DNA copy number and viral titers peaked on Day 3 after viral injection and the values decreased gradually (Figure 5a and b). Importantly, these assays indicated that virus persisted at least 2 weeks after viral injection. Similarly, LacZ staining showed that transgene expression was evident in tumor homogenates between Day 3 and Day 15 after viral injection, however, no LacZ staining was observed in Day 21 tumor samples. These experimental results on viral titer and transgene-expression kinetics provide support for the observations that 5-FC administration starting on Day 3 after viral injection was superior to earlier or later initiation, and that 5-FC administration for a longer duration (up to 12 days) enhanced tumor destruction.

Mouse pharmacokinetic studies

A highly sensitive and specific LC-MS/MS assay was used to measure the concentrations of 5-FC and 5-FU in tumor tissue and plasma samples obtained after 3–18 days of daily treatment with 15 mg 5-FC, given by intraperitoneal injection, beginning 3 days after injecting the bMP6-CMVFcumB virus into subcutaneous MC26 flank tumors. The mean plasma concentration of 5-FC, which depends upon the time interval between administration of the prior dose and collection of the sample, ranged from $0.051 \pm 0.020 \mu\text{g ml}^{-1}$ (Day 9) to $2.14 \pm 8.10 \mu\text{g ml}^{-1}$ (Day 6) on the 4 days during which samples were obtained (Figure 6). Intratumoral concentrations of 5-FC were similar to its concentration in plasma and ranged from $0.080 \pm 0.017 \mu\text{g g}^{-1}$ (Day 9) to $1.46 \pm 5.41 \mu\text{g g}^{-1}$ (Day 6). Intratumoral concentrations of 5-FC were similar to its concentration in plasma and ranged from

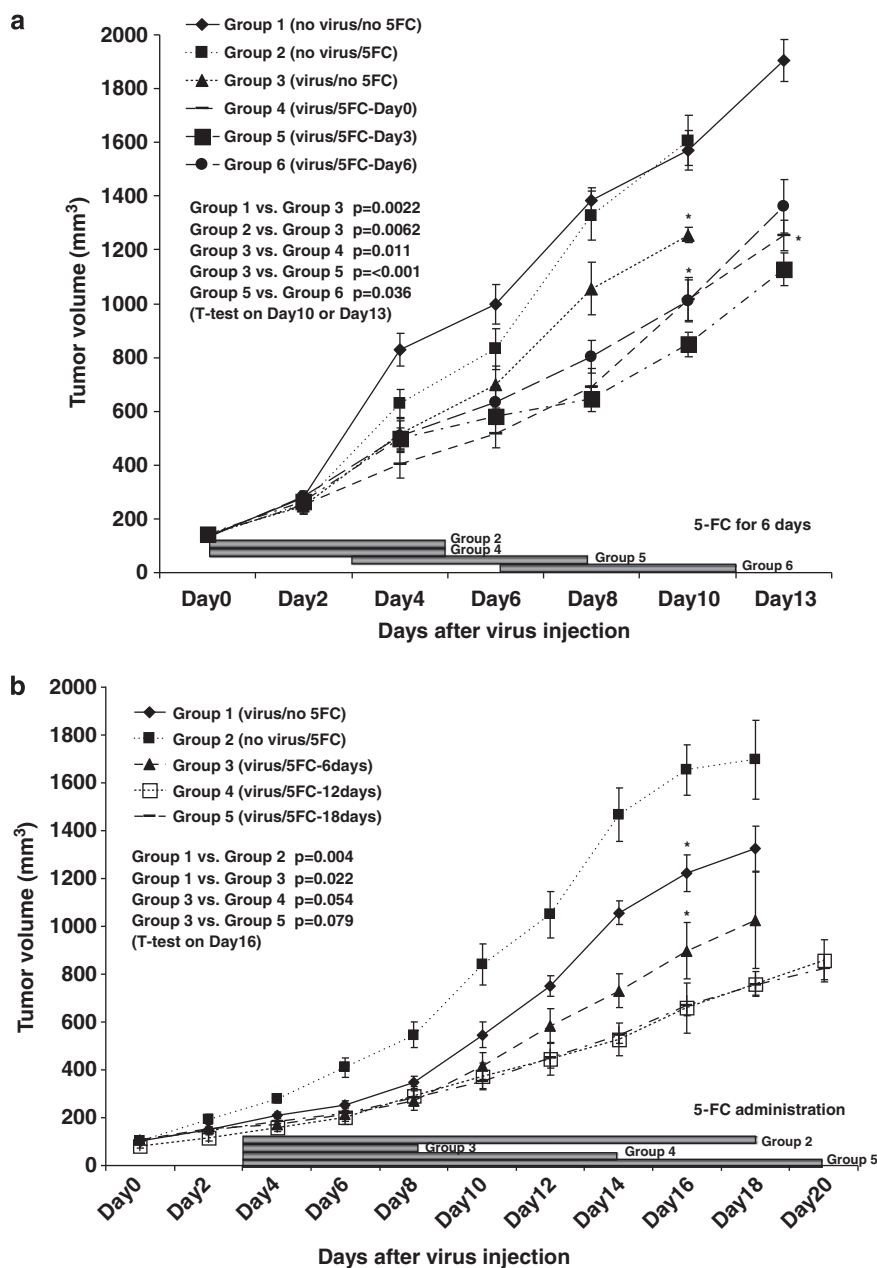


Figure 4 Early 5-FC initiation after viral infection and 5-FC administration for 12 days is the most effective dosing schedule in a mouse model. (a) MC26 flank tumors were treated by intratumoral injections of bMP6-CMVFcumB in combination with intraperitoneal injections of 5-FC at different initiation times over 6 days. (b) MC26 flank tumors were treated by intratumoral injections of bMP6-CMVFcumB in combination with intraperitoneal injections of 5-FC for different durations times over 18 days. 5-FC, 5-fluorocytosine

$0.080 \pm 0.017 \mu\text{g g}^{-1}$ (Day 6) to $1.46 \pm 5.41 \mu\text{g g}^{-1}$ (Day 3). 5-FU was present at measurable concentrations in tumor samples from at least four of the five mice evaluated at each time point. The mean intratumoral concentration of 5-FU relative to the corresponding intratumoral concentration of 5-FC decreased progressively from 0.49 ± 1.32 after 3 days of dosing to 0.038 ± 0.059 after 18 days of dosing. This finding substantiates that the relative extent of 5-FC conversion to 5-FU decreases progressively over time following viral injection, which indeed corresponds

with the kinetics of yCD expression. The concentration of 5-FU in plasma was below the 10 ng ml^{-1} lower limit of quantitation of the LC-MS/MS assay in all samples obtained at each time interval and undetectable ($<1 \text{ ng ml}^{-1}$) in 70% of the samples. These results demonstrate that combination therapy with a cytosine deaminase-expressing virus and the prodrug 5-FC achieves therapeutic intratumoral concentrations of 5-FU without significant systemic exposure to the cytotoxic anticancer drug.

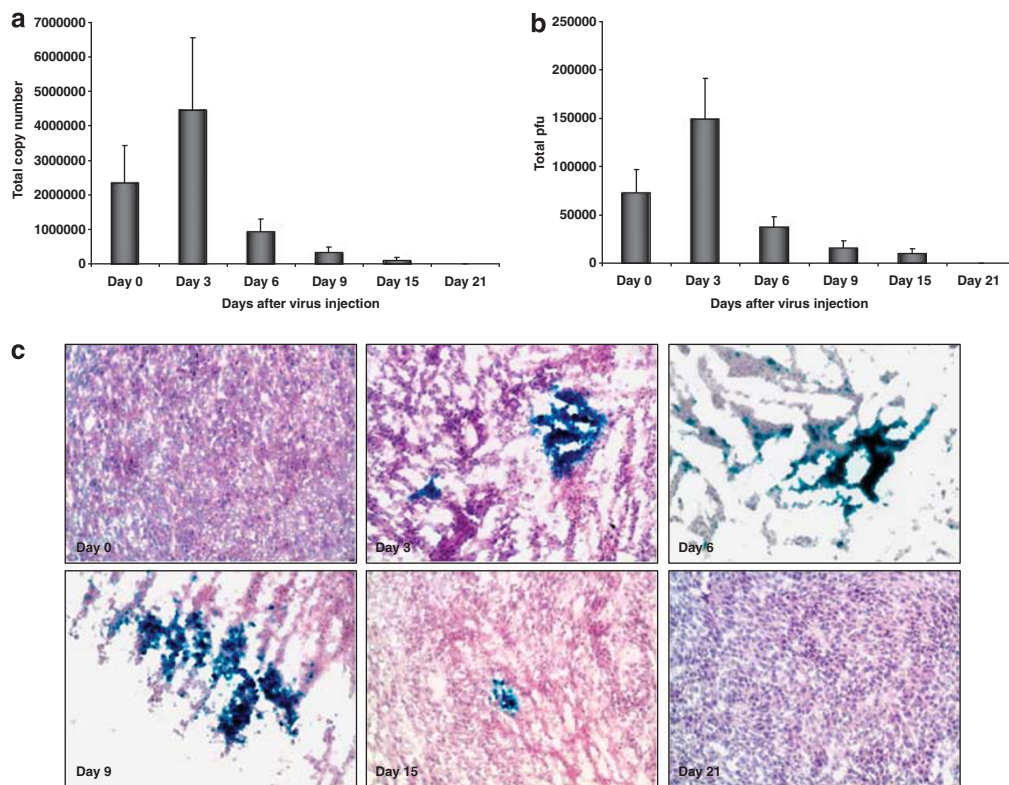


Figure 5 Viral replication and transgene expression are detected up to Day 15 after viral infection. MC26 flank tumors were treated with intratumoral injections of BMP6-CMVFcumB, and tumors were harvested at the indicated time points and analyzed. (a) Viral genomic DNA copy number was assessed by quantitative real-time PCR. (b) Viral titers were assessed by plaque assay. (c) Transgene expression was assessed by LacZ staining (magnification, $\times 200$).

Discussion

Results of several recent clinical trials of HSV-1 mutants for viral oncolysis have demonstrated safety of these viruses for cancer therapy. In each of these clinical trials, the HSV-1 mutant tested had been genetically altered to attenuate the pathological virulence. This strategy not only enhances safety for clinical application, but also attenuates the oncolytic efficiency compared with wild-type HSV-1. Therefore, we and others have explored strategies to enhance HSV-1 viral oncolysis by arming HSV-1 mutants with genes to enhance prodrug activation. Both *in vitro* and *in vivo* studies have demonstrated this approach to be more efficacious than treatment with the virus alone.^{30–32} The yCD gene as a prodrug-activation gene has several advantages in this application.¹³ Specifically, 5-FU has been shown to increase viral replication *in vitro* and extend animal survival *in vivo*.³³ In addition, the additive and synergistic effect of combining HSV-1 oncolysis with 5-FU (rather than bioactivation of 5-FC) has been reported as well.³⁴ Several studies have suggested that enhancement of viral replication by chemotherapeutics leading to increased cell death could be a possible explanation for the detected synergistic effects.^{35–37} Inhibition of viral replication by chemotherapy or activated prodrugs may also be observed, and is caused either

by direct interference with viral DNA synthesis or by killing surrounding cells before their infection. In anticipation of designing clinical trials with yCD-expressing HSV-1 mutants, we examined the relationship between the kinetics of viral replication, timing of 5-FU administration and anti-tumor efficacy.

With HSV-1 administered via direct intratumor injection, viral DNA is immediately measurable and following replication viral titers peak 3 days later. Transgene expression is observed in the infected tissue on Day 3 and persists through Day 15, with onset a bit delayed compared with onset of viral replication. On the basis of these kinetics, it is not a surprising observation that making 5-FU available during the height of infection (Day 3) leads to more effective tumor killing than initiating 5-FU before peak viral replication and transgene expression (Day 0) or waiting until Day 6 to introduce the prodrug. In addition, declining concentrations of 5-FU in the tumors beyond Day 6 are an expected observation and correlate with declining intratumoral transgene expression. Nonetheless, it is notable that virus and transgene expression are detectable in the tumors for at least 15 days following a single intratumoral injection, and bioactivated 5-FU (for example, 5-FU) is measurable in the tissue for up to 21 days. HSV-1 replication was not greatly affected by 5-FU at a concentration of $50 \mu\text{g ml}^{-1}$, which is

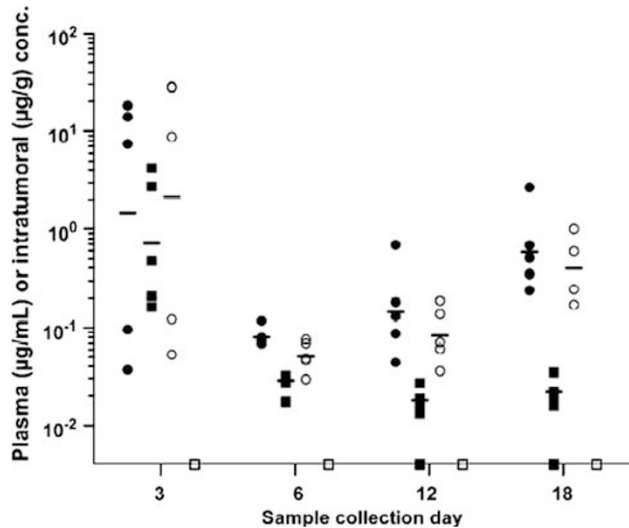


Figure 6 Mouse pharmacokinetic studies. Concentrations of 5-FC and 5-FU in tumor tissue and plasma samples obtained from mice after receiving 15 mg of 5-FC by intraperitoneal injection once a day for 3, 6, 12 or 18 days, beginning 3 days after intratumoral injection of the cytosine deaminase-expressing virus. Samples were obtained from groups of five mice at each sample time. Concentrations determined in samples from individual animals are indicated as follows: (○) 5-FC in plasma, (●) 5-FC in tumor, (■) 5-FU in tumor and (□) 5-FU in plasma. Symbols drawn through the x-axis denote samples with concentrations below the 10 ng ml^{-1} lower limit of quantitation. Horizontal bars represent mean concentrations. 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil.

consistent with previous findings.¹³ These data suggest that the added cytotoxicity achieved by combining BMP6-CMVFcumB with 5-FC exceeds any inhibition of viral replication by 5-FU metabolites. We did not repeat the experiments in a model of multiple viral administrations, but it is reasonable to assume that the principles elucidated in the current experiments would continue to apply.

The inhibition of tumor growth in our mouse model was modest even with prodrug administration. These results may be in part because of the single virus injection used in our study as discussed above. It is more likely though explained by our use of a syngeneic mouse model. Mouse cells are typically less infectable with HSV-1 than human cells and, therefore, cell killing would be expected to be more dramatic in human tumors.

At present, detection of viral replication and transgene expression requires acquisition of tissue followed by analysis using molecular techniques, such as immunohistochemical staining and PCR amplification. These assays are accurate and sensitive, but they require sacrifice of the mouse at each time point, thereby limiting comparison of measurements over time in the same animal. Recently, in order to measure the magnitude and anatomic sites of viral infection or transgene activity *in vivo*, some techniques such as magnetic resonance imaging and positron emission tomography have been developed.^{38–41} However, to our knowledge, there is no study employing these techniques to measure the kinetics

of viral replication and transgene expression in combination with prodrug over a prolonged period of time in a mouse model.

In this prodrug/enzyme system, the levels of 5-FU in plasma and tumors are a matter of concern. It has been suggested that γ CD delivered by an oncolytic HSV-1 vector could be used to reduce the side effects of 5-FU by promoting a localized tumor conversion of 5-FC to 5-FU without raising systemic 5-FU levels.⁴² But, although previous studies in preclinical models have analyzed intratumoral 5-FU concentration in tumors treated with viruses that express γ CD and 5-FC,^{43–45} the relationship between intratumoral 5-FU concentration and plasma 5-FU concentration over time has never been explored. This study is the first to measure the systemic levels in such model and confirms that 5-FU levels were below the level of detection in almost all of the plasma samples. By assessing viral replication and transgene expression kinetics, we were able to establish an optimal dosing schedule for this BMP6-CMVFcumB virus in combination with 5-FC based on the kinetics of viral replication and transgene expression. The approaches used in these studies are useful in the design of clinical trials of armed, oncolytic viruses.

Conflict of interest

The authors declare no conflict of interest.

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