Herpes Simplex Virus Us3(-) Mutant as Oncolytic Strategy and Synergizes with Phosphatidylinositol 3-Kinase-Akt–Targeting Molecular Therapeutics

Ta-Chiang Liu, Hiroaki Wakimoto, Robert L. Martuza, and Samuel D. Rabkin

Abstract

Purpose: Oncolytic herpes simplex virus (HSV) vectors have shown safety in clinical trials, but efficacy remains unsatisfactory. Novel HSV vectors that possess tumor selectivity with enhanced potency are therefore needed. The gene product of HSV Us3 protects virus-infected cells from apoptosis, a cellular pathway frequently dysfunctional in tumors. We hypothesized that Us3 mutants, whose replication would be inhibited by apoptosis in normal cells, would be selective for tumor cells.

Experimental Design: HSV mutants G207 (ribonucleotide reductase$^-$/γ34.5$^-$), R7041 (Us3$^-$), and R7306 (Us3 revertant) were tested in normal and tumor cells for viral replication, antitumoral potency, apoptosis induction, and Akt activation. Safety and biodistribution after systemic administration and antitumoral efficacy after intratumoral (i.t.) or i.v. administration were examined.

Results: Us3 deletion results in up to 3-log replication inhibition in normal cells, which correlates with enhanced apoptosis induction. In contrast, R7041 replicates very well in tumor cells, showing 1 to 2 log greater yield than G207. *In vivo*, R7041 shows no signs of toxicity after systemic delivery in both immunocompetent and immunodeficient mice and shows preferential and prolonged replication in tumors compared with normal tissues. R7041 displays significant antitumoral efficacy after i.t. or i.v. administration. An additional feature of Us3 mutants is enhanced Akt activation compared with wild-type infection, which sensitizes cells to phosphatidylinositol 3-kinase-Akt inhibitors (LY294002, Akt inhibitor IV), shown by synergistic antitumoral activity *in vitro* and enhanced efficacy *in vivo*.

Conclusions: Us3 deletion confers enhanced tumor selectivity and antitumoral potency on herpes simplex virus-1 and provides for a novel mechanism of combination therapy with phosphatidylinositol 3-kinase-Akt–targeting molecular therapeutics.

Replication-selective oncolytic herpes simplex virus (HSV) vectors have emerged as a new platform for cancer therapy (1–4). These vectors replicate in and destroy tumor cells while sparing normal cells. The self-perpetuating nature allows a small amount of input virus to be amplified after several rounds of replication *in situ*. As these viruses mainly kill tumor cells by oncolysis, they do not have cross-resistance with other treatments (e.g., radiotherapy or chemotherapy) and, as such, are able to complement these therapeutic approaches.

Several HSV mutants (e.g., 1716, G207, NV1020, and OncoVex$^{GM\text{-}CSF}$) have been tested in clinical trials of patients with various solid tumors (5–10). Whereas these trials have confirmed the safety of these viruses in patients, therapeutic benefits have thus far remained limited (10, 11). Therefore, mechanisms compromising clinical efficacy need to be elucidated to enhance viral antitumoral potency while preserving tumor selectivity.

Most of the current oncolytic HSVs are constructed by the "gene deletion" approach in which viral genes dispensable for growth in tumor cells but not in normal cells are deleted. The majority of oncolytic HSV vectors in trials have deletions in γ34.5, which is associated with significant attenuation of viral replication in tumor cells. Therefore, an alternative approach is to identify other nonessential viral genes whose mutation will provide tumor selectivity with enhanced potency.

Viral antiapoptotic genes are a group of genes that antagonize apoptotic pathways. Replication of viruses with deletions in these genes is limited in normal cells, which have intact apoptosis pathways, but is only minimally affected in tumor cells, which have defects in apoptosis pathways (12). The HSV

Authors' Affiliation: Molecular Neurosurgery Laboratory, Brain Tumor Research Center, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts

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Note: R. Martuza and S. Rabkin are consultants to MediGene AG, which has a license from Georgetown University for G207.

Requests for reprints: Ta-Chiang Liu, Brain Tumor Research Center, Massachusetts General Hospital, CPZN-3800 Simches Research Building, 185 Cambridge Street, Boston, MA 02114. Phone: 617-726-3040; Fax: 617-643-3422; E-mail: tachiangliu@yahoo.com.

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Us3 gene product is a serine-threonine kinase, and it has been shown to be able to block apoptosis induced by various stimuli including infection (13–18). Other activities of Us3 include phosphorylation of HDAC (19), suppression of cellular respiration, reduction of oxygen consumption through a block in electron transport (20), inhibition of IFN response (21), and interactions with downstream suppressor of c-jun NH_2-terminal kinase in the Cdc42/Rac pathway (22). Previous studies have shown that an HSV-2 Us3(-) mutant is less toxic than wild-type virus, presumably due to enhanced apoptosis in susceptible tissues limiting virus spread (23–25).

Here, we propose deleting Us3 as a new strategy for construction of oncolytic HSV vectors. We show that *Us3* mutant R7041 possesses robust oncolytic activity while retaining safety. It also sensitizes tumor cells to phosphatidylinositol 3-kinase (PI3K)-Akt–targeting small molecules, thus opening up a new approach for combination therapies targeting this cancer pathway.

Materials and Methods

Cells and viruses. Human glioblastoma cell lines U87 and T98, human colorectal carcinoma cell line SW480, human lung adenocarcinoma cell line A549, human cervical carcinoma cell line HeLa, human fibroblast cell line MRC5, and African green monkey kidney cell line Vero were obtained from American Type Culture Collection and grown in DMEM + 10% calf serum. Normal primary human umbilical cord vascular endothelial cells, human prostate epithelial cells, and their culture medium EGM-2 and PrEGM were obtained from Cambrex and maintained as described by the vendor. *Us3*-deleted mutant R7041 and its revertant R7306 were kindly provided by Dr. Bernard Roizman (Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL; ref. 26). Oncolytic HSV mutant G207 has deletions of both copies of γ*34.5* and an inactivating LacZ insertion in *UL39* (encoding ICP6; ref. 27).

Virus replication assays. For virus replication assays, cells were seeded into 12-well plates (1×10^5 per well). Twenty-four hours later, cells were infected with different viruses at 1 plaque-forming unit (pfu)/cell for 2 h. Cells and medium were harvested at the indicated times post-infection, processed with three freeze/thaw cycles and sonication, and then titered on Vero cells. Experiments were repeated at least thrice with each condition in duplicate.

Cell survival assay. Cells were seeded into 96-well plates at 5,000 to 10,000 per well. Twenty-four hours later, cells were infected with 3-fold serial dilutions of viruses (starting from 3 to 30 pfu/cell). Seventy-two hours post-infection, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) assays were done according to vendor instruction. Dose-response curves and 50% effective dose values (ED_{50}) were obtained and compared.

In situ ***active caspase-3 determination.*** Cells were grown in eight-well chamber slides (Becton Dickinson). PBS or viruses (1 pfu/cell) were added and slides were washed with cold PBS and fixed with methanol at 20 h post-infection. The active-form caspase-3 was detected by immunohistochemistry as previously described using rabbit anti-human active-form caspase-3 antibody (0.3 μg/mL; R&D Systems; ref. 28). The percentages of caspase-3–positive cells were determined by counting a total of 1,000 cells for each condition in triplicates.

Activated Akt assay. U87 and A549 cells were mock infected or infected with R7041 or R7306 as described above. Cells were harvested at 8 h post-infection. Akt activation was determined by Western blot analysis with total or phosphorylated Akt (Ser^{473}) rabbit anti-human primary antibodies (1:1,000; Cell Signaling).

Interaction between HSV and PI3K-Akt–targeting small molecules. A549 and U87 cells were seeded into 96-well plates at 10,000 per well. After 24 h, cells were treated with virus alone, small molecules alone, or their combination at different dose levels as previously described using Chou-Talalay analysis (29). Small molecules were given 8 h after virus infection. The cells were incubated for a further 72 h, then a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) assay was done. Experiments were repeated thrice with each condition in triplicate. Dose-response curves were fit to Chou-Talalay lines (29). Fraction affected-combination index correlation was then obtained, with combination index values of 0.8 and 1.2 as cutoff points for synergy and antagonism, respectively.

Safety studies. Two animal models were used for toxicity assessment. Athymic mice (National Cancer Institute) were given PBS or viruses (1×10^7 pfu/ injection) via i.v. route (n = 4-7). Strain F was given on day 0, and R7041 and G207 were given on days 0, 3, and 6. Animals were monitored for signs of toxicities and were sacrificed when moribund. In addition, immunocompetent BALB/c mice (National Cancer Institute) were given PBS or viruses i.p. (1×10^8 pfu/injection; n = 8). Mice were monitored for 5 weeks and sacrificed when found to be moribund. Animal care was in accord with institution guidelines.

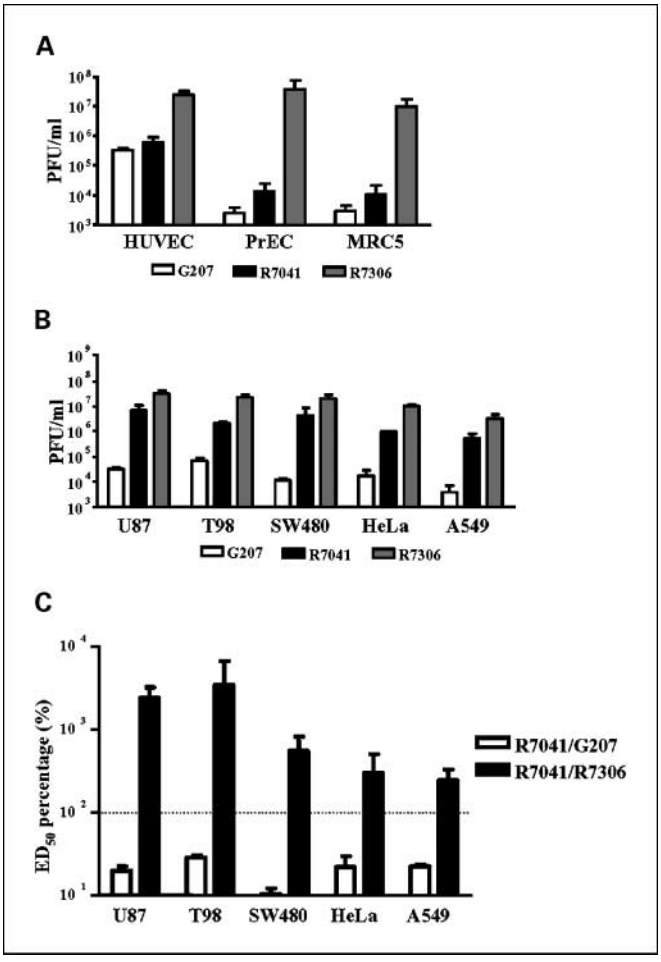

Fig. 1. *A,* comparison of viral replication of G207, Us3(-) mutantR7041, and Us3(+) revertant R7306 in normal cells at 24 h post-infection. White columns, G207; black columns, R7041; gray columns, R7306. $P < 0.05$, between G207 or R7041 and R7306 in all cell lines. *B,* comparison of viral replication of G207 and R7041 in tumor cells. White columns, G207; black columns, R7041; gray columns, R7306. $P < 0.05$, between G207 and R7071 or R7306 in all cell lines and between R7041 and R7306 in T98 and HeLa. *C,* R7041 showed enhanced antitumoral potency over G207. ED_{50} values of R7041 were obtained and compared with those of G207 and R7306. ED_{50} percentages were shown. White columns, R7041/G207 percentages; black columns, R7041/R7306 percentages.

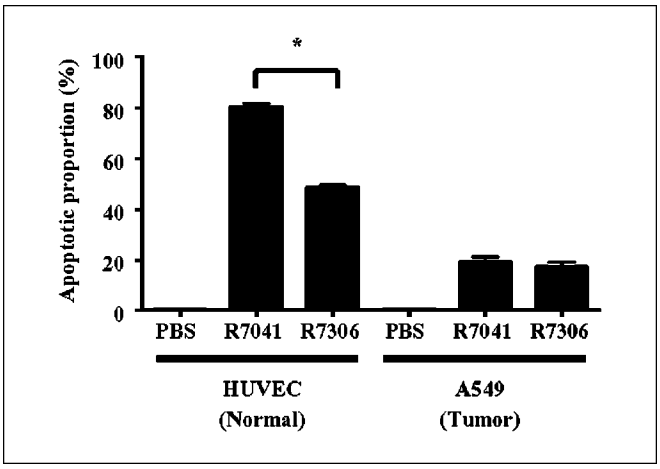

Fig. 2. Enhanced apoptosis induction in normal cells with R7041 infection. Normal cells [human umbilical cord vascular endothelial cells (*HUVEC*)] or tumor cells (A549) were treated with PBS, R7041, or R7306, and active caspase-3 was determined by immunocytochemistry. Both viruses induce greater apoptosis compared with PBS, whereas R7041 showed enhanced apoptosis induction over R7306 in normal cells but not in tumor cells. *, $P < 0.0001$, between R7041 and R7306.

Biodistribution studies. A549 cells were implanted s.c. into the flanks of 6- to 8-week-old athymic mice (National Cancer Institute; 1×10^7 cells per implantation). Once tumor size reached 50 to 100 mm^3, the animals were randomized into four groups ($n = 4$ or 5 per group). A single i.v. injection of R7041 (1×10^7 pfu) was given (day 0), and animals were sacrificed and organs and tumors harvested on days 1, 3, 7, 14, and 21. Tissue samples were minced, subjected to three freeze-thaw cycles and sonication, and titered on Vero cells as described above.

Efficacy studies. U87 cells were implanted s.c. into the flanks of 6- to 8-week-old athymic mice (1×10^6 cells per implantation). Once tumor size reached 50 to 100 mm^3, the animals were randomized into three groups (mock, G207, or R7041; $n = 7$ per group) on day 0. Intratumoral (i.t.) injections of PBS or viruses (3×10^6 pfu/injection) were given on days 1, 4, and 7. The A549 tumor model was the same as in the biodistribution studies. Mice were randomized into three groups as above. Intravenous injections of PBS or viruses (1×10^7 pfu/injection) were given on days 1, 4, and 7 ($n = 7$). For the combination studies, U87 tumors were implanted and randomized into four groups as described ($n = 12$ per group): mock, LY294002 alone, R7041 alone, or LY294002 + R7041. Intratumoral injections of PBS or R7041 (5×10^5 pfu/injection) were given on days 1, 4, and 7, whereas i.p. injections of LY294002 (18 mg/kg) were given daily from days 1 to 8. Tumor volumes and survival were monitored two to three times a week for all studies. Animal care was in accord with institution guidelines.

Statistical analysis. Comparisons of variables (*in vitro* and *in vivo* viral yield, ED_{50} values, percentage of cells undergoing apoptosis, and growth of tumors) were made using two-sided Student's *t* test or one-way ANOVA. Comparisons of Kaplan-Meier curves were made using log-rank tests. $P < 0.05$ was considered statistically significant.

Results

Us3 mutant R7041 is tumor selective with enhanced replication and potency versus G207 in tumor cells. We compared the replication of G207, Us3(-) mutant R7041, and Us3(+) revertant R7306 in a panel of normal cell lines. In these cells, R7041 showed similar viral yield as G207 (Fig. 1A; $P > 0.05$, between G207 and R7041 in all cell lines). Both G207 and R7041 showed significant reduction in burst size versus R7306 (Fig. 1A; $P < 0.05$, between G207 or R7041 and R7306 in all cell lines).

We next compared the replication of G207 and R7041 in a panel of tumor cells. In these cells, the virus yield of G207 ranged from 4,000 to 56,000 pfu/mL at 24 h post-infection and from 5,000 to 67,000 pfu/mL at 48 h post-infection. In contrast to our findings in normal cells, R7041 showed 6- to 217-fold increase in burst size versus G207 at 24 h post-infection, and 34- to 681-fold increase at 48 h post-infection (Fig. 1B; $P < 0.05$, between G207 and R7041 or R7306 in all cell lines and between R7041 and R7306 in T98 and HeLa).

The antitumoral potency of the viruses was then tested. ED_{50} values of the viruses were determined for each cell line and compared. R7041 was attenuated compared with R7306 (Fig. 1C; $P < 0.05$, for U87, T98, and SW480), but it was significantly more potent than G207 (Fig. 1C; $P < 0.05$, for all cell lines). Therefore, enhanced viral replication of R7041 in tumor cells correlated with enhanced antitumoral potency.

Us3(-) R7041 induces apoptosis preferentially in normal cells. Previous studies describing oncolytic viruses engineered by deletions in antiapoptotic genes show that these viruses induce apoptosis preferentially in normal cells, resulting in limited viral replication and spread (12, 28, 30). We therefore tested if R7041 exerts similar phenotypes. As shown in Fig. 2, using caspase-3 activation as readout, tumor cells treated with R7041 or R7306 showed similar levels of apoptosis (Fig. 2; $P > 0.05$). In contrast, normal cells infected with R7041 showed

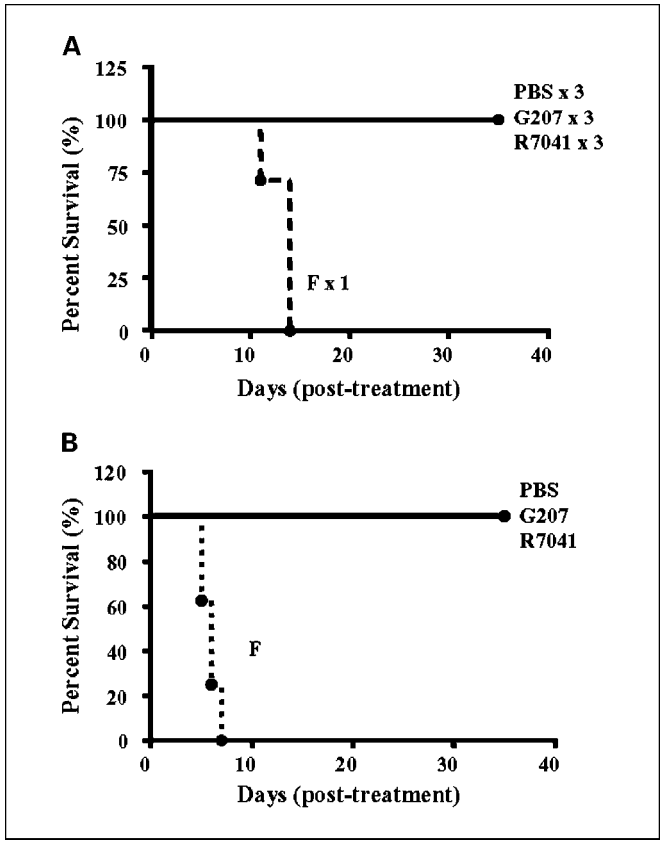

Fig. 3. Toxicity studies of HSV vectors. *A,* PBS or viruses were injected into *nu/nu* mice via tail vein. Whereas strain F treatment group all died within 14 d, no mortality or clinical symptoms were found in G207 and R7041 groups up to 35 d posttreatment ($P < 0.0001$). *B,* PBS or viruses were injected into immunocompetent BALB/c mice via single i.p. injection. Strain F treatment group all died within 7 d, whereas all other groups showed 100% survival up to 35 d posttreatment ($P < 0.0001$).

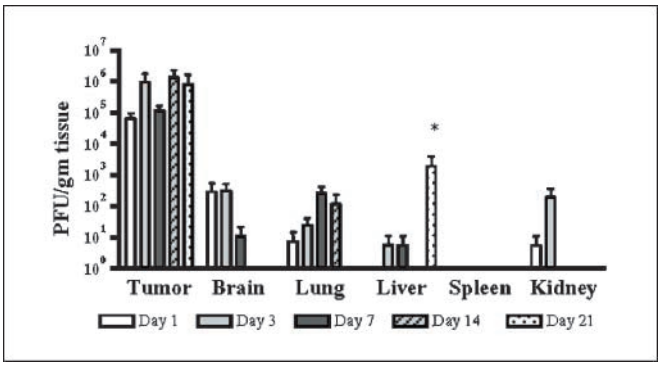

Fig. 4. R7041 preferentially replicates in tumor tissues after systemic delivery. Viruses were recovered from tumor and various normal organs at different time points after i.v. administration. Virus titers in tumors were significantly greater and prolonged compared with normal organs ($P < 0.01$). *, virus was recovered in only one of four animals.

increased apoptosis induction compared with R7306-treated cells (Fig. 2; $P < 0.0001$).

Us3 deletion significantly enhances safety in both immunodeficient and immunocompetent animals. To evaluate the safety profile of R7041, we first injected PBS, G207, R7041, or strain F (wild-type) i.v. into athymic mice. As shown in Fig. 3A, whereas single strain F injection with 1×10^7 pfu resulted in animal death within 14 days, three injections (given on days 0, 3, and 6) of G207 or R7041 did not cause animal death ($P < 0.0001$) or any clinical symptoms up to 5 weeks. In a separate study, immunocompetent BALB/c mice were given a single i.p. administration of PBS or viruses (1×10^8 pfu). Strain F–treated mice all died within 1 week of injection, whereas neither the G207 nor the R7041 treatment group showed disease symptoms, similarly with the PBS treatment group (Fig. 3B; $P < 0.0001$). Therefore, R7041 showed similar safety profiles to G207 after i.v. and i.p. administrations in both immunocompetent and immunodeficient mice.

R7041 shows preferential persistence/replication in tumor tissues after systemic delivery. We then explored biodistribution and replication of the virus after systemic delivery (i.e., *in vivo* tumor selectivity). Tumors and organs were harvested at specific time points after single i.v. virus administration. Infectious virus was recovered from tumor tissue over a prolonged period (up to 21 days post-injection), whereas very low levels were detected in normal tissues for mostly less than a week (Fig. 4). In addition, the amount of virus recovered (standardized by weight) in tumor was significantly greater than in other organs (Fig. 4; $P < 0.01$).

R7041 sensitizes tumor cells to Akt-targeted small molecules. Previous reports have shown that R7041-infected cells showed enhanced Akt phosphorylation/activation compared with R7306 wild-type–infected cells (31). Our results showed that whereas R7306 infection minimally (in U87 cells) or moderately (in A549 cells) induced Akt phosphorylation, R7041 infection strongly induced Akt activation (Fig. 5A). Because the Akt pathway is critical in tumorigenesis and has been a target for drug discovery (32–34), we explored whether activation of Akt by virus infection would sensitize tumor cells to small molecules targeting the PI3K-Akt pathway. We infected tumor cells with R7041 or R7306, followed by treatment with PI3K-Akt–targeting small molecules (LY294002, Akt inhibitor IV). As shown in Fig. 5B and C, LY294002 and Akt inhibitor IV synergized with R7041 in both cancer cell lines (combination index: U87 cells, 0.103-0.248; A549 cells, 0.224-0.775), whereas the effect with

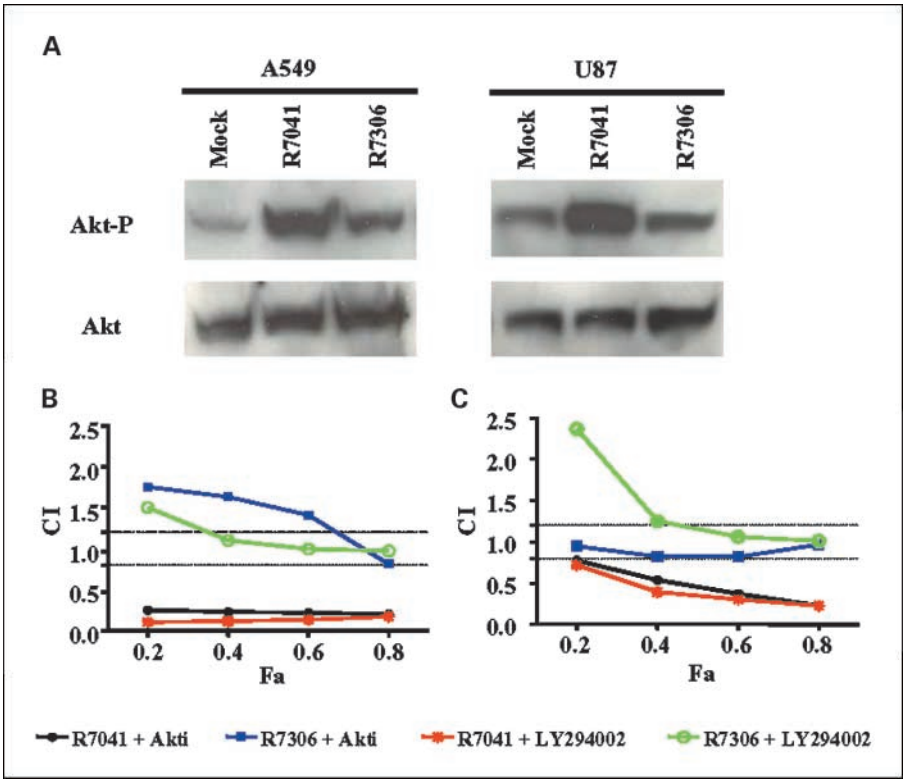

Fig. 5. R7041 infection activates the PI3K-Akt pathway and sensitizes tumor cells to PI3K-Akt–targeting molecular therapeutics. *A*, Western blot analysis of total and phosphorylated Akt after treatment in U87 and A549 cells. Fraction affected-combination index (*Fa-CI*) plot of U87 (*B*) or A549 (*C*) cells receiving HSV and compounds targeting the PI3K-Akt pathway. Whereas synergy was seen in R7041 and LY294002 or Akt inhibitor IV, R7306 showed additive to antagonistic effect with these agents.

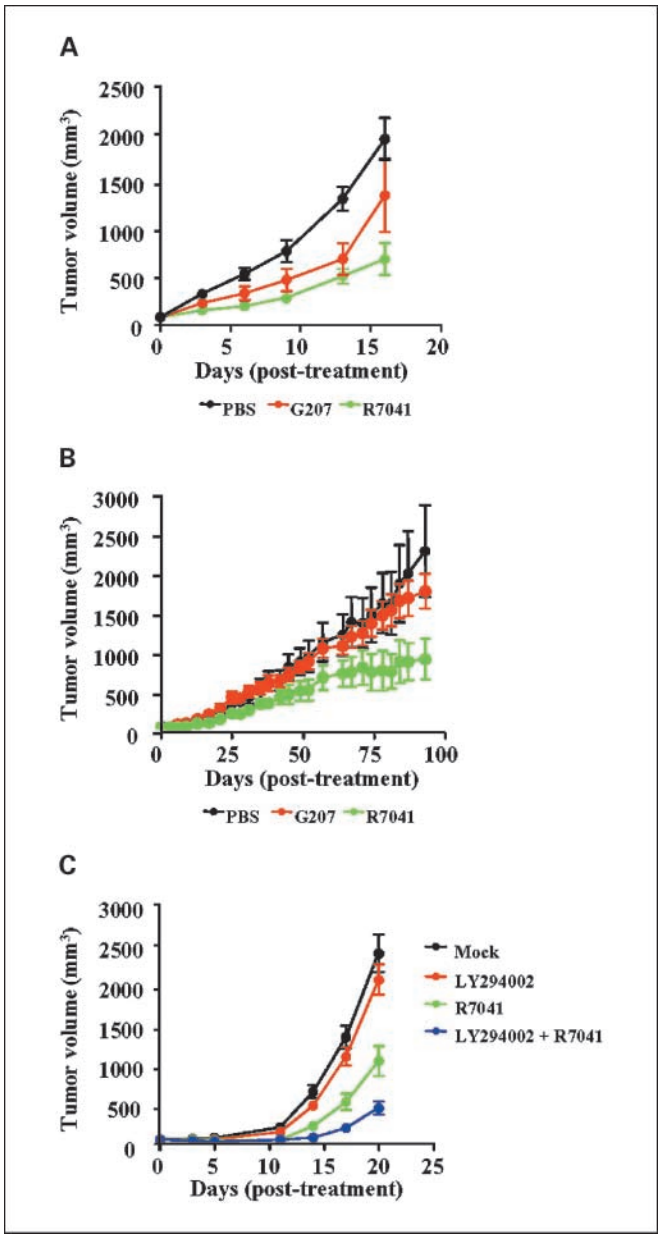

Fig. 6. Antitumoral efficacy of R7041. *A,* U87 xenograft model. Animals received i.t. injections of PBS, G207, or R7041. R7041 treatment group showed enhanced tumor inhibition compared with both PBS and G207 groups ($P < 0.005$). *B,* A549 xenograft model. The R7041 treatment group showed enhanced efficacy compared with both PBS and G207 groups ($P < 0.01$). *C,* U87 xenograft model. Animals were mock treated or treated with LY294002 alone, R7041 alone, or their combination. Whereas R7041 treatment showed significant tumor growth inhibition ($P < 0.001$), combination treatment group showed further enhanced efficacy ($P < 0.01$).

R7306 was mostly additive to antagonistic (combination index: U87 cells, 0.811-1.750; A549 cells, 0.815-2.370).

***R7041 shows enhanced antitumoral efficacy* in vivo.** Two human tumor xenograft models were used to test the efficacy of the viruses. We first tested the viruses in U87 glioma tumors with i.t. injections. R7041 showed greater tumor inhibition than PBS and G207 in this model (Fig. 6A; $P < 0.005$).

One of the greatest hurdles in clinical virotherapy is the lack of systemic efficacy. This is especially so with oncolytic HSV vectors in which most of the currently available mutants have significant attenuation that limits their efficacy. We therefore tested the viruses in A549 xenograft model using i.v. delivery. In this model, G207-treated animals showed no difference in tumor growth compared with PBS-treated animals ($P > 0.05$), whereas the R7041 treatment group showed significant tumor growth inhibition (Fig. 6B; $P < 0.01$). Therefore, after both i.t. and i.v. administration, R7041 showed greater efficacy than G207.

We next investigated whether combining R7041 with an Akt-targeting small molecule would enhance the therapeutic effect *in vivo*. The U87 xenograft model was tested by treating the tumors with i.t. R7041 and/or i.p. LY294002. Whereas LY294002 showed minimal effect on tumor growth, treatment with R7041 significantly inhibited tumor growth ($P < 0.001$), and combination treatment further inhibited tumor growth (Fig. 6C; $P < 0.01$, between single-agent treatment groups and combination treatment group). Therefore, enhanced efficacy was seen with Us3(-) HSV and LY294002.

Discussion

In this report, we identified an HSV mutant with deletion in *Us3* as a tumor-selective oncolytic virus with improved antitumoral efficacy compared with the earlier generation virus G207, which has been tested in clinical trials. The Us3(-) mutant R7041 showed up to a 3-log reduction in replication in normal cells compared with its revertant R7306. Although replication of G207 in normal cells is equally inhibited, in tumor cells R7041 showed up to a 700-fold increase in replication compared with G207. Enhanced replication is correlated with enhanced antitumoral potency. Importantly, enhanced apoptosis induction is noted preferentially in normal cells in which apoptosis pathways are intact, thus limiting replication and spread of R7041 in normal cells. Safety of R7041 was confirmed in both immunocompetent and immunodeficient animals, which was similar to that of G207. Specific activation of the PI3K-Akt pathway by R7041 also enables synergistic cell killing when combined with PI3K-Akt–targeting molecular therapeutics. Finally, efficacy of R7041 was shown to be superior to that of G207 in both i.t. and i.v. treatment models.

These are important findings that have great impact to the field. Although malignant glioma has been a major target tumor type for oncolytic HSV in which systemic efficacy is not a major concern (patients often succumb to the disease before metastases), previous clinical trials with other viruses and tumor types (e.g., melanoma, metastatic cutaneous tumors) failed to show systemic efficacy (10, 11). One of the limiting factors for unsatisfactory efficacy is that the viral genetic modifications that are associated with tumor selectivity also significantly attenuate the replication and spread of viruses in tumor cells. Therefore, novel viral genes essential for growth in normal cells but dispensable in tumor cells without losing efficacy need to be identified. The HSV *Us3*(-) mutant belongs to a growing family of oncolytic viruses that have one or more viral antiapoptotic genes deleted (12). This group of viruses takes advantage of dysregulated apoptosis pathways in cancer cells to facilitate their replication, which is significantly limited in normal cells in which apoptosis pathways remain intact.

Importantly, tumor selectivity of *Us3*(-) R7041 is shown both *in vitro* and *in vivo*. Our findings in toxicity and biodistribution studies are consistent with previous publications comparing

the toxicities of *Us3*(-) and *Us3*(+) viruses, in which the *Us3*(-) mutant was found to be severely attenuated in toxicity (up to 100,000-fold reduction in LD_{50}; refs. 23–25).

Previous studies have identified inhibition of the Akt pathway as one of the mechanisms for Us3 antiapoptotic activity (31). Our findings are consistent with this in that infection with R7041 showed significantly enhanced Akt activation. Activation of this pathway is common in most human cancers and has been shown to play important roles in cell survival, proliferation, antiapoptosis, and cell cycle arrest and has been a target for new anticancer therapeutics development (32–37). However, as the PI3K signaling pathway is also critical in maintaining various functions of normal cells, drugs targeting this pathway will likely need an expanded therapeutic window for success, such as when PI3K signaling is hyperactive (33). Our results are the first to show complementation of a genetically engineered oncolytic virus and molecular therapeutics through the P13K-Akt pathway.

Us3 deletion has recently been studied as a new oncolytic strategy (38). Although the data confirmed our findings that *Us3*(-) HSVs are tumor-selective oncolytic agents, several important questions remained unanswered. Mechanism(s) that inhibited *Us3*(-) mutant replication in normal cells were not shown. *In vivo* tumor selectivity was shown only at one early time point after i.t. injection; selectivity (by recovering infective viral particles) after systemic administration over an extended period was not studied. Importantly, although antitumoral efficacy was shown with i.t. injection, there is an unexplained discrepancy between *in vitro* and *in vivo* potency when compared with other oncolytic viruses used in the same study. Furthermore, antitumoral efficacy after systemic delivery was not shown. Finally, the effect of Akt activation in the context of combination therapy was not explored (38).

In summary, our results show that deleting the antiapoptotic *Us3* gene is a promising approach to engineer oncolytic HSVs. It enhances tumor selectivity, antitumoral potency, and efficacy. It also allows synergistic tumor killing with PI3K-Akt–targeting therapeutics. *Us3* deletion therefore has the potential of replacing *γ34.5* deletion for engineering oncolytic HSV vectors.

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