

# Replication-competent, Nonneuroinvasive Genetically Engineered Herpes Virus Is Highly Effective in the Treatment of Therapy-resistant Experimental Human Tumors<sup>1</sup>

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## Abstract

A genetically engineered, nonneurotropic herpes simplex virus (R7020) with a proven safety profile in both animals and humans was found effective in the treatment of large xenotransplanted tumors arising from a radiation- and chemotherapy-resistant human epidermoid carcinoma and a hormone-refractory prostate adenocarcinoma. R7020 replicated to high titer and caused rapid regression of the human tumor xenografts. Tumor destruction was accelerated in animals given both R7020 and fractionated ionizing radiation. Tumors arising from cells surviving one treatment with R7020 were fully susceptible to a second dose of virus. We conclude R7020 is an effective antitumor agent for non-central nervous system tumor xenografts with an excellent safety profile.

## Introduction

The strategy of virus-based anticancer therapy is to develop viruses capable of infecting malignant tumors, of spreading, and of destroying malignant tumors without deleterious effects in normal tissues. The key to the development of such agents is the identification of viral genes whose deletion or modification enables tumor selectivity in cell killing with appropriate genetically engineered viruses. The ability to intervene repeatedly with the same virus is another important aspect of viral therapy. The development of such therapies has been evasive despite much intensive investigation. Attenuated retroviruses encoding HSV<sup>3</sup> thymidine kinase were created to target dividing tumor cells (1). This strategy relies heavily on less efficient bystander effects because only a small fraction of tumor cells are infected. In another strategy, conditional replication-competent adenoviruses lacking the *E1b* 55 kDa gene were proposed to replicate only in tumor cells lacking p53 (2, 3). Recently, the basis of this strategy has been questioned (4, 5). In addition, only 50% of human tumors are estimated to have absent or nonfunctional p53 (6).

Genetically engineered, replication-competent HSVs (HSV-1) have recently been proposed to treat malignant gliomas (7). In antiglioma therapy, HSV-1 mutants were constructed to preferentially replicate in proliferating tumor cells, thereby eliminating the risk of widespread dissemination of the virus in the CNS characteristic of HSV encephalitis in humans. The viruses tested to date were mutants lacking genes

required for viral DNA synthesis in nondividing cells (e.g., thymidine kinase, ribonucleotide reductase; 7, 8). More recent studies were done with mutants lacking,  $\gamma_134.5$ , a gene required for viral replication in the CNS (9, 10). The decrease in viral proliferative potential required for safe intracranial HSV inoculation correlates with a decrease in the oncolytic potential of the virus (11). In this report, we describe initial studies on a genetically engineered virus with greater replication capability than  $\gamma_134.5$  null attenuated herpes viruses for tumors of non-CNS origin (7, 8, 10, 11).

R7020 was designed as a candidate for human immunization against infections with HSV-1 and HSV-2 (12). HSV DNA consists of two stretches of unique double-stranded DNA sequences flanked by inverted repeats (13). The inverted repeats contain five genes,  $\alpha 0$ ,  $\alpha 4$ ,  $\gamma_134.5$ , *ORF P*, and *ORF O*. In R7020, the  $U_L23$  (thymidine kinase) and  $U_L24$  genes were deleted. One set of the inverted repeats was replaced with the HSV-1  $U_L23$  gene under a stronger promoter and HSV-2 DNA sequences encoding the HSV-2 glycoproteins G, J, D, and I (12). Thus, R7020 has only one copy of  $\gamma_134.5$  deleted and is comprised of HSV-1 and HSV-2 genes. R7020 was extensively investigated in rodent and primate studies for genetic stability and safety (12, 14). Because the virus has demonstrated safety in limited human vaccine trials, we tested its potential use as an oncolytic agent for radioresistant, chemoresistant epidermoid carcinoma and hormone-resistant prostate adenocarcinoma xenograft models (15, 16).

The studies described in this report were based on three considerations: (a) previously described genetically engineered herpes viruses have limited antitumor activity in human tumor glioma xenografts resulting in tumor xenograft growth delays (7, 8, 10, 11); (b) studies described elsewhere demonstrated a synergistic destruction of malignant glioma xenografts by IR and genetically engineered HSV lacking both copies of the  $\gamma_134.5$  gene (11). The significant enhancement in tumor cell destruction was due to increased viral replication and spread in tumors irradiated shortly after viral inoculation; and (c) one of the causes of failure in cancer therapy is tumor cell resistance to cytotoxic or hormonal treatment. The genetic instability that leads to cell transformation enhances the evolution of drug and/or IR resistance or hormone resistance. For example, *p53* gene deletion or mutation may decrease tumor cell susceptibility to apoptosis induced by chemotherapy and/or IR (17–19). Mutations in the androgen receptor lead to hormone resistance in prostate cancer (20). Also, gain of function mutations such as activation of the *bcl2* family of genes enhances resistance to a variety of cytotoxic therapies (21). One benefit of using viral lysis as an antitumor strategy is that viral lysis has the potential to overcome tumor resistance to conventional agents. In addition to the intrinsic genetic instability of tumor cells, one consequence of anticancer treatment is the selection and evolution of cells resistant to the therapeutic agent. We investigated whether the exposure

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<sup>3</sup> The abbreviations used are: HSV, herpes simplex virus; CNS, central nervous system; IR, ionizing radiation; pfu, plaque-forming unit(s).

of tumors to HSV could lead to selection of tumors resistant to viral treatment.

We report two important findings in these studies: (a) R7020, a safe, clinically tested, genetically engineered recombinant virus is highly effective as an antitumor agent in chemotherapy/radiotherapy-resistant, hormonally resistant, p53+ and p53- xenograft models of human tumors (15, 16, 22). Moreover, the combination of IR and R7020 resulted in more rapid volume reduction of tumor xenografts than either treatment alone; and (b) R7020 could be sequentially administered to effectively destroy tumors arising from surviving cancer cells. No evidence of tumor resistance to oncolytic effects of R7020 was noted.

## Materials and Methods

**Cells and Viruses.** The African green monkey Vero cell line and PC-3 cell line were obtained from American Type Culture Collection (Rockville, MD). SQ-20b is an epidermoid cancer cell line isolated from a patient after radiotherapy as described previously (15). HSV-1(F) is the prototype wild-type HSV-1 virus used in these laboratories. The construction of R7020, was described elsewhere (12). Viruses were titered on Vero cells as described previously (9).

**Tumor Regression Studies.** SQ-20b tumor cells in amounts of  $5 \times 10^5$  cells per mouse were suspended in 100  $\mu$ l of sterile PBS and injected into the right hind limb of 5–6-week-old athymic nu/nu mice and grown to 200–1000 mm<sup>3</sup> (Fredrickson Cancer Research Institute, Bethesda, MD). Mice were randomized into two treatment groups: (a) controls were given 10  $\mu$ l of a buffer solution; and (b) mice were given  $2 \times 10^6$  pfu of R7020 in 10  $\mu$ l of buffer with a Hamilton syringe. The tumor mass was measured biweekly or until the tumor volume reached 2000 mm<sup>3</sup>. Tumor volumes were calculated using the formula (length  $\times$  width  $\times$  height)/2 which is derived from the formula of an ellipsoid ( $\pi d^3$ )/6. Animal studies were done in accordance with a protocol approved by the Animal Resource Center at the University of Chicago. Fractional tumor volume is defined as the tumor volume at the specified time point divided by the initial tumor volume ( $V/V_0$ ). Animals were killed when the tumor volume exceeded 2000 mm<sup>3</sup>. Similar experiments were carried out with PC-3, with the only exception that  $2 \times 10^7$  cells in 100  $\mu$ l of PBS were injected per xenograft. Irradiation of xenografts was done as described previously (11). Briefly, tumor-bearing hind limbs were exposed to IR using a GE 250 kV maxitron generator (191 cGy/min, 150 kVp). Irradiation was given in 400-cGy fractions Monday, Tuesday, Thursday, and Friday for 2 weeks to a total dose of 3200 cGy starting 6 h after infection with R7020. Statistical analysis was performed between buffer and viral-infected groups using a *t* test and ANOVA for comparing the effects of combining IR and R7020.

**Harvesting SQ-20b Xenografts for Viral Quantification.** Tumors were aseptically harvested at specified time points after infection and snap-frozen in liquid nitrogen and stored at  $-70^\circ\text{C}$ . Tumors were homogenized in 1 ml of 199V and 1 ml of sterile skim milk for 20 s on ice using a Polytron tissue homogenizer (Kinematica, Switzerland). The homogenate was sonicated three times for 15 s each and titered on Vero cells.

**Tumor-resistance Studies.** Tumors were grown as described above. When tumors were  $>200$  mm<sup>3</sup>,  $2 \times 10^6$  pfu of R7020 in 10  $\mu$ l of buffer were injected on day 0. Tumors were measured biweekly. As tumors regrew to their starting tumor volume (volume at day 0), they were randomized and 10  $\mu$ l of buffer, or  $2 \times 10^6$  pfu of R7020, or  $2 \times 10^6$  pfu of HSV-1(F) in the same volume of buffer were reinjected. Animals with tumor volume  $>2000$  mm<sup>3</sup> were killed in accordance with institutional guidelines.

## Results

**R7020 Is a Highly Effective Antitumor Virus.** Tumor cells comprised from SQ-20b, a chemotherapy/radiation-resistant epidermoid carcinoma, with a homozygous p53 mutation, or PC-3, a hormone-independent prostate adenocarcinoma that expresses functional p53 protein were injected into the hind limb of nude mice (15, 16). The experiments in this investigation were performed using tumor xenografts with a mean initial volume of 630 mm<sup>3</sup> corresponding roughly to 3% of mouse body weight, which exceeds the tumor:weight ratio in almost all cancer patients. The experimental design is in contrast, previous studies on viral oncolysis that were performed with tumor masses of approximately 30–100 mm<sup>3</sup> (3, 7, 8). Initially, a viral dose-response study was performed on SQ-20b

xenografts. SQ-20b xenografts were grown, and milk buffer or  $2 \times 10^4$  to  $2 \times 10^7$  pfu (in one log increments) were injected and xenograft volume was measured biweekly. Tumor regression began to occur at viral doses of  $2 \times 10^4$  pfu or greater (data not shown). Subsequent tumor xenograft studies used  $2 \times 10^6$  pfu of R7020. SQ-20b xenografts were grown, and on day 0, mice were randomized into two treatment groups. One group received buffer injections, whereas the second group received  $2 \times 10^6$  pfu of R7020 injected directly into the tumor mass. Fig. 1a shows the mean fractional tumor volume of buffer-injected and virally infected tumors. Buffer-injected tumors doubled by day 6, and animals were killed when their tumor volume was  $>2000$  mm<sup>3</sup>. SQ-20b xenografts treated with R7020 began to regress 13 days after infection and reached a nadir at 41 days postinfection at which time the mean tumor volume reduction was 5-fold from the initial tumor volume (Fig. 1;  $P = 0.007$  30 days postinfection, *t* test). SQ-20b xenografts treated with R7020 resulted in 72% (8 of 11) of the tumor xenografts regressing to  $<10\%$  of their initial tumor volume by day 41, and 7 of the 8 retained this size ( $<10\%$  of their initial tumor volume) for  $>80$  days. In previous studies (15),<sup>4</sup> SQ-20b xenografts that regressed to  $<10\%$  of the initial tumor volume did not regrow and had no histological evidence of tumor regrowth. Fig. 1b shows that R7020 was effective in tumor volume regression of hormone-resistant prostate adenocarcinoma xenografts as well ( $P = 0.003$ , 30 days postinfection, *t* test). Buffer-injected tumors doubled by day 23, whereas R7020-injected xenografts regressed and achieved a nadir approximately 20–30 days after infection. Taken together, these data demonstrate that R7020 is an effective antitumor virus in the treatment of therapy-resistant human tumor xenografts.

**Kinetics of Viral Replication in SQ-20b Xenografts.** R7020 ( $2 \times 10^6$  pfu) or buffered saline was injected into SQ20b xenografts. The tumor was excised and homogenized, and the virus was titered on Vero cell monolayer cultures. Fig. 2 shows the recovery of virus from tumors inoculated with R7020. Viral titers peaked at 7 days after infection with  $124 \times 10^6$  pfu/tumor, *i.e.*, a 62-fold increase in virus titer over the amount injected into the tumors. It is of interest that R7020 ( $>10^5$  pfu) was recovered as late as 30 days after infection. Previous results (14) with a more attenuated herpes virus with both copies of  $\gamma_134.5$  deleted (R3616) did not result in either efficient viral proliferation or prolonged proliferation within glioma xenografts. These results indicate replication-competent antitumor viruses must retain sufficient proliferative capability to result in significant tumor regression.

**SQ-20b Tumor Xenografts Do Not Develop Resistance to Oncolytic Effects of R7020 Virus.** In this series of experiments,  $2 \times 10^6$  pfu of R7020 were injected into tumors on day 0. Tumors were measured biweekly, and as tumors that initially responded regrew to their starting volume (volume on day 0), the mice were randomized and were given reinjections with buffer,  $2 \times 10^6$  pfu of R7020, or  $2 \times 10^6$  pfu of wild-type HSV-1(F). The results, (Fig. 3) were as follows:

(a) all of the three buffer-reinjected tumors continued to increase in size (Fig. 3a);

(b) Fig. 3, b and c, shows the response of R7020 tumors when either R7020 or wild-type HSV-1(F) were reinjected as the fractional tumor volume approached 1 (day 0 tumor volume). The fractional tumor volume decreased after the second viral injection of either R7020 or wild-type HSV-1(F);

(c) tumors continued to show sensitivity for R7020 viral oncolysis though two cycles of R7020 injection and did not recur for at least 120 days from the initiation of the experiment; and

(d) mice that were given reinjections of HSV-1(F) died 4–6 weeks after wild-type virus injection, whereas mice that were given repeated injections of R7020 thrived.

<sup>4</sup> H. J. Mauceri, personal communication.

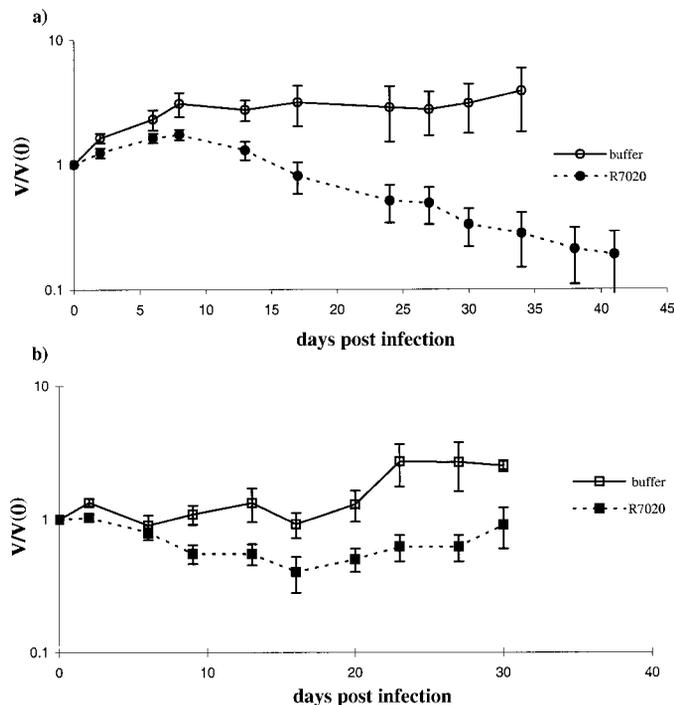


Fig. 1. Mean fractional tumor volumes of SQ-20b (a) or PC-3 (b) xenografts treated with buffer or R7020. Tumors were injected with  $10 \mu\text{l}$  of buffer or  $10 \mu\text{l}$  of  $2 \times 10^6$  pfu of R7020 intratumorally on day 0. Tumor volumes were measured biweekly.

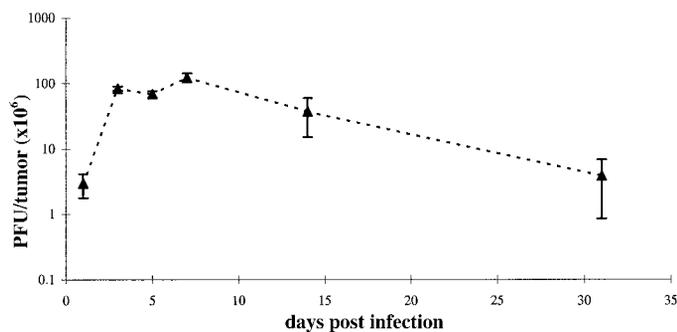


Fig. 2. Recovery of R7020 virus from SQ-20b tumors. R7020 ( $2 \times 10^6$  pfu) was injected into tumors on day 0, and tumors were then harvested and assayed for virus on days 1, 3, 5, 7, 14, and 31 postinfection.

Because the murine model is indicative of HSV neuroinvasiveness, these data further demonstrate the safety of R7020 compared with wild-type HSV infection in the murine model. These data demonstrate that experimental human SQ-20b tumor xenografts do not become resistant to reinfection with genetically engineered herpes virus R7020.

**R7020 Interacts with IR.** To determine whether irradiation of the radiation-resistant SQ-20b cell lines enhanced the oncolytic effect of R7020, xenografts were infected and subjected to the fractionated IR protocol as described in "Materials and Methods." Buffer-treated tumors doubled by day 6, and irradiated xenografts had a mean doubling time of 13 days. The modest growth delay exhibited in xenograft growth compared with control tumors demonstrates the relative radioresistance of the SQ-20b cell line (Fig. 4). The combination of IR with R7020 was significantly better than either R7020-alone or IR-alone treatment ( $P < 0.001$ , ANOVA). Although the time to reach 50% tumor regression in R7020-treated mice was 23 days, the combination of IR with R7020 resulted in a 50% tumor-volume regression by day 16. By day 20, xenografts treated with R7020 and

IR had a mean fractional tumor volume of 0.25 compared with 0.72 for viral-alone treated tumors ( $P = 0.10$ ,  $t$  test).

## Discussion

HSVs offer many advantages as oncolytic agents. These viruses replicate well and destroy a variety of cancer cells. In addition, the functions of many viral genes are known and, therefore, are easily attenuated by genetic engineering of specific deletions. The undesirable properties of HSV-1 are neuroinvasiveness, ability to establish latency, and reactivation from a latent state. R7020, the virus used in the studies of this report, has been attenuated by genetic engineering and extensively tested in a variety of rodent and nonhuman primate models (12, 14). These studies have shown that the virus is attenuated in all of the species tested. A key property of interest is the lack of neuroinvasiveness even in the most susceptible species tested to date: the owl monkeys (14).

In this report, we demonstrate the substantial efficacy of the recombinant virus, R7020, in the treatment of epidermoid and prostate adenocarcinoma subtypes of experimental human tumors frequently resistant to common cancer treatments. The tumor model that we have used in these studies consisted of inserting human cancer cells into the mouse hind limb (11). A major experimental modification introduced in this study compared with previous studies of viral oncolysis was to

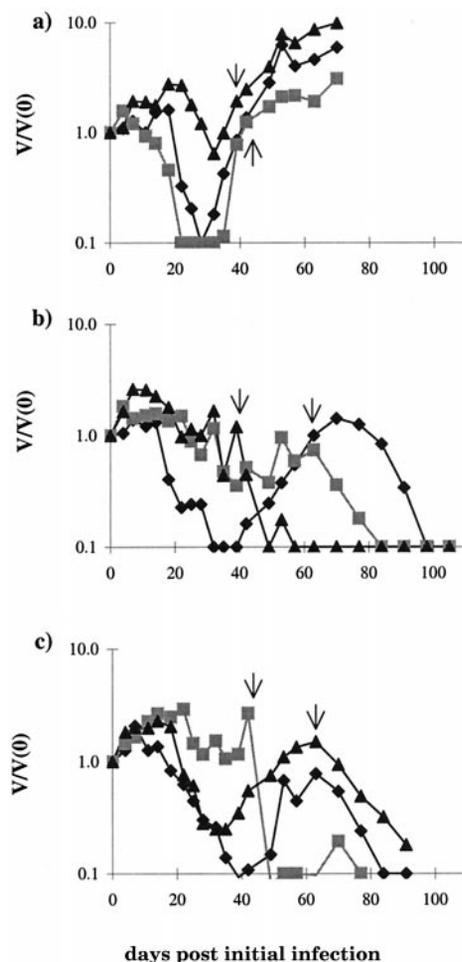


Fig. 3. Tumors remain sensitive to repeated treatments with R7020. R7020 ( $2 \times 10^6$  pfu) was injected into SQ-20b xenografts on day 0. Tumor volume was measured biweekly. As tumors regrew back to the initial starting volume ( $v/v(0) = 1$ ), mice were randomized and given reinjections with buffer (a),  $2 \times 10^6$  pfu of R7020 (b), or  $2 \times 10^6$  pfu of wild-type HSV-1(F) (c); and tumors were measured biweekly. Arrows, times at which buffer, R7020, or wild-type HSV-1(F) was reinjected into tumors. Each line represents an individual tumor xenograft.

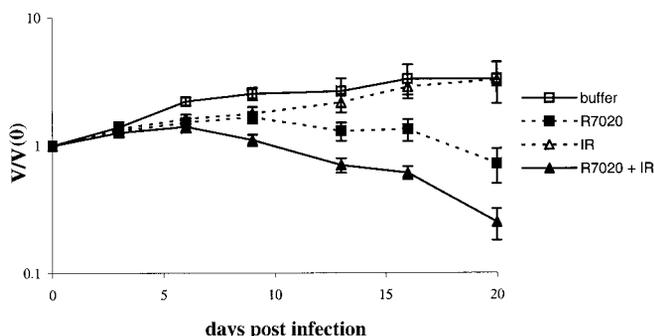


Fig. 4. Mean fractional tumor volumes of SQ-20b xenografts treated with buffer, IR, R7020, or IR + R7020. On day 0, buffer was injected into tumors that had been treated with buffer and IR; and 10  $\mu$ l of  $2 \times 10^6$  pfu of R7020 were injected into virally treated tumors. Irradiated tumors received 400 cGy daily to a total dose of 3200 cGy.

increase the mean volume of the xenograft to 630 mm<sup>3</sup> at the time at which treatment by virus injection was initiated. The objective of this modification was to simulate the ratio of virus:tumor cells that is observed in clinical cancer therapy. Our results demonstrate that R7020 replicates to high titers and causes tumor destruction after a single intratumor injection. These results are in striking contrast to previously studied genetically engineered herpes viruses that resulted in tumor growth delay without evidence of tumor regression (7, 8, 10, 11). Studies using replication-conditional adenovirus rely on multiple viral injections to achieve an antitumor effect in xenograft models, which indicates the poor replication potential of such viruses to outpace tumor growth (3). The studies reported here also show that SQ-20b tumors arising from residual cells in tumors previously treated with R7020 retain their susceptibility to infection. This observation is consistent with the evidence that mutations that lead to the selection of cells resistant to infection are rare and that repeated application of R7020 may be feasible in clinical protocols.

We have also tested in this model the effects of combining virus inoculation and IR. As expected, IR by itself had a minimal effect on the xenograft arising from implantation of radioresistant SQ-20b cells. Although R7020 is an effective antitumor agent by itself, combining IR with R7020 resulted in more rapid and complete tumor cell destruction. A 3-fold reduction in tumor volume of combined-treated (R7020 with IR) versus R7020-alone-treated tumors shows the potential efficacy of combining irradiation with genetically engineered herpes in future clinical protocols. This combination was shown to be synergistic in its antitumor effects in an earlier report (11). The modification that we have introduced in this report was to apply fractionated IR approximating the doses applied in clinically relevant protocols. The addition of IR to attenuated HSV anticancer therapy may prove to be especially beneficial in clinical situations in which the tumor burden may be too large for single-agent therapy. Although the mechanism by which IR leads to enhanced tumor-cell destruction by recombinant HSV is still under investigation, recent studies have shown that IR enhances gene expression of the reporter gene *luciferase* whose expression was driven by the immediate early promoter of the human cytomegalovirus, implying viral promoters may be up-regulated by IR (23). Cellular promoters also have previously been reported to be up-regulated by IR (24).

Barriers to introducing viral vectors into clinical use stem in part from the apprehension of using live attenuated viruses that may revert to wild type or cause severe viremia. Extensive studies on R7020 have established its safety in many animal systems including humans. In summary, R7020 has potent oncolytic activity and interacts with IR. Also, SQ-20b tumor xenografts do not develop resistance to the cytolytic effects of R7020 (a major limitation of conventional cancer therapies), which suggests that resistance to HSV therapy may not develop.

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