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The effects of radiation on antitumor efficacy of an oncolytic adenovirus vector in the Syrian hamster model

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Abstract

We report that radiation enhances the antitumor efficacy of the oncolytic adenovirus vector VRX-007 in Syrian hamster tumors. We used tumor-specific irradiation of subcutaneous tumors and compared treatment options of radiation alone or combined with VRX-007 and cyclophosphamide (CP). Radiation therapy further augmented the VRX-007-mediated inhibition of tumor growth, in both CP-treated and non-CP-treated hamsters, even though radiation did not lead to increased viral replication in tumors when compared to those treated with VRX-007 alone. Moreover, tumor growth inhibition was similar in tumors irradiated either one week before or after injection with VRX-007, which suggests that radiation exerts its antitumor effect independently from vector therapy. Thus, our results demonstrate that these two therapies do not have to be provided simultaneously to enhance their combined effectiveness against subcutaneous hamster tumors.

Keywords

tumor; virotherapy; hamster; radiation; replicating; adenovirus

Introduction

Virotherapy has emerged as a promising therapeutic treatment for cancer. Replication-competent, oncolytic viruses can selectively infect and damage tumor cells, with their replication cycle ultimately resulting in cell lysis. In theory, viral progeny can infect neighboring cells, and this oncolytic process could continue until the tumor is eliminated. Adenoviruses (Ads) are some of the most well-known oncolytic vectors and they are being used in numerous clinical trials.[1-3] Most of these trials include vectors that can be directly injected into tumors and are almost always used in combination therapy.[4-6]

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Conflict of Interest: The VRX-007 vector was provided by VirRx, Inc., and W.S.M.W. and K.T. own stock in the company.

Supplementary information is available at *Cancer Gene Therapy's* website.

A number of Ad serotype 5-based vectors have been engineered by our group to overexpress the Adenovirus Death Protein (ADP) in a mostly deleted E3 region.[7-10] These replication-competent viruses have enhanced oncolytic potential when compared to wild-type Ad5.[8] One such vector, VRX-007, has been shown to suppress tumor growth in nude mice,[11] cotton rats,[12] and hamsters[13-15] and is currently in a Phase I clinical trial (Protocol # 0510-732). Syrian hamsters have been described as a permissive animal model for human Ad5 replication, and, unlike studies in nonpermissive murine models, hamsters can be studied whilst maintaining their competent immune systems.[16-18]

Although oncolytic Ad vectors are highly efficient at killing tumor cells *in vitro*, their activity in clinical studies has been more modest.[2, 3] Thus, combination therapy is often used where monotherapy has failed.[3, 19, 20] Radiotherapy, alone, is effective in decreasing tumor burden by inducing cellular damage and death. However, high dosages of radiation are potentially toxic to normal tissue, and fractionated dosing is preferred to decrease potential damage.[21]

We report here that radiotherapy enhanced the oncolytic effect of VRX-007 in the Syrian hamster tumor model without increasing vector replication or altering the antiviral immune response. These results agree with previously published data in cell culture and in human xenografts in immunodeficient mice.[19, 22] We have previously reported enhanced antitumor efficacy when the chemotherapeutic and immunosuppressive drug, cyclophosphamide (CP), is combined with VRX-007 in hamster tumors.[13, 14] The absence of complete eradication of tumors led us to pursue triple combination therapy, including VRX-007, CP, and radiotherapy, which inhibited nearly all tumor growth. Lastly, we followed the replication of an intratumorally injected luciferase-expressing Ad vector using *in vivo* imaging.

Materials and Methods

Cell culture

The Syrian hamster kidney cancer cell line (HaK) and the human cell lines HEK293 and A549 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS) at 37°C in a CO_2 incubator.[23] The HEK293 cell line was purchased from Microbix (Toronto, Ontario, Canada), and the A549 and HaK cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). The SHPC6 (Syrian hamster pancreatic cancer) cell line[15] was cultured in DMEM containing 15% FBS, 1 mM nonessential amino acids, and 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA).

Viruses

The construction of VRX-007 is explained elsewhere.[8, 9, 24] It is an oncolytic Ad vector identical to Ad5, except it lacks most of the E3 region and overexpresses the E3-11.6K Adenovirus Death Protein (ADP). VRX-007 is designated 007 in the figures and figure legends. 007-Luc is identical to VRX-007 except that the luciferase gene has been incorporated just downstream to *adp*.

Animals

Female Syrian hamsters (5-6 weeks old) were purchased from Harlan Sprague Dawley (Indianapolis, IN) or Charles River Laboratories (Wilmington, MA). The Institutional Animal Care and Use Committee of Saint Louis University gave approval for these studies, which were executed in accordance with federal and institutional guidelines.

Cyclophosphamide treatment

Cyclophosphamide (Sigma-Aldrich, St. Louis, MO) was given biweekly starting one week before the first vector treatment. This initial dose of CP was 140 mg/kg body weight and all other doses were 100 mg/kg.[13] With this dosing schedule, hamsters are severely immunosuppressed after one week.[23]

Radiotherapy

To reduce the damage to surrounding normal tissue, we constructed a holding box in which hamsters are covered by lead sheets, exposing only the tumor to X-ray radiation (Supplementary Figure 1). The X-Rad 320 (Precision X-Ray, North Branford, CT), especially designed for delivering specific radiation dosages to small animals, was used. Hamsters in the radiotherapy groups with HaK tumors received 8 Gray (Gy) and with SHPC6 tumors received 6 Gy, always localized to the tumor.

Antitumor efficacy

Subcutaneous tumors were generated by injecting 2×10^7 HaK cells or 1.5×10^7 SHPC6 cells (in 200 μl PBS) into the right hind flank of the hamsters.[23] Tumor volumes were measured biweekly with digital calipers and calculated as 0.5 × length × width × width. Once the tumors reached 200-300 μl in size, animals were randomized by tumor size into groups and the treatment series started. The tumors were injected with VRX-007 or 007-Luc; all mock groups were injected with vehicle (PBS). The vector dose that was given is designated in the figure legends. At the specified time points, hamsters were sacrificed via CO_2 asphyxiation and organs were harvested. Experiments were terminated as indicated in Figures 1, 3-5 for ethical reasons (e.g. the tumors were ulcerated).

Quantification of bioluminescence imaging

Animals were given one intratumoral injection of a luciferase-expressing virus. For each image, 1 ml of D-luciferin, potassium salt (Gold Biotechnology, St. Louis, MO) dissolved in PBS at 15 mg/ml, was injected intraperitoneally per animal. After 15 min, animals were anesthetized and placed into the IVIS Spectrum optical imaging system (PerkinElmer, Waltham, MA). Living Image Software was used to quantify and analyze luminosity as photon emission, which is calculated as follows: photons/sec/cm^2/steradian.

Virus quantification in tissues

Hamster tumors or livers were homogenized in PBS using the TissueLyser (Qiagen, Valencia, CA). Homogenates were freeze-thawed three times, then sonicated for 6 min. The samples were diluted onto HEK293 cells (DMEM 5%FBS), and the 50% tissue culture infective dose ($TCID_{50}$) was calculated 14 days later by counting the number of wells

positive for cytopathic effect (CPE).[23] Samples that were under the limit of quantifiability yet had at least one positive well were marked “unquantifiable,” and samples from which no viral cytopathic effect was detected in any wells were marked “undetectable.”

Quantification of neutralizing antibody titers

Serum samples were incubated at 56°C for 30 min to inactivate complement. Two serum samples were assayed per plate. Serum samples (in four replicate wells) were diluted twofold across a 96-well plate in DMEM containing 10% FBS.[25] One row contained no serum samples in order to observe the effect of virus only. Dilutions of sera were incubated with 100 PFU per well of VRX-007 for 1 h at 37°C. After 1 h of incubation, A549 cells were added (5×10^5 cells/plate). Plates remained at 37°C for 10 days, after which, media was replaced with 200 μl of neutral red (Sigma-Aldrich, St. Louis, MO) dissolved in PBS at 30 μl/ml and incubated at 37°C for 1 h. Plates were washed twice with PBS, and 100 μl of acidified ethanol solution (50% ethanol, 1% acetic acid in water) was added. After 10 min, absorbance was measured at 550 nm and a colorimetric assay was performed on a microplate reader to determine cell viability (Biotek, Winooski, VT).

Single step growth curve

HaK cells were plated onto 35 mm dishes and irradiated with 20 Gy at 24 h before or after infection with VRX-007. At 1 h post infection, monolayers were washed thrice, and at indicated time points, both the medium and monolayer were harvested for $TCID_{50}$ assay on HEK293 cells.[23]

Statistical evaluation

All statistical analyses were performed using GraphPad Prism 4 for Windows (GraphPad Software, Inc., San Diego, CA). Because the distribution of data did not satisfy the requirements for parametric analysis, statistical significance was assessed using nonparametric tests. The treatment effect was detected by the Kruskal-Wallis test, and pairwise comparisons were made using the Mann-Whitney U test to determine statistical significance. $P\leq0.05$ was considered to be significant. For samples in which infectious virus titers were unquantifiable, the limit of quantification was used to calculate statistical significance.

Results

Combined therapy with VRX-007 and radiation inhibits HaK tumor growth

Radiotherapy is nonselective to tumor cells,[26] so we constructed a holding box that exposed only the subcutaneous tumors (Supplementary Figure 1). To determine the effectiveness of VRX-007 plus radiation in hamster tumors, we divided hamsters with HaK tumors into 4 groups (mock, radiation, VRX-007, and VRX-007 + radiation). Intratumoral (i.t.) injection with 1×10^{10} PFU of VRX-007 was administered daily for 6 consecutive days. On the 7^{th} day, tumors were irradiated with 8 Gy; the same radiation dose was also given on days 39 and 55. The dates of radiotherapy were chosen based on tumor growth throughout the study. Animals from the mock group were sacrificed at day 41 due to large tumor burden (>10 000 μl). The other groups were sacrificed on day 61 post infection (p.i.). As shown in Figure 1a,

the tumors treated with either single therapy (radiation or VRX-007) did not differ significantly from each other in volume (P=0.46), and both groups had smaller tumors than did the mock group (P<0.03). Importantly, the tumors given both VRX-007 and radiotherapy had the greatest inhibition of growth, and were significantly smaller than either single therapy group from days 41 to 61 (P<0.05).

$TCID_{50}$ assays were performed on livers, tumors, and blood samples from hamsters sacrificed on day 61; no infectious virus was detected in samples of any of the groups (data not shown), which was expected in immunocompetent animals inasmuch as there is a strong neutralizing antibody response to intratumorally injected VRX-007 that begins by about one week post injection. Following these results, neutralization assays were performed on serum samples taken at the time of sacrifice at 61 days. Hamsters in both VRX-007 groups produced similar levels of anti-Ad neutralizing antibodies (Figure 1b). Thus, VRX-007 + radiation did not produce a greater antiviral immune response than VRX-007 alone. The similar serum antibody titers found in both the VRX-007 and VRX-007 + radiation groups also suggest that the tumor-specific radiation did not markedly affect VRX-007 replication. Due to the limitations of the Syrian hamster model, an immune response specific to tumor antigens would be difficult to detect.[14, 27] However, the lack of radiation-induced induction of antiviral immunity may suggest that no increase in antitumor immunity occurred either.[28]

Radiation does not enhance VRX-007 replication in HaK tumors

The lack of infectious virus in the tumors at 61 days p.i. does not exclude the possibility that radiation enhanced viral replication earlier in the study—meaning soon after infection. We performed a short-term experiment to address whether radiation affects vector replication within the tumors shortly after i.t. injection of vector. Hamsters were divided into the same 4 groups shown in Figure 1. Subcutaneous HaK tumors were irradiated with 8 Gy, then injected with 1×10^{10} PFU of VRX-007 for 6 consecutive days. Animals were sacrificed on days 4 and 7 after the last vector injection. Infectious virus persisted in the tumors for 7 days in the irradiated and non-irradiated animals, with the titers being slightly lower (P=0.10) in the irradiated tumors (Figure 2a). There was no detectable infectious virus in the liver on days 4 or 7 (data not shown). As shown in Figure 2b, animals with irradiated tumors did not produce a higher level of anti-Ad neutralizing antibodies, and this result supports the data shown in Figure 1b, which was taken at 61 days post infection. Thus, radiation did not induce greater viral replication or increase the host antiviral immune response.

Radiation does not enhance viral replication *in vitro*

Radiation therapy and oncolytic Ad treatment combined inhibited tumor growth significantly better than either treatment alone, yet we did not observe an increase in viral replication in the HaK tumors or the liver in our studies with irradiated animals. More experiments were performed to determine the effect of radiation on virus infection and replication in HaK cells *in vitro*. HaK cells were infected with VRX-007 *in vitro* and the progeny virus that was produced at multiple time points p.i. was quantified on HEK293 cells to generate a single step growth curve (Figure 2c). HaK cells produced about 10-fold less infectious virus when infected and then irradiated 24 h later, and 100-fold less virus when irradiated and then infected 24 h later. Thus far, we have shown that radiation enhanced

antitumor efficacy without increasing viral replication in HaK tumors *in vivo* or HaK cells *in vitro*. In actuality, radiation decreased VRX-007 replication *in vitro* (Figure 2c).

Triple combination therapy with VRX-007, radiation, and cyclophosphamide results in greater tumor suppression

We have previously reported that hamsters treated with CP have an augmented antitumor response when combined with VRX-007.[13, 14, 29] The antitumor efficacy induced by CP may be due to its immunosuppressive activity inhibiting clearance of the virus, or its chemotherapeutic activity causing tumor cell death. The addition of CP treatment allows us to study the antitumor efficacy of three therapies combined—VRX-007, radiation, and CP. For this study, HaK tumor-bearing hamsters were divided into 8 groups: mock; CP; radiation; CP + radiation; VRX-007; VRX-007 + CP; VRX-007 + radiation; VRX-007 + CP + radiation. The tumors were irradiated once with 8 Gy; after 24 h, 1×10^{10} PFU of VRX-007 was injected i.t. for 6 consecutive days. CP treatment was started one week prior to vector injection and was dosed biweekly throughout the study to ensure the hamsters were immunosuppressed before infection and throughout the study. Animals were sacrificed at 44 days post radiation. As shown in Figures 3a and b, tumors from the mock group grew significantly larger than those from any double therapy group ($P<0.015$), and the greatest tumor inhibition was seen in the tumors given triple therapy ($P<0.0006$ compared to mock). The combination of three modalities significantly ($P<0.05$) increased antitumor efficacy over the efficacy of the combination of any two modalities (i.e. vector + CP, vector + radiation, or radiation + CP).

As mentioned, the combination of VRX-007 + radiation was more effective than either therapy alone (Figure 3a and b), which is consistent with the data shown in Figure 1a. The two experiments differ in that tumors were irradiated one day before VRX-007 was injected (Figure 3) or were irradiated one day after VRX-007 was injected (Figure 1). Therefore, the enhanced efficacy of the combination is similar whether radiation is given before or after vector injection.

In agreement with our previous experiments, radiation did not induce greater viral replication. The tumors from the hamsters sacrificed at 44 days (Figure 3) were analyzed for the presence of virus. Substantial virus titers were found in the two groups immunosuppressed by CP, but notably, the VRX-007 + CP group and the VRX-007 + CP + radiation group had similar amounts of virus, indicating that radiation did not affect virus replication or persistence in the tumors of CP-treated animals (Figure 3c). No virus was found in the VRX-007 or VRX-007 + radiation groups without CP (Figure 3c), which is consistent with the results in Figure 1 and is expected inasmuch as these hamsters were immunocompetent. Both the VRX-007 + CP and VRX-007 + radiation groups had similar tumor sizes throughout the study, which suggests that long-term viral replication is not necessary for the enhanced antitumor efficacy that results from the triple combination therapy.

Radiation and VRX-007 do not need to interact to enhance antitumor efficacy

We used a luciferase-expressing oncolytic vector, 007-Luc, to further investigate how radiation affects HaK tumor growth and vector replication. 007-Luc expresses luciferase almost exclusively when it has replicated and gone into the late phase of infection (data not shown). Thus, luciferase expression can be used as a surrogate for vector replication, which can be followed in the same animal throughout the study. Notably, we altered the dosing regimen to one single intratumoral injection of vector instead of the 6 consecutive daily injections as done in Figures 1 and 3. Also, we included groups in which radiotherapy and 007-Luc were given simultaneously or separately (one week apart). One dose of tumor-specific radiation was given either immediately (within several min) before 007-Luc intratumoral injection (007-Luc + radiation-before), or one week after infection (007-Luc + radiation-after). Vector replication had decreased by about 2 orders of magnitude at 7 days p.i. when the animals in the 007-Luc + radiation-after group were irradiated; thus, interaction between vector and radiation, if any, should be limited (see Figure 4b). As shown in Figure 4a, after 35 days p.i., the mock-treated animals had significantly larger tumors than those in either of the 007-Luc + radiation groups ($P<0.02$). All single therapy groups had similar antitumor efficacy that trended toward improvement ($P=0.18$) when compared to the mock group. In agreement with Figures 1 and 3, both double therapy groups (007-Luc + radiation) had greater antitumor efficacy than either single therapy group ($P<0.013$). Importantly, in the double therapy groups, radiation enhanced the tumor suppression to the same degree regardless of whether it was administered before (007-Luc + radiation-before) or after (007-Luc + radiation-after) infection ($P>0.08$ at any time point). 007-Luc replication, as demonstrated by luciferase expression, did not differ significantly among any vector-injected groups (Figure 4b). For the group which received treatment one week after vector injection, the virus did not reactivate in response to X-ray radiation as luminosity did not increase at any time during the study and was down to baseline levels by approximately 15 days p.i.

A similar experiment was performed using subcutaneous SHPC6 tumors, which come from a Syrian hamster pancreatic cancer cell line.[15] These tumors grow more rapidly than the HaK tumors used in earlier experiments. Radiation has a stronger effect on SHPC6 tumors (Supplementary Figure 2) than on HaK tumors, so a lower dose of radiation (6 Gy vs. 8 Gy) was administered. In the experiment shown in Figure 5, Many animals were sacrificed prematurely due to increased tumor burden, including the mock group at day 28 and both the radiation-before group and the 007-Luc group at day 33 (Figure 5a). At day 28, the mock group had significantly larger tumors than all other groups ($P<0.05$), and the 007-Luc group had significantly larger tumors than either double therapy group ($P<0.05$). Both of the 007-Luc + radiation groups had the smallest tumors, regardless of whether the radiation was given one week before or after vector injection. At no time during the study were tumors from the 007-Luc + radiation-before group and the 007-Luc + radiation-after group significantly different from each other ($P>0.40$). In agreement with the data obtained with HaK cells (Figure 4b), luciferase expression—meaning viral replication—did not differ among any groups (Figure 5b). It is important to note that the luciferase expression in SHPC6 tumors is much lower overall than it is in HaK tumors (approximately 100-fold), and the subcutaneous SHPC6 tumors seem to be less susceptible to 007-Luc replication *in vivo*.

We conclude from the data in Figures 4 and 5 that radiation therapy before or after vector injection enhances antitumor efficacy without inducing increased viral replication at any time post infection in the two cell lines tested. Importantly, the combined effect did not require that both therapies be given at the same time.

Discussion

Almost 50% of cancer patients will undergo radiotherapy.[30] Oncolytic vectors have been used successfully in combination with radiation. The theory behind multimodality treatments is that attacking tumor cells via different mechanisms may prevent cells from having time to develop resistance to any single treatment.[31] There are conflicting reports on the best method for administering the combined therapies. Some suggest that radiation must be given prior to vector infection for synergy to occur,[32-35] and some advise that radiation may kill the virus.[35] Meanwhile, others contend that the vector genome is less susceptible to radiation-induced damage because it is much smaller compared to cellular DNA.[19] Results from other research groups indicate that virus injection leads to increased apoptosis and radiosensitivity, but only when radiation is administered after vector injection.[34, 36, 37]

We have investigated if the sequence of radiation and vector administration influences the antitumor efficacy in the Syrian hamster model and have examined possible mechanisms of action. Previously, we reported that radiation produces an increase in cell lysis in studies with VRX-007 *in vitro* and that radiation of subcutaneous human A549 tumors in nude mice enhanced the oncolytic effect of a related vector, KD3.[22] Here we report the results of radiation and VRX-007 combination therapy on subcutaneous tumors in hamsters.

The enhanced antitumor efficacy that we observed with the combination therapy in our model was not the result of increased viral replication (as shown in Figures 1, 3, 4 and 5). Others have reported previously that radiation can induce greater vector replication, causing increased oncolytic activity,[38-40] but it has also been reported that high radiation doses might even decrease vector replication.[35] Our data agree with previous findings which suggest that radiation treatment does not interfere *significantly* with vector replication *in vivo*.[19, 32, 34]

Another potential mechanism by which radiotherapy enhances vector-induced oncolysis is to improve the distribution of the vector throughout the tumor. According to Lamfers, *et al*., radiation may accelerate cell lysis in gliomas, allowing earlier release of progeny virus.[41] Furthermore, according to L. Wein *et al*., the spatial distribution of a replication-competent vector within the solid tumor is the most important factor in determining treatment outcome.[42] Histolological staining of cervical cancer tumors infected with an E1B-19K-plus E1B-55K-deleted Ad vector also revealed better distribution with radiotherapy.[43] It has been reported that radiation allows the virus to propagate further away from the injection needle track to improve efficacy in gliomas.[40] Our experiments did not produce histological differences among irradiated or non-radiated groups, however (data not shown). With our system, the increased dissemination effect may be obscured by the enhanced viral spread of VRX-007 due to its overexpression of ADP.[7] Furthermore, HaK tumors, unlike gliomas, are similar to most human solid tumors in that they are quite heterogeneous, with intervening

blood vessels, fibrous connective tissue, and necrotic cells,[44] which can impede virus spread.

Here, we report that tumor-specific radiotherapy enhanced the efficacy of an oncolytic Ad vector, whether tumors were irradiated before or after vector injection. The improvement in tumor growth suppression was not mediated by increased viral replication or an effect on antiviral immunity. Our data suggest that the enhanced antitumor efficacy from this double combination is exerted by independent actions of the two modalities. This was evidenced by the increase in antitumor efficacy when radiation was applied one week p.i.—after the immune response has eliminated most of the virus. As anticipated, triple therapy with VRX-007, radiation, and CP yielded almost no HaK tumor growth. CP is an immunosuppressive agent, and there was increased tumor suppression when CP treatment was added to VRX-007 and radiation therapy. Thus, it is not likely that an enhanced radiation-induced immune response is part of the mechanism by which radiation increases the efficacy of VRX-007 plus CP in this model.

Based on our data, we suggest that radiation does not sensitize tumors cells to infection or vice versa, but that the combination is beneficial for antitumor efficacy overall. Also, the extent or duration of vector replication does not influence the efficacy of the vector. Thus far, the studies reported here are limited to hamster tumor cells lines growing in Syrian hamsters, and further experiments using other animal models and cell lines are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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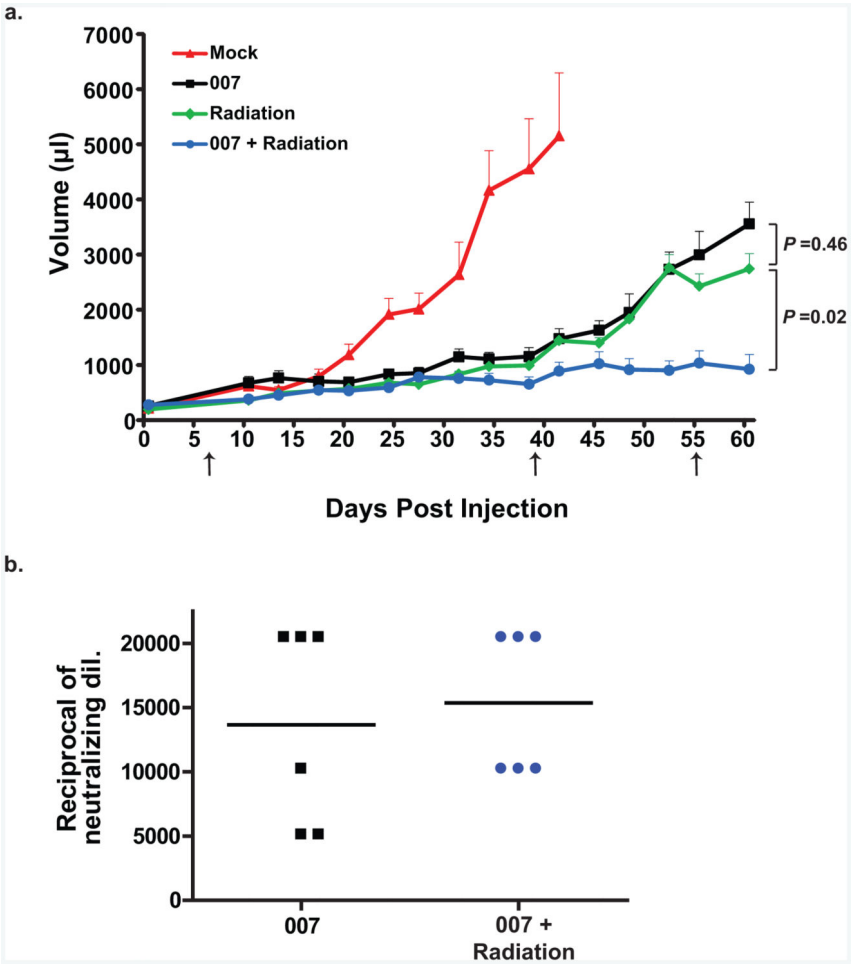

Figure 1.

The effects of combined therapy with radiation and VRX-007 on tumor size. HaK tumors were injected for 6 days with 1×10^{10} PFU of VRX-007 and then irradiated with 8 Gy on days 1, 39, and 55 p.i. (arrows). (**a)** Mean tumor volume. The number of animals per group were: mock (n=9), VRX-007 (n=8), radiation (n=7), VRX-007 + radiation (n=8). There was a significant difference ($P< 0.03$) between mock and all other groups starting at day 24, and there was a significant difference ($P< 0.05$) between each single therapy and combined therapy starting at day 41. Error bars represent mean + SE. (**b)** Serum neutralizing antibody titers. An anti-Ad antibody assay of the serum was performed at time of sacrifice, 61 days p.i. (P=0.6991).

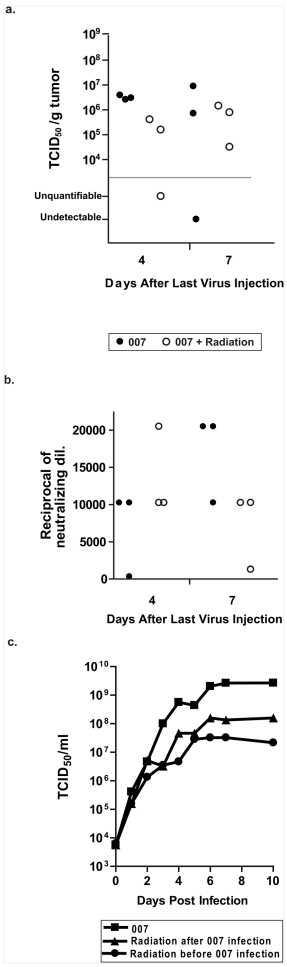

Figure 2.

The effect of radiation on infected HaK cells *in vivo* and *in vitro* in short-term studies. Animals were irradiated with 8 Gy 24 h before intratumoral injection with VRX-007 for 6 consecutive days. Samples were collected at days 4 and 7 after the last virus injection. There were 3 animals per group. **(a)** $TCID_{50}$ assay of virus extracted from HaK tumors. There was no significant difference between both groups on days 4 or 7 (P>0.100). **(b)** Serum neutralizing antibody titers. An anti-Ad antibody assay of the serum was performed at time of death indicated. There was no significant difference between both groups on days 4 or 7 (P>0.200). **(c)** Single step growth curve demonstrating the effect of radiation 24 h before or after VRX-007 infection. HaK cells were irradiated with 20 Gy at 24 h before or after infection with VRX-007. The level of infectious virus was tested from the total cells and media from the dish and is represented as $TCID_{50}$/ml as determined on HEK293 cells.

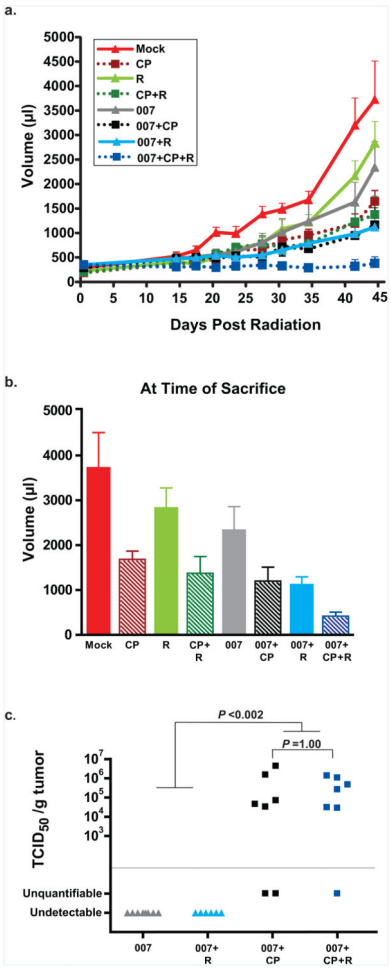

Figure 3.

The combination of VRX-007, cyclophosphamide (CP), and radiation treatments results in the least amount of tumor growth. HaK tumors were irradiated with 8 Gy at 1 day before 6 consecutive days of VRX-007 intratumoral injections. Intraperitoneal injections of CP were given biweekly starting one week before infection and for the duration of the study. The number of animals per group were: mock (n=7), CP (n=7), radiation (n=7), CP + radiation (n=9), VRX-007 (n=9), VRX-007 + CP (n=8), VRX-007 + radiation (n=7), VRX-007 + CP + radiation (n=7). Radiation is abbreviated as "R." **(a)** Mean tumor volume measured biweekly throughout the study. **(b)** Mean tumor volume at time of sacrifice, 44 days p.i. **(c)** $TCID_{50}$ of virus extracted from the tumors, collected at time of sacrifice. There was significantly more infectious virus in both CP-treated groups compared to both non-CP treated groups (P=0.0012). There was no difference in the amount of virus in the tumors of the VRX-007 + CP and VRX-007 + CP + R groups.

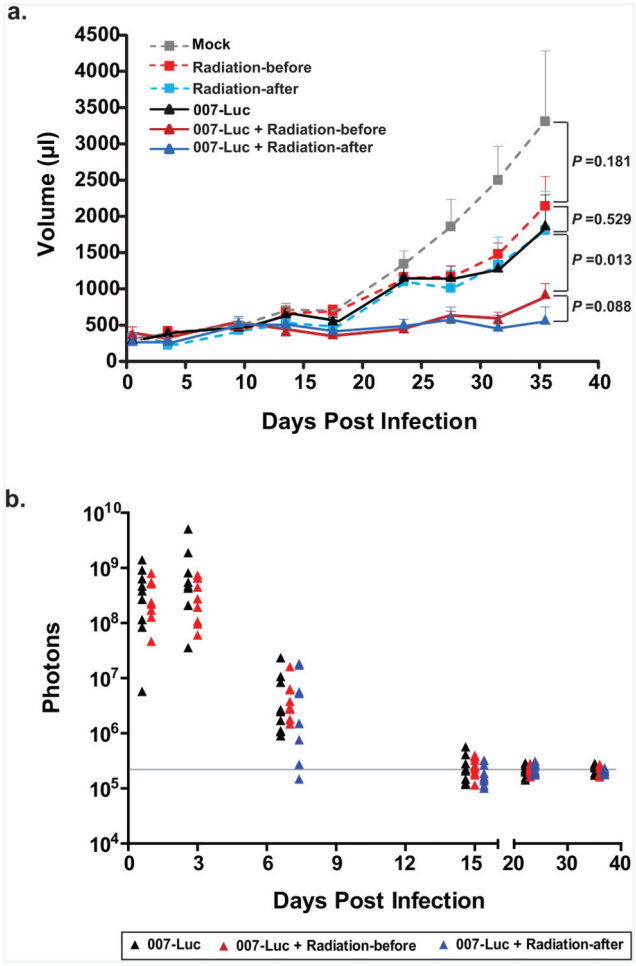

Figure 4.

Radiation and 007-Luc work independently to inhibit HaK tumor growth. HaK tumors were injected once with 1×10^{10} PFU of 007-Luc. Irradiated groups were given 8 Gy either immediately before infection (007-Luc + radiation-before) or one week after infection (007-Luc + radiation-after). The number of animals per group were: mock (n=8), radiation-before (n=9), radiation-after (n=6), 007-Luc (n=6), 007-Luc + radiation-before (n=6), 007-Luc + radiation-after (n=9). **(a)** Mean tumor volume. Error bars represent mean + SE. After 35 days post infection, the single therapy groups (007-Luc only, radiation only) had significantly larger tumors than those in either of the double therapy (007-Luc + radiation) (P<0.02). Importantly, tumor growth suppression was similar (P=0.088) in the 007-Luc + radiation-before and 007-Luc + radiation-after groups. **(b)** Luciferase expression in tumors, measured by total flux of photons. The gray line suggests the background intensity (approximately 10^5 photons). The 007-Luc + radiation-after group could not be imaged at the day 1 and day 3 time points because the infection and radiation are done in BSL-2 isolation and the imaging is done in a BSL-3 facility; once the hamsters enter the BSL-3 area for imaging, they cannot be brought into the BSL-2 area for radiation. However, up to the point of receiving radiation treatment, these hamsters were treated identically to those in

the vector only (007-Luc) group, so their luciferase expression data is expected to be the same as for animals in the 007-Luc group.

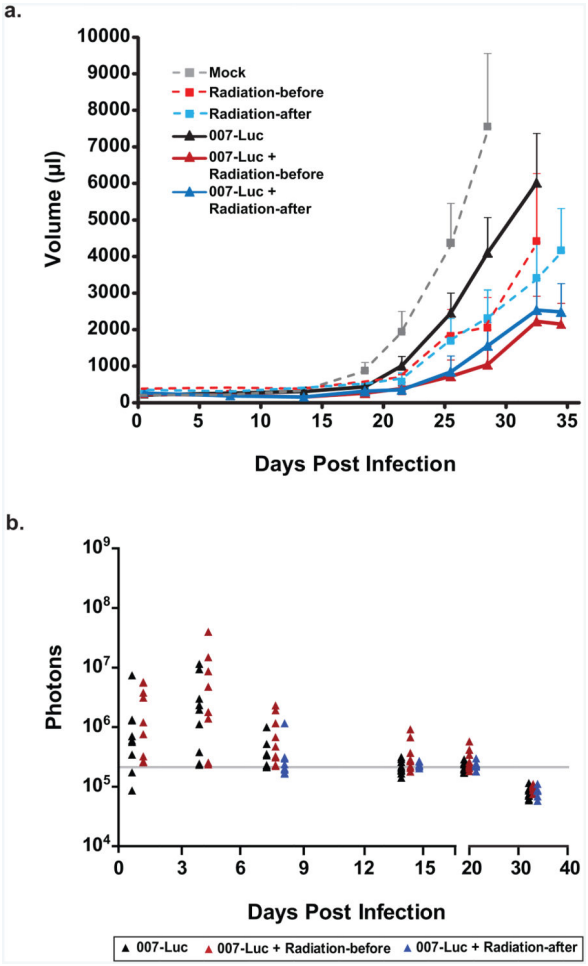

Figure 5.

Radiation and 007-Luc work independently to inhibit SHPC6 tumor growth. SHPC6 tumors were injected once with 1×10^{10} PFU 007-Luc. Irradiated groups were given 6 Gy either immediately before infection (radiation-before) or one week after infection (radiation-after). The number of animals per group were: mock (n=9), radiation-before (n=8), radiation-after (n=7), 007-Luc (n=9), 007-Luc + radiation-before (n=9), 007-Luc + radiation-after (n=9). **(a)** Mean tumor volume. Error bars represent mean + SE. Tumors in the mock group were significantly larger ($P<0.05$) than all other groups on day 28, and tumors in the 007-Luc group were larger than either double combination on day 28 ($P<0.05$). Tumor suppression was similar in the 007-Luc + radiation-before and 007-Luc + radiation-after groups ($P>0.40$ at all time points). **(b)** Luciferase expression in tumors, measured by total flux of photons. The gray line suggests the background intensity (approximately 10^5 photons).