ARTICLE

ATN-224 enhances antitumor efficacy of oncolytic herpes virus against both local and metastatic head and neck squamous cell carcinoma

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Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequent cancer worldwide, and the 5-year survival rates are among the worst of the major cancers. Oncolytic herpes simplex viruses (oHSV) have the potential to make a significant impact in the targeted treatment of these patients. Here, we tested antitumor efficacy of RAMBO, an oHSV armed with the antiangiogenic Vst120, alone and in conjunction with ATN-224, a copper chelator against HNSCC in vitro and in vivo animal models. We found that all tested HNSCC cells responded well to virus treatment and were sensitive to RAMBO-mediated oncolytic destruction. In vivo, RAMBO had a significant antiangiogenic and antitumorogenic effect. Physiologic levels of copper inhibited viral replication and HNSCC cell killing. Chelation of copper using ATN-224 treatment significantly improved serum stability of RAMBO and permitted systemic delivery in HNSCC tumor xenografts models. Furthermore, our results show that the combination of ATN-224 and RAMBO strongly inhibits lung metastases in a mouse model of HNSCC. These findings suggest that combining ATN-224 with RAMBO has potential for clinical trials in both early and advanced HNSCC patients.

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC), which occurs in the oral cavity, oropharynx, larynx, and hypopharynx, is the sixth most common cancer worldwide and the 5-year survival rates are among the worst of the major cancer. There are over 35,000 new cases of oral and oropharyngeal squamous cell carcinomas diagnosed in the United States each year, resulting in 12,000 deaths.1 Currently, patients with locally advanced stage I/II of HNSCC are treated with surgery, radiotherapy, chemotherapy, and molecular targeted therapies.2 Despite initial clinical responses, 50% of these patients relapse and develop advanced and metastatic disease. Despite significant improvements in the therapy, the long-term survival rates in patients with advanced stages of HNSCC have not increased significantly. Treatment often leads to severe and permanent functional deficits with a negative impact on patients’ quality of lives.3 Therefore, there is a great need for the development of novel therapies to improve survival of recurrent and metastatic HNSCC patients while limiting treatment-related toxicities.

Oncolytic herpes simplex viruses (oHSV) are genetically engineered to specifically replicate in tumor tissue and avoid infection and propagation in normal cells.4 oHSV have shown antitumor efficacy in vitro and in vivo animal models of head and neck cancers.5,6 In the few clinical trials investigating safety of oHSV administered to patients, no dose-related toxicities were identified.3 However, complete responses or therapeutic efficacy have rarely been observed; so, significant improvements in oHSV therapy are necessary. Currently, there are two HSV-1-derived oncolytic viruses that are being tested for safety and efficacy in patients with head and neck cancers (NCT01017185, and NCT00931931).

Angiogenesis has a well-recognized role in HNSCC progression, resistance to drugs, and radiotherapy. Many clinical trials have been conducted with antiangiogenic agents in this disease, even if they often showed limited efficacy.7 Copper is an essential cofactor for the function of many angiogenesis-promoting enzymes and plays a key role in multiple steps along the angiogenesis pathway, leading to the activation of many molecules involved in angiogenesis.8 Furthermore, raised serum copper concentrations were observed in nearly 40% of the HNSCC patients.9 Therefore, copper suppression therapy may improve HNSCC patient survival. Apart from supporting angiogenesis, serum copper also inhibits wild-type HSV infection and replication via DNA damage induced by copper (II) ions. Additionally, strategies to combat tumoral angiogenesis have been

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shown to improve oHSV therapy.\textsuperscript{10,11} Tetrathiomolybdate (TM) functions by creating a complex with copper and serum albumin, effectively restricting cellular uptake of copper and has shown to strongly suppress increases in inflammatory and immune-related cytokines. TM has been approved by FDA for the treatment of Wilson’s disease and is currently under investigation in several Phase II trials as an antiangiogenic and antineoplastic agent in a variety of cancers (NCT00383851 and NCT00405574, respectively). ATN-224 is a second-generation analog of TM that is orally available and has superior stability and a faster onset of action. While angiogenesis plays a significant role in the progression of HNSCC, the impact of oHSV in combination with antiangiogenic strategies has not been tested in preclinical or clinical HNSCC. We have previously shown that copper inhibit oHSV and sequestration of copper by ATN-224 is effective preclinical or clinical HNSCC. We have previously shown that copper combination with antiangiogenic strategies has not been tested in significant role in the progression of HNSCC, the impact of oHSV in

RESULTS

RAMBO is cytotoxic and antiangiogenic toward human squamous cell carcinoma (SCC) cells

Vstat120 is the cleaved and secreted extracellular fragment of brain-specific angiogenesis inhibitor 1 (BAI1), and has been shown to be a potent antiangiogenic and antitumorigenic factor. Oncolytic herpes simplex virus (oHSV) RAMBO is an antiangiogenic Vstat120 expressing oncolytic virus. First, we tested the expression of Vstat120 in SCC cells infected with RAMBO. Western blot analysis using CAL27 and UM-SCC-74A cells treated with phosphate-buffered saline (PBS), control virus rHSVQ1, or RAMBO showed efficient production of Vstat120 in cells infected with RAMBO (Figure 1a). Next, we compared antitumor efficacy of rHSVQ1 and RAMBO in subcutaneously implanted CAL27 xenograft model. When CAL27 tumor size reached a volume of around 150 mm$^3$, animals were injected intratumorally with 1 x 10$^6$ pfu of rHSVQ1 or RAMBO and then were closely monitored for tumor growth. There was significant increase in progression free survival in RAMBO-treated mice compared to rHSVQ1 ($P$...
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Figure 2 Antitumor efficacy of RAMBO against CAL27 SCC flank model. Athymic nude mice were subcutaneously implanted with CAL27 cells. When tumor size reached a volume of around 150 mm³, animals were injected intratumorally with PBS or 1 × 10⁶ pfu of RAMBO. Tumor volume was measured regularly after treatment. (a) Tumor growth in individual mice treated with PBS (left panel) or RAMBO (right panel) over a period of time after treatment. (b) Average tumor volume of mice treated with PBS or RAMBO over periods of study. Data points represent the mean of the tumor size for each group at the indicated time points. (c) Representative images of CAL27 tumor bearing mice treated with PBS or RAMBO. (d) Kaplan–Meier survival curves of the data in (b). The percentage of surviving mice was determined by monitoring the death of mice over a period of 60 days. *P < 0.05 compared with mice treated with PBS. The arrow indicates the time of treatment with RAMBO.
Physiologic level of copper inhibits RAMBO-mediated SCC cell killing and ATN-224 rescues its effect.

We have previously shown that physiologic levels of copper can inhibit oHSV replication and killing of glioma cells.12 To test whether copper could inhibit RAMBO-mediated killing of SCC cells, we measured the viability of UM-SCC-74A cells infected with RAMBO with and without copper. Figure 3a demonstrates that the ability of RAMBO to destroy UM-SCC-74A cells is significantly reduced when treated with physiologic levels of copper. Quantification of viable cells revealed a dose dependent and significant reduction in cell viability of cells treated with RAMBO (Figure 3b). Average physiologic human levels of copper are above 10 µmol/l and complete inhibition of RAMBO-mediated cell killing was observed when RAMBO was treated with amounts greater than 10 µmol/l copper. Since copper inhibits oHSV activity in vitro, we tested whether copper chelation with ATN-224 could rescue RAMBO-mediated oncolytic SCC cell killing. Oncolytic efficacy of RAMBO treated with copper in the presence or absence of ATN-224 was tested by measuring the viability of UM-SCC-74A cells (Figure 3c). Crystal violet staining of viable cells shows efficient oncolysis of cells treated with RAMBO in the absence of copper (Figure 3c, top panel lane 2). However, the addition of copper eliminated the ability of RAMBO to kill UM-SCC-74A cells (Figure 3c, top panel lane 4). The addition of ATN-224 at concentrations of 8 µmol/l or higher showed complete rescue of RAMBO’s oncolytic affects (Figure 3c, top panel lanes 10–12). In the absence of copper, ATN-224 did not affect oncolytic ability of RAMBO to destroy UM-SCC-74A cells in vitro (Figure 3c, bottom panel lane 5–12). Quantification of viable cells revealed that ATN-224 treatment efficiently rescued copper-mediated inhibition of RAMBO cell killing in multiple SCC cells (Figure 3d).

ATN-224 rescues copper-mediated inhibition of RAMBO replication in vitro

To evaluate whether the inhibition of RAMBO induced SCC cell killing by copper was due to inhibition of viral replication, we measured the viral replication in the presence and absence of copper and ATN-224 (Figure 4). As RAMBO encodes GFP, fluorescence microscopy was utilized to visualize GFP positive RAMBO-infected cell. Copper found in serum has been shown to inhibit wild-type HSV infection, and its topical use is currently being evaluated as an antitherpetic agent in patients with herpes skin lesions.13–15 Consistent with these previous reports, a complete absence of GFP-positive cells was apparent when cells were infected in the presence of copper. However, the

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Figure 3  Copper-mediated inhibition of SCC cell killing induced by RAMBO is rescued by ATN-224. (a) Microscopic images of crystal violet stained UM-SCC-74A cells infected with RAMBO ± copper. (b) Quantification of cell viability of UM-SCC-74A cells infected with RAMBO at the indicated MOI ± physiologic levels of copper. (c) Images of crystal violet stained UM-SCC-74A cells infected with RAMBO treated with ATN-224 in the presence (upper panel) or absence (bottom panel) of copper. (d) Quantification of cell viability of the indicated cells infected with RAMBO at the indicated MOI ± copper in the presence or absence of ATN-224. Copper mediated reduced SCC cell killing induced by RAMBO was significantly rescued by ATN-224. Data shown are representative results from a total of three separate experiments. Data were analyzed by the paired student’s t-test and error bars indicate ± SD for each group. *indicates P value <0.05.

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the liver and circulating in blood, and serum Cp measurements have Ceruloplasmin (Cp) is a copper-containing oxidase, synthesized in ATN-224 treatment of mice could reduce serum copper levels. To test the in vivo relevance of these findings, we first tested whether in vivo ATN-224 rescues copper-mediated inhibition of RAMBO replication. Combination treatment with ATN-224 and RAMBO increases antitumor efficacy in vivo Since increased tumor growth is associated with higher serum copper levels and copper sequestration has been shown to have some therapeutic efficacy, we evaluated the therapeutic efficacy of ATN-224 in two phenotypically different types of SCC (UM-SCC-74A and CAL27) xenograft models established in nude mice. UM-SCC-74A is a rapidly growing SCC cell line, while CAL27 has a slower growth rate. When the subcutaneously implanted CAL27 tumors reached an average size of 150 mm³ in volume, mice were randomized and ATN-224 (0.7 mg/day) or PBS was systemically administrated via daily gavage (n = 10/group) until finishing experiment. At day 7 from ATN-224 or PBS daily gavage, animals were injected intratumorally with RAMBO (1 × 10⁶ pfu) or PBS and tumor growth was measured twice a week (Figure 6a,b). As presented in Figure 6a, control tumors which received PBS showed rapid tumor growth, leading to a tumor volume of 663 ± 72.5 mm³ by day 15. In contrast, the mean tumor volumes in mice treated with RAMBO, ATN-224, or ATN-224 plus RAMBO were 211.1 ± 23.5, 346.2 ± 34.9, and 138.9 ± 18.5, respectively. These data correlate to 68.2, 47.8, and 79.1% tumor growth inhibition, respectively, as compared to PBS control. By day 60 following treatment, 80% of the animals in the ATN-224 plus RAMBO-treated group were still viable as compared to 0, 30, and 30% in the PBS-, RAMBO-, and ATN-224-treated groups, respectively (Figure 6b). The tumor growth suppression that was observed in the CAL27 xenograft tumor model was also observed in the rapidly growing subcutaneous UM-SCC-74A xenograft model (n = 7/group) (Figure 6c,d).
Specifically, one out of seven tumors treated with ATN-224 plus RAMBO completely regressed. A single intratumoral injection of RAMBO demonstrated significant decreased tumor growth (median survival of 20 compared to 16 days for RAMBO versus PBS, <0.05). While treatment with ATN-224 alone also had significant antitumor effects, the combination of ATN-224 and RAMBO showed improved therapeutic efficacy over either agent alone (median survival of 40 versus 27 days for RAMBO versus ATN-224, <0.01) (Figure 6c,d).

Combination treatment with ATN-224 and RAMBO inhibits tumor angiogenesis and spontaneous lung metastasis

The apparent enhanced antitumor efficacy and survival benefits resulting from combination treatment with ATN-224 and RAMBO was further investigated by immunohistochemical examination for H&E and CD31 staining of CAL27 tumors (Figure 6e). Mice treated with both ATN-224 and RAMBO had significantly increased tumor necrosis (black arrow in Figure 6e). Furthermore, there was a significant difference in inhibition of angiogenesis between RAMBO alone and ATN-224 plus RAMBO-treated tumor (P < 0.01) (Figure 6e,f). Similarly, significantly reduced angiogenesis was also observed in UM-SCC-74A subcutaneous tumor bearing mice (Supplementary Figure S2). Treatment with angiogenesis inhibitors and induction of hypoxia is frequently associated with increased tumor cell entry into circulation and metastasis. Thus, we evaluated whether combination treatment with ATN-224 and RAMBO would have an effect in blocking on metastasis in our UM-SCC-74A tumor model (Figure 6g,h). We frequently observe lung metastases in mice subcutaneously implanted with UM-SCC-74A. Thus, we harvested lungs and sectioned amongst the different in vivo groups with UM-SCC-74A flank tumors. Significant reduction in the number of mice showing metastasis was observed in mice treated with ATN-224 plus RAMBO compared to PBS treated mice (1/7 versus 5/7 mice, P < 0.05) (Figure 6g,h). Taken together, these results indicate that combination treatment with ATN-224 and RAMBO strongly inhibited lung metastasis of SCC and suggests that this combinatorial therapy has global implications for clinical trials in advanced metastatic stage III/IV HNSCC patients.
Figure 6  In vivo antitumor effect of the combination treatment with ATN-224 and RAMBO (a). Subcutaneously implanted tumors derived from CAL27 (a,b) and UM-SCC-74A (c,d) cells were treated with PBS, or ATN-224 by daily gavage, when tumor size reached around 150 mm³. Seven days later from ATN-224 treatment, mice were treated with PBS or RAMBO (1 × 10⁵ pfu on day 1 and day 28 for CAL27 and 1 × 10⁶ pfu on day 7 for UM-SCC-74A) and tumor volume was monitored over time. On day 15 after treatment, the tumor sizes were compared (a and c). RAMBO combined with ATN-224 significantly enhanced antitumor effect compared to the treatment with ATN-224 or RAMBO alone. (b and d) Kaplan-Meier survival curves following same treatments. The percentage of surviving mice was determined by monitoring the death of mice (tumor size > 1,000 mm³) over a period of 60 days. There was a statistically significant longer survival in mice treated with combination therapy (*P < 0.05). The arrows indicate the time of RAMBO injection and ATN-224 treatment. (e) Representative histological characterization of CAL27 xenograft tumor after treatment of 1 week is displayed in Figure 6a and b. Control (PBS) tumor tissue showed dilated blood vessels and microvessels (arrows). ATN-224 treated tumor exhibited fewer microvessels and some necrotic foci (arrow heads). Tumors from mice treated with both RAMBO and ATN-224 showed more extensive necrosis and reduced blood vessel density than RAMBO alone treated tumors. (f) Quantification of CD31 immunostained vessels. There was significant difference in microvessel numbers between PBS control and ATN-224 treatment (n = 3/group) (*P < 0.05). Combination of ATN-224 and RAMBO further reduced microvessel number than RAMBO treated alone (PBS versus ATN-224, *P = 0.015; PBS versus RAMBO, **P = 0.015; RAMBO versus ATN-224+RAMBO, *P = 0.033, PBS versus ATN224+RAMBO, **P = 0.01). (g) The proportion of lung metastases noted in UM-SCC-74A flank tumor model with the different treatment paradigms. (h) Table for lung metastasis incidence of subcutaneously implanted UM-SCC-74A tumor (n = 7/group). Data were analyzed by the paired student’s t-test.
Oncolytic herpes simplex virus (oHSV) has been widely used in clinical trials and proven to be safe with minimal dose-limiting toxicities. However, the effectiveness of oHSVs in patients has not lived up to the expectations generated from the results observed in preclinical studies. Combining oHSV with chemotherapeutic agents presents an opportunity to increase oncolytic activity by either bolstering virus replication or suppressing systemic innate immune responses that can lead to rapid virus clearance. Despite augmenting virus replication, combination of oncolytic viral therapy with chemotherapy has only been well tolerated with few dose limiting toxicities. While these studies have remain promising, a better elucidation of factors that can limit in vivo efficacy of oHSV vectors can help design improved treatment strategies.

Our in vitro and in vivo assays demonstrated that physiological levels of copper inhibit oHSV therapeutic efficacy. Average serum copper concentrations in adults are 18–20 µmol/l and our in vitro data reveal that concentrations of copper above 8–10 µmol/l inhibits oHSV infection and replication. Based on this finding, we speculated that physiologic copper significantly hinders oHSV therapeutic efficacy in clinical trials. Therefore, we hypothesized that by reducing serum copper levels, we can improve systemic delivery and replication of oHSV, resulting in enhanced therapeutic efficacy.

ATN-224, bis (2-hydroxethyl) trimethylammonium, is a second-generation analog of ammonium terathiomolybdate (TM), that is US Food and Drug Administration approved for Wilson’s disease. The drug is orally available and sustained copper reduction is well tolerated. Copper chelation in humans with ATN-224 is also a safe and effective method of reducing physiologic levels of copper such that it inhibits angiogenesis but not basic cell cycle functioning. Additionally, ATN-224 has been shown to have copper independent antiangiogenic effects by directly inhibiting tumor cell invasion and inducing cancer cell anoikis. Its anticancer efficacy has also been imparted to the inhibition of SOD1 and multiple cofactors of the angiogenesis cascade and is currently being investigated as an anticancer agent in several clinical trials (NCT00383851, NCT00405574). Furthermore, the usage of ATN-224 has been approved in Europe.

Here, we tested the impact of an oHSV that encodes for an antiangiogenic gene on head and neck squamous cell carcinoma (HNSCC) alone and in combination with ATN-224. RAMBO is an oHSV that encodes for antiangiogenic Vstat120 and has shown therapeutic efficacy against brain tumors in mice. In this study, we showed that HNSCC is sensitive to RAMBO-induced viral replication and cell killing and RAMBO can be an effective treatment strategy for head and neck cancer. Moreover, the addition of ATN-224 greatly enhanced serum stability, viral replication, and cell killing of RAMBO, resulting in enhanced in vivo survival and decreased lung metastasis. While we have shown increased serum stability of RAMBO in mice treated with ATN-224, we have not tested its systemic delivery efficacy in HNSCC bearing mice in vivo. Future studies evaluating systemic delivery efficacy, safety, and biodistribution for HNSCC will be needed before its clinical investigation in patients.

This study is limited by the lack of testing in an immunocompetent host, which has been documented to play a major role in oncolytic viral clearance. ATN-224 and its parent compound, TM, is a triple threat when used in combination with oHSV therapy. As we demonstrated here, reduction of physiologic levels of copper significantly enhances oHSV therapy through (i) reduction of direct copper mediated inhibition of oHSV, (ii) decreased angiogenesis leading to reduced tumor growth and viral clearance, (iii) increased oHSV replication and cell killing. Decreased angiogenesis and increased viral replication may be able to overcome viral clearance prompted by the innate immune response.

In conclusion, these experiments support the future use of ATN-224 in combination with oHSV for HNSCC. We plan to pursue phase 1 clinical trials as further testing in patients will uncover the potential of combining oHSV with ATN-224, which has the ability to make significant improvements in efficacy of oncolytic viral therapy.

**MATERIALS AND METHODS**

**Reagents**

ATN-224, choline tetrathiomolybdate, was kindly provided by Dr. Andrew P. Mazar (Chemistry of Life Processes Institute, Northwestern University, Evanston, IL). ATN-224 stocks (50 mg/ml) were prepared in water, aliquoted, and frozen until use. ATN-224 was diluted to the desired concentration using PBS or medium just before use. For in vitro experiments, ascorbate buffer was used as a reducing agent as described. Copper chloride (CuCl₂·H₂O) and ascorbic acid were obtained from Sigma (St Louis, MO).

**Cell culture and oncolytic viruses**

Six human squamous cell carcinoma (SCC) cell lines (UM-SCC-7A, UM-SCC-1, UM-SCC-11A, UM-SCC-47, UM-SCC-2, and ATCC CAL 27) were used and cultured in Dulbecco modified Eagle medium (DMEM, Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco/Invitrogen), 50 µg/ml of penicillin G, 50 µg/ml of streptomycin sulfate, and 1× MEM NEAA (Gibco/Invitrogen), Vero (African green monkey kidney cells, ATCC) cells were maintained in DMEM containing 10% FBS without 1× MEM NEAA. All cell lines were maintained at 37 °C in a humidified atmosphere at 5% CO₂.

The construction and generation of both control rHSVQ1 and RAMBO (Rapid Antiangiogenesis Mediated By Oncolytic Virus) viruses have been previously described. Viruses were propagated in Vero cells, purified, and infectious virus titers (plaque forming unit per ml (pfu/ml)) were determined by standard plaque forming unit assay on Vero cells.

**Western blot analysis**

Immunoblots were probed with rabbit anti-N-terminal BAI1 to probe for Vstastat120 or mouse anti-GAPDH (Abcam, Cambridge, MA) followed by goat anti-rabbit (Dako, Carpinteria, CA) or sheep anti-mouse (Amersham Healthcare, Piscataway, NJ).

**Cytotoxicity assay**

Cells were seeded into a 96-well plate (2% FBS media), and were infected with oHSV at the indicated multiplicity of infection (MOI) preincubated with ascorbate buffer ± copper (1 mg/l) ± ATN-224 (32 µmol/l) for 30 minutes at RT. Forty-eight hours postinfection, cell viability was measured using a standard crystal violet assay as described.

**In vitro viral replication assays**

Cells were seeded into six-well plates, and were infected with oHSV in three groups: RAMBO only (0.1 MOI), RAMBO + Cu (virus 0.1 MOI + copper 20 µmol/l) and RAMBO + Cu + ATN-224 (virus 0.1 MOI + copper 20 µmol/l + ATN-224 32 µmol/l). Infection proceeded for 2 hours, and then unbound virus was washed away and fresh 2% FBS DMEM media was added to each well. Forty-eight hours post-oHSV infection, cells and media were harvested and the amount of infectious virus particles were determined by performing a standard plaque forming unit assay on Vero cells, as previously described.

**Animal xenograft**

All mice studies were performed in accordance with the Subcommittee on Research Animal Care at The Ohio State University guidelines, and have been approved by the Institutional Review Board. Four- to 5-week-old female athymic nu/nu mice (NIH-NCI) were injected subcutaneously with 1.5 × 10⁷ of UM-SCC-7A or CAL27 in 100 µl volume into the rear flank. When tumors reached average size of 150 mm³, mice were randomized to receive either ATN-224 (0.7 mg per day for each mouse) or sterile PBS, delivered by daily
oral gavage. At day 7 from gavage, RAMBO (2 × 10⁶ pfu) or PBS was administered by direct intratumoral injection. The ATN-224 and PBS treatment was continued until the end of the experiment and the tumor volume was calculated using the following formula: Volume = 0.5 LW², where W and L are the two maximum dimensions. Measurements of tumor volumes were taken as indicated, and mice were killed when tumor volumes exceeded 2,000 mm³ or >20% of body mass was lost.

Ceruloplasmin assay
Ceruloplasmin levels were measured weeks 1, 2, 4, and 6 during the study period. Nontumor bearing mice were treated with ATN-224 (0.7 mg/kg) or PBS by daily gavage and blood was collected from facial vein every weeks. Ceruloplasmin was assayed based on its oxidase activity. Two tubes each containing 25 μl mouse serum and 375 μl of 0.1 mol/l sodium acetate buffer (pH 5.0) were incubated for 5 minutes at 37 °C, and the amount of virus particles was measured by a standard plaque forming unit assay on Vero cells. This assay was run using serial diluted RAMBO 37 °C, and the amount of virus particles was measured by a standard plaque

Ex vivo and in vivo serum rescue assay
For ex vivo serum rescue assay, CAL27 tumor-bearing mice were treated with ATN-224 (0.7 mg/kg) or PBS by daily gavage. On day 14 after ATN-224 treatment, blood was collected from facial vein and serum was harvested as described. Twenty microliters of serum diluted with 20 μl of Hank’s buffered salt solution (HBSS) were incubated with RAMBO (2 × 10⁶ pfu) for 1 hour at 37 °C, and the amount of virus particles was measured by a standard plaque forming unit assay on Vero cells. This assay was run using serial diluted RAMBO and plaque versus pfu number was plotted to give the linear regression standard curve. For in vivo serum rescue assay, mice were fed with PBS/ATN-224 (0.7 mg/kg) for 10 days and then RAMBO (5 × 10⁶ pfu) was administered by tail vein injection. Twenty minutes postvirus injection, serum was harvested and the number of infectious virus particles present was determined.

Viral gene copy assay
To measure systemic oHSV (RAMBO) delivery efficacy in vivo, quantitative real-time PCR analysis of viral gene copy was determined. CAL27 tumor-bearing mice were treated with ATN-224 (0.7 mg/kg) or PBS by daily gavage and injected with RA virus using the cloning vector containing the ICP4 primers were used: sense, 5′-CGACACGTCGACGACCCG-3′ and anti-sense, 5′-GATCCCCCTCCCGCTTCGTTCG-3′. Viral gene copy present in the tumors was measured by determining the total number of copies of the HSV-specific ICP4 gene using absolute quantitative real-time PCR (qPCR) analysis. A linear regression curve was generated by diluting the plasmid to various concentrations ranging from 0.001 μg/5 μl to 1 μg/5 μl DNA. The number of plasmids per μg of DNA was calculated by determining the μg of DNA per plasmid using the total number of nucleotides in the plasmid (amounting to 6.022 × 10⁹/23(length ° 1 × 10⁹ * 660)). QPCR with the diluted concentrations of the plasmid was performed as previously described and the CT value versus copy number (Log transformed) was plotted to give a linear regression standard curve. The linear regression line formula generated was used to calculate the oHSV gene copy number.

Evaluation of tumor xenograft by histological analysis
Tumor sections were fixed in 4% paraformaldehyde, embedded in paraffin and cut into 5-μm sections. Representative sections were stained with Hematoxylin and Eosin (H&E), and then examined by light microscopy. For immunohistochemistry of CD31, sections were stained using antimonius CD31 antibody at a 1:250 dilution (BD Pharmingen, San. Jose, CA). Appropriate negative and positive controls were done. The total number of stained vessels was determined in five random high-power fields (×400 magnification), and then the mean was reported in a blinded fashion for each tumor.

Lung metastasis
At the time of dissection, lungs were excised and tumor nodules were evaluated. Lung tissues were fixed in 4% paraformaldehyde for 2 days, and 5-micrometer tissue sections were cut and stained with H&E. The entire sections were examined microscopically at low powers (×40 and ×100 magnifications) for the presence of metastases.

Statistical analysis
GraphPad software was used for all statistical analysis. Data are presented as mean ± SD. Data were analyzed using unpaired, two-tailed t-tests when comparing two variables. ANOVA with Tukey’s post-test was used to compare data in experiments where more than two variables were compared simultaneously.

CONFLICT OF INTEREST
The authors declared no conflict of interest.

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Supplementary Information accompanies this paper on the Molecular Therapy—Oncolytics website (http://www.nature.com/mto)