Published in final edited form as:
Surgery. 2013 September ; 154(3): 486–495. doi:10.1016/j.surg.2013.06.004.

An Oncolytic Vaccinia Virus Expressing the Human Sodium Iodine Symporter Prolongs Survival and Facilitates SPECT/CT Imaging in an Orthotopic Model of Malignant Pleural Mesothelioma

Laurence J. Belin, MD, MPH[1], **Justin W. Ady, MD**[1], **Christina Lewis, BA**[1], **Drew Marano, BA**[1], **Sepideh Gholami, MD**[1], **Kelly Mojica, BA**[1], **Clarisse Eveno, MD**[1], **Valerie Longo, BA**[2], **Pat B. Zanzonico, PhD**[2], **Nanhai G. Chen, PhD**[3,4], **Aladar A. Szalay, PhD**[3,4,5], and **Yuman Fong, MD**[1]

[1]Department of Surgery, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, United States

[2]Departments of Medical Physics and Radiology, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, United States

[3]Genelux Corporation, San Diego Science Center, San Diego, CA 92109, United States

[4]Department of Radiation Medicine and Applied Sciences, Rebecca & John Moores Comprehensive Cancer Center, University of California San Diego, San Diego, CA 92093, United States

[5]Department of Biochemistry, Rudolf Virchow Center for Experimental Biomedicine, and Institute for Molecular Infection Biology, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany

Abstract

Background—The purpose of this original work is to examine the ability of an oncolytic vaccinia virus expressing the human sodium iodine transporter (hNIS) to provide real time monitoring of viral therapy and effective treatment of malignant pleural mesothelioma (MPM).

Methods—Infectivity and cytotoxic effect of GLV-1h153 on mesothelioma cell lines of all histologic subtypes was assayed *in vitro*. Viral replication was examined by standard viral plaque assay. Orthotopic MPM xenografts were generated in athymic nude mice and treated with

Corresponding Author, Yuman Fong, MD, Department of Surgery, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065, Phone: (212) 639-2016 Fax: (212) 639-4031, Fongy@mskcc.org.

No competing financial interests exist for Laurence J Belin, Justin W Ady, Christina Lewis, Drew Marano, Sepideh Gholami, Kelly Mojica, Clarisse Eveno, Valerie Longo, Pat Zanzonico and Yuman Fong. Nanhai Chen, and Aladar Szalay are affiliated with Genelux Corporation.

intrapleural GLV-1h153 and assessed for effect on tumor burden and survival. Orthotopic tumors were also imaged on SPECT/CT after ^{131}I administration.

Results—GLV-1h153 infected and killed all cell lines in a time and concentration dependent manner. Viral replication demonstrated over a 2.5 log increase in titer over 4 days. Intrapleural treatment of orthotopic MPM xenografts resulted in a significant reduction in tumor burden one week after treatment and an improvement in survival. Infection of orthotopic xenografts was both therapeutic and facilitated monitoring by ^{131}I-SPECT/CT via expression of hNIS in infected tissue.

Conclusions—Our results suggest GLV-1h153 is a promising therapeutic agent for MPM and warrants further investigation.

Background

Malignant pleural mesothelioma (MPM) is an aggressive malignancy that arises from the pleura. The pathogenesis of mesothelioma is linked to a chronic inflammatory response resulting from inhalational asbestos exposure. While excision via pleurectomy/decortication or extra- pleural pneumonectomy is considered to be the mainstay of therapy for localized disease, mesothelioma is rarely detected at a time when it is amenable to surgical excision alone. Thus, most interventions are ultimately palliative. The median overall survival of locally advanced and metastatic MPM without treatment is 6-9 months.[1] Tri-modal therapy with neo-adjuvant platinum and anti-folate based chemotherapy regimens, surgical cytoreduction, and adjuvant radiotherapy are first line of treatment in stage II and III disease but confer only modest survival benefits. Novel forms of therapy are desperately needed for this terrible disease.

Oncolytic viral therapy selectively destroys tumor tissue through multiple mechanisms including direct cellular lysis and tumor vaccination. Among viruses currently under active anti-cancer investigation, there is great enthusiasm for oncolytic vaccinia virus (VACV). Vaccinia possesses a large 192-kb genome that readily accepts sizable insertions of foreign DNA without compromising viral replication. Furthermore, vaccinia undergoes replication in the cytoplasm, thereby decreasing the likelihood of recombination events with the host genome. Most importantly, vaccinia is arguably the most successful and extensively delivered biologic agent in the history of human disease. The administration of vaccinia virus as a live vaccine to millions during the WHO's successful smallpox eradication campaign has resulted in a well-characterized safety profile.[2]

GLV-1h68 is a replication-competent oncolytic vaccinia virus with interruptions in thymidine kinase, hemagglutinin, and *F14.5L* that confer tumor selectivity and decrease virulence to normal host cells.[3] At present, multiple stage I clinical trials using GLV-1h68 among patients with assorted solid tumors, peritoneal carcinomatosis and cancers of the head and neck are ongoing.[4] We have previously reported on the development of GLV-1h153 derived from GLV-1h68.[5] GLV-1h153 differs from its parent virus by the insertion of the human sodium iodine symporter (hNIS). Human NIS is a membrane bound glycoprotein present on the baso-lateral surface of thyroid follicular cells that facilitates the transport of iodine into the cytoplasm where it is organified in the process of thyroid

hormone synthesis.[6] This receptor is responsible for the utility of radio-iodine in imaging well-differentiated thyroid cancer. In this paper, we report that GLV-1h153, a novel vaccinia virus carrying hNIS, kills mesothelioma *in vitro*, confers improved 30-day survival in an orthotopic model of malignant pleural mesothelioma, and facilitates PET imaging for real-time tracking of viral therapy.

Materials and Methods

Cell Lines

MSTO-211H, H-MESO, JMN, VAMT, and CV1 cells were obtained from American Type Culture Collection (ATCC). Human MSTO-211H cells stably transfected with a luciferase reporter gene were a generous gift from the laboratory of Dr. Prasad Adusumilli at Memorial Sloan Kettering Cancer Center. Mesothelioma cells were maintained in RPMI with 10% fetal calf serum with penicillin (100,000 U/L) and streptomycin (100mg/L), and incubated in a 5% C02 humidified incubator at 37 degrees. CV1 cells for viral plaque assay were maintained in Minimum Essential Medium (MEM) with 10%FBS, penicillin and streptomycin.

Virus

GLV-1h153 is a replication-competent recombinant vaccinia virus derived from the parent vaccinia virus LIVP strain (Lister strain, Institute for Research on Virus Preparations, Moscow). It was reconstituted from its parent virus, GLV-1h68 with the insertion of the human sodium iodine symporter vector using Fugene (Roche, Indianapolis, IN), as described previously. PCR sequencing was used to confirm the genotype of hNIS expressing GLV-1h153. Additionally, GLV-1h153 contains an enhanced green fluorescent protein (eGFP) transgene under the control of a constitutively active VACV early/late promoter.

In Vitro Vector Spread Imaging

MPM cells representing epithelial (H-MESO), sarcomatoid (VAMT), bi-phasic (MSTO-211H and mixed (JMN) histologic subtypes were plated at a concentration of 5×10^3 cells in 1 ml of media per well in 24-well flat bottom plates and allowed to adhere over 24 hours. Cells were then treated with media alone (control), or infected with GLV-1h153 at an MOI (multiplicity of infection, ratio of viral particles to cell in culture) of 1.0. Infection was carried out initially in 200ul of media for 30 minutes at room temperature followed by the addition of 800ml media per well and incubated at 37°C in a 5% C02 incubator for 5 days. As GLV-1h153 carries an eGFP transgene, GFP microscopy may be used as a marker of viral infection and propagation in mesothelioma cells. Cells were examined with a fluorescence-inverted microscope (Nikon Eclipse TE300, Nikon, Japan) for GFP expression. Control and infected cells were visualized with both brightfield and GFP filters. Representative images were obtained and images were merged to identify the infected mesothelioma cells. Serial images were obtained over a 5 day period to track viral propagation.

Cytotoxicity Assay

MPMcell lines MSTO-211H, H-MESO, JMN, and VAMT were plated and treated with media alone (control), or GLV-1h153 at an MOI of 0.1, 1.0, and 5.0. At 24 hour intervals post-infection, percent survival for each group was determined using a standard lactate dehydrogenase (LDH) release bioassay. To accomplish this, supernatants were removed and cells are washed with PBS. Cells were then lysed with Triton X-100 (1.35%, Sigma, St. Louis, MO) at 37°C for 5 minutes. The intracellular LDH release of the lysed samples is then measured with a Cytotox 96 kit (Promega, Madison, WI) on a spectrophotometer (EL321e, Bio- Tek Instruments) at 490 nm. Results are expressed as surviving fraction, based on the measured absorbance of cellular lyastes compared to the lysates of untreated controls. All conditions were performed in triplicate and results were averaged.

In Vitro Viral Replication Analysis

The ability of GLV-1h153 to replicate within mesothelioma cells was evaluated by standard viral titration assays. A total of 5×10^3 MSTO-211H, H-MESO, JMN and VAMT cells in 1mL of media were plated separately in 12 well plates. Cells were infected with GLV-1h153 at an MOI of 1.0 and incubated for 5 days. At 24-hour intervals after viral infection, supernatants were collected and frozen at -80 degrees until all samples were available. At this time, 1:10 serial dilutions of supernatants were prepared and a standard viral plaque assay was performed on confluent CV-1 fibroblast cells. Viral titer was calculated and plotted over time. Samples were performed in triplicate.

In Vitro Radio-uptake Assay

To demonstrate that infected mesothelioma cells of all histologic subtypes express functional hNIS protein capable of radioisotope transport, HMESO, VAMT, MSTO211H and JMN cells were plated separately at a density of 5×10^5 in six-well plates for 24 hours. Cells were subsequently infected with GLV-1h153 at an MOI of 1.0 or mock-infected with media for 24 hours. After this period, cells were incubated for 1 hour with 0.5 μCi ^{131}I per well of ^{131}I and 0.1mM sodium iodide (NAI) with, or without, 1mM sodium perchlorate (NaCl04) (Sigma Aldrich, St. Louis, MO), a specific competitive inhibitor of hNIS. Supernatants were collected and cells were washed with cold PBS then lysed with TPE buffer. Supernatants and lysates were measured in a gamma-counter (Perkin Elmer, Waltham, MA). A Bradford protein assay was conducted on the lysates. The ^{131}I activity in the cell was then calculated as CPM/GM protein. All assays were performed in triplicate.

Establishment of Orthotopic MPM

All animals received humane care and maintained in accordance with the “guide for the care and use of laboratory animals (NIH).” Animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Memorial-Sloan Kettering Cancer Center. Homozygous athymic female mice were purchased from Taconic Farms Inc. and were provided food and water *ad libitum*. Animals were anesthetized in an induction chamber with a mixture of isoflurane (2L/minute) and oxygen (2L/minute). Mice were transferred to a sterile surgical stage and anesthesia was maintained via nose cone. Mice were placed in left lateral position and the right chest was prepared with 10% povidone Iodine solution. A

5mm incision was made over the fourth or fifth intercostal space and 1×10^5 cells in 200μl of pre-suspended MSTO-211H transduced with Luciferase reporter were injected into the pleural cavity with care not to violate the underlying lung. After the needle was withdrawn, the skin was closed with surgical staples. Animals were warmed and recovery was observed for 15 minutes before the mice were returned to their cage.

Bioluminescent Imaging (BLI) of Orthotopic MPM Xenografts

One week after tumor inoculation, mice were anesthetized with isoflurane and underwent intraperitoneal injection of 150 μl of luciferin (15mg/ml). Five minutes after injection, mice were imaged with an Ivis 200 optical imaging system (Caliper Life Sciences, Hopkinton, MA). Capture time was 40 seconds. Average radiance was quantified ($p/s/cm^2/sr$) with Living Image software version 4. Mice were then quantitatively randomized to untreated control and viral treatment groups. Serial bioluminescent images were obtained and quantified at one-week intervals after treatment for the duration of the survival study. Results were averaged and plotted weekly in Microsoft Excel.

Treatment of Malignant Pleural Mesothelioma

Following randomization, mice were again anesthetized and placed on the surgical stage in lateral decubitus position. The prior thoracotomy incision was re-opened and 1×10^4 or 1×10^5 PFU (plaque-forming units) of GLV-1h153 in 200μL of PBS was injected via insulin syringe into the pleural space as described above. Control animals received 200ul PBS intrapleurally. The wound was again closed with surgical clips, mice were warmed and recovery was observed for 15 minutes prior to returning the cage.

Confirmation of Viral Transfection of Mesothelioma *In Vivo*

Orthotopic mesothelioma was established and treatment with intrapleural GLV-1h153 was performed as described previously. Approximately 48 hours after treatment, xenografts were euthanized and tumor burden was collected from pleural and mediastinal surfaces by blunt dissection. Tumor tissue was fixed with 4% paraformaldehyde, paraffin embedded, and sliced into 5 micron sections on paraffin microtome. Tissue sections were then blocked for 30 minutes in 10% normal goat serum and 2% BSA and incubated for five hours in anti-smallpox A27L (Abcam, Cat# ab53311) at 1:100 dilution followed by a 60 minute incubation with biotinylated goat-anti rabbit IgG (Vector labs, cat#PK6101) at 1:200 dilution. Detection was performed with Streptavidin-HRP D (Ventana Medical Systems), followed by incubation with Tyramide Alexa Fluor 488 (Invitrogen, cat#T20922). DAPI counterstain was performed and rabbit IgG was utilized as a negative control.

SPECT/CT Imaging of Treated MPM Xenografts

48 hours after intra-pleural treatment with GLV-1h153, 3 representative mice from treatment groups and 1 untreated control were delivered ~ 200 μCi of carrier free ^{131}I via tail vein injection. Four hours after administration of ^{131}I, mice were anesthetized with isoflurane and individually imaged on a dedicated small-animal SPECT/CT scanner (nanoSPECT/CT, Bioscan, Washington, DC). Reconstructed SPECT images were fused with the registered helical CT scan images.

Survival Analysis

In order to determine the efficacy of intrapleural treatment with GLV-1h153 on prolonging survival in a mouse model of MPM, mice with established tumor burden were quantitatively randomized into treatment groups (n = 6). Following randomization, mice were assessed regularly for weight, dyspnea or tachypnea, or the development of cachexia. Mice who met humane survival endpoints were euthanized with $C0_2$. Survival data was analyzed and plotted with GraphPad Prism 4.0 (GraphPad Software, San Diego, California). At the time of death or at termination of the 30 day survival study, all mice underwent sternotomy to confirm the presence or absence of gross mesothelioma. Representative mice were submitted for necropsy by a certified pathologist for microscopic evaluation.

Statistical Analysis

All in vitro experiments were performed in triplicate and results expressed as mean +/- standard error of the mean. Comparisons between groups were made using the two-tail Student's T- test. Survival difference between treated and untreated groups were analyzed with Log-rank test.

Results

GLV1h53 Infectivity is Time Dependent Across a Spectrum of Mesothelioma Cell Types *In Vitro*

Following viral infection with an MOI of 1.0, GFP expression was assessed by fluorescent microscopy in four mesothelioma cell lines representing epitheliod (H-Meso), sarcomatoid (VAMT), biphasic (MSTO-211H), and mixed (JMN) histologies. GFP expression increases with time in all four cell lines. Viral cytotoxicity and consequent cell death at later time points resulted in a subsequent decline in GFP expression observed in MSTO-211H, H-Meso and JMN lines at 120 hours (Figure 1).

GLV-1h153 Demonstrates Time and Concentration Dependent Cytotoxic Effect on MPM Cell Lines *In Vitro*

Mesothelioma cell lines of all histologic subtypes demonstrated sensitivity to the cytotoxic effect of GLV-1h153. GLV-1h153 killed all cell lines in a dose dependent fashion with all lines demonstrating near complete eradication by five days post-infection at an MOI of 5.0 (Figure 2A). The epithelial mesothelioma cell line H-MESO demonstrated particular sensitivity with an MOI of 0.1 resulting in near 80% cell kill at 5 days post-infection.

GLV-1h153 Replicates Efficiently in MPM Cells *In Vitro*

Viral proliferation of GLV-1h153 in H-Meso, JMN, MSTO-211H, and VAMT mesothelioma cell lines was assessed by standard viral plaque assay of supernatants collected daily over a four-day period following infection with GLV-1h153. Epithelial cell line H-MESO, and the biphasic line MSTO-211H supported an excess of 2.5-logarithmic increase in viral titer at peak replication. JMN cells similarly exceeded a 2-log increase in titer by day 4 when compared to the initial treatment dose of 5×10^3 viral plaque forming units (Figure 2B). The sarcomatoid line VAMT evidenced the least efficient replication but

even in this line viral titer doubled by 72 hours. These results demonstrate that mesothelioma cells support efficient viral replication of GLV-1h153 in-vitro.

GLV-1h153 Infects Mesothelioma *In Vivo*

Immunofluorescent detection with an antibody specific for the intracellular mature virion form of vaccinia virus evidenced clear fluorescence in the mesothelioma tumor deposits of a treated mouse 48 hours after intrapleural delivery of GLV-1h153 (Figure 3). This confirms that GLV-1h153 is effectively transduced into orthotopic mesothelioma via intrapleural delivery.

Intrapleural Treatment with GLV-1h153 Reduces Tumor Burden in an Orthotopic Model of MPM as Measured by Bioluminescent Imaging

Treatment with 1×10^5 PFUs of GLV-1h153 resulted in a progressive and significant reduction in tumor burden for the duration of the survival study. By 3 weeks after randomization, mice treated with 1×10^5 PFUs of GLV-1h153 demonstrated a nearly 3-log decrease in average bioluminescent radiance relative to controls ($p<0.001$). (Figures 4 and 5A) Decreasing treatment titer to 1×10^4 also resulted in significant reduction of tumor burden on BLI three weeks after treatment ($p =0.04$.) On complete pathological exam at time of euthanasia, 6/6 mice treated with 1×10^5 PFUs and 5/6 mice treated with 1×10^4 PFUs of GLV-1h153 evidenced no residual tumor burden while 6/6 control mice had gross and histologic confirmation of MPM (Figure 5B).

Intrapleural Treatment with GLV-1h153 Results in Improved 30-day Survival in an Orthotopic Model of MPM

All mice (n = 6 per group) treated with intrapleural injection of 1×10^4 or 1×10^5 PFUs of GLV-1h153 were alive 1 month after tumor establishment. Mean survival in untreated controls was 21 days and all mice had expired by day 29. Conversely, all animals in both treatment dose groups were alive at day 30. These survival differences were significant on Kaplan-Meier analysis (1×10^4 PFUs vs control log-rank $p=0.0009$, 1×10^5 PFUs vs control log rank $p=0.0009$) (Figure 6).

Mesothelioma Cells Infected with GLV-1h153 Express Functional, Blockable HNIS Facilitating Uptake of Radio-iodine *In Vitro*

H-Meso, MSTO-211H, JMN and VAMT cells infected for 24 hours with GLV-1h153 followed by one hour of incubation with carrier-free ^{131}I all demonstrated significantly increased radiouptake compared to uninfected controls as measured by gamma scintigraphy (Figure 7A). The epithelial cell line H-Meso and biphasic line MSTO-211H both demonstrated nearly 10-fold increased ^{131}I uptake in infected cells relative to uninfected controls while mixed line JMN and sarcomatoid line VAMT demonstrated 6- fold and 2.5-fold more internalization respectively. Furthermore, this effect was completely abrograted by competitive inhibition of hNIS with NaCl04, resulting in iodine internalization at a level equivalent to that of uninfected controls. These results demonstrate that GLV-1h153 facilitates transport of radio-iodine via specific, blockable, hNIS mediated transport.

Intrapleural treatment with GLV-1h153 facilitates ^{131}I SPECT/CT imaging of orthotopic mesothelioma xenografts

Two animals from the orthotopic treatment group and one control were administered ^{131}I via tail vein injection. At approximately 4 hours following radio-iodine administration, mice were imaged on SPECT/CT. The two mice that had received intrapleural GLV-1h153 demonstrated clear ^{131}I uptake in pleural tumor burden (Figure 7B) while no such signal was evident on the PBS treated control animal.

Discussion

Malignant Pleural Mesothelioma remains a disease with few effective treatment options. MPM is a local disease in early stages, and primary curative treatment strategies are aimed at margin negative local control with surgery and adjuvant irradiation. Despite these modalities, disease most often recurs locally and progresses to mortality. In most instances, however, mesothelioma typically presents in later stages. As such, many patients are not surgical candidates at the time of diagnosis. There is a clear need for novel local and systemic control of mesothelioma. Unfortunately, to date, trials examining intra-cavitary administration of novel chemotherapeutics have been characterized by high rates of toxicity, local recurrence and conflicting results.[7] Intra-cavitary oncolytic viral therapy is an exciting new possibility supported by a wealth of pre-clinical work. Three different constructs of oncolytic herpes simplex virus delivered intrapleurally were able to selectively infect MPM in-vivo, induce GFP expression allowing thoracoscopic identification of tumor tissue, and prolong survival in an orthotopic mouse model.[8] In a second study, intrapleural treatment with a genetically engineered Newcastle disease virus (NDV) resulted in complete response to viral therapy in 65% of the animals within 14 days. Long-term survival exceeded 50 days in treated animals compared to 23 days in controls and multiple intrapleural administrations resulted in significantly superior response than single dosing.[9] It should be noted that all cell lines utilized in the present work are derived from chemo and radiation resistant cell lines which suggests a role for viral therapy in multi-modal treatment strategies. In fact, there is prior evidence that viral therapy can have synergistic effect with platinum based chemotherapy in the treatment of MPM. In one in vitro study, an oncolytic herpes virus in combination with cisplatin enhanced viral replication 4 to 11-fold, cell kill 2 to 3-fold and resulted in dose reductions for both agents as high as 600-fold.[10]

Oncolytic VACV has been proven to have efficient tumoricidal effect both *in vitro* and *in vivo* in our laboratory in a number of tumor models. GLV-1h68 is the parental virus to the current construct and its engineering has been previously described. It is a replication-competent, recombinant vaccinia virus derived from the wild type Lister strain and expressing transgenes for Renilla-luciferase, beta-galactosidase, and green fluorescent protein.[11] For these studies, we have modified the GLV-1h68 virus to carry hNIS, an imageable gene in an attempt to produce a therapeutic virus that we may be tracked by non-invasive imaging. We have previously published our success in utilizing this viral construct to both treat and provide real-time, non-invasive imaging of thyroid[12], pancreatic[13] and triple negative breast cancer (TNBC) xenografts[14]. Most recently, we have demonstrated that GLV-1h153 can treat orthotopic TNBC underline tumors, prevent metastasis, and

facilitate ^{131}I imaging of positive surgical margins after resection. The present work describes encouraging results utilizing GLV-1h153 to treat malignant pleural mesothelioma while providing real-time, hNIS mediated imaging of viral delivery.

In vitro work presented here demonstrate the ability of GLV-1h153 to efficiently infect, replicate within, and kill mesothelioma cells of epithelial, bi-phasic, sarcomatoid and mixed histologic subtypes. Infection was demonstrated to occur in a time dependent fashion as evidenced by a progressive increase in GFP expression in all cell lines tested over the course of 72 hours. The time-course of infection correlated with the tumoricidal effect of GLV-1h153 as well as the efficient viral replication demonstrated on viral plaque assay. Interestingly, while epithelial, biphasic, and mixed lines were rapidly infected and sustained an excess of 2 – 2.5 logarithmic increase in viral titer, the sarcomatoid line VAMT appeared to have a delayed rise in GFP which had not yet reached its peak by 120 hours. Furthermore, viral titer in this line reached only twice the starting dose and hNIS specific ^{131}I radiouptake 24 hours after infection was expectedly lower than that seen among epithelial, biphasic and mixed histologies. Conversely, the epithelial H-Meso cell line demonstrated particular sensitivity to viral oncolysis with doses as low as MOI 0.1 resulting in complete eradication which may reflect the favorable tumor characteristics observed in this subtype clinically. In fact, the varying permissiveness to infection and viral gene expression observed *in vitro* may reflect the distinct tumor biologies of the different histologic subtypes of mesothelioma. Interestingly, despite differences in infectivity and replication, all lines demonstrated near-complete cell kill by GLV-1h153 over five days at an MOI of 5.0 suggesting that delivering adequate dose of virus may be a determining factor in effective therapy.

Intrapleural treatment with GLV-1h153 significantly diminished tumor burden on both bioluminescent imaging and at gross necropsy. Microscopic exam of paraffin embedded tumors collected from representative treatment and control mice showed clear viral transduction of tumor tissue at 48 hours following infection, and treated animals were confirmed to be free of microscopic evidence of residual mesothelioma after 30 days. This reduction in tumor mass translated to improved 30-day survival at both treatment doses tested relative to PBS treated controls. While the *in vivo* work presented here was undertaken only in tumors derived from the bi-phasic line MSTO-211H, the *in vitro* data demonstrate equivalent or greater sensitivity of epithelial and mixed cell lines to viral infection, replication and radio-uptake in comparison to MSTO-211H. Furthermore, all lines tested, including sarcomatoid line VAMT, demonstrated equivalent tumoricidal response. In sum, both the *in vitro* and *in vivo* work presented here is encouraging pre-clinical data but true variations in treatment response to oncolytic vaccinia virus based on tumor biology will need to be elucidated by clinical trials in humans.

The addition of the human sodium iodine symporter to oncolytic vaccinia virus is the novel facet of this therapeutic strategy for mesothelioma. Traditionally, biopsy has been the gold standard for the tracking of viral delivery to target tissues. As oncolytic viral therapies increasingly enter into clinical trials, the ability to monitor therapy non-invasively and in real time will become paramount in allowing clinicians to correlate viral delivery with treatment response and toxicity. GLV-1h153 is engineered with hNIS inserted under the control of an early vaccinia synthetic promoter. Infected tumor tissue begins to express the

iodine symporter within 6 hours of infection (immunoblot data not shown), long before the virus begins to kill tumor cells. During this time, as evidenced by our radiouptake assay *in vitro* and confirmed by our successful imaging *in vivo*, infected tumor cells express functional, viral-mediated hNIS on their cell surface. Thus, viral transduction of tumor may be imaged non-invasively with radio-iodine or other isotopes transported by hNIS, such as technetium. To extrapolate to a clinical scenario, here we have reported on the development of a therapeutically effective oncolytic vaccinia virus that will provide clinicians with the ability to track delivery and transduction of an oncolytic virus into mesothelioma burden in real-time via standard PET and SPECT imaging modalities. This could allow the titration of treatment doses and frequency to obtain optimal, patient-specific tumor transduction and monitoring of treatment effect.

This is not the first report of an oncolytic virus inducing hNIS expression in infected mesothelioma, however, we believe several attributes of vaccinia virus make it a more attractive candidate for human therapy. Previously published work utilized an attentuated oncolytic measles virus encoding interferon β and hNIS to provide immune stimulation and non-invasive I-125-PET imaging and treatment in a mouse model of MPM.[15] The data demonstrated a potent immunostimulatory response in infected mesothelioma tissue as well as prolonging survival in vivo. We believe VACV to be preferable to other oncolytic vectors such as measles virus and adenovirus due to a large genome that easily accepts transgenes without compromising viral replication, a favorable and well-established safety profile for both recipients and medical professionals, and the availability of targeted treatment in the unlikely event of systemic dissemination in an immunocompromised cancer patient.

In conclusion, this study demonstrates that an oncolytic vaccinia virus carrying hNIS efficiently infects, replicates within, and kills treatment-refractory mesothelioma cells of all histologic subtypes. Furthermore, the delivery of the hNIS receptor to the cell surface of infected tumor cells is functional *in vitro* and *in vivo*. Intrapleural treatment with GLV-1h153 prolongs survival and provides a non-invasive means to track oncolytic viral therapy in an orthotopic mouse model of malignant pleural mesothelioma. These findings support the human investigation of this potentially exciting therapy as a treatment option for this terrible disease.

Acknowledgments

This study was supported by: William H. and Alice Goodwin and the Commonwealth Foundation for Cancer Research, the Experimental Therapeutics Center of Memorial Sloan-Kettering Cancer Center, and the Flight Attendant's Medical Research Institute (FAMRI) as well as Genelux Corporation.

References

1. Rusch VW. Diagnosis and treatment of pleural mesothelioma. Semin Surg Oncol. 1990; 6(5):279–85. [PubMed: 2237087]
2. Fenner, F.; Henderson, DA.; Arita, I.; Jezek, Z.; Ladnyi, ID. Smallpox and its eradication. Geneva, Switzerland: World Health Organization; 1988.
3. Zhang Q, Yu YA, Wang E, Chen N, Danner RL, Munson PJ, et al. Eradication of solid human breast tumors in nude mice with an intravenously injected light-emitting oncolytic vaccinia virus. Cancer Res. 2007 Oct 15; 67(20):10038–46. [PubMed: 17942938]

4. identifier: NCT00794131. Phase I Study of the Safety, Tolerability, and Tumor-Specific Replication of the Intravenous Administration of Green Fluorescent Protein Encoded Genetically Engineered Attenuated Vaccinia Virus, GL-ONC1, in Patients With Advanced Solid Organ Cancers. www.clinicaltrials.gov ClinicalTrials.gov processed this record on January 02, 2013
5. Haddad D, Chen NG, Zhang Q, Chen CH, Yong Y, Lorena G, et al. Insertion of the human sodium iodine symporter to facilitate deep tissue imaging does not alter oncolytic or replication capability of a novel vaccinia virus. J Transl Med. 2011 Mar 31.9:36. [PubMed: 21453532]
6. Spitzweg C, Joba W, Eisenmenger W, Heufelder AE. Analysis of human sodium iodine symporter gene expression in extrathyroidal tissues and cloning of its complementary deoxyribonucleic acids from salivary gland, mammary gland, and gastric mucosa. J Clin Endocrinol Metab. 1998 May; 83(5):1746–51. [PubMed: 9589686]
7. Tsao AS, Mehran R, Roth JA. Neoadjuvant and intrapleural therapies for malignant pleural mesothelioma. Clin Lung Cancer. 2009 Jan; 10(1):36–41. [PubMed: 19289370]
8. Adusumilli PS, Stiles BM, Chain KK, et al. Imaging and therapy of malignant pleural mesothelioma using replication-competent herpes simplex viruses. J Gene Medicine. 2006; 8:603–615.
9. Silberhumer GR, Brader P, Wong J, Serganova IS, Gönen M, Gonzalez SJ, et al. Genetically engineered oncolytic Newcastle disease virus effectively induces sustained remission of malignant pleural mesothelioma. Mol Cancer Ther. 2010 Oct; 9(10):2761–9. [PubMed: 20858727]
10. Adusumilli PS, Chan MK, Chun YS, Hezel M, Chou TC, Rusch VW, Fong Y. Cisplatin-induced GADD34 upregulation potentiates oncolytic viral therapy in the treatment of malignant pleural mesothelioma. Cancer Biol Ther. 2006 Jan; 5(1):48–53. [PubMed: 16294031]
11. Kelly KJ, Woo Y, Brader P, Yu Z, Riedl C, Lin SF, et al. Novel Oncolytic Agent GLV-1h68 Is Effective Against Malignant Pleural Mesothelioma. Human Gene Therapy. 2008 Aug.19:774–782. [PubMed: 18754710]
12. Gholami S, Haddad D, Chen CH, Chen NG, Zhang Q, Zanzonico PB, et al. Novel Therapy for Anaplastic Thyroid Carcinoma Cells Using an Oncolytic Vaccinia Virus Carrying the Human Sodium Iodine Symporter. Surgery. 2011 Dec.150:1040–47. [PubMed: 22136819]
13. Haddad D, Zanzonico PB, Carlin S, Chen CH, Chen NG, Zhang Q, et al. A vaccinia virus encoding the human sodium iodine symporter facilitates long-term image monitoring of virotherapy and targeted radiotherapy of pancreatic cancer. J Nucl Med. 2012 Dec; 53(12):1933–42. [PubMed: 23139088]
14. Gholami S, Chen CH, Lou E, De Brot M, Fujisawa S, Chen NG, Szalay AA, Fong Y. Vaccinia virus GLV-1h153 is effective in treating and preventing metastatic triple-negative breast cancer. Ann Surg. 2012 Sep; 256(3):437–45. [PubMed: 22868370]
15. Li H, Peng KW, Dingli D, Kratzke RA, Russell SJ. Oncolytic measles viruses encoding interferon β and the thyroidal sodium iodine symporter gene for mesothelioma virotherapy. Cancer Gene Ther. 2010 Aug; 17(8):550–558. [PubMed: 20379224]

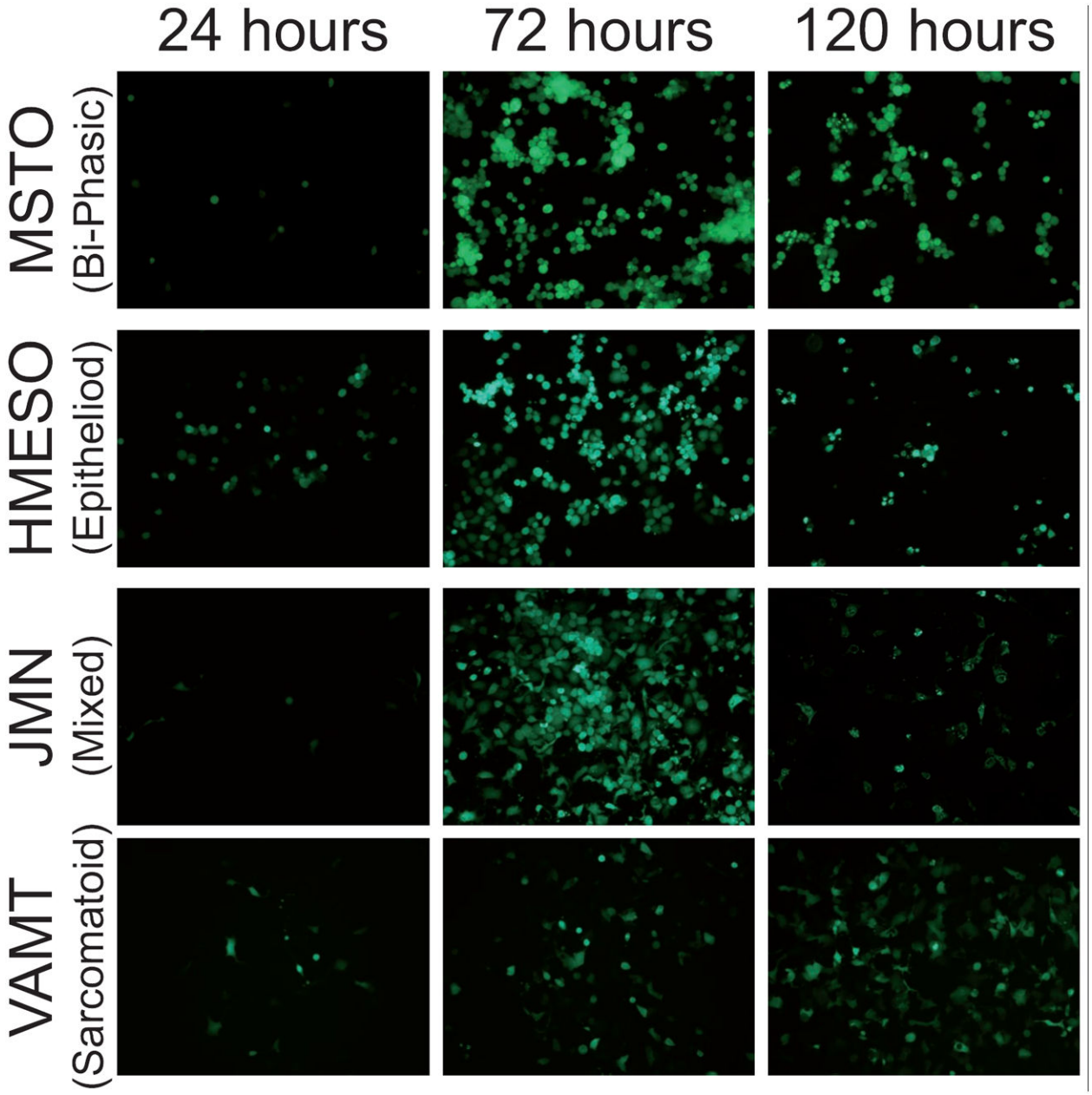

Figure 1. GLV-1h153 infection of MPM (MSTO-211H) is both time and concentration dependent in vitro

Fluorescent microscopy demonstrates increasing viral-mediated GFP expression at increasing time intervals for all histologic subtypes of mesothelioma in-vitro. Viral mediated tumor cell killing at longer time points results in a subsequent decrease in GFP expression as seen in H-MESO, MSTO, and JMN cell lines.

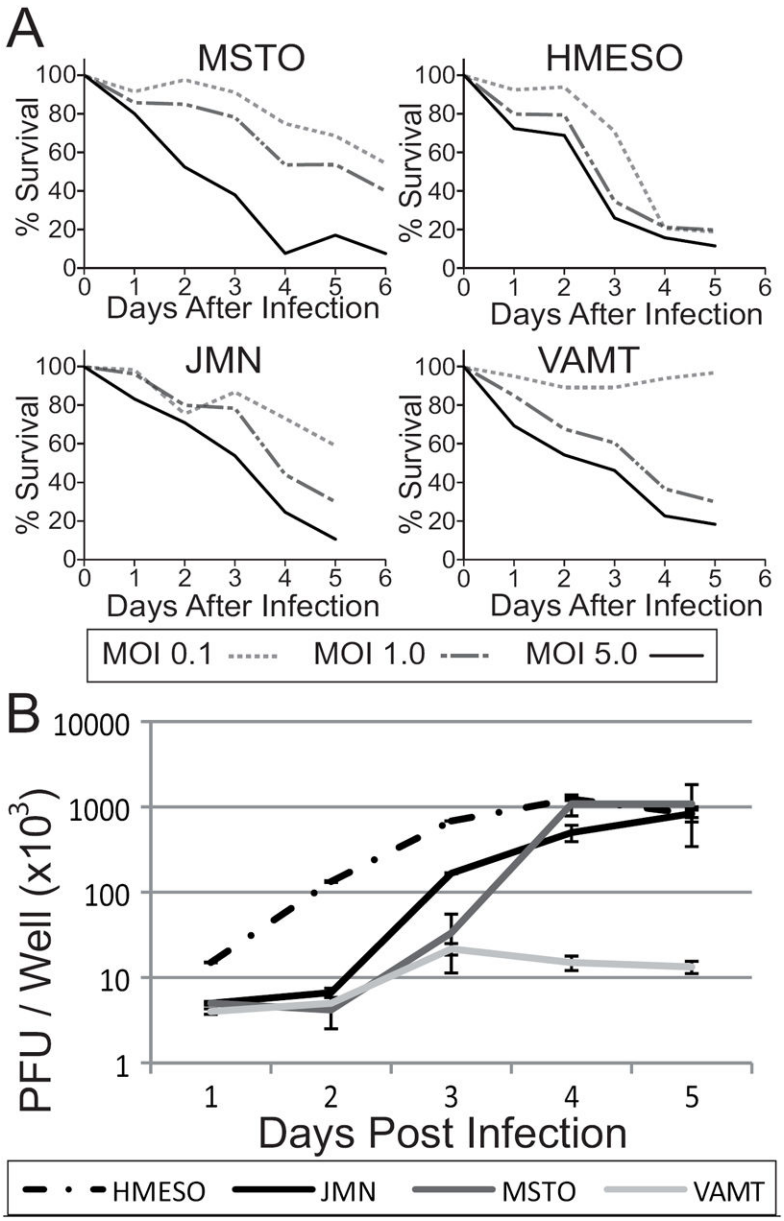

Figure 2. GLV-1h153 demonstrates strong cytotoxic effect on mesothelioma cell lines and replicates efficiently *in vitro*

Cytotoxicity assays of four mesothelioma cell lines infected with GLV-1h153 at MOIs of 0.1, 1.0, and 5.0 demonstrate near-complete cell kill by day 5 in all cell lines (A). Viral replication is supported by mesothelioma cells in-vitro. Standard viral plaque assay of cells infected with an MOI of 1.0 demonstrate 2 - 2.5 log increase in viral titers over four days in three out of four lines tested (B).

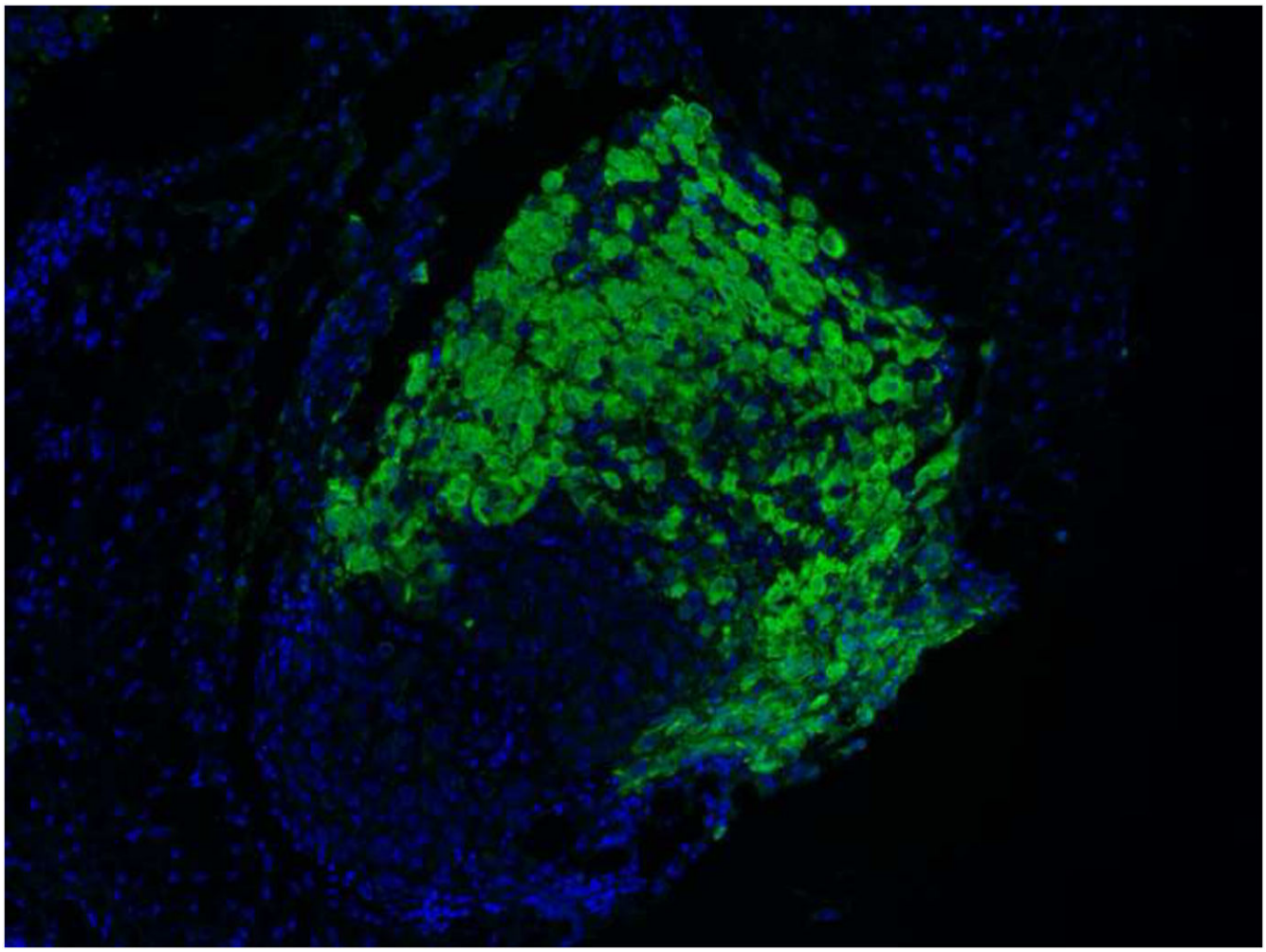

Figure 3. GLV-1h153 infects mesothelioma *in vivo*

Immunofluorescent detection with a monoclonal antibody specific for the intracellular mature form of vaccinia virus demonstrates viral transduction in the tumor deposits of a treated mouse 48 hours after intrapleural delivery of GLV-1h153 (Vaccinia: Green, Blue: DAPI counterstain).

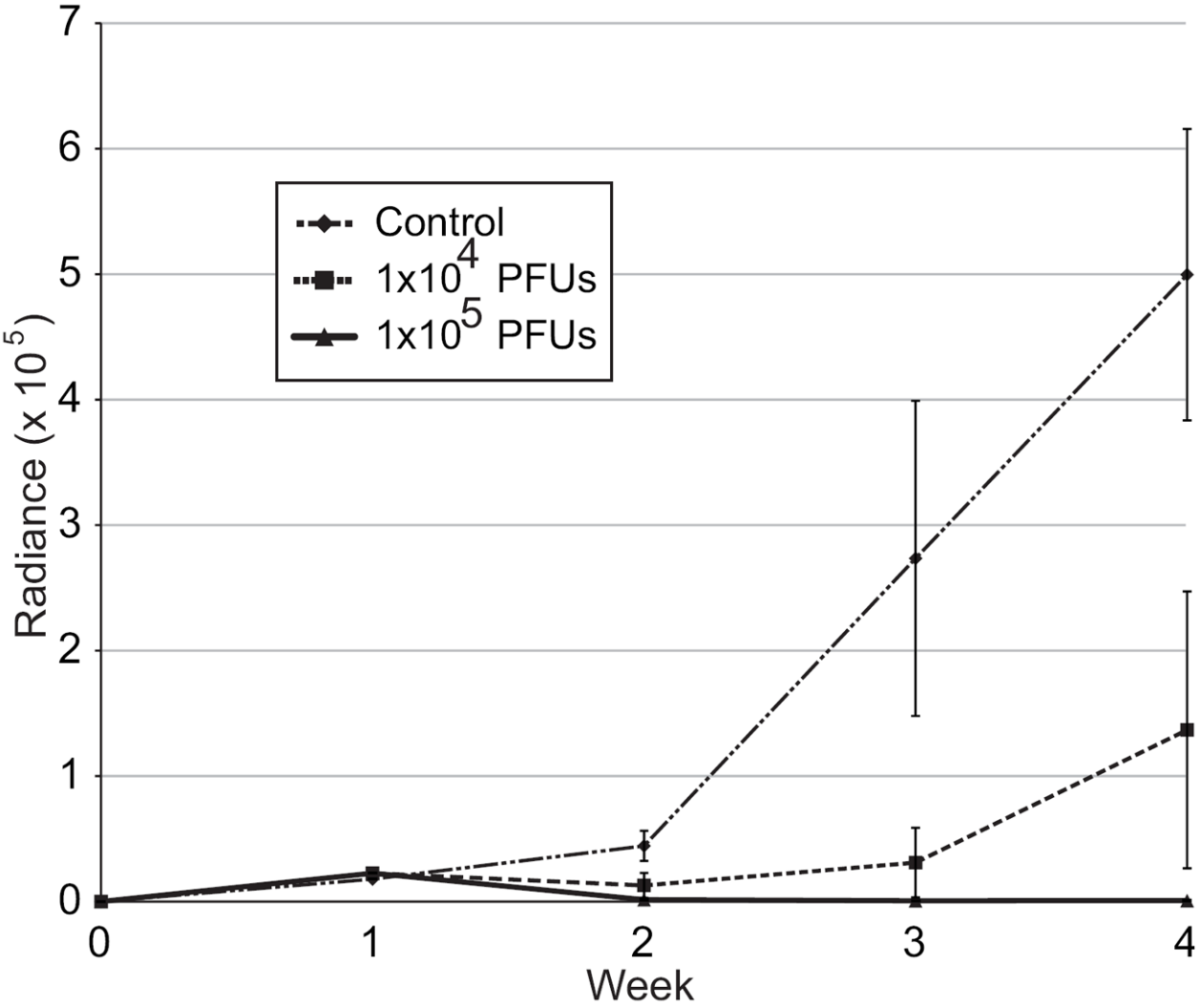

Figure 4. Treatment with intrapleural GLV-1h153 results in decreased tumor burden in treated animals compared to untreated controls as measured by bioluminescence

Control animals demonstrate progressive increase in mean radiance over three weeks after randomization. Treatment with 1×10^5 PFUs of GLV-1h153 is a threshold dose for eradication of bioluminescent signal as early as 1 week after treatment.

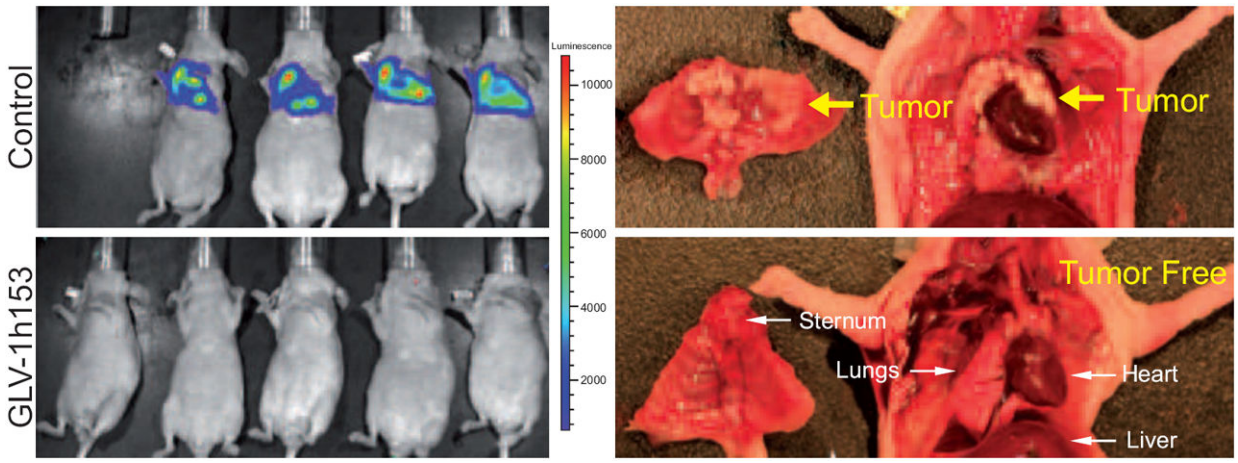

Figure 5. Intra-pleural treatment with GLV-1h153 eradicates tumor burden in a mouse model of MPM

Three weeks after randomization, bioluminescent imaging demonstrates luciferase positive mesothelioma tumor burden in controls but not mice treated with 1×10^5 PFUs GLV-1h153 (A). Representative control mouse euthanized at three weeks after randomization demonstrates macroscopic tumor deposits on all visceral surfaces and chest wall. Mouse treated with intrapleural injection of 1×10^5 PFUs of GLV-1h153 demonstrate no gross disease at the same time point (B).

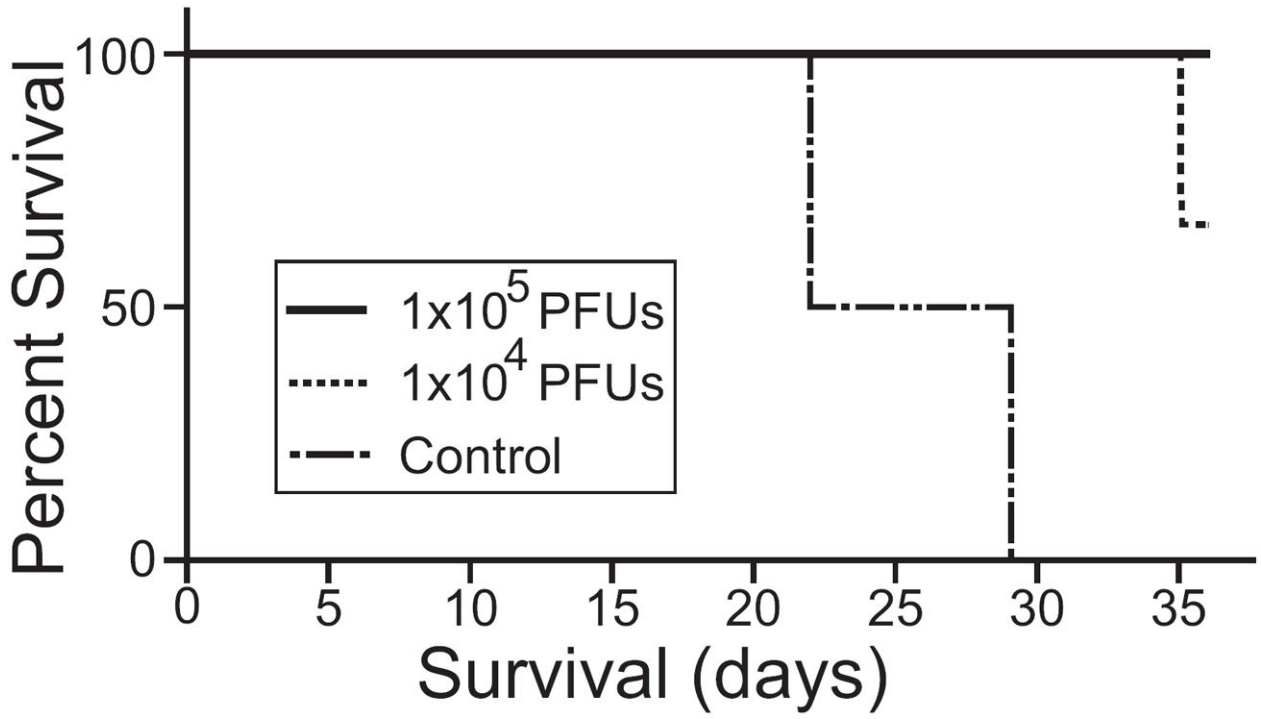

Figure 6. Intra-pleural treatment with GLV-1h153 prolongs survival in an orthotopic mouse model of MPM

Kaplan Meier survival analysis demonstrates that intrapleural treatment with a threshold dose of 1×10^5 PFUs of GLV-1h153 7 days after tumor establishment resulted in improved 30-day survival relative to untreated controls (log-rank p = .0009).

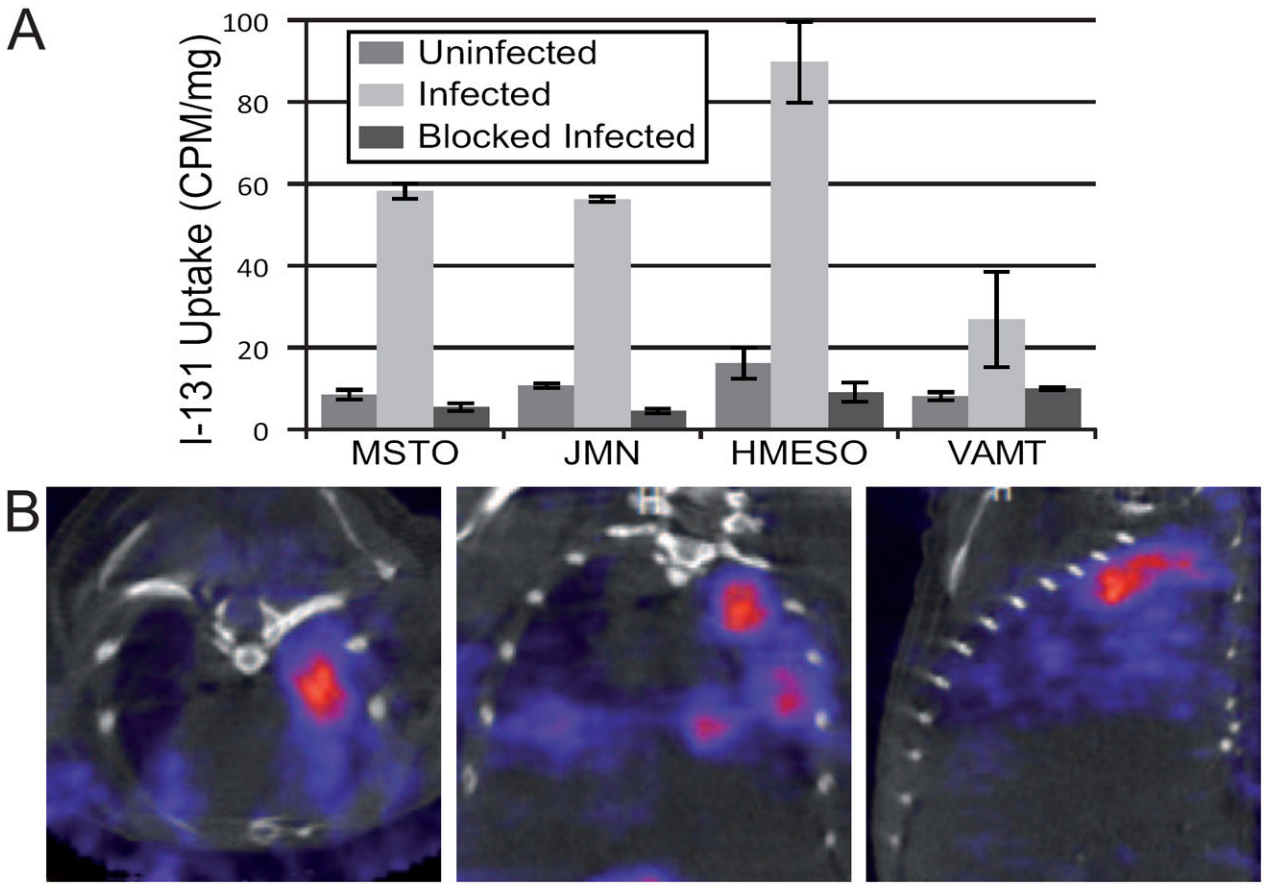

Figure 7. MPM cells infected with GLV-1h153 demonstrate enhanced, hNIS specific uptake of ^{131}I *in vitro* and may be imaged with radio-iodine *in vivo*

Twenty-four hours after infection with GLV-1h153 at an MOI of 1.0, viral mediated hNIS expression results in 131I radiouptake as much as 10-fold higher in infected cells compared to controls. This effect was abrogated by the addition of sodium percholorate (NaCl04), a specific competitive inhibitor of hNIS (A). ^{131}I-SPECT/CT imaging demonstrate radiouptake of ^{131}I in pleural tumor deposits of a treated mouse (B: axial, coronal, sagittal planes).