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Molecular imaging with bioluminescence and PET reveals viral oncolysis kinetics and tumor viability

Darshini Kuruppu,

Department of Surgery, Massachusetts General Hospital, Boston, MA

Anna-Liisa Brownell,

Department of Radiology, Massachusetts General Hospital, Boston, MA

Khalid Shah,

Department of Radiology, Massachusetts General Hospital, Boston, MA

Umar Mahmood, and

Department of Radiology, Massachusetts General Hospital, Boston, MA

Kenneth K. Tanabe

Department of Surgery, Massachusetts General Hospital, Boston, MA

Abstract

Viral oncolysis, the destruction of cancer cells by replicating virus, is an experimental cancer therapy that continues to be explored. The treatment paradigm for this therapy involves successive waves of lytic replication in cancer cells. At current, monitoring viral titer at sites of replication requires biopsy. However, repeat serial biopsies are not practically feasible for temporal monitoring of viral replication and tumor response in patients. Molecular imaging provides a non-invasive method to identify intracellular viral gene expression in real time. We imaged viral oncolysis and tumor response to oncolysis sequentially with bioluminescence and positron emission tomography (PET), revealing the kinetics of both processes in tumor xenografts. We demonstrate that virus replication cycles can be identified as successive waves of reporter expression that occur ~2 days after the initial viral tumor infection peak. These waves correspond to virions that are released following a replication cycle. The viral and cellular kinetics were imaged with Fluc and Rluc bioluminescence reporters plus two 18F-labelled PET reporters (FHBG, 9-(4-18F-fluoro-3-[hydroxymethyl] butyl) guanine) and (FLT, 18F- 3'-deoxy-3'-fluorothymidine), respectively. Correlative immunohistochemistry on tumor xenograft sections confirmed in vivo results. Our findings show how PET can be used to identify virus replication cycles and for real-time measurements of intratumoral replicating virus levels. This noninvasive imaging approach has potential utility for monitoring viral oncolysis therapy in patients.

Keywords

Molecular imaging; PET; HSV-1 oncolysis; bioluminescence; viral kinetics

SENIOR/CONTACT AUTHOR: Kenneth K. Tanabe Massachusetts General Hospital, 55 Fruit Street Boston, MA 02114.

CONFLICT OF INTEREST STATEMENT:

The authors disclose no conflict of interest.

Introduction

Viral oncolysis by Herpes Simplex virus 1 (HSV-1) is currently being investigated for cancers unresponsive to conventional treatment. Replication-conditional HSV-1 mutants are genetically engineered for effective oncolysis. These mutants replicate preferentially in cancer cells rather than in normal cells, which forms basis for safety and efficacy of this approach (1, 2). The process of oncolysis begins when the virus infects a tumor cell. Progeny virions that are liberated at the end of a replication cycle infect neighboring cancer cells to begin another wave of replication. The process continues until the cancer is eliminated or the virus is extinguished by the host defense mechanisms. Thus, the initially injected virus titer is amplified to a larger virus titer to destroy the cancer cells (3, 4). HSV-1 is among several oncolytic viruses that are studied, which include reovirus, vaccinia virus, poxvirus, adenovirus, and vesiculostomatitis virus (5-8). HSV-1 has an advantage over some of the other oncolytic viruses due to its large genome capacity, non-integration of its DNA into the host genome, and high infectivity in cancer cells. The availability of anti-herpetic drugs such as acyclovir and gancyclovir provide added safety as they can help terminate unwanted viral replication if needed (9-11).

Monitoring is needed during viral oncolysis therapy to identify virus titer at sites of virus replication. The current method of biopsy is not feasible for repetitive virus assessment. The absence of a reliable and clinically applicable method to measure viral activity in real-time is a major drawback for monitoring viral oncolysis therapy in clinical trials. The non-invasive and dynamic imaging capabilities of bioluminescence and PET provide a means to image viral replication repetitively *in vivo*, preclinically for bioluminescence and both preclinically and for clinical translation with PET. Using specific reporters for each imaging modality, the kinetics of virus replication can be studied by identifying sites and intensity of reporter expression. And the destruction of cancer cells undergoing viral oncolysis can be simultaneously studied.

Bioluminescence imaging provides rapid, short interval, serial imaging for kinetic studies. Due to its high target to background ratio, bioluminescence offers a sensitive and robust tool for studying gene expression and function (12-14). Through multi-reporter imaging of distinct substrates, temporal relationships between multiple biological processes and their pathological manifestations in cancer, atherosclerosis, and neurological disorders, and treatment response can be defined (15-18). The kinetics of virus replication and the tumor response to lytic replication can be simultaneously studied *in vivo*, in preclinical models using this approach.

In contrast PET is readily translatable to human studies. It is routinely used with [^{18}F] FDG as a sensitive non-invasive imaging method to identify and stage tumors, determine treatment success and disease recurrence at the deep tissue level without background tissue interference. The prototype enzyme reporter, HSV-TK is ideal for imaging virus replication in tumors with PET. Based on the promiscuity of HSV- thymidine kinase (TK), the guanine derivative 9-[3-fluoro-1-hydroxy-2-propoxymethyl] guanine (FHBG) (19, 20) serves as a substrate for viral TK. The phosphorylated [^{18}F -labelled] FHBG which is trapped inside

cells is detected with PET. We have previously reported that HSV-1 oncolysis can be imaged with PET using FHBG as the substrate for HSV-TK (21). However, due to the large viral titer used in the study, PET imaging was restricted to the early stages of virus replication. Imaging beyond this stage was not possible since most of the cancer cells were destroyed. The kinetics of virus replication can be better understood by sequential study of oncolysis. In order to address this in a more clinically relevant scenario, we assessed lower titers and used the replication-conditional oncolytic HSV-1 mutants, hrR3 and HSV-Luc. Due to LacZ gene insertion into the gene locus for viral ribonucleotide reductase, these mutants replicate preferentially in cancer cells where the abundant nucleotides substitute for the absent ribonucleotide reductase gene product (22, 23). HSV-Luc in addition expresses the Fluc gene.

Here we present our investigations on the kinetics of HSV-1 replication and the accompanying tumor response to oncolysis with dual bioluminescence and PET imaging. Through optimization of dose, dosing intervals, and substrate for reporters, we identify, for the first time, virus replication cycles as waves in bioluminescence and PET reporter expression. In addition, by studying the kinetics of tumor cells subjected to oncolysis we show a reduction in tumor burden subsequent to lytic replication. These results support the translatability of monitoring virus replication with serial PET scans using [^{18}F] FHBG.

Methods

Development of stable cell lines for bioluminescence imaging

Human cancer cell lines (MDA-MB-231 breast cancer [ATCC HTB-26], A2058 amelanotic melanoma [ATCC CRL-11147], and HT29 colon cancer [ATCC HTB-38]) and mouse cancer cell lines (4T1 breast cancer [ATCC CRL-2539] and MC26 colon cancer) were engineered to express Rluc. The human cell lines were maintained in RPMI-1640 medium supplemented with 10% FBS, while the murine cell lines were maintained in DMEM supplemented with 10% FBS. The cell lines were obtained from ATCC and were used in this study for less than 6 months after resuscitation. They are authenticated by ATCC after a comprehensive quality control before shipment. MC26 was kindly provided by Dr. Robert L. Martuza. The cell lines were tested for mycoplasma, Hoechst DNA staining, PCR, and culture testing for contaminant bacteria, yeast, and fungi. Authentication procedures used include species verification by DNA bar coding and identity verification by DNA profiling.

The cell lines were infected with lentivirus containing cDNA for Rluc and mCherry as well as puromycin resistance. Within 18 to 24 hrs post infection approximately 90% of cells expressed mCherry fluorescence. These cells were detached with trypsin and plated in 6-well dishes in media containing puromycin (10 $\mu\text{g/mL}$). This allowed growth of cells with the newly incorporated lentivirus DNA containing the mCherry, Rluc and the puromycin resistance gene. Over the following week colonies that developed in media containing puromycin were examined for mCherry expression under an inverted phase contrast fluorescence microscope. The colony that expresses the strongest mCherry signal for each of the cell lines was expanded for our experiment. The Rluc expression was validated with coelenterazine.

Replication-conditional HSV-1 mutants

The replication-conditional HSV-1 mutant that expresses Fluc (HSV-Luc) was generated by a bacterial artificial chromosome (BAC)-based HSV cloning system (kindly provided by Dr. Robert L. Martuza) utilizing Flip-Flop HSV-BAC technology (23). The HSV-Luc contains the luciferase expression cassette driven by the cytomegalovirus (CMV) promoter and the LacZ gene inserted in the middle of the UL39 (ICP6) gene. The replication-conditional HSV-1 mutant hrR3 has the ICP6 inactivated by insertion of the LacZ gene (22). The viruses were propagated in Vero cells and replication assays were performed to determine the titer as previously described (24).

In vitro measurement of virus with Fluc bioluminescence

Cancer cells (MDA-MB-231 and MC26) were plated in 24 well plates at a concentration of 6×10^4 cells/ well. A day later the cells were exposed to different titers of HSV-Luc (1×10^3 to 1×10^8 plaque forming units (pfu)). The virus was imaged with bioluminescence using luciferin. In order to determine the kinetics of virus replication, Fluc expression was imaged over sequential time points at 6hrs, 12hrs, 18hrs, 24hrs, 30hrs and 36hrs post infection. This bioluminescence signal was expressed as the net intensity per area of triplicate wells. A viable cell count was performed using trypan blue exclusion for each well. This was done at each of the time points that were imaged.

In vitro measurement of virus with thymidine kinase PET

Cancer cells (MC26) and Vero cells were plated in 6 well plates at a concentration of 4×10^5 cells/ well. Once the cells were 80% confluent they were infected with 1×10^8 pfu hrR3. Virus expression in the cells was determined by incubating the cells with [^{18}F]FHBG for 2 hrs, followed by two washes. The [^{18}F]FHBG uptake by the cells was measured by a gamma-counter (Packard Cobra 5005), and was expressed per cell. The concentration of cells in each well was determined by a viable cell count.

Flank tumor model

BALB/c nude mice were obtained from the COX institutional animal breeding services (Steele Laboratories). All experiments were performed according to the institutional subcommittee on research animal care. The mice were housed in an authorized animal facility with free access to food and water. Flank tumors were created in mice using each of the stable cell lines that were developed and grown in culture. The cells were trypsinized and washed in medium containing serum to inactivate trypsin. After a PBS wash, the cells were prepared for injections. Each cell line was injected into groups of ten mice. 1×10^5 cells from each cell line in 100 μL PBS were injected bilaterally or to one side of the flank. Experiments were commenced when the tumors reached 5 mm in diameter.

In vivo virus and tumor imaging with dual Fluc and Rluc bioluminescence

The animals were randomized into control and virus treatment groups. Tumors in both groups were imaged for Rluc bioluminescence every other day for 8 days. Images were acquired for 10 min after coelenterazine injection (7.5 μg i.v./ mouse) at 8x8 binning (Carestream Molecular Imaging). HSV-Luc (1×10^8 pfu/ 50 μL in PBS) was injected into

the tumor center and imaged daily for 8 days. The virus treated group underwent dual imaging for Fluc and Rluc. Fluc bioluminescence was acquired for 10 min at 8x8 binning after luciferin injection (3.5 μ g i.p./ mouse). Fluc images were acquired a minimum of 6 hrs after Rluc images were captured to allow for complete clearance of the Rluc signal. Each of the Rluc and Fluc images was overlaid onto an X-ray image captured for 20 sec. Tumor volume was calculated in the control and treatment groups with caliper-measured tumor diameter. Bioluminescence signal was quantified using the imaging software (Carestream). Regions of interest were normalized to background intensity and expressed as net intensity per unit area.

***In vivo* virus and tumor imaging with microPET**

When the bilateral flank tumors reached 5 mm in diameter, virus was injected directly into the left tumor, while the right tumor served as the control. The mice were positioned into the scanner under isoflurane anesthesia (1–1.5% isoflurane with 2 L/min oxygen). We used a microPET scanner with a resolution of 4.1 mm³ (microPET-P4, Concorde Microsystems, Inc., Knoxville, TN). Transmission images were gathered with the aid of ⁵⁷Co to correct for attenuation. Dynamic volumetric data were obtained for 120 min after [¹⁸F]FHBG (100 μ Ci t.v./mouse) injection. Volumetric images were reconstructed with filtered-back projection after the data were corrected for uniformity, scatter, attenuation, decay, and injected activity using AsiPro4.1 software. Time-activity curves were generated from the selected regions of interest from the control tumor, virus injected tumor, the liver, and the heart. PET signal was expressed as the % radiolabel accumulation/ injected dose/cc. Imaging studies with [¹⁸F]FHBG were conducted at 2, 6, 24, 48, and 72 h for different virus titers (hrR3 at 1×10^9 , 1×10^7 , 1×10^5 , and 1×10^3 pfu). The background activity was measured before injecting the [¹⁸F]FHBG at each time point to correct for any residual signal from the dose that preceded it. Although negligible, this measurement was corrected with the injected dose. The tumor response to lytic replication was imaged with [¹⁸F]FLT-PET.

Identification of virus with LacZ in frozen tumor sections

Frozen tumors were sectioned at 5 μ m and mounted on superfrost-coated microscope slides (Fisher Scientific). The sections were fixed in 3% glutaraldehyde prior to LacZ staining for β -galactosidase. The sections were incubated in 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) solution at 37°C overnight. The X-gal solution was prepared as follows: X-gal at 20 mg/mL in dimethyl formamide, 0.1 M potassium ferricyanide, 0.1 M potassium ferrocyanide, and 0.1 M MgCl₂ (Sigma). Areas of LacZ expression appeared blue. Sections were cover-slipped and studied under a light microscope.

Hematoxylin and eosin (H&E), and immunohistochemical (IHC) staining for HSV-TK, proliferating cell nuclear antigen (PCNA) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of tumors

Formalin-fixed tumors were processed, paraffin embedded and serial sections were mounted for H&E, HSV-TK, PCNA, and TUNEL staining. HSV-TK IHC was performed after antigen retrieval in citrate buffer. Endogenous peroxidase and serum were blocked before HSV-TK antiserum was applied at 1:5000 dilution overnight at 4 °C. The sections were incubated in biotinylated goat anti-rabbit secondary antibody, followed by horseradish

peroxidase, before chromogen development with peroxidase substrate 3,3'-diaminobenzidine (DAB). HSV-TK-positive cells appeared brown. PCNA IHC was performed using the PCNA antibody (raised in mice) (Millipore) overnight at 1:1,000 at 4°C. The antibody was detected with biotinylated anti-mouse secondary antibody as described above. PCNA positive cells appeared brown. Apoptosis staining was performed using the ApopTag Fluorescein In-Situ Apoptosis Detection Kit (Millipore), which detects DNA strand breaks by the TUNEL method. After proteinase K treatment, the sections were incubated with terminal deoxynucleotidyl transferase enzyme, followed by incubation with antidigoxigenin-peroxidase conjugate and counterstained with propidium iodide. ApopTag-positive cells were detected under fluorescence.

Statistical Analysis

Data were expressed as the mean $\pm$ standard deviation (SD), (unless otherwise specified as the mean $\pm$ standard error of the mean (SEM)) and were compared using an unpaired two-tailed t test. The P values were compared with the controls data set.

Results Stable cell lines and virus can be detected through bioluminescence and PET imaging

The cell lines (human MDA-MB-231, A2058, HT29, and mouse 4T1 and MC26), stably transformed to express mCherry and Fluc were detected through red fluorescence and bioluminescence, respectively (Fig 1B). The replication-conditional HSV-1 mutant (HSV-Luc), which contains the Fluc and TK genes, can be imaged with bioluminescence and PET (Fig 1C). Virus replication in MC26 cancer cells was imaged with Fluc bioluminescence. The Fluc signal was comparable to that of MC26 cells which stably express Fluc (Fig 1C – upper graph). Virus replication was identified with [^{18}F]FHBG-PET. Radiolabel uptake in MC26 cells infected with virus was comparable to MC26 cells that stably express the mutant viral TK (MC26-sr39TK) (Fig 1C –lower graph).

Kinetics of virus replication measured *in vitro* with bioluminescence and PET tracers

The kinetics of virus replication was studied *in vitro* with Fluc bioluminescence. The Fluc signal intensity correlated with the virus titer while it increased over time. In the highest virus titer (1×10^8 pfu HSV-Luc), two peaks were identified at 18 hrs and 30 hrs post infection, which corresponded to virus replication cycles (Fig 2A). The cell counts decreased over time suggestive of lytic replication. Low titers (1×10^3 , 1×10^4 , and 1×10^5 pfu), were undetectable with bioluminescence, while the cell counts increased over time, similar to the uninfected controls (Fig 2B). The kinetics of virus replication (hrR3 at 1×10^8 pfu) studied *in vitro* with the PET tracer [^{18}F]FHBG demonstrated a marked radiolabel uptake in virus infected MC26 and Vero cells (Fig 2C). This was accompanied by a reduction in cell concentration (Fig 2D), indicative of lytic replication.

Identification of virus replication cycles *in vivo* with bioluminescence and PET imaging

The kinetics of HSV-1 replication was studied sequentially with bioluminescence and PET in mice bearing tumor xenografts (MDA-MB-231 and MC26). Virus (HSV-Luc at 1×10^9 , 1×10^8 and 1×10^7 pfu) replication in MDA-MB-231 tumors imaged with bioluminescence

showed peaks in Fluc reporter expression over time. The intense peak at day 1 was followed by two smaller peaks at day 3 and day 5 as the signal decreased (Fig 3A). The peaks correlated with virus released following a replication cycle, confirmed by plaque assay. While the 'wave' pattern was consistent, the signal intensity correlated with the virus titers. Virus (hrR3 at 1×10^9 , 1×10^7 , 1×10^5 and 1×10^3 pfu) replication in MC26 flank tumors imaged with PET showed a profile similar to that observed with bioluminescence. The [^{18}F]FHBG reporter accumulation in cancer cells infected with 1×10^5 pfu hrR3 expressed a 'wave' like pattern over 3 days. The peaks were identified at 6 hrs (0.3 days) and 72hrs (3 days) and correlated with virus titers confirmed by plaque assays on tumors post imaging. The [^{18}F]FHBG signal in the highest titer (1×10^9 pfu) decreased after the initial peak at 6 hrs (Fig 3B), while infection with 1×10^3 pfu was undetectable.

Tumor growth kinetics identified with bioluminescence correlates with *in vitro* volumetric data

Growth kinetics of human (MDA-MB-231-Rluc, A2058-Rluc, and HT29-Rluc) and mouse (4T1-Rluc and MC26-Rluc) cancer cells growing in the flanks were studied with sequential Rluc bioluminescence imaging over 8 days. Tumor volume was calculated from caliper measurements at each time point for comparison. The Rluc expression in the bioluminescence scans that defines tumor borders increased over time in all 3 tumors xenografts (Fig 4A, 4C, and 4E). The calculated Rluc signal intensities for each tumor xenograft correlated with external tumor volume measured from caliper readings (Fig 4B, 4D, and 4F). These parameters define tumor growth. We observed similar growth dynamics with bioluminescence and correlation with volumetric measurements from caliper readings in flank tumors from mouse breast cancer cells (4T1-Rluc) (Supplemental-1).

Dynamics of virus replication and tumors undergoing oncolysis imaged with dual bioluminescence

The dynamics of virus replication and tumor response was studied with dual Fluc and Rluc bioluminescence following HSV-Luc infection of MDA-MB-231-Rluc tumors. Mice were randomized into treatment and control groups when the tumors reached 5 mm in diameter. Virus (1×10^8 pfu) was injected to the treatment group once baseline Rluc readings were obtained. Both groups were imaged over 8 days. The area of Rluc expression that defines tumor borders in the control group increased (Fig 5A), while that in the virus treated group decreased over time (Fig 5B). The corresponding calculated Rluc signal intensities rose exponentially in the control group while it was markedly inhibited in the virus treated group ($P < 0.004$; Fig 5C). The decrease in reporter expression is suggestive of lytic replication. The caliper-measured tumor volume showed a profile similar to Rluc measurements for the control group, but diverged for the treatment groups (Fig 5D). This difference is attributable to the process that is measured in each case: Rluc defines a functional measurement that identifies viable cells, while caliper measurements are from external tumor diameters that also incorporate the necrotic tumor core (viable and non-viable tumor). The Fluc signal peaked 6 hrs after virus injection and decreased over time (Fig 5E). Quantified signal intensities revealed peaks every 2 days after the initial peak expression (Fig 5F), which are indicative of newly released virions following a replication cycle as measured in plaque assays. Lytic replication reduces the number of viable cells for virus replication, which

accounts for the decreasing Fluc signal. A similar response in virus and tumor dynamics was observed in the mouse colon cancer cell line (MC26-Rluc) (Supplemental-2).

Dynamics of virus replication and tumor response to lytic replication imaged with PET

Virus replication in MC26 flank tumors was imaged with PET using [^{18}F]FHBG over 3 days after injecting 1×10^5 pfu hrR3 into the right flank tumor. Sites of virus replication were identified by intracellular phosphorylated [^{18}F]FHBG accumulation. The intensity of the signal correlated with the magnitude of replicating virus (Fig 6A). The control tumor had no [^{18}F]FHBG accumulation on PET. The time activity curves showed [^{18}F]FHBG accumulation in the virus-infected tumor while the radiolabel was washed out of the control tumor, heart, and the liver (Fig 6B). The fate of the cancer cells undergoing oncolysis was studied with [^{18}F]FLT-PET imaging which identified a highly proliferative tumor prior to virus injection. After virus injection, the signal decreased and the site of lytic replication was identified as central photopenia, which correlated with the area of tumor cell lysis (Fig 6C).

Molecular PET and bioluminescence imaging data correlates with *in vitro* findings

Tumors that were imaged *in vivo* with bioluminescence and PET were excised to study growth characteristics before and after viral replication by immunohistochemical staining. Tumor cell proliferation imaged with [^{18}F]FLT-PET, mCherry fluorescence, and Rluc bioluminescence correlated with PCNA immune-staining of *ex vivo* tumor sections (Fig 7A). Tumor destruction following virus replication was identified as a central loss of signal when imaged with Rluc bioluminescence, mCherry fluorescence, and [^{18}F]FLT-PET. On *ex vivo* H&E staining this central region was necrotic with a marked reduction in proliferating tumor cells (PCNA) (Fig 7B). Viral replication was identified with [^{18}F]FHBG-PET and Fluc bioluminescence. Virus was detected with LacZ and HSV-TK IHC on *ex vivo* tumor sections (Fig 7B). The central core consisted of apoptotic cells.

Discussion

Successful treatment outcomes in preclinical animal models have set the stage for clinical trials of viral oncolysis as a novel cancer therapy (25). In these studies, the virus must be closely monitored to determine the accuracy of virus delivery to the tumor, as well as assessing viral titer locally and following viral replication at non target sites. Viral replication kinetics and biodistribution can be studied noninvasively by imaging virus gene expression that occurs intracellularly. Such information can be used to modulate virus dose, delivery routes, and the viral construct. In this study we identified virus replication cycles as waves in the reporter gene expression in real-time with bioluminescence and PET imaging. The peaks in these waves correspond to virions that are released at the end of a replication cycle. To our knowledge this is the first report of sequential measurement of virus replication with bioluminescence and PET, and the first to report on *in vivo* assessment of intratumoral viral waves that provide continued tumor lysis after the initial wave of infection and oncolysis has occurred.

Bioluminescence is widely used in tumor biology to study growth characteristics and treatment response in cancers (26-29). In these models, bioluminescence reporters identify

tumor growth, angiogenesis and apoptosis in response to different treatments. And, bioluminescence studies have been conducted for oncolytic vesicular stomatitis virus on prostate (26) and bladder cancers (30); oncolytic vaccinia virus on pancreatic (18); and oncolytic adenovirus therapy of gliomas (31) and ovarian cancer (32); as well as HSV-1 strains on HCC (33). These studies limit their investigations to evaluating tumor response to lytic replication, without the study of viral replication itself. In addition, the properties of HSV-1, such as the infectivity of wild type virus in healthy mice (34), host immune responses to infection (35), transcriptional activity of early and late promoters (36), and the infectivity of various strains for cancer therapy (33) have been studied with bioluminescence. In these studies virus was measured at a specific endpoint of the experiment. In contrast, we have dynamically studied HSV-1 replication and oncolysis with sequential imaging.

In most imaging studies involving HSV-1, the virus is used as an immunotherapy (e.g. T lymphocytes) for cancers (37) or as an amplicon to deliver cell based therapies (e.g. TNF-related apoptosis-inducing ligand [TRAIL]) (38). The TK gene of the virus is often used for prodrug activation with gancyclovir, or as the prototype PET reporter to image tumor growth and response to treatment. Most often the HSV-TK gene is inserted into the genome of other oncolytic viruses to image these viruses with PET (17). However, the use of HSV-TK to track the replication kinetics of its own virus, that of HSV-1, is limited. We previously demonstrated that HSV-1 oncolysis can be imaged with PET using the HSV-TK as the reporter (21). In the present study we show that HSV-1 oncolysis and virus replication cycles can be studied with dual reporter imaging with bioluminescence and PET. We did not focus on imaging HSV-1 replication on metastases. However, in other studies we have identified metastases at distant sites in the gut with bioluminescence and PET while imaging viral oncolysis treatment of liver metastases. With regards to immunity, we have previously reported no difference in anti-tumor activity of hrR3 in immunocompetent and immunocompromised mice (22). The active immune system in immunocompetent mice neither enhanced nor attenuated anti-tumor effect of the virus.

We imaged two replication-conditional HSV-1 viruses, HSV-Luc and hrR3, with bioluminescence and PET. These mutants selectively replicate in proliferating tumors (10, 22, 23). We have analyzed tumor and normal liver parenchyma after hrR3 was injected into the spleen to deliver virus to treat liver metastases. No virus was detected in the normal liver. This observation has also been confirmed with *in vivo* imaging with bioluminescence and PET. Virus (hrR3 or HSV-Luc) injected into a normal mouse (via the tail vein) was observed in the liver within 6 to 24 hrs with a signal in the entire liver. This signal had completely resolved by 48hrs. However, in the case of a mouse-bearing liver metastases, virus was observed in the tumors beyond 48 hrs while the signal had resolved in normal liver.

Out of the two replication-conditional HSV-1 viruses, used in this study, hrR3 is more robust and has a greater replication rate than HSV-Luc, explaining the difference between the two initial imaging peaks for the viruses. The more robust hrR3 replication generated a replication peak at 6 hrs instead of 24hrs as seen with HSV-Luc. An additional contribution is likely related to different parent tumor lines undergoing therapy in these studies. What is

striking is that replication cycles can be monitored non-invasively with bioluminescence and PET, irrespective of the type of virus mutants or the type of cancer, suggesting the broad applicability of this approach to follow viral oncolysis *in vivo*, both preclinically and for clinical translation. The ability to assess oncolytic viral replication kinetics and spatial distribution *in vivo* has the potential to help optimize this therapeutic paradigm and accelerate clinical trials using targeted viral therapy.

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Fig 1. Schematic characterization of molecular reporters, cells and virus

A: The dual bioluminescence detection system is based on the enzymatic reaction of Fluc and Rluc where luciferin and coelenterazine serves as substrates. The PET ligand [¹⁸F]FHBG, serves as the substrate for HSV-TK, which phosphorylates and traps it intracellularly. **B:** Stable cancer cells lines that express Rluc and mCherry can be identified with Rluc bioluminescence, and mCherry fluorescence. We show transformed MC26 cells imaged for Rluc *in vitro*. **C:** HSV-Luc expresses the Fluc and TK genes. The replicating virus was identified in MC26 cells with Fluc bioluminescence *in vitro* (MC26+HSV-Luc), and the signal was comparable to MC26 cells that transiently express Fluc (MC26-Fluc). The virus replicating in MC26 cells was detected with the PET tracer [¹⁸F]FHBG *in vitro* (MC26+hrR3). The [¹⁸F]FHBG signal was comparable to that of MC26 cells which express the TK gene transiently (MC26sr39TK).

Fig 2. Kinetics of virus and cells *in vitro* determined with bioluminescence and PET substrates
A: The replication kinetics of HSV-Luc in MC26 cells studied with Fluc bioluminescence. Two peaks (arrows) were observed in the Fluc signal in the high virus titers (1×10^8 , 1×10^7 , and 1×10^6 pfu) whereas the signal was undetectable in the lower virus titers. Fluc signal is expressed as the mean net intensity/area $\pm$ SD. **B:** Viability of MC26 cells infected the virus. The cell counts decreased when infected with high viral titers (1×10^8 , 1×10^7 , and 1×10^6 pfu). Cell count is expressed as the mean $\pm$ SD. **C:** [^{18}F]FHBG uptake in MC26 and Vero cells 24 hrs after HSV-Luc (1×10^8 pfu) infection. Data are the mean % of ID of [^{18}F]FHBG uptake/cell $\pm$ SD. **D:** Changes in MC26 and Vero cell concentration once infected with virus. The MC26 cell concentration was markedly reduced compared to cells not infected with virus. Cell concentration is expressed as the mean $\pm$ SD.

Fig 3. Dynamics of virus replication *in vivo* imaged with bioluminescence and PET

A: Kinetics of virus replication in flank tumors studied with Fluc bioluminescence. The Fluc signal expressed 3 peaks (arrows) that decreased in intensity over time. Fluc signal is presented as the mean net intensity/area $\pm$ SD. **B:** Kinetics of virus replication imaged with [¹⁸F]FHBG- PET. The [¹⁸F]FHBG uptake in tumors peaked (arrows) at 6 hrs (0.3 days) in the high virus titers. It decreased in highest virus titer (1×10^9 pfu), while it peaked again at 3 days in the lower titer (1×10^5 pfu). The lowest titer (1×10^3 pfu) had no signal. The radiolabel uptake is presented as the mean $\pm$ SD of the % injected dose/cc.

Fig 4. Tumor growth imaged with Rluc bioluminescence correlates with volumetric measurements obtained with caliper readings

The growth of human tumor xenografts imaged with Rluc bioluminescence. The Rluc signal areas in scans of bilateral MDA-MB-231-Rluc flank tumors increased over time (**A**). The Rluc signal intensity increased and corresponded with caliper-measured tumor volume (**B**). A similar expression was observed where the Rluc signal increase correlated with caliper-measured tumor volume in A2058-Rluc (**C, D**), and HT29-Rluc (**E, F**) flank tumors. Data are expressed as the mean $\pm$ SEM.

Fig 5. Tumor growth and viral replication kinetics imaged with dual bioluminescence

A: Growth of MDA-MB-231-Rluc flank tumors imaged with Rluc. **B:** Growth inhibition of MDA-MB-231-Rluc tumors undergoing viral oncolysis (1×10^8 pfu) imaged with Rluc. **C:** Rluc signal intensities in the control and virus-treated groups. Rluc signal is shown as the mean net intensity/area $\pm$ SD. Asterisks indicate $P < 0.004$. **D:** Tumor volume measured with caliper readings in the control and virus treated groups. Tumor volume is shown as the mean $\pm$ SD. Asterisks indicate $P < 0.002$. **E:** Sequential imaging of virus replication with Fluc. **F:** The Fluc signal intensities peaked (arrows) several times as it decreased over time. Fluc signal is expressed as the mean net intensity/area $\pm$ SD.

Fig 6. Dynamics of viral replication and cell proliferation imaged with microPET

A: Localization of sites of virus replication in tumors with [^{18}F]FHBG-PET. Virus was injected into the left of bilateral flank tumors. [^{18}F]FHBG uptake was seen in the virus infected tumor but not in the control tumor. **B:** Time activity curves obtained from dynamic PET scans after [^{18}F]FHBG injection. [^{18}F]FHBG accumulated in the virus infected tumor, while it was washed off from the heart, liver and control tumor. **C:** Identification of proliferating tumor with [^{18}F]FLT-PET. Photopenia identifies the site of virus replication in the tumor core.

Fig 7. Correlation of bioluminescence, fluorescence and microPET imaging data *in vitro*
A: Tumor characteristics before virus administration. The tumor is identified with Rluc bioluminescence, mCherry fluorescence, and [^{18}F]FLT-PET (small arrows). H&E and PCNA are show highly proliferative tumors *in vitro*. **B:** Changes in tumor characteristics following lytic virus replication. A destroyed tumor core is identified with Rluc, mCherry and [^{18}F]FLT-PET (arrows), 48 hrs after intra tumor virus (1×10^8 pfu) injection. The changes are seen on H&E and PCNA where the destroyed tumor core (arrow) is surrounded by rim of viable tumor (asterisk). **C:** Replicating virus is identified with Fluc bioluminescence and [^{18}F]FHBG-PET. IHC staining for LacZ and HSV-TK (arrow heads) in excised tumors confirm the presence of virus. Apoptosis is seen in the destroyed tumor core.