# Characterization of an Oncolytic Herpes Simplex Virus Drug Candidate

**OZAN S. KUMRU,[1] SANGEETA B. JOSHI,[1] PREM THAPA,[2] NIGEL PHEASEY,[3] PAUL S. BULLOCK,[4] HOUMAN BASHIRI,[5] CHRISTINE S. SISKA,[5] BRUCE A. KERWIN,[5] FENG HE,[5] DAVID B. VOLKIN,[1] C. RUSSELL MIDDAUGH[1]**

[1]Department of Pharmaceutical Chemistry, Macromolecule and Vaccine Stabilization Center, University of Kansas, Lawrence, Kansas 66047

[2]Microscopy and Analytical Imaging Laboratory, University of Kansas, Lawrence, Kansas 66047

[3]Amgen Inc., Abingdon, England OX14 4RX, UK

[4]Amgen Inc., Woburn, Massachusetts 01801

[5]Amgen Inc., Seattle, Washington 98119

*Received 26 June 2014; revised 8 September 2014; accepted 19 September 2014*

*Published online in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.24211*

**ABSTRACT:** The structural integrity and conformational stability of a genetically modified live, oncolytic herpes simplex virus (o-HSV) were investigated across a wide pH (5.5–8.0) and temperature (10°C–87.5°C) range. A combination of circular dichroism, intrinsic and extrinsic fluorescence, and static light scattering results was visualized using an empirical phase diagram approach to provide a global assessment of physical stability. Distinct phases were identified including the native state of the virus, an intermediate phase that could represent gradual swelling and/or shedding of the viral envelope, and a highly disrupted, aggregated phase. The nature of these altered forms of the virus was further evaluated by transmission electron microscopy and viral plaque assays. The effect of freeze–thaw (F/T) stress on o-HSV was also examined. After one F/T cycle, a loss of infectious virus titers was observed. In addition, the monomeric virus particle concentration decreased during F/T stress, whereas there was a concurrent increase in larger particles (2–10 μm). The comprehensive biophysical characterization of viral stability conducted in this study identified major degradation events leading to loss of infectivity of o-HSV and represents an important step toward stabilization of the virus against thermal and F/T stresses. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

**Keywords:** physical stability; particle size; biotechnology; cancer; preformulation; formulation; phase diagram; viral vectors

## INTRODUCTION

The use of oncolytic viruses has recently emerged as a promising approach for cancer treatment by selective destruction of cancerous cells.[1] Examples that have shown potential for oncolytic viral therapy include herpes simplex virus (HSV), vesicular stomatitis virus, Newcastle disease virus, adenovirus, and poxvirus.[2] Although the oncolytic potential of viruses in general has been observed for over 50 years, only after the advent of recombinant DNA technology has the full potential of this technology been explored.[3–7] One such promising oncolytic viral treatment is Talimogene laherparepvec (also known as OncoVex-$^{GM\text{-}CSF}$), which uses a genetically modified HSV (HSV-1).[8] The virus contains deletions in the *ICP34.5* and *ICP47* genes of HSV-1, which effectively constrains virus replication to cancer cells (*IPC34.5*) and facilitates antigen presentation in infected cells (*ICP47*).[8–10] The virus is also engineered to produce the cytokine human granulocyte macrophage-stimulating factor (GM-CSF). Upon cancer cell lysis, GM-CSF is released and recruits antigen-presenting cells (APCs) to the site of the tumor. The released virus infects nearby cancerous cells, while the APCs process and present tumor-specific antigens to promote a systemic antitumor humoral immune response. The virus can also infect noncancerous cells, but is unable to complete its replication cycle thus leaving the noncancerous cell unharmed.[8,11] A reduction in tumor size in patients with cancer of the head and neck as well as malignant melanoma has been observed during human clinical trials.[12–16]

Herpes simplex virus-1 is a member of the family Herpesviridae, which are a class of enveloped viruses that contain a relatively large (~152 kb) dsDNA genome (~74 open reading frames).[17] The icosaherdral capsid is attached to the envelope via a tegument resulting in a virion ~200 nm in diameter.[18] Live, attenuated viruses, in the absence of stabilizing excipients, are generally unstable for extended periods of time when stored at ≥4°C, when subjected to elevated temperatures, or after multiple freeze–thaw (F/T) cycles.[19–23] Because of the inherent instability of live virus, stabilizers are typically required to preserve the biological activity.[21,22,24,25] In the case of other live virus products such as Varicella Zoster live vaccine, measles, and HSV-2, the use of stabilizing excipients to preserve biological activity is required.[22,25–29] Prior to the identification of stabilizing excipients, however, it is important to understand the physicochemical and biological behavior of the virus in response to exposure to different environmental stress conditions to better understand and identify the causes and mechanism of the degradation pathways.

In this study, we characterized the physical stability of a genetically modified oncolytic HSV in response to different

*Correspondence to*: C. Russel Middaugh (Telephone: +785-864-1919; Fax: +785-864-5814; E-mail: middaugh@ku.edu); Feng He (Telephone: +206-265-6917. Fax: +206-217-0346; E-mail: fhe@amgen.com)

This article contains supplementary material available from the authors upon request or via the Internet at http://onlinelibrary.wiley.com/.

Journal of Pharmaceutical Sciences

solution pH, temperatures, and F/T stress. We used a variety of biophysical techniques to characterize the overall secondary and tertiary structure of the major viral proteins, the hydrodynamic diameter of the virus, its aggregation tendency, and the accessibility of viral nucleic acid. The biophysical results were correlated with electron micrographs and the biological activity of the virus. The combined results provide a comprehensive representation of the physical degradation pathways of the virus in response to the aforementioned stress conditions.

## EXPERIMENTAL METHODS

### Materials and Sample Preparation

o-HSV was produced and purified by Amgen Inc. (Thousand Oaks, CA, USA) and provided at a concentration of $10^8$ plaque forming units (pfu)/mL. For initial characterization, o-HSV was dialyzed (2 × 1 L for ~18 h) against CP buffer (20 mM citrate phosphate buffer with a total ionic strength of 0.15 that was adjusted with NaCl) using Slide-A-Lyzer dialysis cassettes [10 kDa molecular weight cutoff (MWCO)] (Thermo-Fisher, Rockford, Illinois). The pH was adjusted from 5.5 to 8.0 in 0.5 pH unit increments. In experiments that required more concentrated virus, aliquots were pooled and pelleted by ultracentrifugation (22,400 × g for 2 h at 4°C) using a Beckman Optimax preparative ultracentrifuge with an MLA-80 rotor and polyallomer thick wall ultracentrifuge tubes (Beckman-Coulter, Brea, California; part #366640). The viral pellet was washed with ~10 mL of CP buffer at 4°C followed by incubation at 4°C with gentle agitation for 20 ± 4 h. Protein concentration was measured using a BCA assay kit (Thermo-Fisher) following the manufacturer's instructions and generating a standard curve employing known concentrations of bovine serum albumin.

### Viral Plaque Assay

The amount of infectious o-HSV was quantified by titrating test samples onto susceptible indicator cells, observing the cytopathic effect and counting the subsequent pfu [limit of detection ≥ 2.08 log(pfu/mL)]. Briefly, baby hamster kidney (BHK; ATCC, Manassas, Virginia) cells were propagated in DMEM (Life Technologies, Carlsbad, California) supplemented with L-glutamine (Life Technologies), 10% fetal bovine serum (Thermo-Fisher, Waltham, Massachusetts) and antibiotics streptomycin and penicillin (Life Technologies). BHK cells were seeded in 12-well plates 1-day prior to testing. Test samples were serially diluted and used for infection of the monolayer. After an initial incubation period to allow for virus adsorption, the cells were covered by an overlay medium containing carboxymethylcellulose and growth medium and incubated for 72 h at 37°C and 5% $CO_2$. The cells were subsequently fixed using a 0.01% glutaraldehyde solution (Sigma–Aldrich, St Louis, Missouri) after aspirating the inoculum and washing with phosphate-buffered saline (PBS). The cells were then stained using a 2% crystal violet solution (Sigma–Aldrich) to visualize the plaques. To determine the viral titer plaques formed for each dilution of the test, samples were counted and the final titer was determined ($\log_{10}$ pfu/mL) from the average of the duplicates tested.

### Far-UV Circular Dichroism Spectroscopy

Circular dichroism (CD) analysis was performed using a Chirascan-plus CD spectrometer (Applied Photophysics Ltd., Leatherhead, UK) equipped with a peltier temperature controller and a 4-position cuvette holder (Quantum Northwest, Liberty Lake, Washington). Far-UV CD spectra of duplicate samples were collected in the range of 260–200 nm using a 0.2 cm path length quartz cuvette sealed with a Teflon stopper (Starna Cells Inc., Atascadero, California). For scans, a sampling time per point of 1 s in 0.5 nm increments were used. For thermal melts, a sampling time per point of 1 s in 1 nm increments were used. The CD signal at 222 nm was monitored as a function of temperature from 10°C to 87.5°C in 2.5°C intervals. The heating rate was 1°C/min, and the equilibration time at each temperature was 1 min. The absorbance of the buffer was subtracted from all measurements. All data were subjected to a five-point Savitzky–Golay smoothing filter using the Chirascan software, and normalized by the min/max function in Microsoft excel (Microsoft Corporation, Redmond, Washington). Transition temperatures ($T_m$) were calculated by fitting the data to a Boltzmann distribution using the non-linear curve fitting function in Origin software (Origin Lab, Northampton, Massachusetts).

### Intrinsic Tryptophan Fluorescence Spectroscopy

The intrinsic fluorescence of o-HSV was measured using a QM-40 Spectrofluorometer (Photon Technology International (PTI), Inc., Birmingham, New Jersey) equipped with a four-position cell holder and peltier temperature control device (Quantum Northwest). Fluorescence emission spectra of o-HSV were recorded as a function of temperature (10°C–87.5°C) and pH (5.5–8.0). Duplicate samples were measured using an excitation wavelength of 295 nm (>95% Trp emission). Emission spectra were collected from 305 to 405 nm with a step size of 1 nm and an integration time of 1 s. The excitation and emission slits were set at 4 and 6 nm, respectively. The spectra were collected at 2.5°C intervals with a 3 min equilibration time at each temperature. Quartz 0.2 × 1 $cm^2$ path length cuvettes were used in all experiments. The position of the emission wavelength maximum was determined using a mean spectral center of mass method (msm) executed in *Origin 7.0* software after buffer subtraction. This method increases the signal to noise ratio, but the peak positions are generally red shifted between 10 and 14 nm from their actual values.

### Extrinsic Fluorescence Spectroscopy using Propidium Iodide

The accessibility of viral DNA was measured using propidium iodide (PI) (Life Technologies), which is a membrane impermeable dye that binds the major groove of double stranded DNA (dsDNA).[30,31] PI (suspended in ultrapure water) was mixed with o-HSV at a final concentration of 20 μM. Duplicate samples were measured using an excitation wavelength of 535 nm with the emission spectra monitored from 550 to 650 nm as a function of temperature (10°C–87.5°C) and pH (5.5–8). The excitation and emission slits were set at 4 nm. Step size and integration time were 1 nm and 0.5 s, respectively. The spectra were collected at 2.5°C intervals with a 3 min equilibration time at each temperature. Cuvettes, instrument setup, and data analysis were identical to the intrinsic fluorescence experiments. Fluorescence intensity at 613 nm was plotted as a function of temperature. The emission spectrum of the buffer with 20 μM PI was subtracted from all measurements.

 DOI 10.1002/jps.24211