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The D-form of a novel heparan binding peptide decreases cytomegalovirus infection *in vivo* and *in vitro*

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ABSTRACT

Human cytomegalovirus (HCMV) infection *in utero* can lead to congenital sensory neural hearing loss and mental retardation. Reactivation or primary infection can increase the morbidity and mortality in immune suppressed transplant recipients and AIDS patients. The current standard of care for HCMV disease is nucleoside analogs, which can be nephrotoxic. In addition resistance to current treatments is becoming increasingly common. In an effort to develop novel CMV treatments, we tested the effectiveness of the D-form of a novel heparan sulfate binding peptide, $p5R_D$, at reducing infection of ganciclovir (GCV) resistant HCMVs *in vitro* and MCMV *in vivo*. HCMV infection was reduced by greater than 90% when cells were pretreated with $p5R_D$. Because $p5R_D$ acts by a mechanism unrelated to those used by current antivirals, it was effective at reducing GCV resistant HCMVs by 85%. We show that $p5R_D$ is resistant to common proteases and serum inactivation, which likely contributed to its ability to significantly reduced infection of peritoneal exudate cells and viral loads in the spleen and the lungs *in vivo*. The ability of $p5R_D$ to reduce HCMV infectivity *in vitro* including GCV resistant HCMVs and MCMV infection *in vivo* suggests that this peptide could be a novel anti-CMV therapeutic.

1. Introduction

Human cytomegalovirus (HCMV) infection or reactivation from latency causes severe disease in immune-suppressed transplant recipients, AIDS patients, and newborns (Crough and Khanna, 2009). Infection can lead to mononucleosis-like symptoms, interstitial pneumonia, gastroenteritis, retinitis, or organ transplant rejection (Cheung and Teich, 1999; Mocarski et al., 2013; Ramanan and Razonable, 2013; Schleiss, 2013; Singh, 2006). *In utero* infection can lead to microcephaly, hepatosplenomegaly and sensorineural hearing loss (SNHL) in newborns (Cheeran et al., 2009; Pass, 2005).

Due to the limited success of HCMV vaccines (Griffiths et al., 2013; Sung and Schleiss, 2010), HCMV treatment consists of antiviral drugs like ganciclovir (GCV), valganciclovir (i.e., an oral prodrug of GCV), cidofovir (CDV), and foscarnet (FOS). These antivirals are nucleoside analogs that target viral DNA synthesis (De Clercq, 2004b). Although effective, these drugs can be nephrotoxic and induce leukopenia (Biron, 2006). In addition long-term use of these antivirals has lead to the evolution of HCMV resistant strains (Lurain et al., 2002; Lurain and Chou, 2010). One approach to develop novel anti-CMV therapeutics is to target other aspects of the HCMV lifecycle other than viral DNA synthesis.

A potential target for anti-CMV therapeutics is the initial, critical attachment step during viral entry in which HCMV binds to cell surface heparan sulfate (HS) (Compton et al., 1993; Vanarsdall and Johnson, 2012). HS consists of glucosamine and glucuronic acid or iduronic acid moieties that are N-acetylated as well as N- or O-sulfated, which allows for incredible diversity in the structures on different cell types (Esko and Lindahl, 2001; Liu and Thorp, 2002; Rabenstein, 2002). Heparan sulfate proteoglycans (HSPGs) participate in physiological processes including embryonic development,

Abbreviations: CMV, cytomegalovirus; GCV, ganciclovir; HCMV, human cytomegalovirus; MCMV, murine cytomegalovirus; p.i., post infection; pfu, plaque forming units; i.p., intraperitoneal; i.v., intravenous; dpi, days post infection; GAGs, glycosaminoglycans; HSPGs, heparan sulfate proteoglycans; HS, heparan sulfate; CDV, cidofivir; FOS, foscarnet; PEC, peritoneal exudate cell; BAC, bacterial artificial chromosome; hpi, hours post infection.

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binding of growth factors, chemokine transcytosis, cell adhesion and lipid metabolism (Bishop et al., 2007). CMVs utilize HSGPs for their initial attachment to the cell and subsequent initiation of infection (Compton et al., 1993). Previous studies have shown that HCMV may preferentially bind to 6-O-sulfated or 3-O-sulfated heparan sulfate moieties during viral entry (Baldwin et al., 2015; Borst et al., 2013). The distribution of HS on host cells in addition to viral preference for specific subtypes of HS, make this structure a potential anti-CMV therapeutic target.

One approach to limit HS-mediated viral entry is through the use of peptides designed to preferentially bind HSPGs. Peptides offer several benefits as therapeutics. They can be readily synthesized, designed to be highly specific, easily modified to enhance biological activity, and less toxic because they are catabolized into amino acids (Castel et al., 2011). However, their susceptibility to proteases and short serum half-life have historically limited the use of peptides as therapeutics (McGregor, 2008). Recently several HS-binding peptides have been tested *in vitro* and *in vivo* for their ability to inhibit herpesvirus infections. The HS reactive peptide, G2, a 10-mer derived from a phage display library, inhibits HSV-1 infection *in vivo* (Tiwari et al., 2011). Additionally, a peptide known to bind to hypersulfated HS, p5+14, was shown to effectively inhibit HS-mediated entry of both murine and human CMV as well as HSV *in vitro* (Dogra et al., 2015). In this study we characterize a related peptide, p5R, which is 14 amino acids shorter and has a higher propensity to form an α-helix. Furthermore, we hypothesized that the D-form of peptide p5R, $p5R_D$, would still inhibit CMV infection *in vitro* and more efficacious *in vivo* due to its resistance to proteolytic cleavage. Finally, we tested whether $p5R_D$ inhibited GCV-resistant HCMVs *in vitro* in an effort to show its efficacy against clinical strains of HCMV.

2. Materials and methods

2.1. Peptide synthesis

Using p5 as a template to design p5R and $p5R_D$ the lysine resides were replaced with arginine residues to yield p5R - GGGYS RAQRA QARQA RQAQR ARGAR Q. Both peptides have a positive net charge of +8 and a predicted alpha helical secondary structure according to ITASSER predictions (Roy et al., 2010; Zhang, 2008). Peptides p5R and $p5R_D$ were purchased from Anaspec (Fremont, CA) and purified as previously described (Dogra et al., 2015).

2.2. Cells and mice

All cells used in our experiments were low passage-number (less than 22 passages). Human lung fibroblasts (MRC-5) were cultured in MEM (Corning, Manassas, VA and HyClone, Logan, UT) supplemented with 10% Fetal Bovine Serum – Premium (FBS; Atlanta Biologicals, Atlanta, GA), and 2 mM L-glutamine (HyClone, Logan, UT). Human foreskin fibroblasts (HFF; ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, HyClone, Logan UT) with 2 mM sodium pyruvate (Corning, Manassas, VA and HyClone, Logan, UT) supplemented with 10% FBS, and 2 mM L-glutamine. Human aortic endothelial cells were cultured in EGM-2 Bullet Kit (Lonza, Walkersville, MD) supplemented with 6% FBS, and 2 mM L-glutamine. Human pigment epithelial cells (ARPE-19) were cultured in DMEM:F12 medium (Corning, Manassas, VA and HyClone, Logan, UT) supplemented with 10% FBS. Mouse embryonic fibroblasts 10.1 (MEF 10.1) were cultured in DMEM (Lonza, Rockland, ME) supplemented with 10% Fetal Clone III serum (FCIII) (Hyclone, Logan, UT), 100 U/ml Penicillin/Streptomycin and 2 mM L-glutamine. All cells were grown at 37 °C and 5.0% CO_2. Male and female BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, ME) and bred in the University of Tennessee Laboratory Animal Facility. Eight to 12 week old mice were used in all experiments. All mice were housed under specific pathogen-free conditions and the experiments were performed under the auspices of University of Tennessee IACUC-approved protocols.

2.3. Viruses

HCMV TB40E expressing luciferase under the control of the UL18 promoter was a gift from Drs. Christine O'Connor and Eain Murphy (University of Buffalo and FORGE Life Science, LLC). Virus was cultured on HFF cells and titered using plaque assay or luciferase/luminescent expression assay using a Synergy 2 plate reader (BioTek, Winooski, VT). Based on a standard curve, approximately fifteen plaques are equivalent to 1000 relative light units (RLU).

Recombinant, Wild Type and GCV resistant HCMVs (T3261, T3252, T3265 and T3429) were generated as described (Chou, 2010, 2011; Chou and Bowlin, 2011; Chou et al., 2005). These recombinant viruses were a kind gift from Dr. Sunwen Chou (Oregon Health Sciences University). Recombinant viruses were titered using plaque assays and secreted alkaline phosphatase (SEAP) assay (Chou et al., 2005). HCMV clinical isolates, CH19 and CH-13 (Lurain et al., 2002), were a kind gift from Dr. Nell S. Lurain (Rush University). All viruses were titered using 0.5% agarose overlay in a plaque assay.

MCMV K181 (Booth et al., 1993) was cultured *in vitro* in MEF 10.1 cells. The viral titer was determined via plaque assay. All viruses were stored at −80 °C until use.

2.4. Luciferase assay

Peptide reduction of HCMV TB40E infection was measured using a luciferase assay. Briefly, cells were seeded into 24-well plates. After the cells reached 80–85% confluency, peptide, suspended in 10% FBS/PBS, was added. Control treatments included 10% FBS in PBS without peptide. After 30 min virus was added to cells at ~35–50 pfu or ~2500–3000 RLU and allowed to incubate at 37 °C for 60 min. Following virus incubation, the peptide and virus was removed and fresh media was added. Plates were incubated at 37 °C in 5% CO_2 for 3 days.

On day 3, medium was removed and cells were washed once with PBS. Cells were lysed using the passive lysis buffer (Promega, Madison, WI). Cell lysate was pelleted and luciferase assay reagent was combined with equal amounts of supernatant into an opaque 96-well microplate. Luminescence was measured as RLU using a Synergy 2 plate reader (BioTek). The RLU values for peptide-untreated and uninfected treatment served as a negative control and were subtracted as background. Peptide-untreated virally-infected cells served as the positive control and normalized to 100%. Data was analyzed using Prism 5.0 (GraphPad Software, La Jolla, CA). Data was expressed as percent infection (100× (number of RLU after treatment/RLU in the PBS treated cells)).

2.5. Proteolytic stability

Proteolytic stability was measured following incubation with proteases or serum. The peptides were incubated with trypsin (Lonza, Walkersville, MD) or elastase (Sigma, St. Louis, MO) at 100 μg/ml, or FBS, or human serum for 30 or 60 min then added to cells 30 min prior to infection with virus.

2.6. Plaque reduction assay

Peptides were screened for their ability to reduce infection of the BAC-derived recombinant GCV-resistant HCMV and clinically