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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.

Keywords

cytomegalovirus; heparan sulfate; ganciclovir; protease resistance; MCMV; HCMV; peptide

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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, binding of growth factors, chemokine transcytosis, cell adhesion and lipid metabolism (Bishop et al., 2007). CMVs utilize HSPGs 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 his current 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 D- form of peptide p5R, p5R_D, would still

inhibit CMV infection *in vitro* and would be 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 residues 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 normal 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 2mM L-glutamine (HyClone, Logan, UT). Human foreskin fibroblasts (HFF; ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, HyClone, Logan UT) with 2mM sodium pyruvate (Corning, Manassas, VA and HyClone, Logan, UT) supplemented with 10% FBS, and 2mM L-glutamine. Human aortic endothelial cells were cultured in EGM-2 Bullet Kit (Lonza, Walkersville, MD) supplemented with 6% FBS, and 2mM 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), 100U/ml Penicillin/Streptomycin and 2mM L-glutamine. All cells were grown at 37°C and 5.0% CO₂. 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 titrated 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 titrated 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 minutes virus was added to cells at ~ 35 –50 pfu or ~ 2500 –3000 RLU and allowed to incubate at 37°C for 60 minutes. 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 ($100 \times (\text{number of RLU after treatment} / \text{RLU in the PBS treated cells})$).

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 $100\text{ }\mu\text{g/ml}$, or FBS, or human serum for 30 or 60 minutes then added to cells 30 minutes 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 isolated GCV-resistant strains using a plaque reduction assay on MRC-5 and HFF cells. Cells were treated with peptide or PBS for 30 minutes prior to the addition of virus. After one hour of incubation, the mixture was removed and replaced with a 0.5% agarose in complete MEM (MRC-5 cells) or DMEM (HFF cells) medium. The plates were incubated at 37°C and 5.0% CO_2 for 7 to 10 days when plaques began to develop. Plaques were manually counted using a dissection microscope after staining with Coomassie blue. For MCMV *in vivo* experiments, MEF 10.1 cells were infected with serial dilutions of homogenized organs or infected peritoneal exudate cells (PECs). Following a 1 hr. incubation, the media was removed and fresh 2% carboxymethyl cellulose (Sigma

Aldrich, St. Louis, MO; CMC) in complete DMEM was added. Cells were incubated at 37°C and 5.0% CO₂ for 5 days then stained with Coomassie stain and plaques counted.

2.7 Biodistribution of peptides

To determine the distribution of p5R_D among the relevant organs, mice were injected i.v. in the lateral tail vein, with I¹²⁵ labeled peptides (< 120 µCi, 20 µg of peptide). At 1, 2, 4 or 24 hr. post-injection mice were euthanized with isoflurane inhalation overdose and the spleen, liver, lung, and eight other tissues were harvested and the tissue radioactivity measured as previously described (Wall et al., 2011). The biodistribution of radiolabeled peptide was expressed as percent injected dose per gram of tissue (%ID/g).

2.9 Data and statistical analysis

All data were normalized to 100% using the peptide untreated and infected positive control data. The data are the combined from three or more independent experiments with at least three replicates in each experiment. Error bars represent the standard deviation (SD). Statistical significance was calculated using a Student's t test, Man Whitney, or 2 way ANOVA followed by Bonferroni posttests. The IC₅₀ values were calculated using a linear regression sigmoidal dose dependent test. All analyses were performed using Prism 5.0 (GraphPad). A p-values less than 0.05 were considered statistically significant. Significant values are labeled as *=p<0.05, **=p<0.01, ***=p<0.001, NS=non-significant reduction in infection.

3 Results

3.1 Peptide Efficacy *in vitro*

Previously we have shown that the p5-related peptides bind amyloid deposits via interactions with hypersulfated HS and amyloid fibrils and can acts as an inhibitor of herpesvirus entry (Dogra et al., 2015; Martin et al., 2013; Wall et al., 2011). The synthetic peptides, p5R and p5R_D, are comprised of the heptad repeat [AQRAQAR] in the L and D-forms, respectively. Both have an overall +8 net charge and reduce MCMV infection by >75% (data not shown) (Dogra et al., 2015). We also showed that the peptides do not affect cell viability (Supplemental Fig. 1). Furthermore, the peptides have a similar IC₅₀ for blocking infection of human fibroblasts with a luciferase-expressing TB40 HCMV (48.5µM p5R and 60.2µM p5R_D). At a concentration >200 µM, both p5R and p5R_D reduce HCMV infection by 90% (Fig. 1A).

HCMV infects a wide range of host cells and its tropism varies depending on the cell types used for propagation (Adler and Sinzger, 2013; Hahn et al., 2004; Ryckman et al., 2008; Scrivano et al., 2011; Sinzger et al., 2006; Vanarsdall et al., 2011; Vanarsdall and Johnson, 2012; Wang and Shenk, 2005). To examine the breadth of HCMV peptide inhibition, we tested peptide blockage of infectivity using fibroblasts from another tissue (HFF), endothelial cells (HAEC), and epithelial cells (ARPE-19). Both p5R and p5R_D, when added at least 30 minutes prior to infection, reduced HCMV infection by >80% in all cell types (Fig. 1B). Although similar to each other, both were more efficient at preventing HCMV infection in fibroblast cells (~10% more effective) than on other cell types (Kruskal-Wallis

test $p < 0.000$; t test: $p = 0.024$ for p5_D on ARPE-19 vs MRC5). This is in contrast to the previously characterized p5+14, which was most effective on ARPE-19 cells (Dogra et al., 2015). This implies that these peptides could be 1) have different affinities for HS. 2) the cell lines express different HS moieties 3) the viruses use different entry mechanisms for the different cell types. Previous data show that p5R has a higher affinity for heparin, amyloid, and blocks MCMV infection 2x more efficiently than p5 (Dogra et al., 2015; Martin et al., 2013; Wall et al., 2011). These data suggest peptides containing arginine in place of lysine may bind to an HS that is important for HCMV entry into the different cell types. Our results demonstrate that both p5R and p5R_D have similar IC₅₀ values and efficiency to reduce HCMV infection in different cell types *in vitro*.

3.2 Proteolytic stability of p5R_D

D-form peptides are resistant to many of the body's proteases. This proteolytic resistance would prolong peptide survival within the host and potentially increase its efficacy (McGregor, 2008). To measure the proteolytic stability of p5R_D, we incubated both peptides with trypsin and elastase serine proteases. Peptides p5R and p5R_D were pre-treated with the different proteases for 30 or 60 minutes. After which, the peptides were added to cells 30 minutes prior to the addition of virus. Peptide p5R_D retained its ability to limit HCMV infection by ~90%, whereas p5R exhibited only a 20% inhibition after incubation with the serine proteases (Fig. 2A and B). To mimic a biologically relevant environment, peptides were also incubated in human serum or FBS for 30 minutes or 60 minutes prior to addition to MRC-5 cells. Under these conditions, peptide p5R_D reduced HCMV infection by 90% after incubation with FBS while p5R was no longer inhibitory (Fig. 2C). Similarly, p5R_D incubated with human serum reduced HCMV infection by 85%, while serum incubated p5R only achieved a 50% reduction (Fig. 2D). The differences between human serum and FBS treatment could be due to differences in protease concentrations or other inhibitors of the peptide/HS interaction (Lu et al., 2006). These data suggest the D-amino acids in p5R protect it from proteolysis making it a better option for *in vivo* evaluation.

3.3 Efficacy of p5R_D *in vivo*

Our data suggests that p5R_D effectively reduces HCMV infection *in vitro* and was resistant to serum proteases and degradation when exposed to serum. Before evaluating the anti-viral efficacy in mice we characterized the peptide's biodistribution in healthy animals. Biodistribution experiments of ¹²⁵I-labeled peptide in BALB/c mice showed that, ¹²⁵I-p5R_D was retained at high levels in the spleen, liver, and kidneys of mice for at least 24 hours post injection (Table 1). In contrast, the majority of ¹²⁵I-p5R was excreted or degraded by 4 hours. Only radioiodide, which is liberated during renal and hepatic catabolism of the peptide, is visible in the stomach of the mice. To assess the anti-viral efficacy of p5R_D *in vivo*, cohorts of mice were injected i.v. with 500 µg of p5R_D or PBS at 1, 2, or 4 hour prior to i.p. administration of MCMV (1×10⁶ pfu). The spleen and liver, which are sites where peptide p5R_D was retained for >24 h, were harvested 4 days post infection (dpi) for titering of virus. The p5R_D-treated mice show reduced viral titers for both the liver and spleen when the animals were treated with peptide prior to infection (Fig. 3). However, the greatest reduction occurred in the spleen (~25% reduction) when the peptide was administered 1 hour prior to infection (Fig. 3C). There is a statistically significant reduction of the viral load

in the spleen and liver with ~15–25% decrease in viral titers between peptide treated and PBS treated mice at all timepoints.

To address whether peptide p5R_D was capable of inhibiting initial MCMV entry but failing to inhibit the subsequent infection of new cells days after administration, we focused on the infection of peritoneal exudate cells (PECs) early after infection. 500 µg of peptide was administered i.v. 4 or 1 hr. prior to i.p. infection of MCMV. The PECs were harvested 2 hrs. post infection and washed to remove excess virus. An infectious centers assay was used to measure infected PECs. Our data show that administration of peptide 4 hrs. (Fig. 4A) or 1 hr. (Fig. 4B) prior to infection significantly reduced the number of infected PECs ($p = 0.007$ and 0.003 , respectively), suggesting that p5R_D likely inhibits the initial entry of MCMV into host cells.

3.4 Peptide efficacy against GCV^R HCMV

The long-term use of antivirals has contributed to the development of HCMV resistance to the current antivirals (Lurain and Chou, 2010). For example, it is estimated that between 2–27.5% of HCMVs are now resistant to GCV (Jabs et al., 1996; Jabs et al., 1998; Liu et al., 2000; Scott et al., 2004). We next examined the efficacy of peptide p5R_D as an inhibitor of infection using GCV^R HCMV. Bacterial artificial chromosome (BAC)-derived HCMVs with mutations in either the *UL54* gene (T3429) that encodes the DNA polymerase targeted by GCV or the viral *UL97* gene (T3252) that encodes the kinase required for phosphorylation of GCV were tested against p5R_D. These point mutations are representative mutations found in clinical isolates and incorporated into these BAC derived strains (Chou, 2010; Chou and Bowlin, 2011; Chou et al., 2005; Limaye, 2002). Notably mutations in *UL54* also confer resistance to CDV and FOS (Lurain and Chou, 2010). We tested the hypothesis that mutations that contribute to GCV resistance would not confer “resistance” to the inhibitory effects of the p5R_D peptide treatment. First, we confirmed, using the SEAP assay, that T3429 and T3252 strains are resistant to high concentrations of GCV (Supplemental Table 1) (Chou, 2011; Chou and Bowlin, 2011). To test the efficacy of peptide p5R_D at inhibiting these GCV-resistant viruses, MRC-5 cells were treated with 200 µM peptide prior to infection. Using the SEAP assay to quantify infection as a surrogate for the conventional plaque assay, treatment with p5R_D reduced the infection of MRC-5 fibroblasts by recombinant GCV^R HCMVs, (T3429 and T3252) by ~80% (Fig. 5A).

GCV^R HCMV has been isolated from organ transplant recipients as well as other settings (Chou et al., 2002; Lurain et al., 2002; Lurain and Chou, 2010; Ramanan and Razonable, 2013; Singh, 2006). Given that peptide p5R_D reduced the infectivity of recombinant GCV^R HCMV *in vitro*, we next tested whether this peptide was similarly effective on clinically isolated GCV^R HCMV. These strains, from patients CH-13 and CH-19, were isolated from seropositive recipients during lung transplantation (Lurain et al., 2002). The strain from patient CH-19 (CH-19) has a point mutation A594V making it GCV^R. Notably T3252 recombinant virus from the previous experiment (Figure 5A) contains the same point mutation, A594V. The CH-13 A isolate (CH-13 A) contains a deletion in codons 597–603 within the *UL97* gene which confers resistance to GCV (Chou et al., 1995b). Using the *in*

vitro plaque assay, peptide p5R_D reduced CH-13 A and CH-19 infection of MRC-5 cells by >84% (Fig. 5B).

3.5 Effects of p5R_D with decreasing concentrations of GCV

To examine the effect of both p5R_D and GCV treatment in combination, MRC-5 cells were incubated with either GCV, p5R_D, or a combination of GCV and p5R_D with decreasing concentrations of GCV. Cells treated with 3 μ M of GCV alone showed ~95% reduction in HCMV infection (Fig. 6). Similarly, cells treated with 200 μ M of p5R_D showed ~90% reduction in HCMV infection (Fig. 1 and 6). The combination therapy of p5R_D (200 μ M) and GCV, at either 1.5 μ M or 0.75 μ M lead to a 94% reduction in HCMV infection (Fig. 6). These data suggest that the IC₅₀ value for GCV was reduced when cells are pre-treated with 200 μ M of peptide p5R_D prior to infection. The combination of the two anti-CMV treatments should reduce prolonged GCV usage, making the development of GCV^R HCMV less likely and limiting GCV's toxic side effects.

4 Discussion

Generation of an effective reagent, such as a synthetic peptide, that prevents cellular tethering of CMV and thereby hindering infection would benefit 20 to 60% of transplant patients that are at risk for developing HCMV disease (Scott et al., 2004). Furthermore, an effective prenatal prophylactic anti-CMV peptide will benefit the 0.5% to 2% of newborns at risk for contracting HCMV *in utero* (Schleiss, 2013). Novel treatments for HCMV must address host organ toxicity and antiviral resistance currently associated with GCV, CDV, and FOS treatments. The current study aims to address those concerns by testing the ability of a D-amino acid, proteolytically-resistant peptide, p5R_D, to reduce CMV infection. Our data show that p5R and p5R_D reduce HCMV infection of not only fibroblasts, but also endothelial and epithelial cells *in vitro* (Fig. 1). A broad spectrum CMV inhibitor is important as CMV has an expansive cell-type tropism for replication and latency (Vanarsdall and Johnson, 2012). While p5R and p5R_D reduce HCMV infection in all cell types, there are subtle differences in the percent reduction that potentially reflects differences in HS expression on the cell. It is known that CMV uses a 6-O-sulfated and 3-O-sulfated heparan sulfate for entry (Baldwin et al., 2015; Borst et al., 2013). Our data suggest that p5R_D binds to a specific subset of HS, that is different from HS targeted by the previously characterized p5+14 and the HSV-1 entry inhibitor peptide, G2 (Dogra et al., 2015; Tiwari et al., 2011). This is supported by our previous observation that, in contrast to p5R_D, ¹²⁵I-labeled p5+14 is not retained in the kidneys, liver, and spleen of WT mice at high concentrations for >4 h post injection (Wall et al., 2015).

In order for p5R_D to effectively reduce CMV infection *in vivo*, it must resist hydrolysis from host proteases. Our data indicates that the anti-CMV function of p5R_D, but not p5R, was unaffected after incubation with the serine proteases, trypsin and elastase, or with bovine or human serum (Figure 2). Using radiolabeled peptide, we also show that p5R_D is retained in the spleen, liver, and kidney of WT mice for >24 h while the L-form, p5R, is rapidly dehalogenated and/or excreted. These data suggest that the D-amino acids used to synthesize

p5R_D confer protection from hydrolysis and enhances its potential to reduce HCMV infection *in vivo*.

In our *in vivo* experiment, p5R_D was administered i.v. before i.p. MCMV infection. Peptide was administered i.v. to mimic clinical administration of antiviral reagents. Additionally i.v. administration results in widespread biodistribution of p5R_D (Table 1.). MCMV i.p. injection results in a systemic infection that involves a variety of tissues and organs (Shellam et al., 2006). In a systemic infection model of MCMV infection followed by primary dissemination to the spleen and liver, we found that p5R_D reduces infection in these organs at 4 dpi. There are several possible explanations for why the reduction was not as great as the *in vitro* reduction. One possibility is that CMV infects host cells by a variety of mechanisms including cell-to-cell spread or pH-dependent endosomal entry, which do not require cell surface HSPGs (Gerna et al., 2016; Gerna et al., 2008; Lemmermann et al., 2015; Ryckman et al., 2006; Vanarsdall and Johnson, 2012; Wang et al., 2007). MCMV intra-tissue spread is mediated by a specific glycoprotein complex (gH/gL/MCK-2) that differs from the glycoprotein complex (gH/gL/gO) used in initial MCMV entry (Scrivano et al., 2010). The MCMV gH/gL/gO complex is responsible for entering fibroblasts through a fusion event, which presumably initially involves HSPG tethering (Wagner et al., 2013). The MCMV gH/gL/MCK-2 complex facilitates infection in macrophages via the endocytic pathway (Hahn et al., 2004; Scrivano et al., 2010). It is not known whether this utilizes HSPGs for entry. If intra-tissue spread occurs without the use of HSPGs, p5R_D would be ineffective at reducing infection of tissue cells *in vivo*. Another possible explanation for p5R_D's lack of efficacy *in vivo* could be due to its lack of efficacy once infection has been initiated. At 4 dpi MCMV has likely undergone at least two rounds of exponential replication (Mocarski, 1996). Even at 90% reduction of infection, two rounds of growth would only decrease the viral load by little more than a log, which is at the limit of detection of plaques assay. Whether this degree of reduction in viral load would result in reduced pathology in the humans, remains open to speculation.

Another factor that could affect p5R_D efficacy is the turnover rate for the HS ligand that to which it binds. The turnover rate for HS and HSPGs is relatively rapid ($T_{1/2} < 4$ hours) for murine epithelial cells (David and Van den Berghe, 1989). To examine whether p5R_D limits initial MCMV entry, mice were injected with p5R_D prior to MCMV infection of PECs early after infection (Zhang et al., 2008). We chose an i.v. treatment with p5R_D in order to be consistent with previous data (Figure 3 and Table 1) and to mimic potential clinical utility. Peptides are small enough to penetrate blood vessel walls. Therefore, we predicted p5R_D would still be able to access the virus in the peritoneum (Fosgerau and Hoffmann, 2015). An infectious centers assay revealed that p5R_D significantly reduced the amount of infected PECs when administered 1 or 4 hours before MCMV infection (Figure 4). Because 1 hr. pretreatment was more effective than the 4 hr. treatment (in both the i.p. and PEC infections), this could point to HS turnover as being important efficacy. As the long the time interval between peptide administration and virus increases, the blocked HS would get internalized and subsequently replaced with de novo or recycled unblocked HS. While ~50% of PECs are macrophages (Ray and Dittel, 2010), we did not assay whether the peptide is active against macrophages, their subsets, or other cells found in peritoneal cavity. Thus, the

timing of p5R_D administration appears to be important *in vivo* which may be due to the relatively quick turnover rate of HSPGs on different cell types.

The increasing frequency of GCV^R HCMV has warranted the development of novel therapeutics and approaches to dealing with CMV infection (Chou et al., 1995a; Li et al., 2007; Limaye, 2002; Lurain et al., 2002; Lurain and Chou, 2010). The current HCMV antiviral agents target and prevent viral DNA replication (De Clercq, 2004a). Given this clinical need we hypothesized and demonstrated that p5R_D could inhibit GCV^R HCMV infection of cells because the peptide is thought to inhibit HSPG-mediated viral entry instead of viral DNA replication (Figure 5). Often HCMV antiviral agents are used to treat organ transplant recipients for months following transplantation (Li et al., 2007; Limaye, 2002; Lurain et al., 2002; Lurain and Chou, 2010). Among transplant recipients and AIDS patients there is a 5%–10% rate of therapeutic resistance (Lurain et al., 2002). Therefore peptides such as p5R_D and similar reagents that act by inhibiting the initial CMV-cell interaction may provide new benefits for these patients. In addition, we have demonstrated that p5R_D may be effectively used in combination therapy with traditional anti-CMV nucleoside analogs, such as GCV. Pre-treatment using p5R_D would require less GCV to achieve therapeutic benefit. This would decrease the likelihood of developing GCV-resistant strains of HCMV and minimize the organ toxicity associated with high concentrations of GCV. This peptide, and similar reagents, could provide the basis for a novel approach to treating CMV diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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	cidofovir
FOS	foscarnet
PEC	peritoneal exudate cell
BAC	bacterial artificial chromosome

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Highlights

- The D form of p5R prevents MCMV infection *in vitro* and *in vivo*
- P5R_D prevents infection of HCMV GCV resistant mutants
- P5R_D allows for a decrease in the concentration of GCV to prevent HCMV infection

Figure 1. *In vitro* analysis of p5R and p5R_D

(A) The IC₅₀ was determined by adding successive peptide concentrations (10μM, 50μM, 100μM, 200μM, and 250μM) to MRC-5 cells 30 minutes prior to the addition of TB40E-Luc. Each point is the average of three replicates $\pm$ SD from 3 separate experiments. (B) 200μM of p5R and p5R_D were added to either MRC-5, HFF, ARPE-19, or HAEC cells 30 min prior to the addition of TB40E-Luc. Each point represents a well of an experiment. The horizontal bars represent the average of the percent reduction from at least three separate experiments with three replicates in each experiment $\pm$ SD.

Figure 2. Proteolytic stability of p5R and p5RD

200 μ M of each peptide was pre-treated with 100 μ g/ml of trypsin, elastase, fetal bovine serum (FBS) or human serum for 30 minutes and 60 minutes. Protease treated peptides were added to MRC-5 cells 30 minutes prior to the addition of virus. Data represents the average of the percent reduction compared to PBS-treated controls from three separate experiments with repeated three times. Statistical significance was determined by 2 way ANOVA with Bonferroni posttest: ** p-value<0.01, *** p-value<0.001 $\pm$ SD.

Figure 3. p5RD reduces the viral load in spleen and liver of peptide in mice

Mice were pretreated with 500 μ g of p5RD or PBS intravenously at 4 (A), 2 (B), 1 (C) hrs before infection with 1×10^6 pfu of WT MCMV (K181). Organs were harvested 4dpi. Data represent the average of peptide treated or PBS-treated controls from three experiments with 5–6 mice in each. P-values were calculated using a Mann Whitney test.

Figure 4. Inhibition of PEC infection after treatment with p5R_D

Mice were pretreated with 500 μ g of p5R_D or PBS intravenously 4 hrs. (A) or 1 hrs. (B) before i.p. infection with 1×10^6 pfu of WT MCMV (K181). PECs were harvested 4hpi. 2×10^5 PECs were placed over a confluent layer of MEF 10.1 cells. Horizontal bars represents the average $\pm$ SD of peptide or PBS-treated controls from two independent experiments with 5 repeats in each. Statistical significance was determine using a t-test: ** p-value<0.01, *** p-value<0.001.

Figure 5. p5R_D efficacy against GCV^R HCMV

200μM of p5R_D was added to MRC-5 cells 30 min prior to the addition of BAC parental (WT), recombinant GCV^R (i.e., mutant Pol or mutant UL97) (A) or GCV^R clinical isolates (B). Titers of the viruses were assayed with an SEAP assay (RLU) or plaque assays for clinically isolated GCV^R HCMV. All horizontal bars ±SD represent that average of the % expression of SEAP or titers/untreated controls of three independent experiments with three to six replicates in each. $P>0.72$ using Kruskal-Wallis analysis.

Figure 6. The combination of GCV and p5RD is more efficient at preventing HCMV replication MRC-5 cells were either treated with PBS (control) or 200μM of p5RD 30 min prior to the addition of TB40E-Luc. After infection, virus media was removed and cells were treated with four different GCV concentrations (3μM, 1.5μM, 0.75μM and 0.38μM) or no GCV (control). Horizontal bars± SD represent the average from four experiments in duplicate. Statistical significance was determined by 2 way ANOVA with Bonferroni posttest: *** p-value<0.001.

Table 1

p5R and p5RD biodistribution in BALB/c mice

p5R	p5RD									
	Tissue distribution (%ID/g)									
	1hpi		4hpi		2hpi		24hpi			
	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
muscle	0.8	0.19	0.28	0.07	0.17	0.03	0.07	0.01	0.07	0.01
liver	2.96	0.58	1.25	0.26	40.04	5.06	35.69	1.28	35.69	1.28
pancreas	2.64	0.77	0.7	0.25	0.28	0.04	0.12	0.01	0.12	0.01
spleen	1.95	0.42	1.02	0.47	7.86	1.5	6.87	0.6	6.87	0.6
left kidney	3.72	0.17	1	0.26	50.9	6.4	46.29	2.08	46.29	2.08
right kidney	2.97	1.41	0.97	0.2	56.02	8.73	48.76	4.68	48.76	4.68
stomach	19.69	3.52	5.43	0.58	1.64	0.27	0.51	0.14	0.51	0.14
upper intestine	2.12	0.66	0.68	0.18	0.91	0.13	0.44	0	0.44	0
lower intestine	1.68	0.26	0.77	0.08	0.64	0.07	0.3	0.02	0.3	0.02
heart	1.85	0.43	0.76	0.27	0.66	0.13	0.32	0.01	0.32	0.01
lung	3.03	0.45	1.02	0.21	3.22	0.4	1.73	0.3	1.73	0.3