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Gallic Acid-Based Small-Molecule Inhibitors of JC and BK Polyomaviral Infection

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Abstract

JCPyV and BKPyV are common human polyomaviruses that cause lifelong asymptomatic persistent infections in their hosts. In immunosuppressed individuals, increased replication of JCPyV and BKPyV cause significant disease. JCPyV causes a fatal and rapidly progressing demyelinating disease known as progressive multifocal leukoencephalopathy. BKPyV causes hemorrhagic cystitis and polyomavirus associated nephropathy in bone marrow transplant recipients and in renal transplant recipients respectively. There are no specific anti-viral therapies to treat polyomavirus induced diseases. Based on detailed studies of the structures of these viruses bound to their receptors we screened several compounds that possessed similar chemical space as sialic acid for their ability to bind the virus. Positive hits in the assay were restricted to gallic acid based compounds that mimic the viruses known cellular glycan receptors. Pre-treatment of virions with these inhibitors reduced virus infection in cell culture and as such may form the basis for the development of virion specific antagonists to treat these infections.

Keywords

JCPyV; polyomavirus; BKPyV; progressive multifocal leukoencephalopathy; hemorrhagic cystitis; polyomavirus nephropathy; virus receptors; gallic acid

1. Introduction

The human polyomaviruses, JCPyV and BKPyV, infect greater than 50% of the human population worldwide (Kean et al., 2009; Knowles, 2006; Knowles et al., 2003). These viruses establish life-long persistent infections that are typically asymptomatic. In people

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whose cellular idrachenbergimmune responses are blunted, JCPyV and BKPyV cause significant disease. JCPyV causes a devastating and rapidly progressing central nervous system (CNS) demyelinating disease known as progressive multifocal leukoencephalopathy (PML)(Aksamit, 2008; Berger, 2011; Berger and Clifford, 2011; Ferenczy et al., 2012). PML occurs when the virus gains access to the CNS and infects oligodendrocytes and astrocytes. The disease occurs in 2–5% of AIDS patients and in approximately 1% of patients being treated with immunomodulatory drugs for multiple sclerosis and other autoimmune diseases (Bloomgren et al., 2012; Hellwig and Gold, 2011; Simpson, 2011). To date there are no specific treatments for JCPyV infection or PML and the time from diagnosis to death is one to two years.

Complications of BKPyV infection include the development of polyomavirus associated nephropathy (PVN) in kidney transplant recipients and hemorrhagic cystitis (HC) in cancer patients undergoing bone marrow transplantation (Bedi et al., 1995; Hirsch, 2005; Hirsch and Steiger, 2003; Xie et al., 2009). Reactivation of BKPyV in transplant patients leads to increased viral growth and significant cytopathology in the kidney (Hirsch et al., 2002). The incidence of PVN in renal transplant patients is between 5% and 10%, and approximately 20% of these patients will lose the graft (Drachenberg et al., 2007; Ramos et al., 2002; Schaub et al., 2010). The incidence of HC can be as high as 50% in adult bone marrow transplant patients. It is currently impossible to predict which patients will develop these complications and there are limited treatment options for those who develop the disease.

The cell surface receptor of the human polyomavirus JCV is a complex consisting of a serotonin receptor and the linear sialic acid containing pentasaccharide LSTc (Assetta et al.; Fonseca-Elphick et al., 2004; Maginnis et al., 2010; Neu et al., 2010b). The BKPyV recognizes disialic acid gangliosides GD1b, GT1b, GD3, and GD2 as receptors (Low et al., 2006; Tsai et al., 2003). The atomic structures of these interactions have been characterized in detail and serve as the basis for screening small molecules that recognize the virus surface (Neu et al., 2013; Neu et al., 2010a). A small set of chemical compounds that mimic sialic acid were screened by passing them over magnetic beads coated with the JCPyV VP1 protein. These compounds included molecules that occupy a similar chemical space as sialic acid, as well as compounds containing polyhydroxyl and polyphenolic groups. Compounds that bound to VP1 were then assayed for their ability to inhibit virus infection and virus binding to cells. We found that –(–)epigallocatechin gallate (EGTG), –(–)epigallocatechin (EGT), and tannic acid all reduced virus binding and infection, at doses that were relatively non-toxic. These molecules are potential drug leads that may advance to novel therapeutics to reduce the burden of polyomavirus induced disease.

2. Materials & Methods

2.1 Cell Lines, Viruses, Solvents and Chemicals

293FT cells are derived from human embryonal kidney cells transformed with the SV40 large T antigen (Invitrogen Life Technologies), SVGA cells are subclones of the SV40 origin-defective transformed human glial cell line SVG (Major et al., 1985). Vero cells are a monkey kidney epithelial cell line. 293FT were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented to contain 10% fetal bovine serum (FBS) (Mediatech,

Inc.), 0.1 mM non-essential amino acids (NEAA), 6 mM L-glutamine, 1 mM sodium pyruvate, and 500 mg/ml geneticin. Vero cells were grown in MEM with 5% FBS. All cells were grown in a humidified incubator at 37°C and 5% CO₂. JCPyV and BKPyV were propagated and purified as previously described (36). JCPyV and BKPyV were labeled with Alexa Fluor 488 according to manufacturer's instructions (Invitrogen). Briefly, purified JCPyV or BKPyV was dialyzed extensively against 0.1M carbonate buffer, pH 8.3, and then labeled with Alexa Fluor 488 at a molar ratio of 200:1 for 1 h at 21°C. Labeled JCPyV and BKPyV were then dialyzed against Buffer A, aliquoted, and stored at -80°C until use. Pseudoviruses were produced by as previously described (Gee et al.). Pseudovirus infection was measured at 72 hours post-infection from supernatant using the BioLux® *Gaussia* Luciferase Assay Kit (NEB cat # E3300L). Cell toxicity was measured using the Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay (MTS) kit (Promega cat # G5421). Viral infection was quantified by immunofluorescent staining of VP1, using the monoclonal antibody Pab597 and Alexa fluor 488, and counting the number of positive nuclei per field.

To measure binding, directly labeled JCPyV or BKPyV was incubated for 1h on ice in the presence or absence of compound at the indicated concentrations. Virus-compound complexes were then bound to 10⁶ SVGA or Vero cells for an additional hour on ice. Cells were washed in PBS to removed unbound virus, fixed with 1% paraformaldehyde and analyzed by flow cytometry (BD FACSCanto II).

Antibodies used in magna bead experiments were purchased from GE Healthcare Life Sciences. MagnaBind carboxyl derivatized beads (Product No. 21353) and EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride] (Product No. 22980) were from Pierce. Phosphate buffered saline (PBS) was purchased from Thermo Scientific (Product No. 28372). The other reagents were obtained from Sigma unless otherwise noted.

2.2 Magna Bead Assay

A panel of small molecules that are similar in chemical space to sialic acid were prepared as 10 mM stock solutions in LC-MS grade water (Fisher) on the day of the experiment and kept in ice. These molecules included LSTc, sialic acid, gallic acid, tannic acid, (-)-epigallocatechin gallate, (-)-epigallocatechin, acetobromo-alpha-D-galactose, shikimic acid, arabic acid, and ellagic acid. JCPyV VP1 pentamers were either directly conjugated to beads (tube 1) or were indirectly conjugated to beads (tube 2) using an antibody to the HIS tag present on the c-terminus of VP1 according to the manufacturers directions. In short, 200 µl MagnaBind carboxyl derivatized beads (Pierce) were transferred to two microcentrifuge tubes each (tube 1 and tube 2) and placed near a magnet for 1 min. After aspirating and discarding the supernatant, the beads were washed four times with PBS pH 7.2 and then incubated with 500 µL of EDC-NHS (1:1, 1.5 equiv) mixture in *N*-Methylpyrrolidone (NMP) for 2 h at RT. The beads were removed using a magnet and washed with PBS. 200 µL of JCPyV VP1 pentamers (4.363 mg/mL in PBS) were added to tube 1 and 500 µL of a 1:10 dilution anti-His antibody (40 µg/µL in PBS, GE Healthcare Life Sciences) to tube 2; both were incubated with gentle shaking for 2 hours in ice. Following incubation, the supernatant was aspirated from the coupled beads using a magnet and beads were washed three times with PBS. The same concentration of JCPyV pentamers was then incubated with

tube 2 containing the anti-HIS conjugated beads for 2 hours on ice with gentle shaking. These beads were then washed 3X in PBS. Beads from both preparations were suspended in 1ml PBS and divided into two sets of 4 (tube 1-A to -D and tube 2-A to -D). 50 μ L of each compound was added to each tube as a mixture and incubated for 15 h at 4 °C. Following incubation, supernatant was removed and the beads were agitated and washed two times with PBS. Then, 100 μ L of LC-MS grade methanol was added to each tube and the solutions were heated to 40 °C for 10 min to denature the protein and release the small molecules. The beads were removed using the magnet and the supernatant was analyzed by LC/MS. Similar results were obtained from both sets of beads (directly or indirectly conjugated).

2.3 LC–MS analysis

Chromatographic analysis was carried out with a Shimadzu 8030 LC-MS equipped with an online solvent degasser unit, 7-port, 6-position high pressure column switching valve allows multi-column selection, a refrigerated auto sampler, and a column oven set to 40 °C. Separation was achieved on an Aeris Widespore XB-C18 column (150 mm $\times$ 4.6 mm, 200 Å, 3.6 μ m) from Phenomenex (Torrance, CA, USA), using mixtures of solutions A (0.1% formic acid in LC-MS grade water) and B (0.1% formic acid in LC-MS grade methanol) as mobile phase. The amount of solution B in the mobile phase (expressed as % v/v) was linearly increased from 25% to 75% during the first 30 min, followed by a second linear increase to 95% B from 30 to 32 min. The composition of the mobile phase was kept constant at 95% B for 3 min, and then returned to the initial conditions in 0.1 min. To ensure equilibration, a post-run time of 10 min at 25% B was defined. The total chromatographic analysis time per sample was 35 min. Samples were analyzed with an injection volume of 20 μ L and a constant flow rate of 500 μ L/min. The compounds were analyzed using positive (Q3 scan), negative ion (Q3 scan) modes and optimized selected ion monitoring (SIMs). The library compounds were previously analyzed using the LC-MS to obtain the retention time with mass spectral confirmation. All appropriate controls were carried out including MagnaBind carboxyl derivatized beads alone as a negative control. The method was validated using LSTc as a positive control.

3. Results

3.1 Magnetic Bead Screening

JCPyV VP1 pentamers were coupled to magnetic beads either directly or indirectly and the beads were used to competitively pan a mixture of ten small molecules with the potential to bind JCPyV VP1 (Fig. 1). The beads were washed and the small molecules released by gentle heating in methanol. The beads were removed using a magnet and the supernatant injected into the LC-MS system to identify molecules that bound. Three molecules (EGTG, EGT, and tannic acid) from the mixture of 10 compounds were identified as positive binders (Fig. 2).

3.2 Screening hits using luciferase expressing pseudovirus

The compounds were screened for their ability to inhibit transduction of cells by both JC and BK luciferase expressing pseudoviruses (JCPsV, BKP sV). BKP sV was used in addition to JCPsV as there is considerable conservation of amino acid sequence and structure between

these viruses and both require sialic acid for cell binding. EGTG, EGT, and tannic acid significantly inhibited transduction by both JCPsV and BKPsV in a dose dependent manner (Fig. 3). We also screened the other seven molecules for their ability to inhibit transduction. LSTc and sialic acid both inhibited transduction and this is consistent with previously published work so we did not include those data here. The other molecules were either highly toxic to the cells or had no effect on transduction by either virus (data not shown).

3.3. Infection assay with authentic virus

We next asked whether these same concentrations of compounds could inhibit infection of cells by authentic virus. SVGA cells and Vero cells were incubated with JCPyV and BKPyV in the presence and absence of EGTG, EGT, tannic acid, or vehicle control. The cells were then washed and scored at 72 hours post-infection for the expression of the late viral protein VP1. EGTG, EGT, and tannic acid significantly inhibited infection of both viruses at doses that were not toxic to the cells (Fig. 4).

3.4 Virus binding assay

We next asked whether the compounds ability to inhibit infection with authentic virus would correlate with a decreased ability of the treated viruses to bind the cells. JCPyV and BKPyV were labeled with Alexa-fluor 488 and incubated in the presence and absence of each compound. Untreated and treated viruses were then incubated with cells and binding scored by flow cytometry. EGTG, EGT, and tannic acid all reduced JCPyV virus binding to cells. EGTG and TA, but not EGT, reduced binding of labeled BKPyV to cells. (Fig 5).

In summary, we developed an assay to rapidly screen compounds that bind to virus pentamers immobilized on magnetic beads. The three compounds that were positive hits in the assay all reduced virus infection by interfering with initial attachment of the virus to the cells.

4. Discussion

Human polyomaviruses cause significant morbidity and mortality in immunosuppressed patients and treatment options are limited. We sought to develop a screen that would allow us to select for small molecules capable of binding to the viruses' outer shell that is made up of a single viral protein known as VP1. A truncated version of VP1 when expressed in *E. coli* and purified will self assemble into pentameric capsomeres that recapitulate the early events involved in virus infection (Nelson et al., 2012; Salunke et al., 1986). Pentamers of polyomaviruses have been used extensively to characterize virus structure in their native state or when bound to ligands such as LSTc (Stehle et al., 1996; Stehle and Harrison, 1996; Stehle and Harrison, 1997; Stehle et al., 1994). We therefore used pentamers as a model system to pan for small molecules capable of binding. To do this the pentamers were conjugated to magnetic beads either directly or indirectly using an anti-HIS antibody and then both sets of beads were used to pan molecules that were similar in structure to the JC virus attachment protein LSTc. Ten molecules were screened as a mixture and three of these bound well to the immobilized pentamers. The same results were obtained whether we used beads with directly coupled pentamers or beads with pentamers coupled using an antibody

against a HIS tag on the c-terminal tail of VP1. These three compounds as well as the other compounds that did not bind were tested for their ability to inhibit transduction of cells by luciferase expressing pseudoviruses. We used JCPyV and BKPyV based pseudoviruses initially thinking that the BKPyV would serve as a negative control as we didn't screen the compounds for binding to BKPyV derivitized beads. Interestingly the three hits in our bead binding assay reduced transduction of cells by both JC and BK pseudoviruses and inhibited infection of cells by both viruses. In hindsight this is not surprising as both viruses share a requirement for sialic acid binding, and all of these small molecules mimic interactions that might be shared between these viruses. The binding data however clearly show differences between JCPyV and BKPyV as only one of the compounds was able to reduce binding of labeled BKPyV to cells whereas all three reduced JCPyV binding to cells. There may be several explanations for this. First, the compounds were not screened against BKPyV pentamers bound to beads but only against JCPyV pentamers bound to beads with the JCPyV receptor (LSTc) as a positive control. This receptor is not shared with BKPyV which uses disialic acid gangliosides to bind and infect cells. It could be that these two compounds bound to regions on the BKPyV capsid that did not occlude the receptor binding pocket but still disrupted infection by sterically interfering with factors that could influence internalization. It could also be that subsequent downstream effects are disrupted by having these chemicals bound to the capsids. We saw a similar phenomenon when we treated virus with human alpha defensins. In the case of JCPyV the defensins bound to the virus and reduced infection at a step downstream of cell surface binding (Zins et al., 2014, J. Virol., 88:948–960). This same defensin inhibited BKPyV infection by disrupting binding (Dugan et al., 2008, JBC. 283:31125–31132). The compounds that did not bind to the beads did not inhibit transduction so these were not tested further in our assays.

Our data suggest that viral attachment proteins can be coupled to beads and used to effectively screen large families of compounds in a high throughput fashion to identify potential antiviral targets.

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Highlights

- JCPyV and BKPyV are common polyomaviruses that cause disease in immunocompromised patients.
- Magnetic bead based screening coupled to MassSpec analysis identified compounds that bound to pentameric JC VP1.
- Several of the compounds reduced binding and infection of cells by both JCPyV and BKPyV.
- This is an efficient way to screen for compounds capable of inhibiting sialic acid dependent viruses.
- These compounds may be developed into effective anti-virals.

Figure 1. Assay Scheme for Magnetic Bead Screening

JC VP1 pentamers were immobilized on magnetic beads either directly or indirectly. Small molecules were then incubated as a mixture with the beads overnight at 4°C. The beads were pelleted, washed 3 times, and small molecules were then released by heating to 40°C in methanol. The beads were removed using the magnet and the supernatant was injected into a Shimadzu 8030 LC-MS-MS system.

Figure 2. Three molecules identified by LC-MS analysis

LC Chromatogram of the supernatant from magna-bead-assay after incubation of a mixture of EGTG, EGT, and TA with JCPyV VP1-coupled magnetic beads that was injected into a Shimadzu 8030 LC-MS system. The MS spectra of respective peaks are shown. Each peak was analyzed using positive (Q3 scan), negative ion (Q3 scan) modes and selected ion monitoring (SIMs).

Figure 3. JC and BK Pseudovirus Assay

JCPsV (Panel A, SVGA cells) or BKPsV (Panel B, Cos-7 cells) were incubated with vehicle control (No Drug-DMSO) or with increasing doses of EGTG, EGT, or tannic acid as indicated. Cells were then challenged with the pseudoviruses and relative luciferase expression was assayed at 72 hours post-infection. At least three replicates were tested for each sample.

Figure 4. JC and BK Infectivity

JCPyV (Panel A, SVGA cells) or BKPvV (Panel B, Vero cells) were incubated with vehicle control (No Drug-DMSO) or with increasing doses of EGTG, EGT, or tannic acid as indicated. Cells were then challenged with the viruses and infection scored 72 hours later by indirect immunofluorescence assay for the late viral protein VP1. Three replicates were tested for each sample. The compounds were not toxic in either SVGA cells (Panel C) or Vero cells (Panel D).

Figure 5. JC and BK binding assay

Labeled JCPyV (panel I, SVGA cells) or BKPvV (panel II, Vero cells) were incubated with cells in the presence and absence of increasing concentrations of EGTG, EGT, and tannic acid (TA). Binding to cells was assayed by flow cytometry. EGTG, EGT, and TA all reduced binding of JCPyV to cells in a dose dependent manner. EGTG and TA, but not EGT, reduced binding of labeled BKPvV to cells.