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USC-087 Protects Syrian Hamsters Against Lethal Challenge with Human Species C Adenoviruses

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Abstract

Human adenoviruses (AdV) cause generally mild infections of the respiratory and GI tracts as well as some other tissues. However, AdV can cause serious infection in severely immunosuppressed individuals, especially pediatric patients undergoing allogeneic hematopoietic stem cell transplantation, where mortality rates are up to 80% with disseminated disease. Despite the seriousness of AdV disease, there are no drugs approved specifically to treat AdV infections. We report here that USC-087, an *N*-alkyl tyrosinamide phosphonate ester prodrug of HPMPA, the adenine analog of cidofovir, is highly effective against multiple AdV types in cell culture. USC-087 is also effective against AdV-C6 in our immunosuppressed permissive Syrian hamster model. In this model, hamsters are immunosuppressed by treatment with high dose cyclophosphamide. Injection of AdV-C6 (or AdV-C5) intravenously leads to a disseminated infection that resembles the disease seen in humans, including death. We have tested the efficacy of orally-administered USC-087 against the median lethal dose of intravenously administered AdV-C6. USC-087 completely prevented or significantly decreased mortality when administered up to 4 days post challenge. USC-087 also prevented or significantly decreased liver damage caused by AdV-C6 infection, and suppressed virus replication even when administered 4 days post challenge. These results imply that USC-087 is a promising candidate for drug development against HAdV infections.

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Supplementary data (Appendix A)

USC-087 preliminary analysis (NMR and LC/MS spectra); flash chromatography purification details; NMR and MS spectra, combustion analysis data for purified sample; body weight changes.

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INTRODUCTION

Human adenoviruses (HAdV) have a linear duplex DNA genome that encodes about 35 genes and is enclosed in an icosahedral protein capsid without a lipid membrane (reviewed in (Berk, 2013; Ison and Hayden, 2016; Lion, 2014; Wold and Ison, 2013). There are more than 70 types (genotypes, previously referred to as serotypes) of HAdV that are grouped into 7 species (A-G). Two Species C types discussed in this article are named HAdV-C5 and HAdV-C6 (for the sake of brevity, we will refer to these viruses as Ad5 and Ad6, respectively). Ad5 and Ad6 are ubiquitous, infect most children, and generally cause asymptomatic or fairly mild infection of the respiratory, gastrointestinal, ocular, or other tissues; the infections usually are self-limiting in healthy individuals.

The most serious HAdV infections occur in immunocompromised individuals (reviewed in (Ip et al., 2013; Ison and Hayden, 2016; Lion, 2014; Martinez-Aguado et al., 2015; Wold and Ison, 2013). Especially at risk are young children undergoing allogeneic hematopoietic stem cell transplantation (allo-HSCT), where the incidence of infection ranges from 5%–6% to 42%–47% depending on the study (Ison and Hayden, 2016; Lion, 2014; Sandkovsky et al., 2014). Mortality rates are as high as 26% with symptomatic infection and 80% for disseminated disease. Many AdV types can be isolated from these patients but Species C types predominate (Lion, 2014). With pediatric solid organ transplants, incidence is 4% to 38% in liver transplants, 7%–50% in heart and lung transplants, and 4% to 57% in small bowel recipients. Mortality rates are up to 54% in liver transplants and 18% in kidney recipients. In adult allo-HSCT, the incidence ranges from 3% to 15% (Lion, 2014).

With disseminated AdV disease, multiple organs are involved, and AdV DNA can be detected by PCR in peripheral blood, urine, bronchoalveolar fluid, and cerebral spinal fluid; death is associated with persisting or increasing AdV levels in peripheral blood and multi-organ failure (Lion, 2014). Risk factors include young age, receipt of T cell-depleted and/or mismatched grafts, use of the anti-CD52 antibody alemtuzumab, severe lymphopenia with low CD3⁺ T cell counts, acute graft versus host disease, early and persistent isolation of AdV from multiple sites, high initial AdV levels in the blood, and increasing AdV levels in serial stool samples beyond 10⁶ virus copies per gram (Ison and Hayden, 2016; Lion, 2014; Lion et al., 2010; Sandkovsky et al., 2014).

There are no drugs approved specifically to treat AdV infections, despite the seriousness of disseminated AdV disease in immunocompromised individuals and in otherwise healthy individuals with life-threatening respiratory infections. Intravenous gamma globulins have been employed, and cidofovir (CDV) is used routinely in many transplant clinics (Ison and Hayden, 2016; Lion, 2014; Lion et al., 2010). CDV, an acyclic nucleoside phosphonate, is an analog of cytidine monophosphate. Following entry into the cell, it is converted by cellular anabolic kinases into CDV-diphosphate, which is an analog of cytidine triphosphate and used as a substrate by the AdV DNA polymerase more efficiently than by cellular DNA polymerases. Although it is not an obligate chain terminator, incorporation of the compound into AdV DNA eventually leads to termination of DNA synthesis. Recent studies report that CDV appears to be effective against HAdV not only in immunocompromised transplant patients, but also in adult immunocompetent patients with HAdV respiratory infections (Kim

et al., 2015; Lee et al., 2017). A significant problem with CDV is poor cellular uptake (because of the charged phosphate group at physiological pH) (Holy, 2003). Also, it is a substrate for organic anion transporter 1, which leads to accumulation of CDV in renal tubules and nephrotoxicity (Cundy, 1999). Treatment with CDV is also associated with neutropenia and leukopenia that contribute to its dose limiting toxicity (Lalezari et al., 1997).

Brincidofovir (BCV, previously named CMX001) is being developed as an antiviral against AdV and a number of other DNA viruses (reviewed in (Florescu and Keck, 2014; Hostetler, 2009)). BCV (3-hexadecyloxy-1-propanol-cidofovir) is a lipophilic derivative of CDV (Hostetler, 2010). The lipid moiety not only improves oral bioavailability, but also facilitates the uptake of BCV into cells. Within cells, the lipid group is cleaved off by cellular phospholipase C, leaving charged CDV which cannot exit cells easily (Hostetler, 2010). Unlike CDV, BCV is not a substrate for the human organic anion transporter 1 and does not accumulate in renal tubules or cause nephrotoxicity (Ciesla et al., 2003). BCV inhibits the replication of almost all DNA viruses (Lanier et al., 2010), including multiple AdV types in cell culture, with EC_{50} values close to 0.02 μ M (Hartline et al., 2005). In a case report, BCV suppressed a disseminated HAdV-C2 infection in a pediatric allo-HSCT patient (Paolino et al., 2011). In a recent randomized placebo-controlled multicenter phase II clinical trial, preemptive treatment with BCV was evaluated in 48 pediatric and adult allo-HSCT patients with asymptomatic HAdV viremia (Grimley et al., 2017). BCV treatment biweekly was reported to reduce all-cause mortality and also appeared to be associated with an early and marked decline in HAdV viremia in subjects with HAdV levels of >1,000 genome copies/ml at baseline, although the results were not statistically significant (Grimley et al., 2017). The most common adverse event associated with oral BCV treatment was diarrhea. The most common HAdVs observed in this trial were members of Species C (Grimley et al., 2017).

USC-087, an *N*-alkyl tyrosinamide phosphonate ester prodrug of HPMPA (Holy and Rosenberg, 1987a, b), the adenine analog of cidofovir, was evaluated against infection in cell culture by multiple HAdV types from different species. Orally administered USC-087 (Fig. 1A) was further evaluated for oral efficacy following intravenous (i.v.) infection by Ad5 or Ad6 in the permissive immunosuppressed Syrian hamster model developed in the Wold laboratory (Toth et al., 2008; Wold and Toth, 2012). In this model, young (to reflect the age of the target human population) Syrian hamsters are immunosuppressed by treatment with high-dose cyclophosphamide. The hamsters are then infected i.v. with Ad5 or Ad6, which replicate to high levels in the liver and other organs, causing pathology or mortality depending on the infecting dose of virus. Using this model, which closely resembles the disseminated HAdV infection and HAdV disease seen in immunocompromised human patients, we previously showed that BCV (Tollefson et al., 2014; Toth et al., 2008), CDV (Tollefson et al., 2014), ganciclovir (Ying et al., 2014b), and valganciclovir (Toth et al., 2015) are active against Ad5 replication and pathogenesis. We now report that orally-administered USC-087 is highly efficacious at a non-toxic dose against Ad5 or Ad6 replication, pathogenesis, and mortality following i.v. infection.

MATERIALS AND METHODS

Cells and viruses

A wild-type human Ad6 isolate (VR-6; Tonsil 99) was purchased from ATCC and cultured and purified as described in (Tollefson et al., 2007). A wild-type Ad5 (named wt500) was plaque purified from a virus stock purchased from the ATCC. This isolate has been used in previous studies (Tollefson et al., 2014; Tollefson et al., 2017; Toth et al., 2015; Ying et al., 2014b). The titer of the virus stocks was determined by plaque assay (Tollefson et al., 2007). For the *in vitro* studies, wild-type stocks of HAdV-B3, HAdV-C5, HAdV-C6, HAdV-B7, and HAdV-D8 were purchased from ATCC and propagated on human foreskin fibroblast cells. Low passage primary human foreskin fibroblast cells were prepared and passaged as described previously (Prichard et al., 2013).

Antiviral compounds

USC-087 was prepared from commercially purchased HPMPA (Rasayan, Inc.; Angene, Inc) and Boc-L-tyrosine (Sigma-Aldrich) using the synthetic route of McKenna (McKenna et al., 2017). A sample of USC-087 made by the same route on a multigram-scale was supplied by a CRO, SRI International (Menlo Park, CA, USA) and then further purified at the USC laboratory by an automated flash chromatography method described herein (Supplemental data; 99%: ^1H and ^{31}P NMR, LC/MS, C,H,N elemental analysis; used in the *in vivo* studies). The CDV control for the *in vitro* studies was kindly provided by Gilead Sciences, Foster City, CA. CDV for *in vivo* studies was obtained from the National Institute of Allergy and Infectious Diseases (NIAID).

Antiviral Assays

In vitro antiviral assays were performed in primary human foreskin fibroblast cells and included a concurrent assessment of cytotoxicity using the same number of cells and at equivalent levels of compound exposure such that accurate selective index (SI) values could be obtained.

The cytopathic effect (CPE) reduction assays were performed in assay medium consisting of MEM with Earle's salts, 2% FBS and standard concentrations of L-glutamine, penicillin, and gentamycin. Cells were seeded into 384-well microtiter plates and were subsequently incubated at 37°C in a humidified 5% CO₂ incubator for 24 h to allow the formation of confluent monolayers. Dilutions of test compounds were prepared in the plates in a series of 5-fold dilutions in duplicate wells to yield final concentrations that range from 300 to 0.1 μM or from 10 to 0.003 μM . Monolayers were then infected at a multiplicity of infection of 0.005 TCID₅₀/cell with Ad5 and incubated further until 100% CPE was observed in the virus control wells. Cytopathology was determined by the addition of CellTiter-Glo reagent (Promega, Madison, WI) according to the manufacturer's suggested protocol. Concentrations of test compounds sufficient to reduce CPE by 50% (EC₅₀) were interpolated from the experimental data. Cytotoxicity was also determined with CellTiter-Glo and concentrations of the compounds that decreased cell viability by 50% (CC₅₀) were also calculated from the data and selective index (SI) values were calculated as the CC₅₀/EC₅₀ as a measure of antiviral activity.

Syrian hamsters

Syrian hamsters (*Mesocricetus auratus*) were purchased from Envigo (Harlan, Indianapolis, IN) at approximately 80 g body weight. All studies were approved by the Institutional Animal Care and Use Committee of Saint Louis University and were conducted according to federal and institutional regulations.

Dosing Solution Preparation

For biological tests, USC-087 was formulated as a stirred suspension (24 h before use, experiments depicted in Figs. 2 and 3) or stable dispersion (experiments depicted in Fig. 4) in 0.5% aqueous CMC (carboxymethylcellulose sodium salt) after brief sonication (Branson 2510 sonicator) at room temperature. In the preferred procedure, CMC (low viscosity), (Sigma-Aldrich [Lot # SLBS7273]) (10.0 g) was added carefully to 1990 mL of deionized water in portions, and the mixture was gently shaken until homogenous (4 h at room temperature). To evaluate the stability of this dispersion, 12 mg of USC-087 in 12 mL of the CMC solution was prepared as described and monitored visually at room temperature (Fig. S8). No loss of homogeneity could be observed visually after 48 h.

Infection of hamsters with adenovirus; treatment with USC-087 or CDV

The hamsters were immunosuppressed using cyclophosphamide (CP) (Toth et al., 2008). CP was administered intraperitoneally (i.p.) twice weekly for the duration of the study, starting with an induction of 140 mg/kg, and then at a dose of 100 mg/kg for all subsequent injections.

Virus challenge—After 3 injections of CP, Ad5 or Ad6 was injected i.v. (via the jugular vein) after anesthetizing the animals with ketamine/xylazine. Three experiments were performed; a pilot prophylactic experiment, a dose range-finding prophylactic experiment, and a therapeutic experiment, for which we used different challenge schedules, described below.

In the pilot experiment, we used female hamsters and Ad5 as the challenge virus. The challenge dose was 2×10^{11} plaque forming units (PFU)/kg, which is the LD₅₀ for Ad5 with female hamsters.

In the dose range experiment we used Ad6, because it is more pathogenic than Ad5 (Tollefson et al., 2017). The challenge dose was 3×10^{10} PFU/kg, which is the LD₉₀ of Ad6 for female hamsters.

For the therapeutic experiment, we used the most stringent conditions; challenging male hamsters, which are more susceptible to HAdV infection than females (Ying et al., 2018), with the more pathogenic Ad6 (Tollefson et al., 2017). The challenge dose was 1.7×10^{10} PFU/kg, which is the LD₅₀ of Ad6 for male hamsters.

Treatment with antiviral compounds—USC-087 was administered through oral gavage (p.o.) in 1 ml of 0.5% carboxymethyl-cellulose (CMC) in an aqueous solution. For the prophylactic-type experiments (Pilot and Dose range), USC-087 administration started

one day before virus challenge and continued daily for the duration of the experiment (Figs. 2A and 3A). For the therapeutic experiments, drug administration started one day before, or 1, 2, 3, or 4 days post challenge, and then continued daily for the course of the study (Fig. 4A).

For experiments in which CDV was used as a positive control, CDV was injected i.p. in phosphate buffered saline (PBS) starting one day before virus challenge, and then 3 times weekly for the duration of the experiment (Fig. 3A).

Endpoints—Treatment groups consisted of 15 animals. All hamsters were observed and weighed daily. Five hamsters of each group were sacrificed at 5 days (for the prophylactic study) or at 7 days (for the therapeutic study) post Ad6 challenge. At necropsy, serum and liver were collected. The virus was extracted from the liver and was quantified by 50% Tissue Culture Infectious Dose (TCID₅₀) assay in HEK-293 cells (Toth et al., 2008), and serum was assayed for liver transaminase levels. The remaining ten hamsters in each group were used for a survival study.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software). Two-way ANOVA was used to compare body weight changes. For serum transaminase levels and virus burden in the liver, the overall effect was calculated using Kruskal-Wallis test, and comparison between groups was performed using Mann-Whitney U-test. $P = 0.05$ was considered significant.

RESULTS

Chemistry

¹H and ³¹P NMR (Varian VNMRS-500 NMR spectrometer) and LC/MS (LCQ Deca XP Max). Analysis of the multigram scale sample of USC-087 supplied by the CRO indicated the presence of ~7% of phosphorous-containing impurities (Fig. S1). ¹H NMR analysis (Fig. S2) also identified several impurities. LC/MS analysis (Fig. S3) suggested that one impurity was *N*-hexadecyl tyrosine amide, presumably from unreacted synthon (vd.(McKenna et al., 2017)).

To obtain highly purified USC-087 suitable for biological studies requiring multigram amounts of the prodrug, we used automated flash chromatography (ISCO Teledyne CombiFlash Rf+) on a silica gel column (Fig. S4). This method is convenient and practical for gram-scale preparations of highly purified USC-087. The isolated product satisfied C,H,N combustion analysis, was >99% pure by ¹H (Fig. S5) and ³¹P NMR (Fig. S6) and had the expected MS peak (Fig. S7). It was unchanged after 1 month storage at room temperature or at 4 °C.

Tissue culture

The antiviral activity of USC-087 against Ad6 was evaluated in a series of *in vitro* CPE assays to identify the most potent analogs of S-HPMPA. These studies indicated that

USC-087 was more than 600-fold more potent than HPMPA and more than 75-fold more potent than the CDV control (Fig. 1B). The enhanced potency of the compound was greater than the increased cytotoxicity and resulted in an improvement of the SI values from 3.8 to >20 when compared to HPMPA. The antiviral activity of the compound was also assessed against HAdV-B3, HAdV-B7, HAdV-C5, and HAdV-D8 with EC₅₀ values of 0.015, 0.002, 0.025, and 0.035 μ M, respectively; these values are similar to the antiviral activity against Ad6 (EC₅₀ of 0.025 $\pm$ 0.026 μ M). These data taken together indicated that USC-087 was a potent inhibitor of HAdV and that it warranted further study in animal models of infection.

Prophylactic administration of USC-087 prevents morbidity and mortality induced by Ad5 infection in Syrian hamsters

To assess the efficacy of USC-087 *in vivo*, we performed an experiment with hamsters challenged i.v. with Ad5 (Fig. 2A). The animals were immunosuppressed with CP, and sorted into 5 groups, two uninfected and three Ad5-infected. The uninfected (injected with virus vehicle, i.e. PBS) groups were: (1) virus vehicle i.v. and drug vehicle p.o. (Vehicle-Vehicle), and (2) virus vehicle i.v. plus 10 mg/kg USC-087 p.o. (Vehicle-USC-087). Animals in the infected groups received 2×10^{11} PFU of Ad5 per kg i.v. There were three such groups, in which the animals received (1) Ad5 i.v. and drug vehicle p.o. (Ad5-Vehicle), (2) Ad5 i.v. plus 10 mg/kg USC-087 p.o. (Ad5-USC-087), or (3) Ad5 plus CDV i.p. (Ad5-CDV). CDV treatment was started with a 37 mg/kg inducer dose and continued at 20 mg/kg, 3 times weekly. Both USC-087 and CDV treatments started at 1 day before virus challenge, and then continued daily (q.d.) or 3 times weekly, respectively.

In-life observations—There were 5 treatment-related deaths in the study, all in the Ad5-Vehicle group (Fig. 2B). HAdV infection induced body weight loss, which was reversed by both USC-087 and CDV treatment (Fig. S9). A small, but significant decrease in body weight gain was observed for the Vehicle-USC-087 group compared to the Vehicle-Vehicle group (data not shown).

Necropsy—At 5 days post challenge, 2 of the 5 Ad5-infected, vehicle-treated hamsters had yellow mottled liver with enlarged gall bladder. The Ad5-infected hamsters sacrificed moribund had yellow, mottled, friable liver with enlarged gall bladder. The mottled appearance of the liver can indicate ongoing hepatocellular necrosis, while the enlarged gall bladder can result from reduced food intake or compression of the bile duct, all consequences of adenovirus infection. At 13 days post challenge, no significant findings were noted for any of the animals.

Clinical chemistry—At the Day 5 necropsy, serum was collected and was analyzed for transaminase levels. The serum alanine transaminase (ALT) levels were elevated for all the Ad5-infected, vehicle-treated hamsters (Fig. 2C). USC-087 or CDV treatment mitigated liver pathology; the serum ALT levels were only mildly elevated for the Ad5-infected, USC-087- and CDV-treated hamsters (Fig. 2C).

Virus burden in the liver—At 5 days post challenge, liver samples were collected at necropsy and were analyzed for infectious virus burden. Vehicle-treated, Ad5-infected

animals had very high virus burden in the liver (Fig. 2D). Oral USC-087 (dosed at 10 mg/kg) or CDV (dosed at 20 mg/kg) i.p. reduced the virus burden in the Ad5-infected animals (Fig. 2D).

Orally dosed USC-087 is more efficacious than equimolar doses of i.p. dosed CDV

Female Syrian hamsters were treated with 1, 3, or 10 mg/kg of USC-087 p.o. (Ad6-USC-087 1, 3, and 10 mg/kg groups) or CDV doses i.p. that were molar equivalents of CDV matching the three USC-087 doses (Ad6-CDV 0.8, 2.4, and 8 mg/kg groups), or left untreated (Ad6-Vehicle group) (Fig 3A). Additionally, a group of animals was treated with the known effective i.p. dose of CDV (Ad6-CDV 20 mg/kg group). During the study, treatment with USC-087 and CDV continued daily or 3 times weekly, respectively. One day after the initiation of treatment with the drugs, the hamsters were injected with the LD₉₀ (3×10^{10} PFU/kg) of Ad6. We chose Ad6 as the challenge virus to increase the rigor of the experiment because it is more pathogenic in hamsters than Ad5 (Tollefson et al., 2017). Hamsters were observed and weighed daily. At 5 days after challenge, 5 animals were sacrificed from each group, while the remaining 10 hamsters were sacrificed at 14 days after challenge or when they became moribund.

In-life observations—There were 35 treatment-related deaths in the study: 11 in the Ad6-Vehicle group, 5 in the Ad6-USC-087 1 mg/kg group, 12 in the Ad6-CDV 0.8 mg/kg group, 6 in the Ad6-CDV 2.4 mg/kg group, and 1 in the Ad6-CDV 8 mg/kg group (Fig. 3B). Notably, there were no deaths in the Ad6-USC-087 3 mg/kg group or the Ad6-USC-087 10 mg/kg group. Hamsters in the Ad6-infected groups lost weight from the onset of the study; USC-087 and CDV treatment reversed body weight loss for Ad6-challenged hamsters in a dose-dependent manner (Fig. S10). A small, but significant decrease in body weight gain was observed for the Vehicle-USC-087 group compared to the Vehicle-Vehicle group (data not shown).

Necropsy—At 5 days post challenge, 5 of the 5 Ad6-infected, vehicle-treated hamsters scheduled for sacrifice had yellow mottled liver with enlarged gall bladder. There were 4 of 5, 5 of 5, 3 of 5, and 2 of 5 animals with such pathology in the Ad6-USC-087 1 mg/kg, Ad6-CDV 0.8 mg/kg, Ad6-CDV 2.4 mg/kg, and Ad6-CDV 8 mg/kg groups, respectively. No gross pathology was observed with the animals in the Ad6-USC-087 3 mg/kg, Ad6-USC-087 10 mg/kg, or Ad6-CDV 20 mg/kg groups. The Ad6-infected hamsters sacrificed moribund had yellow, mottled, friable liver with enlarged gall bladder. At 14 days post challenge, no significant findings were noted for any of the animals.

Clinical chemistry—At the Day 5 necropsy, serum was collected and was analyzed for transaminase levels. Data collected from animals that were sacrificed moribund between Days 5 and 8 are also included. The serum alanine transaminase (ALT) levels were elevated for all the Ad6-infected, vehicle-treated hamsters (Fig. 3C). USC-087 or CDV treatment mitigated liver pathology in a dose-dependent manner (Fig. 3C).

Virus burden in the liver—At 5 days post challenge, liver samples were collected at necropsy and were analyzed for infectious virus burden. Data collected from animals that

were sacrificed moribund between Days 5 and 8 are also included. Vehicle-treated, Ad6-infected animals had very high virus burden in the liver (Fig. 3D). USC-087 or CDV treatment of the Ad6-infected animals reduced the virus burden in a dose-dependent manner (Fig. 3D).

Therapeutic administration of USC-087 prevents morbidity and mortality induced by disseminated Ad6 infection in immunosuppressed male Syrian hamsters

To test for possible sex differences, this experiment was performed using male animals. The animals were immunosuppressed, and then injected i.v. with vehicle or the LD₅₀ of Ad6 (for male hamsters: 1.7×10^{10} PFU per kg). We administered USC-087 p.o. at 10 mg/kg q.d., starting at 1 day before, or 1, 2, 3, or 4 days after Ad6 injection (groups Ad6-USC-087 D -1, D +1, D +2, D +3, D +4, respectively) (Fig. 4A). An Ad6-infected group that did not receive drug, and groups that received virus vehicle and drug vehicle only or virus vehicle plus drug (started at 1 day before challenge) were used as controls.

The body weights and any signs of morbidity of the animals were recorded daily. At 7 days post challenge, 5 hamsters of each group (designated at the start of the experiment) were sacrificed; the remaining 10 hamsters were sacrificed at 14 days post challenge. Hamsters that became moribund before Day 14 were sacrificed as needed.

In life observations—We observed 55% mortality in the Ad6-Vehicle group, while no deaths occurred in the groups that received USC-087 starting from 1 day before or 1 or 2 days after challenge (Fig. 4B). We saw 10% and 15% mortality in the groups in which treatment started 3 or 4 days post challenge, respectively (Fig. 4B). Ad6-injected animals started losing weight at around 4 to 5 days post challenge (data not shown). The body weight loss was greatest in the Ad6-Vehicle group. By 8 days post challenge, Ad6-infected hamsters that received USC-087 starting from 1 day before or 1 or 2 days after challenge had mostly recovered and showed minimal body weight loss compared to untreated animals (Fig. S11). Drug treatment starting 3 or 4 days post challenge had a mitigating effect.

Necropsy—At 7 days post challenge, all the animals in the Ad6-Vehicle group, 1 in the Ad6-USC-087 D +3 group, and 2 in the Ad6-USC-087 D +4 group had yellow mottled liver with enlarged gall bladder. No such pathology was seen in the Ad6-USC-087 D -1, D +1, or D +2 groups. The hamsters sacrificed moribund had yellow, mottled, friable liver with enlarged gall bladder. At 14 days post challenge, no significant HAdV infection-related findings were noted for any of the animals.

Serum chemistry—Serum was collected at the 7 day sacrifice time point and from animals sacrificed moribund up to that time point. USC-087 treatment nearly completely prevented liver damage by Ad6 when administration started up to 2 days post challenge (Fig. 4C). The treatment was partially effective when started at 3 or 4 days after infection (Fig. 4C).

Virus burden in the liver—At 7 days post challenge, liver samples were collected at necropsy and were analyzed for infectious virus burden. Vehicle-treated, Ad6-infected animals had very high virus burden in the liver (Fig. 4D). Remarkably, USC-087 treatment

of the Ad6-infected animals inhibited virus replication even when administered 4 days post challenge (Fig. 4D).

At the 10 mg/kg dose, USC-087 is slightly toxic—We have observed that uninfected, USC-087-treated hamsters gained weight at a slower pace than their untreated counterparts (Fig. 4E). Further, upon gross necropsy, we observed that the kidneys of USC-087-treated hamsters were paler and enlarged compared to vehicle-treated ones, irrespective of whether the animals was infected with HAdV or not. Histopathological evaluation of the kidneys revealed minimal to mild degree of tubular degeneration.

DISCUSSION

HAdV infections can cause multi-organ disease in immunocompromised patients. The high mortality rate of the disease in this patient population makes it imperative to develop efficacious, antiviral drugs to fight HAdV infections. Here we demonstrated that oral administration of USC-087, an *N*-alkyl tyrosinamide phosphonate-ester of HPMPA, is a potent inhibitor of HAdV infections and is effective against i.v. challenge with Ad5 or Ad6 in the immunosuppressed Syrian hamster model. These two species C Ads are frequently found in transplant patients with disseminated HAdV infections (Lion, 2014). The compound reduced virus burden and pathology in the liver, the main target for HAdV replication in the hamster model. As a result, mortality was prevented completely or reduced significantly for USC-087-treated hamsters, even when the administration of the compound started at 4 days after virus challenge. It is known that a significant amount of progeny virus can be isolated from the liver of infected animals by that time (Fig. 4), suggesting that USC-087 treatment can be successful in patients with ongoing HAdV infection and showing that USC-087 is one of the very few compounds exhibiting good efficacy against HAdVs.

A number of compounds and agents have been reported to have anti-HAdV activity in cell culture (reviewed in (Wold and Ison, 2013)). Ribavirin is a nucleoside analog of guanosine that is active against Species C Ads in culture (Morfin et al., 2005), but its efficacy is controversial and it is not recommended for clinical use (Lion, 2014). Ribavirin is ineffective against Ad5 in the immunosuppressed Syrian hamster model (Tollefson et al., 2014). Ganciclovir, an analog of 2-deoxyguanosine, has been employed occasionally, with uncertain success, in the clinic (Ison, 2006; Lenaerts et al., 2008; Lindemans et al., 2010; Matthes-Martin et al., 2013; Sandkovsky et al., 2014; Wold and Ison, 2013). Ganciclovir is active against many herpes viruses (Field and Vere Hodge, 2013); after entering cells, it is phosphorylated by a viral-coded thymidine kinase or UL97 protein kinase, then is converted to the triphosphate form (Prichard and Kern, 2011). However, HAdV does not encode a kinase that is known to phosphorylate the drug, and phosphorylated GCV was not detected in Ad5-infected cells (Ying et al., 2014a). Nevertheless, the human mitochondrial deoxyguanosine kinase does phosphorylate it to a limited extent and it is possible that a homologous enzyme in the hamster might also exhibit similar activity (Sjoberg et al., 1998). Regardless, ganciclovir (Ying et al., 2014a) as well as valganciclovir (Toth et al., 2015) had good activity against Ad5 in the immunosuppressed Syrian hamster model. These compounds inhibit Ad5 DNA replication, possibly by inhibiting the Ad5 DNA polymerase (Ying et al., 2014a; Ying et al., 2015).

Very recently, a number of other compounds and agents have been shown to have anti-HAdV activity in cell culture. These include cardiotonic steroids (Grosso et al., 2017), 3-hydroxy-quinazoline-2,4(1*H*,3*H*)-diones (Kang et al., 2016), phenols in black tea extract (Karimi et al., 2016), dioscin, a compound extracted from air potato (Liu et al., 2013), 6-azacytidine (Alexeeva et al., 2015), and pyrrole and pyrrolopyrimidine compounds (Hamdy and El-Senousy, 2013; Mohamed et al., 2015).

Another promising approach for treating HAdV infections is anti-HAdV adoptive T-cell therapy. For the immunocompromised patients, the loss of HAdV-specific CD4 and CD8 T cells is a critical factor to sensitivity to opportunistic viral infections (Lion, 2014). To remedy this, researchers attempted to adoptively transfer virus specific T cells into T-cell-depleted patients. Because Ad-specific T cells are present in the peripheral blood of healthy adults only at low frequencies, researchers developed processes to enrich Ad-specific T cells (Feucht et al., 2015; Khanna and Smith, 2013; O'Reilly et al., 2016). Therapies based on RNA interference are also under development. While these methods are in the early phase of research, the results are encouraging (Kneidinger et al., 2012; Pozzuto et al., 2015; Schaar et al., 2016).

As opposed to large DNA viruses like herpesviruses and poxviruses, HAdVs have only two virus encoded enzymes, the DNA polymerase and the protease. Besides approaches that target the host (Hutterer et al., 2015), these two viral proteins are the only obvious targets of inhibitors. Thus, it is clear that generating efficacious anti-adenoviral small molecule compounds is a difficult task. We believe that USC-087, only the third compound to show oral efficacy *in vivo* against HAdV infection in the hamster model, is a very promising lead that can be the basis of further development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- Adenovirus infections of immunocompromised patients can cause serious multi-organ disease
- The immunosuppressed Syrian hamster is an adequate animal model to test the efficacy of antiadenoviral drugs *in vivo*
- USC-087, a novel antiviral drug, is efficacious in suppressing HAdV replication and pathogenesis in hamsters

Figure 1. Structures (A) and *in vitro* anti-HAdV activity (B) of CDV, HPMPA and USC-087 Antiviral activity (against HAdV-C5) and cytotoxicity were evaluated in 3–5 separate experiments and EC_{50} values (open symbols) and CC_{50} values (solid symbols) are shown with horizontal bars representing the mean and standard deviation of the data.

Figure 2. USC-087 administered prophylactically reverses Ad5-induced mortality and morbidity, mitigates Ad5-induced liver damage, and decreases virus burden in the liver of immunosuppressed female Syrian hamsters

Ad5-infected hamsters were administered with vehicle or 10 mg/kg of USC-087 p.o. q.d., or with 20 mg/kg of CDV i.p. 3 times weekly; starting 1 day before challenge. **A.** Diagram of the experimental design. **B.** Survival. Ad5-Vehicle v. all other groups $p=0.0161$ (Log rank). **C.** Serum alanine aminotransferase levels. For this and similar subsequent figures, each symbol represents the value from an individual animal; the horizontal bar signifies the mean. **D.** Virus burden in the liver. NQ: not quantifiable (below the quantification threshold of 5×10^4 TCID₅₀/g); *: $p < 0.05$, **: $p < 0.01$

Figure 3. Prophylactically administered USC-087 is more effective in counteracting the effects of disseminated Ad6 infection in female Syrian hamsters than equimolar doses of CDV

USC-087 was administered p.o. starting at one day prior to infection and continued daily for the course of the experiment. CDV was administered i.p. starting with an induction dose one day before virus challenge and continued with 3 weekly injections for the duration of the experiment. **A.** Diagram of the experimental design. **B.** Survival. Ad6-Vehicle v. Ad6-USC-087 1 mg/kg $p=0.0047$; Ad6-Vehicle v. Ad6-USC-087 3 mg/kg or Ad6-USC-087 10 mg/kg $p<0.0001$; Ad6-Vehicle v. Ad6-CDV 0.8 mg/kg $p=0.4505$; Ad6-Vehicle v. Ad6-CDV 2.4 mg/kg $p=0.0178$; Ad6-Vehicle v. Ad6-CDV 8 mg/kg $p=0.0001$; Ad6-Vehicle v. Ad6-CDV 8 mg/kg $p<0.0001$ (Log rank); **C.** Serum alanine transaminase levels. Empty symbols in this and subsequent graphs indicate that the sample was collected from a moribund animal sacrificed ahead of schedule. **D.** Virus burden in the liver. *: $p<0.05$, **: $p<0.01$; for the purposes of statistical calculations, a value of 5×10^4 TCID₅₀/g was assumed for all not detectable and not quantifiable samples; NQ: not quantifiable; ND: not detectable

Figure 4. USC-087 reduces mortality and morbidity even when administered 3 or 4 days after virus challenge

CP-treated hamsters were infected i.v. with Ad6 and left untreated or treated with USC-087 at 10 mg/kg p.o. q.d. Treatment with the drug started at 1 day before or 1, 2, 3, or 4 days after virus challenge. **A.** Diagram of the experimental design. **B.** Survival. $n=15$. Ad6-Vehicle v. all Ad6-USC-087 $p<0.05$ (Log rank). **C.** Serum ALT levels at 7 days post challenge $n=5$ to 9. *: $p<0.05$. **D.** Virus burden in the liver at 7 days post challenge. Empty symbols represent data collected from moribund animals. **: $p<0.01$, ***: $p<0.001$; for the purposes of statistical calculations, a value of 5×10^4 TCID₅₀/g was assumed for all not detectable and not quantifiable samples), NQ: not quantifiable, ND: not detectable. **E.** Mean body weight changes for the Vehicle-Vehicle and the Vehicle-USC-087 groups. $p<0.0001$ (Two-Way ANOVA).