UNDERSTANDING THE CELLULAR MECHANISM OF ADENO-ASSOCIATED VIRUS GENOME STABLIZATION

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ABSTRACT

The Adeno-Associated Virus (AAV) is a small, single stranded DNA virus that has been developed as a gene transfer vector. Early clinical trials using recombinant AAV vectors (rAAV) have identified the following concerns that need to be addressed in order to increase efficiency of these vectors. It has since been determined that AAV vector efficiency decreases due to the following mechanisms: ineffective endocytosis, endosomal degradation, inefficient trafficking, and the need to convert from a single-stranded AAV genome to a transcriptional active double-stranded form. The purpose of this study is to elucidate mechanisms that help stabilize the AAV genome in order to make it a more efficient vector for gene therapy. Previously, there have been studies using fluorescent labeling to track the movement of AAV into the nucleus. An integral part of AAV genomic stability may be the obstacles it encounters in the cytoplasm prior to entering the nucleus. Previous studies on improving AAV transduction have focused primarily on the nucleus. The study will hopefully shed light on the hurdles AAV encounters as it moves through the cytoplasm. Thus, this project has designed and utilized a new system for specifically tracking the status of AAV genomes in the cytoplasm as well as in the nucleus.

This project utilizes a novel dual luciferase reporter system to track the movement of AAV particles from the cytoplasm into the nucleus to elucidate mechanisms that could contribute to the stabilization of the AAV genome. The novel dual reporter system is comprised of a single-stranded vector containing two different types of secreted luciferases: *Cypridina* Luciferase and *Gaussia* Luciferase. *Cypridina* Luciferase is placed under the control of a nuclear promoter and therefore it is expressed only in the

nucleus. The second, *Gaussia* Luciferase, is under the control of a cytoplasmic promoter that will only be expressed in the cytoplasm upon the presence of T7 RNA polymerase. Using this dual reporter luciferase system along with RT qPCR quantification in a Hek293 cell line expressing the T7 RNA polymerase, demonstrated that genomes are present in the cytoplasm at 18 and 24 hours post infection.

The second part of this study is using the dual reporter system to understand the trafficking patterns of rAAV and looking at ways to enhance transduction. One method could be administering rAAV vectors in conjunction with a drug; this approach may help overcome some of these cellular barriers encountered during infection as well as help stabilize the genome. Cidofovir (CDV) is a monophosphate nucleotide analogue that competitively inhibits the incorporation of deoxycytidine triphosphate into viral DNA via viral DNA polymerase. *In vitro*, CDV actively inhibits a number of DNA viruses including herpes viruses, adenovirus, polyomavirus, papillomavirus, and poxviruses. Cidofovir has already been approved by the Food and Drug Administration (FDA) for the treatment of cytomegalovirus (CMV) retinitis in patients with acquired immunodeficiency syndrome (AIDS). The effects of CDV on small DNA viruses that lack their own viral DNA polymerase, like AAV, has not been documented.

Results have demonstrated that CDV is able to increase single-stranded rAAV transgene expression in the nucleus by 2 to 5 fold, depending on the cell type and concentration, *in vitro*, using both rAAV2 and rAAV8 luciferase reporter vectors. These results have been able to be replicated using other reporter vectors: rAAV2-LacZ reporter, rAAV2-GFP, rAAV2-hAAT, and a rAAV8-GFP *in vitro.* Results have shown a dose-dependent increase in rAAV genomes with CDV pretreatment in HelaS3 cells via

Southern Blot. Also southern blot analysis of cells pretreated with CDV then infected with rAAV revealed no difference in the amount of vector present between 0 and 2 hours post infection, suggesting CDV does not enhance viral entry but that CDV may enhance at steps downstream of viral entry.

Using RNA sequencing and Ingenuity software analysis of HelaS3 cells pretreated with CDV, an increase in several genes of interest including those involved in the mechanism of viral exit were observed. These genes include Actin and vacuolar proteins, these molecules are involved in and associated with endosomal sorting complexes as well as required for transport inside the cell. This finding along with southern blot data supports the theory that CDV may enhance rAAV trafficking since we observed that CDV pretreatment enhances viral accumulation of rAAV vectors in both the cytoplasm and nucleus 24 hours post-infection. Also utilizing the dual luciferase reporter system an increase in transgene expression present with CDV pretreatment compared to PBS in both the cytoplasm and nucleus was observed suggesting that CDV may enhance with rAAV trafficking. These results taken together demonstrate that this dual reporter system is a powerful tool for understanding and improving rAAV trafficking. Also drugs like CDV can greatly contribute to the understanding of rAAV trafficking, and eventually lead to the development of novel strategies to increase overall efficiency of AAV transduction.

This thesis is dedicated to my mother and father, Helen and Stephen Pokiniewski, who have not only instilled but supported my great love of knowledge over the years

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CHAPTER 1

INTRODUCTION

Adeno-associated virus Biology

The Adeno-associated virus (AAV)

The Adeno-Associated Virus (AAV) is a parvovirus containing a single-stranded linear DNA genome (1) (2) (3). The virus was first discovered in 1965 while researchers were analyzing a preparation of simian Adenovirus grown in Rhesus monkey kidney cells (1) (2) (3). It is classified as a depeno-virus (2) (3). Wild-type AAV is replication-deficient thus requiring a helper virus such as Adenovirus or Herpes Simplex Virus in order to efficiently replicate its genome (2) (3) (5) (6). In the absence of a helper virus, AAV becomes latent (3). Humans are a natural host for the wild-type AAV virus (3). A majority of the population are exposed to wild-type AAV during childhood and adolescence; prevalence rates for antibody titers can range from 60-80% in adults (5) (6) (7). The virus can persist in either an episomal state or integrate site specifically at chromosome 19q13.4 (3) (5) (6). AAV is a 25-nm non-enveloped virus that has a 4.7 kb genome that contains two palindromic inverted terminal repeats (ITR) which are required for efficient multiplication of the AAV genome (2) (3) (5) (8). The ITR structure serves as both the origin of replication and the primer used to initiate second strand DNA synthesis of the virus (2) (3). The wild-type virus has only 2 open reading frames: Rep, which encodes non-structural proteins needed for genome replication, and Cap, which encodes the capsid proteins needed for structure (2) (3) **(Ill. 1a)**. Three promoters, P5,

P19, and P40, work to produce seven proteins, four rep proteins from the rep gene and three cap proteins from the cap gene (2) (3) (6) (8). The rep gene proteins, Rep78 and Rep68 are expressed via the P5 promoter and are involved in gene expression and DNA replication (2) (3) (5). While Rep52 and Rep40 are expressed via the P19 promoter, they work to collect single stranded DNA for packaging (2) (6) (9). Both Rep68 and Rep40 are the products of alternate splicing (5) (6).

Post replication, both sense and anti-sense strands of DNA generated by the Rep proteins, which will then be packaged into a viral capsid at equal frequency (3). The cap proteins, VP1, VP2, and VP3, are driven by the P40 promoter and are also subjected to alternate splicing (3). They are present at a molar ratio of 1:1:10 (2). The cap proteins together form the capsid of the AAV virus and are therefore responsible for the viral structure (2). The capsid proteins control cellular tropism and will differ between AAV serotypes as well (10). For recombinant AAV vectors (rAAV) the rep and cap genes are replaced with the therapeutic gene of interest in between the ITRs **(Ill. 1b)**. There are multiple serotypes of AAV available, each having individual tropism and characteristics pertaining to different tissue and organs in the body (10) (11). AAV also infects both dividing and non-dividing cells (10).

Gene therapy

Gene therapy is the insertion or alteration of a gene within a patient's cells or tissue to treat a disease (8). It is a method for correcting defective or missing genes that are responsible for disease development (8). Gene therapy is also being used to treat various cancers as well. Determining the most efficient vector to carry DNA into tissues or organs

is one of the challenges in the gene therapy field today. The most common form of gene therapy involves using a viral vector carrying a functional gene into an organism in order to replace a mutated, nonfunctional, or absent gene (8). This system has been used with some success to treat hereditary diseases linked to a genetic defect (3) (8).

Gene therapy has great potential for the treatment of a variety of monogenetic diseases (3) (8). Unlike other approaches, gene therapy aims to target the various underlying cellular and molecular defects responsible for the disease (8). There are many parameters to take into consideration when choosing the optimal vector for gene therapy, for example, incitement of immune response, infecting both quiescent and/or dividing cells, long-term expression, and producible high titers. There are several candidates for gene therapy vectors, such as adenoviruses, retroviruses, and AAV (8). AAV is one of the best vectors to answer many of the problems listed above.

With regard to gene therapy, ITRs seem to be the only sequences required in cis next to the therapeutic gene. Structural (cap) and packaging (rep) genes can be delivered in trans. This small virus does not encode for a polymerase of its own, but relies on cellular polymerases or other viral polymerases to replicate (2) (6). Thus, three plasmids (a plasmid containing the encoded gene of interest between the ITRs, a viral helper plasmid, and a plasmid containing rep and cap genes) are co-transfected into Hek293 cells then harvested. The virus is extracted from the cells, purified, and delivered to the patient (5).

AAV vectors compared to other viral vectors for Gene therapy

AAV vectors are an optimal vector choice to use compared to the other current viral vector options to deliver a gene of interest. The first reason AAV is a superior

vector is its safety profile. AAV elicits a relatively weak immune response and is not known to cause any disease in humans. Retroviral and lentiviral vectors can integrate at many sites in the human genome highlighting the safety concern of insertional mutagenesis possibly causing oncogenesis (3). Both Adenovirus (Ad) and retroviral/lentiviral vectors elicit strong immune responses that can be detrimental for the treatment of monogenetic diseases in gene therapy (3). For example, in a study comparing Ad to AAV vectors for pancreatic gene delivery *in vivo* it was found that Ad elicited a significant leukocyte infiltration early after delivery into the pancreas, whereas none of the AAV vectors tested elicited a significant leukocyte response (12).

Adenovirus and retroviral/lentiviral vectors are able to deliver much larger genes of interest compared to AAV vectors due to the 4.7kb size limitation of this small virus (12). However, studies are being conducted to split genes between two AAV vectors (3). One construct has a small overlapping sequence to the second construct to allow recombination after nuclear entry and transgene product being properly expressed (3). AAV can exist long-term as concatomers in non-dividing cells; however, it will be lost in replicating cells (3). Unlike the other listed viral vectors, AAV is able to maintain lasting transgene expression in non-dividing cells demonstrating why AAV is a better viral vector choice (3).

Advantages and drawbacks of using AAV for gene therapy

AAV has become popular for use in gene therapy for many reasons. First, AAV is non-pathogenic and causes no known diseases in humans (6). AAV is also replication defective and can be produced at high titers (6). AAV has the ability to infect both dividing

and non-dividing cells, and can transduce a wide variety of cell types including liver, eye, brain, and muscle cells (4) (5) (8) (13) (14). AAV is an easily manipulated virus that is able to produce long-term transgene expression with relatively low genotoxicity to cells (3). AAV can be engineered easily through genetic cloning (3) (5) (15). It can also establish a latent infection and produce consistent transgene expression after a single administration. All of these features make AAV an excellent candidate for gene therapy (3). In several animal models, AAV has shown great success for treating monogenetic diseases. AAV delivery in mouse models of Hemophilia B demonstrated therapeutic levels of factor IX as well as no immune infiltration in the liver (16) (17) (18). Studies with hemophilia B dogs have indicated that there is long-term expression of canine factor IX with no development of inhibitory antibodies after AAV vector administration to the liver (18). The long-term expression of canine factor IX levels could be observed years after infusion to the liver (18). This study also showed that the expression of a transgene from AAV vectors within the liver promotes tolerance to transgene product (18). These animal studies demonstrated the great likelihood that AAV vectors could be used as a treatment for hemophilia patients without developing a detrimental immune response from the host (6) (18).

Currently, AAV vectors have attained positive and successful results in a number of clinical trials including hematologic disorders such as the hemophilia B, Gaucher disease, hemochromatosis, and porphyria diseases. A single infusion of an AAV vector containing the factor IX in men with severe hemophilia B lead to longer than 2 years of therapeutic levels of this gene demonstrating that AAV gene therapy has the potential to improve outcomes and simplify treatment of serious monogenetic disorders for patients (7). The AAV vector product to treat the disease, lipoprotein lipase deficiency, has now

been licensed in Europe (7) (19). The European Medicines Agency has recommended approval of AAV to treat a rare genetic disease, lipoprotein lipase deficiency (19). Lipoprotein lipase deficiency affects only several hundred people in Europe (19). Glybera is the AAV therapy recommended for approval in Europe and was developed by uniQure, a Dutch company (19). A single treatment with Glybera, consisting of injections into multiple spots on the leg muscles of patients, is expected to last for several years (19). Regulatory approval of AAV treatments illustrates the importance and positive impact AAV vectors have for facilitating safe and effective therapies for monogenetic diseases in patients.

While AAV is an ideal vector choice for a number of reasons, there are several severe limitations associated with using this virus for gene therapy (2) (17). AAV is abundant in nature, which means that most people have already been infected with the most commonly studied serotype, AAV2 (11). This can be a problem when choosing the optimal serotype for treatment of patients who have already been exposed to one or multiple AAV serotypes (10) (11). While the virus itself is non-pathogenic, it is still a virus and recognized as foreign by the immune system resulting in a large number of people have pre-existing, circulating antibodies to the capsid protein. The recognition and clearance of AAV vectors can diminish the transduction potential of this virus (21).

AAV has been used as a gene therapy vector in clinical trials to treat various diseases like Hemophilia, Leber Congenital Amaurosis (LCA), Cystic Fibrosis, and Parkinson's disease (3). In the clinical setting, there has been great success for treating LCA with AAV vectors (20). AAV vectors carrying the human RPE65gene restored the vision in an animal LCA model as well as in human clinical trials (20). One clinical study

of 15 patients receiving one or more injections of AAV- RPE65 vectors into areas of the retina of one eye demonstrated visual gains within days after treatment. The vision in the AAV injected eye improved within days compared to the control eye of the participant that was not treated. This same group performed a follow-up report indicating that patients from this study maintained visual improvements three years post AAV treatment (20). The success of this trial in humans may in part be due to the fact that the eye is an immunoprivileged site thus having reduced immune surveillance and regulation. Although a transient increase in neutralizing antibodies to AAV capsid was noted in this study, there was no humoral response to RPE65 protein (20). LCA clinical trials demonstrated that AAV vectors can be a successful and safe choice in the treatment of monogenetic disorders.

Other studies have looked at the role of Toll-Like Receptor 9(TLR9) a pattern recognition receptor (PRR) that recognizes pathogen-associated molecular patterns (PAMPs) (21). TLR 9 recognizes non-methylated, single stranded CpG DNA. It is located in the endosomal compartment in which AAV traffics through to enter into the nucleus. In the studies investigating TLR9, it has been shown that AAV can activate mouse plasmacytoid dendritic cells via TLR9 in a MyD88 dependent manner (21). The triggering of TLR9 leads to the production of type I interferons as well as generation of cytotoxic T cells and AAV specific neutralizing antibodies. These T cells can negatively impact AAV transgene expression. Another member from the TLR family that has been shown to recognize AAV is Toll-Like Receptor 2 (TLR 2) which is located on the surface of immune cells that can bind AAV capsids (22). This study demonstrated that AAV capsids can be recognized as a PAMP by TLR2 in human non-parenchymal cells of the

liver such as Kupffer cells (22). The results of these studies demonstrate that AAV can activate PRRs causing the activation of downstream pathways leading to the production of interferons, inflammatory cytokines, and activation of the adaptive branch that can be detrimental to AAV mediated gene transfer. There has been some success for the clinical trials using rAAV vectors for Hemophilia B; however, the interference of the immune system into the AAV-mediated treatment has been shown. Both humoral and cellular immune responses, including neutralizing antibodies and cytotoxic T cells to the viral capsid and the transgene have been generated (3) (4) (23) (24). For example, recent clinical trials have shown that the capsid-specific cytotoxic T-cell response against the transduced hepatocytes appears to be seen in some patients in the trial (3). The rAAV vectors in some patients in this study activated memory B cells, which produced circulating antibodies to AAV that can become a problem for safety, efficacy, and re-administration of rAAV vectors (3) (23). Results from the first clinical trial using AAV-2 factor IX vectors delivered to hemophilia B patients demonstrated short-term transgene expression only lasting 4-6 weeks before beginning to decline (3) (6) (7). The clinical trial highlighted that an increase in AAV-2 capsid-specific T cells became detectable in the blood suggesting that there was a T cell-mediated response against these vectors (3) (22). These findings indicate that the immune system affects efficacy of AAV transduction *in vivo*. The lack of immune response to transduced cells as well as transgene product is critical for the success of AAV mediated gene therapy. The immune complications present major hurdles for AAV to become a safe and efficient vector for gene therapy, since the immune cells destroy transduced cells.

The second major limitation of AAV for gene therapy is payload size. It is well established that AAV has a packaging capacity of 4.7 kb. Research indicates that sequences up to 5 kb in length can be adequately packaged. Attempting to package larger constructs generates oversized vectors and results in genome truncation (25). The oversized vectors are able to produce therapeutic effects; however, the vector population is heterogeneous (25). The last major limitation is the intracellular trafficking of the vector. The intracellular trafficking of AAV is very inefficient and leads to a large amount of vector being lost thus leading to a decrease in transduction (8). The mechanisms that cause the degradation of AAV as it traffics within the cell are not fully understood (26). For example, inefficient AAV trafficking in the cytoplasm and second-strand synthesis have been identified as being major rate-limiting factors in overall AAV gene expression (17) (27) (28) (29) (30) (31) (32).

AAV trafficking

Despite there being a great variety of AAV serotypes to choose from for gene therapy there are multiple biological barriers that appear to limit viral transduction (8) (10) (33). The understanding of AAV trafficking is an important area of research for improvement of AAV vectors in clinical trials (2) (4) (28). There are seven stages of AAV trafficking; some stages are still poorly understood (2) (4) (26) **(Ill. 2)**. The first step is viral binding to a receptor/co-receptor (2) (8) (10). AAV-2 gains entry into target cells by using the Heparin sulfate proteoglycan receptor as its primary receptor (2) (4) (8) (10). This stage of infection can be most significantly influenced by the choice of AAV serotype or type of capsid variant used to generate the recombinant virus since different serotypes have different viral tropisms (15) (33) (34) (35) (36). For example, comparative analysis of

rAAV1, rAAV2, and rAAV5 transduction in the brain has demonstrated differences in cellular tropisms between these three serotypes (15) (33).

The second stage is receptor-mediated endocytosis of the virus (2) (8). This step can be influenced by the abundance of AAV co-receptors on the target cells; this depends on the serotype chosen as well as on the activation of cellular pathways that trigger endocytosis (8) (33) (36) (37) (38). For example, HSPG-bound rAAV2 appears to enter cells through clathrin-coated pits in a dynamin-dependent process (2) (4) (37). Although AAV5 utilizes a different primary attachment receptor (sialic acid), ultrastructure localization data suggest that it also appears to enter the cell through clathrin-coated pits (34) (36). The step following endocytosis is the intracellular trafficking of the virus through the endosomal compartment (6) (30) (37) (39) (40). Processing of the AAV within the endosomal compartment has been shown to be significantly linked to efficient transduction (4) (36) (37) (40). It has been shown that AAV injected directly into the cytoplasm does not traffic to the nucleus, suggesting the importance of the endosomal events for priming the virus for nuclear transport (2) (4) (25) (30) (37) (40) (41). Other studies have shown that inhibiting the acidification of the endosomal compartment by utilizing bafilomycin A1 significantly decreased the infection of the virus (30) (37). It is generally believed that AAV must escape the endosomal compartment and be released into the cytoplasm in order to traffic to the nucleus (2) (8) (30) (37).

The fifth step is intracellular trafficking of the virus from the cytoplasm into the nucleus (8). The intracellular trafficking through the cytoplasm has been identified as a major rate-limiting step in AAV transduction (37) (40). This trafficking step has been hypothesized as an area where a substantial amount of vector is lost (37) (40). When and

how different AAV serotypes escape the late endosome and enter the cytoplasm is still unknown (6). There have been previous studies using fluorescent labeling to track the movement of AAV into the nucleus (30) (37) (41) (42) (43) (45) (46). An integral part of AAV genomic stability may be the obstacles it encounters in the cytoplasm prior to entering the nucleus. Previous studies on improving AAV transduction have focused mainly on the nucleus; however, the hurdles AAV encounters in the cytoplasm may be as important. Also the amount of AAV lost as it moves through the cytoplasm remains undetermined. The nuclear translocation of AAV has been suggested to be a slow and inefficient process (2) (30) (40). It has been shown that disruption of tubulin, microfilaments, and actin filaments inhibits the movement of AAV2 into the nucleus; however, dissecting the functional involvement of these cytoskeletal elements in the cytoplasmic movement of this virus is an open area of research (2) (10) (25).

The size of an AAV virion is comparable in size to passing through the nuclear pore to gain access to the nucleus (15) (40) (47). The VP2 capsid sequence has been suggested as a signal for nuclear entry (13) (52). However, other studies have suggested that AAV can enter the nucleus via in another route or mechanism (15) (36). Hansen *et al* has reported that intact AAV2 capsids can bind and enter the nucleus; this data was observed using Hek293 cells (45). There was a study showing that fluorescently labelled capsids were detected inside the nucleus after AAV infection (13). The sixth step is virion uncoating. Whether the majority of AAV particles uncoat inside or outside the nucleus has been a topic of great debate (2) (13) (36) (41) (47). The final step is the conversion from a single-stranded viral genome to a double-stranded one capable of expressing an encoded

gene (2) (8) (47). It has been suggested that all these stages appear to be inter-related and may be rate limiting for many AAV serotypes (2) (8).

Enhancing recombinant AAV vector transduction for Gene Therapy

Approaches to enhance rAAV transduction

There are multiple approaches to enhance rAAV transduction. The use of alternative serotypes to increase infectivity to a particular tissue or organ due to receptors present has been explored **(Ill. 3)** (24) (34). AAV2 is the most studied serotype; however, it does not infect every tissue. The approach of utilizing an AAV9 vector to target the lungs instead of AAV2 vector due to AAV9 having higher efficiency of transduction to lungs has been implemented. Engineering and altering the residues on the AAV capsid to improve viral trafficking has been performed (5) (10) (15) (36) (48) (49) (50). Genome alterations such as the generation of self-complementary AAV vectors (scAAV) has been another strategy employed (51) (52) (53) (54). These scAAV vectors are more efficient at transduction than rAAV vectors; however, scAAV vectors elicit a higher immune response and can only package 2.3 kilobases of DNA (51) (52) (53). Efforts to improve the packaging capacity of the rAAV vectors have also been carried out (54). Lastly, various pharmaceuticals have been studied as agents to improve rAAV trafficking (8) **(Ill. 5)**. The use of a drug may lead to a reduction in rAAV particles administered. The combination treatment of AAV and a drug could reduce possible immune response observed in some clinical trials. Moreover, the use of a drug may help overcome some of the cellular barriers AAV encounters during infection as well as assist in genome stabilization leading to lasting results.

Known drugs enhancing AAV transduction

There have been multiple studies investigating the effect of various DNA synthesis inhibitors on rAAV vector transduction for example: Hydroxyurea, Etoposide, and Aphidocholin (14) (42) (57) (58) (59) (60) (55). Many of these drugs enhance rAAV transduction *in vitro*; however, the improved transduction efficiencies seen in cells are not observed in animals (61) (62). There is also the issue of toxicity associated with a large majority of the listed rAAV-enhancing drugs (59) **(Ill. 5)**. Screening for new rAAV enhancing compounds and/or molecules that are safer to use would be beneficial for the improvement of AAV vectors for gene therapy.

Degradation of AAV by the proteasome may occur before AAV can complete infection thus hindering the virus' efficiency (63). MG132 is a peptide aldehyde that effectively blocks the proteolytic activity of the 26S proteasome complex (57). MG132 is a potent, reversible proteasome inhibitor; however, a major drawback is associated cytotoxicity (57). Another documented proteasome inhibitor is N-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL). LLnL inhibits proteasomes as well as other proteases (64) (65). Like MG132, LLnL reversibly inhibits the 26S proteasome activity (64). This inhibitor was also found to prolong the association of the major histocompatibility complex class I molecules with the transporters associated with antigen processing (TAP), and to slow these molecules' transport out of the endoplasmic reticulum (ER) (65). It has been suggested that MG132 and LLnL help rAAV escape proteasomal degradation (15).

Etoposide (ETO) is an inhibitor of DNA topoisomerase II. This drug forms a ternary complex with DNA and the topoisomerase II enzyme (58) (59) (61). Topoisomerase II enzyme aids in DNA unwinding, thus preventing re-ligation of the DNA

strands, and by doing so causes DNA strands to break (52) (53). Topoisomerase inhibitors have been postulated to improve double-strand synthesis of the AAV vector genome (58) (59). Hydroxyurea (HU) is a DNA replication inhibitor that negatively affects both the elongation and initiation phases of replication (56) (57) (58). HU is a DNA synthesis inhibitor, which prevents DNA synthesis by inhibiting ribonucleotide reductase and depleting deoxynucleotide pools (67). HU also triggers the S phase checkpoint of the cell cycle (66) (67). It has been shown that HU can aid in converting ssDNA to dsDNA as well as in enhancing nuclear entry of the virus (57) (58) (59) (60) (66) (67).

Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells (30) (37). Previous studies have found that bafilomycin A_1 blocked infection of HeLa cells by the AAV vector supporting the requirement of endosomal acidification and early endosomal escape for efficient AAV vector infection (30) (37).

Antiviral drugs

The antiviral action of (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine (HPMPC, cidofovir, CDV) against cytomegalovirus (CMV) and other DNA viruses was first discovered in 1986 (68). CDV is an injectable antiviral medication that was the first nucleotide analogue approved for clinical use. CDV competitively inhibits the incorporation of deoxycytidine triphosphate into viral DNA via a viral DNA polymerase **(Ill. 6)** (69) (70) (71) (72) (73) (74). CDV was approved by the Food and Drug Administration (FDA) for the treatment of CMV retinitis in patients with acquired immunodeficiency syndrome (AIDS) in 1996 (69) (75). CDV is marketed worldwide as Vistide® for the systemic treatment of CMV retinitis in AIDS patients (75).

CDV demonstrates in vitro activity against numerous DNA viruses including herpes viruses, adenovirus, polyomavirus, papillomavirus, and poxviruses (69) (71) (72) (75) (76). CDV has also shown effectiveness against acyclovir-resistant HSV infections, and BK virus kidney-tropic polyomarvirus infections in transplant patients. This drug is also a treatment option for progressive multifocal leukoencephalopathy (69) (77) (78). In humans, the drug has been successfully used for the treatment of recalcitrant molluscum contagiosum virus and orf virus, a poxvirus, in immunocompromised patients (76). In animal models of viral infection, CDV has shown anti-poxvirus activity (76). CDV has the potential for treatment and short-term prophylaxis of not only orthopox- but also parapox- and molluscipoxvirus infections (76). It has been suggested that the cellular uptake of CDV occurs via fluid-phase endocytosis, so CDV may accumulate in the lysosome (76). Once in the cytoplasm, CDV needs only two phosphorylation steps to be converted to its antiviral active intracellular metabolite, CDV diphosphate (CDVpp) (76). CDV is phosphorylated in a two-step process to yield CDVpp (73). The phosphorylation of CDV is carried out by cellular enzymes in the absence of viral infection **(Ill.7)** (69) (73) (79). Pyrimidine nucleoside monophosphate (PNMP) kinase I catalyzes the first step of CDV phosphorylation. The second step is catalyzed by nucleoside diphosphate (NDP) kinase, pyruvate kinase, or creatinine kinase (75). The metabolism of CDV was shown to remain unchanged between uninfected and infected cells (75). A general feature of CDV is the long intracellular half-life of its metabolites, CDVp, CDVpp and CDVp-choline, which allows for the infrequent administration of the drug (75). After removal of CDV from the cell culture media, the intracellular levels of CDVp and CDVpp have half-lives of about 24 and 65 hours respectively (80) (81). This occurrence is most likely attributed to the

accumulation of the CDVp-choline metabolite that has a half-life of more than 80 hours (74) (75) (77). CDVp-choline metabolite serves as a reservoir from which the active metabolite CDVpp can be produced (74) (75) (77). CDVpp inhibits viral replication by selectively inhibiting viral DNA polymerases (69) (72). It also inhibits human polymerases but this action is 8 to 600 times weaker than its actions on viral DNA polymerases (72). CDV can also incorporate itself into viral DNA hence inhibiting viral DNA synthesis during reproduction (69) (72) (74).

Cidofovir is dianionic at physiological pH resulting in low oral bioavailability in animals and humans; therefore treatment of CDV in patients is performed intravenously (73). Over 90% of one intravenous dose is recovered unchanged in the urine over a 24 hour period. The metabolism of CDV does not contribute significantly to the total clearance (73). Nephrotoxicity is the major toxicity associated with CDV, which was observed in mice, rats, guinea pigs, rabbits, monkeys, and humans (73) (75). Nephrotoxicity accompanying CDV treatment is exhibited by proteinuria, glycosuria, and decreases in serum phosphate, uric acid, and bicarbonate, increases in serum creatinine (75). Also the degeneration and necrosis of the proximal renal tubule cells was observed with CDV treatment (75). Concomitant treatment with probenecid decreases both the renal clearance of CDV and the incidence of nephrotoxicity, presumably by blocking its active tubular secretion (73). CDV is administered intravenously at a dose of 5 mg/kg once weekly for two weeks followed by 5 mg/kg intravenously once every other week (73). The subject is monitored for renal function before and after commencement of CDV therapy, concomitant administration of probenecid, and intravenous hydration to maintain patient safety

(73). The effects of CDV on small DNA viruses that lack their own viral DNA polymerase, like AAV, have not been studied.

Ganciclovir (GCV) was the first antiviral medication approved to treat CMV infections and is widely prescribed to treat these types of infections in immunocompromised patients who are at higher risk for CMV infections (79) (82). GCV is an acyclic analog of the nucleoside guanosine, which is incorporated into DNA and preferentially inhibits viral DNA polymerases over cellular polymerases, thus disrupting viral DNA synthesis (82). Like CDV, GCV has activity against various viruses like herpes virus, Epstein-Barr virus, and Varicella-zoster virus (79). GCV clearance is dependent on renal function, since this drug is actively eliminated by the kidney (83). GCV is administrated intravenously at doses of 2.5 mg/kg 5 mg/kg or 10mg/kg over an hour span. At the start of treatment, GCV is usually given every 12 hours for the first few weeks, and then either once a day or 5 to 7 times a week after that depending on the disease state and treatment plan for each patient (83). GCV can cause some serious side effects, including granulocytopenia, anemia, and thrombocytopenia. Patients receiving ganciclovir are closely monitored. Complete blood counts as well as platelet counts are performed on patients receiving GCV treatment (83). GCV is phosphorylated to ganciclovir monophosphate by a viral kinase encoded by the CMV gene UL97 during infection (83). Next, cellular kinases catalyze the formation of ganciclovir diphosphate and ganciclovir triphosphate (83). The drug has a half-life between 2 to 6 hours (83). About 90% of GCV is eliminated unchanged in the urine (83). Due to poor adsorption of the GCV, an oral ester prodrug of GCV, valganciclovir, was developed to enhance the oral bioavailability of

ganciclovir (83). The effects of GCV on small DNA viruses that lack their own viral DNA polymerase, like AAV, have not been studied.

ILLUSTRATION 1.

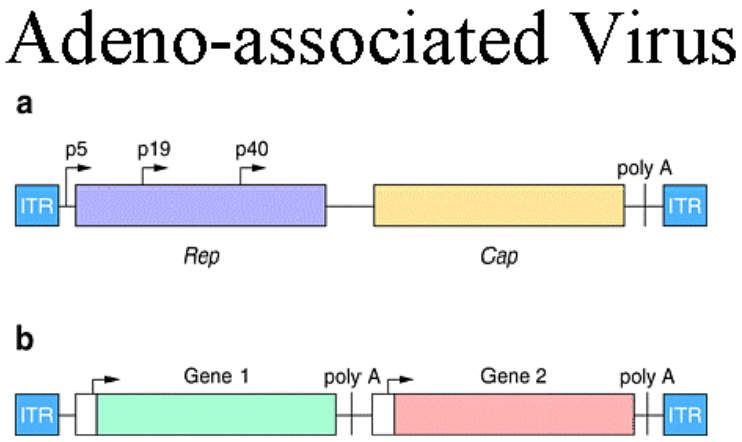


Illustration 1.

(A)Represents wild-type AAV vector which contains two genes rep and cap encoding proteins for replication and encapsidation. The Rep gene contains three promoters: p5, p19, and p40. (B) Represents the rAAV vector used for gene therapy. This vector has the rep and cap genes removed. The therapeutic gene(s) is inserted between the inverted terminal repeats (ITR).

Daya S, Berns KI. Gene therapy using adeno-associated virus vectors. *Clin Microbiol Rev.* Oct 2008;21(4):583-593.

ILLUSTRATION 2.

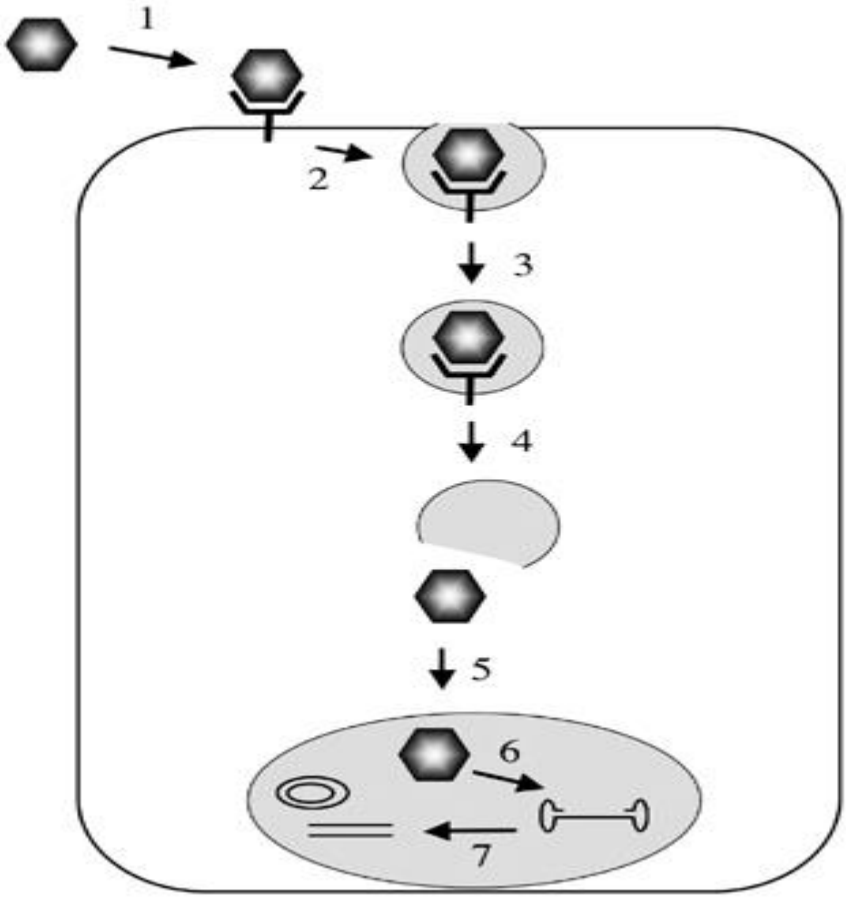


Illustration 2. Intracellular trafficking of adeno-associated viral vectors.

Schematically shown are seven stages of rAAV transduction including: (1) viral binding to a receptor/co-receptor, (2) endocytosis of the virus, (3) intracellular trafficking of the virus through the endosomal compartment, (4) endosomal escape of the virus, (5) intracellular trafficking of the virus to the nucleus and nuclear import, (6) virion uncoating, and (7) viral genome conversion from a single-stranded to a double-stranded genome capable of expressing an encoded gene.

Ding W, Zhang L, Yan Z, Engelhardt JF. Intracellular trafficking of adeno-associated viral vectors. *Gene Ther.* Jun 2005;12(11):873-880.

IILLUSTRATION 3.

AAV receptors and preferential tissue tropism

Virus	Glycan Receptor	Co-receptor/other	Tissue tropism[a]
AAV1	N-linked Sialic acid[122, 123]	Unknown	SM[b][124], CNS[125], Retina[126], Pancreas[127]
AAV2	HSPG[25]	FGFR1[26], HGFR[30], LamR[29], CD9 tetraspanin[128]	VSMC[129], SM[130], CNS[131], Liver[132], Kidney[133]
AAV3	HSPG[18]	FGFR1[134], HGFR[135], LamR[29]	Hepatocarcinoma[136], SM[124]
AAV4	O-linked Sialic acid[137]	Unknown	CNS[138], Retina[139]
AAV5	N-linked Sialic acid[137, 140]	PDGFR[141]	SM[124], CNS[138], Lung[142], Retina[126]
AAV6	N-linked Sialic acid[123], HSPG[122]	EGFR[143]	SM[144], SM (IV)[145], Heart[24], Lung[146]
AAV7	Unknown	Unknown	SM[23], Retina[147], CNS[148]
AAV8	Unknown	LamR[29]	Liver[23], SM[23, 149], CNS[148], Retina[147], Pancreas[150], Heart[149]
AAV9	N-linked galactose[151]	LamR[29]	Liver[152], Heart (I.V.)[152, 153], Brain (I.V.)[154], SM (I.V.)[155], Lungs[156], Pancreas[153], Kidney (I.V.)[156]
BAAV	Ganglioside GM1[37]	Unknown	Unknown

[a] Preferential tissue tropism following local delivery, unless otherwise indicated (I.V.=Intravenous injection)

[b] Abbreviations : SM, Skeletal Muscle; CNS, Central Nervous System; VSMC, Vascular Smooth Muscle Cells

Illustration 3. AAV receptors and preferential tissue tropism.

Listed are the known AAV serotypes along with receptors, co-receptors, and tissue tropisms for each.

Nonnenmacher M, Weber T. Intracellular transport of recombinant adeno-associated virus vectors. *Gene Ther.* Jun 2012;19(6):649-658

ILLUSTRATION 4.

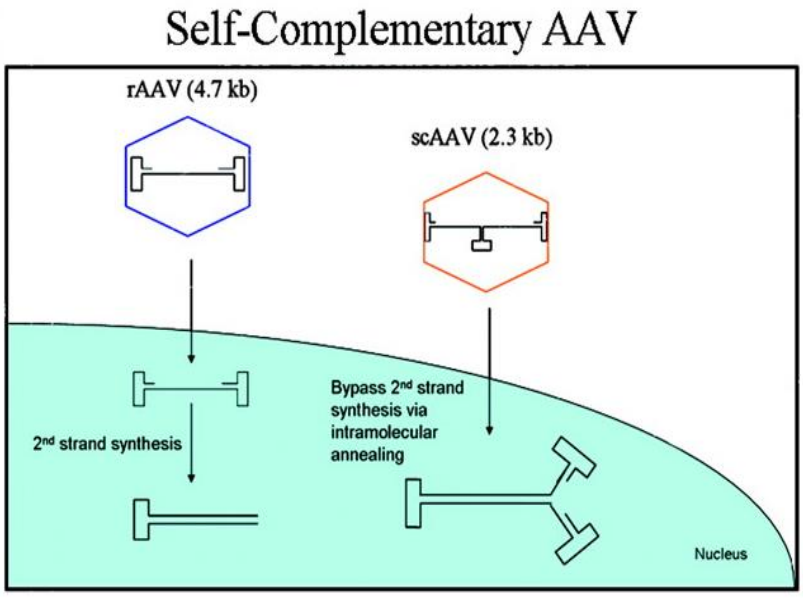


Illustration 4. Comparison of scAAV and rAAV vectors.

scAAV vectors can fold into dsDNA without the requirement for DNA synthesis or base-pairing between multiple vector genomes, thereby bypassing the rate-limiting second-strand DNA synthesis of rAAV vectors. Another difference between the two vectors is that scAAV can only package 2.3kb, while rAAV vectors can package 4.7kb of DNA. scAAV vectors transduce cells more efficiently; however, these vectors are more immunogenic than rAAV vectors.

Daya S, Berns KI. Gene therapy using adeno-associated virus vectors. *Clin Microbiol Rev.* Oct 2008;21(4):583-593.

ILLUSTRATION 5.

Inhibitors and enhancers of AAV trafficking/transduction

Virus	Drug/Protein/Mutation	Effect	Fold enh/inhib[a]	Possible Target
AAV2	Heparin[25]	Inhibitor	- 100-fold	Blocks attachment
AAV2	Bafilomycin A1[39, 51]	Inhibitor	- 10-fold	Blocks endosomal acidification/trafficking
AAV2	Brefeldin A[51]	Inhibitor	- 100-fold	Disrupts the Golgi apparatus, blocks EE-LE transport and Golgi-ER transport/trafficking
AAV2	Chloroquine[65]	Inhibitor	- 100-fold	Blocks endosomal acidification/trafficking
AAV2	Wortmannin[43]	Inhibitor	- 4-fold	Blocks EE-EE and CLIC/GEEC-EE fusion/trafficking
AAV2-8	Cathepsin B-L inhibitors[84]	Inhibitor	- 3-fold	Blocks endosomal capsid processing
AAV2	Rac1 T17N[43]	Inhibitor	- 2-fold	Blocks macropinocytosis/entry
AAV2	Dynamin1 K44A[39, 40]	Inhibitor	- 2-3-fold	Blocks clathrin-mediated endocytosis/entry
AAV2	Rab7 siRNA[67]	Inhibitor	- 2-fold	Blocks late endosome-Golgi transport/trafficking
AAV2	Rab11 siRNA[67]	Inhibitor	- 40%	Blocks recycling endosome-Golgi transport/trafficking
AAV2	AAV HD/AN[b] mutant[53, 75]	Inhibitor	- 500-fold	Endosomal escape
AAV2	AAV BC3 mutant[65, 79]	Inhibitor	- 10000-fold	Viral NLS mutant, blocks nuclear import
AAV2	AAV BR3K mutant[53]	Inhibitor	- 500-fold	Blocks mobilization from the nucleolus
AAV2	Capsid phosphorylation[111]	Inhibitor	- 3-fold	Signal for capsid ubiquitination?
AAV2-5	MG132[51, 97]	Enhancer	+ 10-100-fold	Proteasome inhibitor/capsid protection? Trafficking?/2nd strand synthesis?
AAV2-5	LLnL[97]	Enhancer	+ 10-200-fold	Proteasome inhibitor, similar to MG132
AAV2-5	Doxorubicin[157, 158]	Enhancer	+ 10-100-fold	Topoisomerase inhibitor
AAV2	E3 Ub ligase inhibitor[96]	Enhancer	+ 3-fold	Blocks cellular/capsid ubiquitination?
AAV2	hydroxyurea[159-161]	Enhancer	+ 10-100-fold	Triggers DNA damage response/Additive effect with MG132
AAV2	Tyrphostin-23[98]	Enhancer	+ 20-40-fold	Blocks capsid phosphorylation/ubiquitination? similar to MG132
AAV2	Adenovirus[109]	Enhancer	+ 10-20-fold	Multiple/Unknown, non-additive with MG132)
AAV2	Misfolded CFTR protein mutant	Enhancer	+10-fold	ER stress/Misfolded Protein Response inducer
AAV2	Heat shock[99]	Enhancer	+ 10-fold	2nd strand synthesis? Hsp70 induction? ER stress? Synergistic effect with Tyrphostin23
AAV2	TC-PTP[162]	Enhancer	+ 16-fold	Blocks capsid phosphorylation/ubiquitination?
AAV2	PP5[110]	Enhancer	+ 6-fold	Blocks capsid phosphorylation/ubiquitination?
AAV2-6	AAV Tyrosine Mutants[114, 115, 163, 164]	Enhancer	+ 10-100-fold	Blocks capsid phosphorylation/ubiquitination? Non-additive with MG132

[a] Transduction data are expressed as ratio to control infections and may represent experiments performed in various cell lines/animals/time points.

[b] Abbreviations : HD/AN, VP1 double mutant (^{75}HD>AN) with no PLA2 activity; BC3, VP1/2 basic cluster 3 ^{168}RK>NN mutant; BR3K, highly basic VP1/2 basic cluster 3 ^{167}A>K mutant; CFTR, Cystic fibrosis transmembrane conductance regulator; TC-PTP, T cell protein tyrosine phosphatase; PP5, serine/threonine protein phosphatase 5.

Illustration 5. Inhibitors and enhancers of AAV trafficking/transduction.

Listed are the known inhibitors and enhancers of rAAV vectors.

Nonnenmacher M, Weber T. Intracellular transport of recombinant adeno-associated virus vectors. *Gene Ther.* Jun 2012;19(6):649-658

ILLUSTRATION 6.

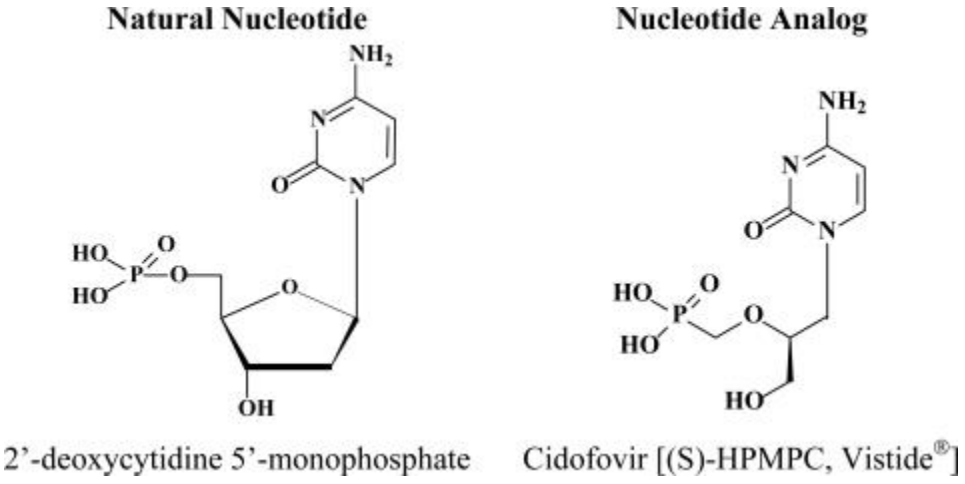
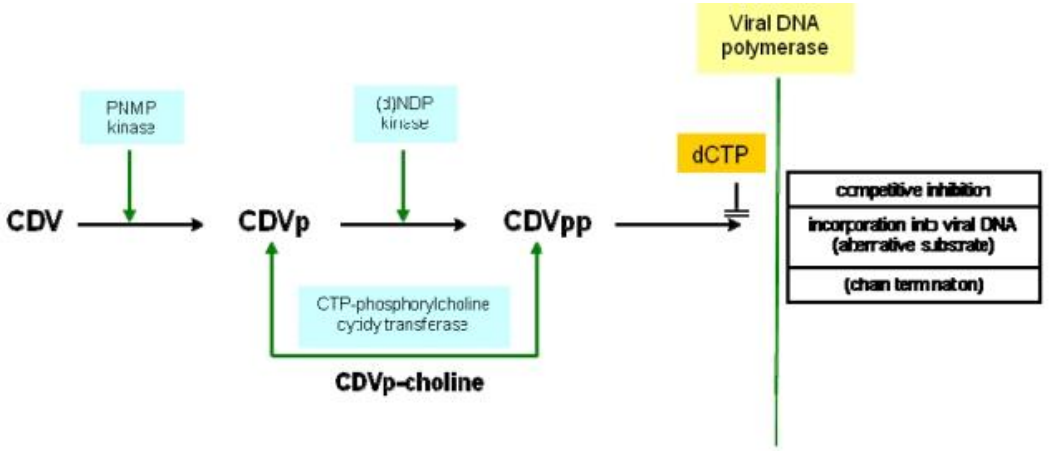


Illustration 6.Chemical structure of

(S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC (CDV)) and its natural nucleotide. CDV is a competitive inhibitor to the natural substrate, 2'-deoxycytidine 5'-monophosphate.

Andrei G, Snoeck R. Cidofovir Activity against Poxvirus Infections. *Viruses.* Dec 2010;2(12):2803-2830.

ILLUSTRATION 7.



Virus DNA polymerase	Active metabolite	Action / result	Blocking of template strand extension	Inhibition of 3'-to-5' exonuclease proofreading activity
CMV (UL54)	CDVpp	Incorporation of 1 CDVpp + any dNTP→ slows chain extension Incorporation of 2 consecutive CDVpp → chain termination	Not determined	+
VACV (E9L)	CDVpp	Incorporation of CDVpp + any dNTP → primer extension significantly reduced	+	+
	(*S*)-HPMPApp	Incorporation of one or more (*S*)-HPMPApp → no effect on primer extension	+	+

Illustration 7. Mechanism of action of CDV.

CDV is activated by cellular enzymes. Pyrimidine nucleoside monophosphate (PNMP) kinase facilitates the conversion of CDV (CDV) to CDV-monophosphoryl (CDVp), which is then further phosphorylated to the active form, CDV-diphosphoryl (CDVpp) by nucleoside 5′-diphosphate (NDP) kinase. CDVp-choline is considered to serve as an intracellular reservoir for the mono- and diphosphoryl derivatives of CDV. The diphosphoryl derivative of CDV (CDVpp) interacts with the viral DNA polymerase as either competitive inhibitors or alternative substrates. For example with human cytomegalovirus infection, chain termination occurs when two consecutive CDVpp are incorporated in the growing DNA chain.

Andrei G, Snoeck R. Cidofovir Activity against Poxvirus Infections. *Viruses.* Dec 2010;2(12):2803-2830.

Project objective and goals

The Adeno-Assoicated Virus(AAV) is a small, non-enveloped, DNA parvovirus that is non-pathogenic to humans (2). It has the ability to infect both dividing and non-dividing cells, and also transduces a wide variety of cell types including liver, eye, brain, and muscle cells (15). AAV is a simple and easily manipulated virus that is able to produce long term transgene expression with relatively low genotoxicity (15). These attributes make AAV an optimal vector choice for the use in gene therapy; however, better understanding of AAV trafficking mechanisms is necessary to further advance the use of AAV to treat genetic disorders (3). The purpose of this study is to elucidate mechanisms that help stabilize the AAV genome in order to make it a more efficient vector for gene therapy. It has since been determined that AAV vector efficiency decreases due to the following mechanisms: ineffective endocytosis, endosomal degradation, inefficient trafficking, and the need to convert from a single-stranded AAV genome to a transcriptional active double-stranded form **(Ill. 8)** (13) (15) (27) (28) (37) (39) (42) (47). A major rate-limiting step in AAV transduction is the intracellular trafficking of the virus from the cytoplasm into the nucleus (27).

To date, little is known regarding the cellular mechanisms controlling intracellular trafficking of AAV through the cytoplasm, or how the vector may be lost during this process. Vector degradation is a major concern for AAV gene therapy because it results in a lower efficiency of transduction due to the loss of viable vector and subsequent genetic expression. Determining where and how the AAV genome is degraded will allow us to increase trafficking and limit degradation. There have been many previous studies using fluorescent labeling to track the movement of AAV into the nucleus (25) (316 (37) (41)

(42) (43). However, an integral part of AAV genomic stability may be understanding how the obstacles AAV encounters in the cytoplasm prior to entering the nucleus relate to overall transduction. Previous studies on improving AAV transduction have focused mainly on the nucleus; however, this study will shed light on the hurdles AAV encounters in the cytoplasm.

The first major aim of this project is to design and implement a novel dual reporter system that will show the status of AAV vectors as they move through the cytoplasm into the nucleus **(Ill. 9)**. This system will be designed to specifically indicate whether or not the AAV genome is exposed before reaching the nucleus, where there is a great potential for DNA degradation. This new system is comprised of a single-stranded AAV reporter vector containing two different types of luciferases: *Cypridina* Luciferase (Cluc) and *Gaussia* Luciferase (GLuc). *Cypridina* Luciferase is placed under the control of a nuclear promoter and therefore it is expressed only in the nucleus. The second, *Gaussia* Luciferase, will be modified and called cytoGLuc. The cytoGLuc is under the control of the T7 promoter a cytoplasmic promoter that must be activated by the T7 RNA polymerase. The cytoGLuc was designed with two introns in the gene **(Ill. 10)**.

Both the original GLuc gene and the cytoGLuc have the same amino acid sequence; however, there are small variations in the three-letter genetic code setting up splice sites if the vector reaches the nucleus. GLuc expression will be detected when AAV is in the cytoplasm in the presence of T7 RNA polymerase. However, inside the nucleus the introns of CytoGLuc will be spliced out making the gene non-functional. This design ensures that there should be no GLuc expression coming from the nucleus. Both of these luciferase

proteins are secreted. Our system will allow us to quantitate how much vector is present in the cytoplasm and in the nucleus at any given time point **(Ill. 11)**.

Our system will be a very powerful tool for studying AAV intracellular trafficking patterns. The second aim of this project is to screen drugs/molecules using our dual reporter system that may enhance rAAV vector trafficking and transduction **(Ill. 12)**. Understanding the cellular trafficking associated with AAV genome stability and the possible cellular interactions will aid in the improvement of AAV vector efficiency for gene therapy.

ILLUSTRATION 8.

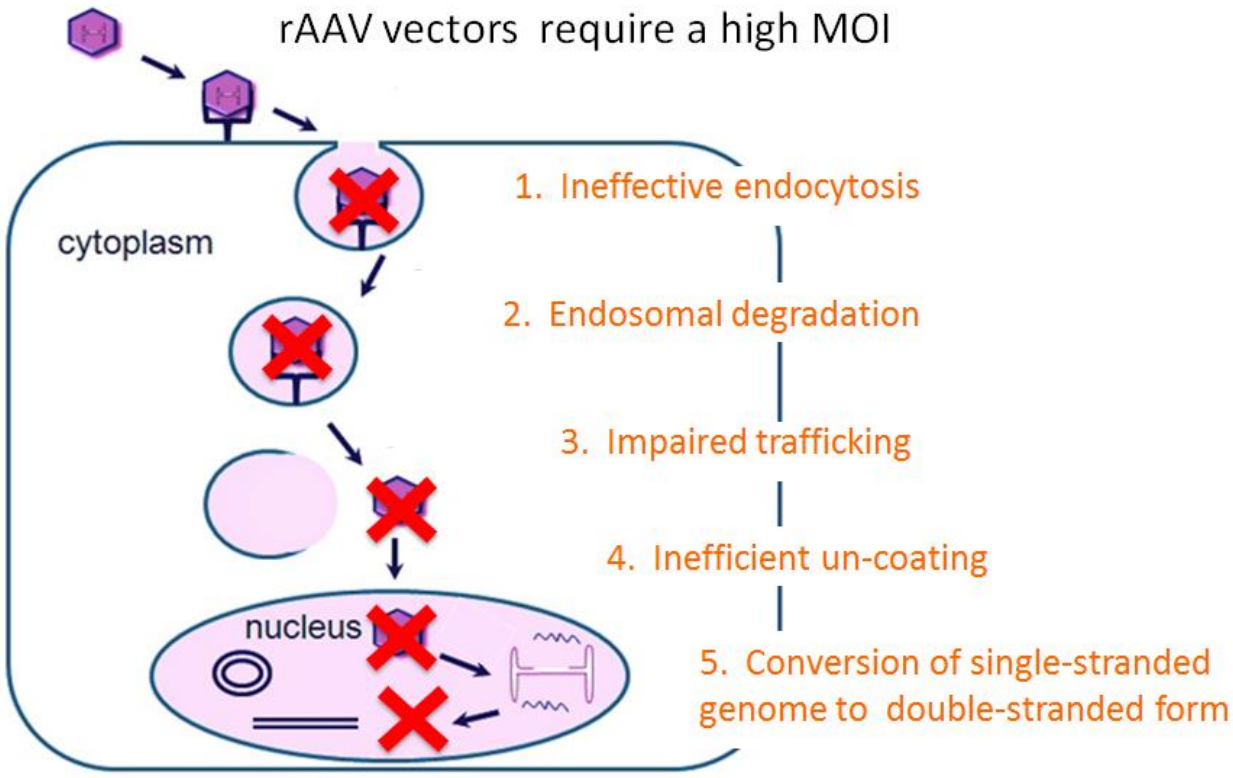


Illustration 8. rAAV vectors require a high MOI due to vector being degraded or lost at various steps during transduction.

It has since been determined that AAV vector efficiency decreases due to the following mechanisms: ineffective endocytosis, endosomal degradation, inefficient trafficking, and the need to convert from a single-stranded AAV genome to a transcriptional active double-stranded form. Due to vector being lost at multiple steps during infection, the virus requires a high MOI to be given to patients.

ILLUSTRATION 9.

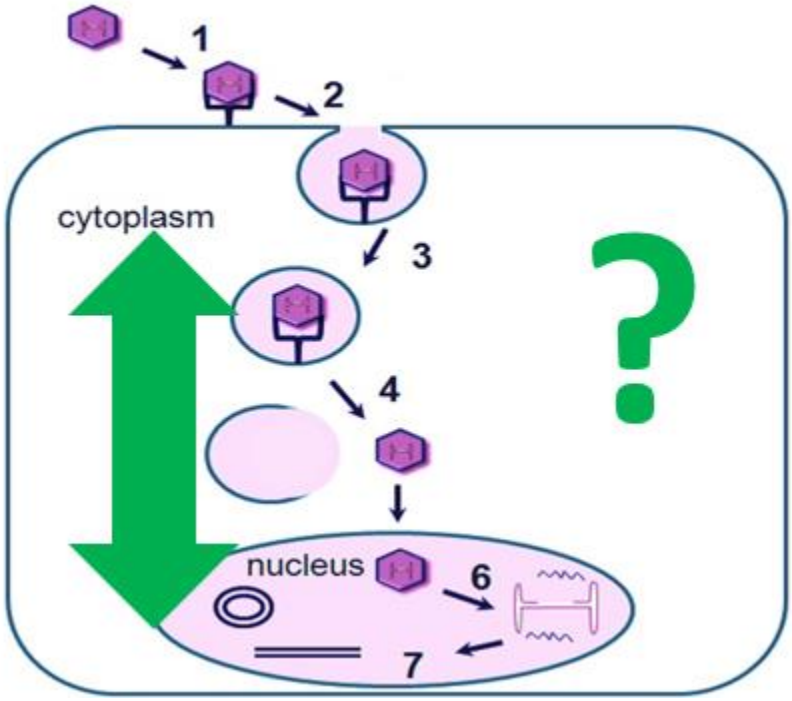


Illustration 9. Determining the fate of AAV vectors in the cytoplasm.

The first aim of this project is to examine the fate and/or role of the AAV genome in the cytoplasm in correlation with the nucleus. This project will determine if the amount of vector in the cytoplasm correlates to overall nuclear transduction.

ILLUSTRATION 10.

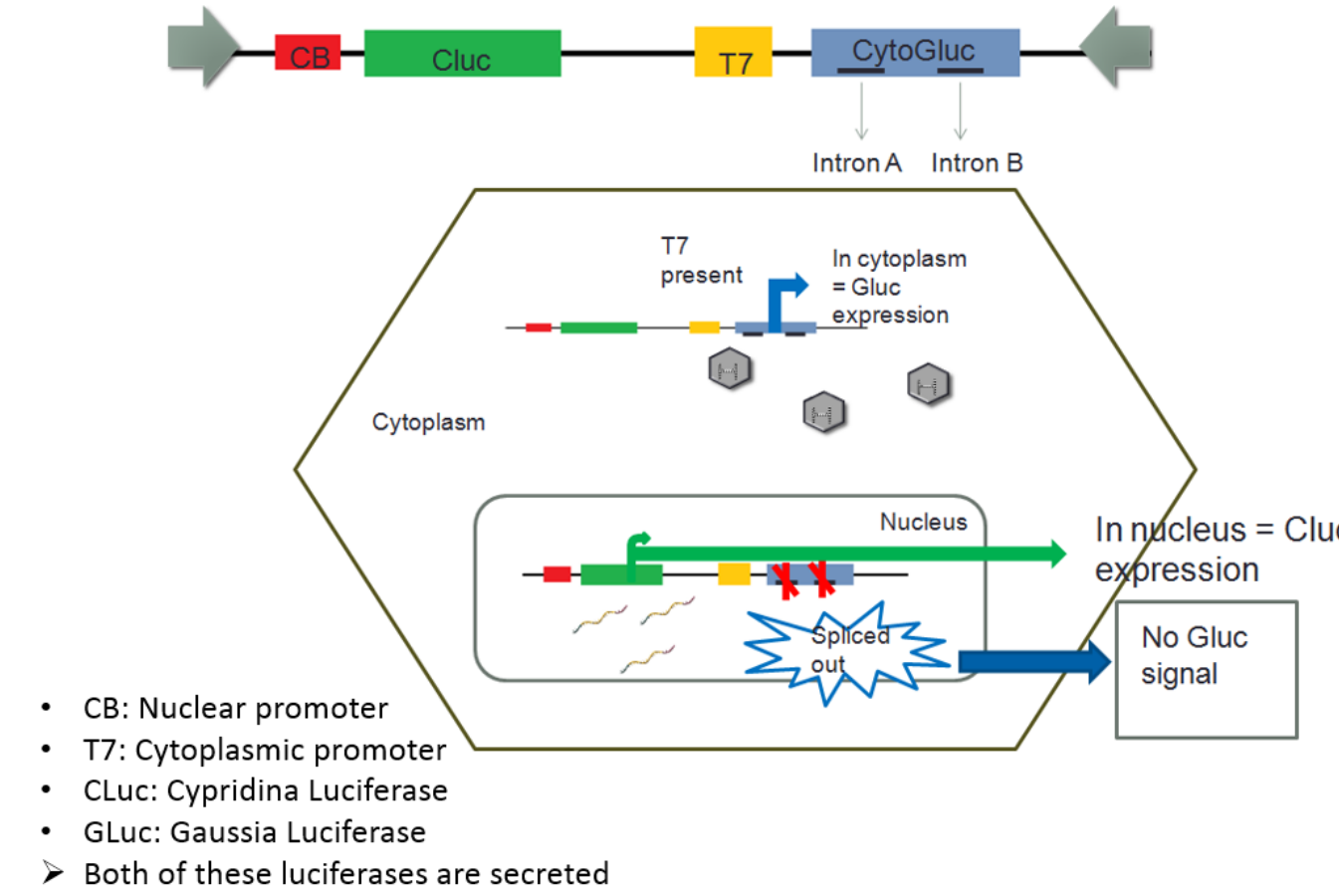


Illustration 10. Novel Dual Reporter System.

This new system is comprised of a single-stranded AAV reporter vector containing two different types of luciferases: *Cypridina* Luciferase (Cluc) and *Gaussia* Luciferase(GLuc). *Cypridina* Luciferase is placed under the control of a nuclear promoter and therefore it is expressed only in the nucleus. The second, *Gaussia* Luciferase, will be modified and named CytoGLuc. The cytoGLuc is under the control of the T7 promoter a cytoplasmic promoter that must be activated by T7 RNA polymerase.

ILLUSTRATION 11.

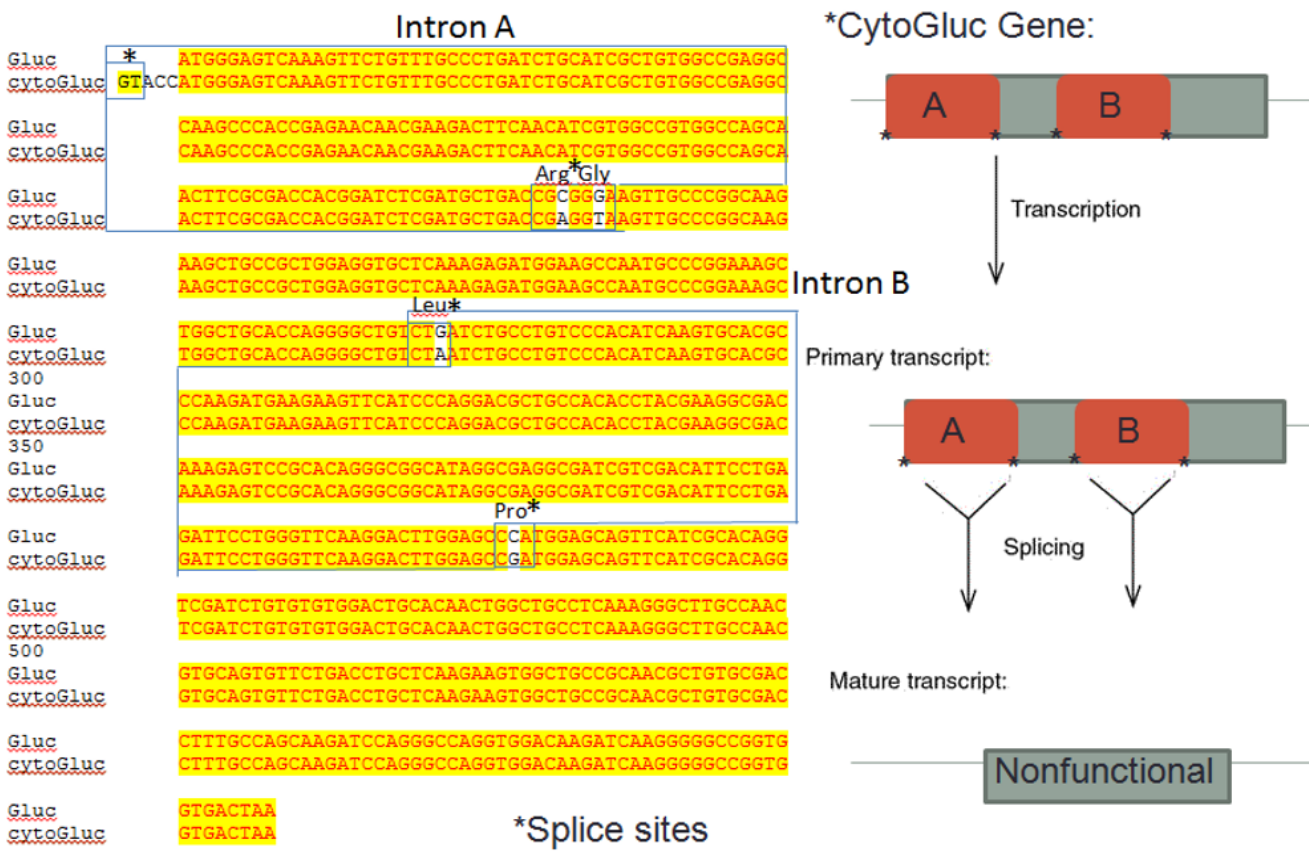


Illustration 11. CytoGLuc Splicing in Reporter System.

The cytoGLuc was designed with two introns in the gene. Both the original GLuc gene and the cytoGLuc have the same amino acid sequence; however, there are small variations in the three letter genetic code setting up splice sites when the vector reaches the nucleus. GLuc expression will be detected when AAV is present in the cytoplasm along with T7 RNA polymerase. However, inside the nucleus the introns should be spliced out and making the gene non-functional. GLuc expression will only be coming from the cytoplasm.

ILLUSTRATION 12.

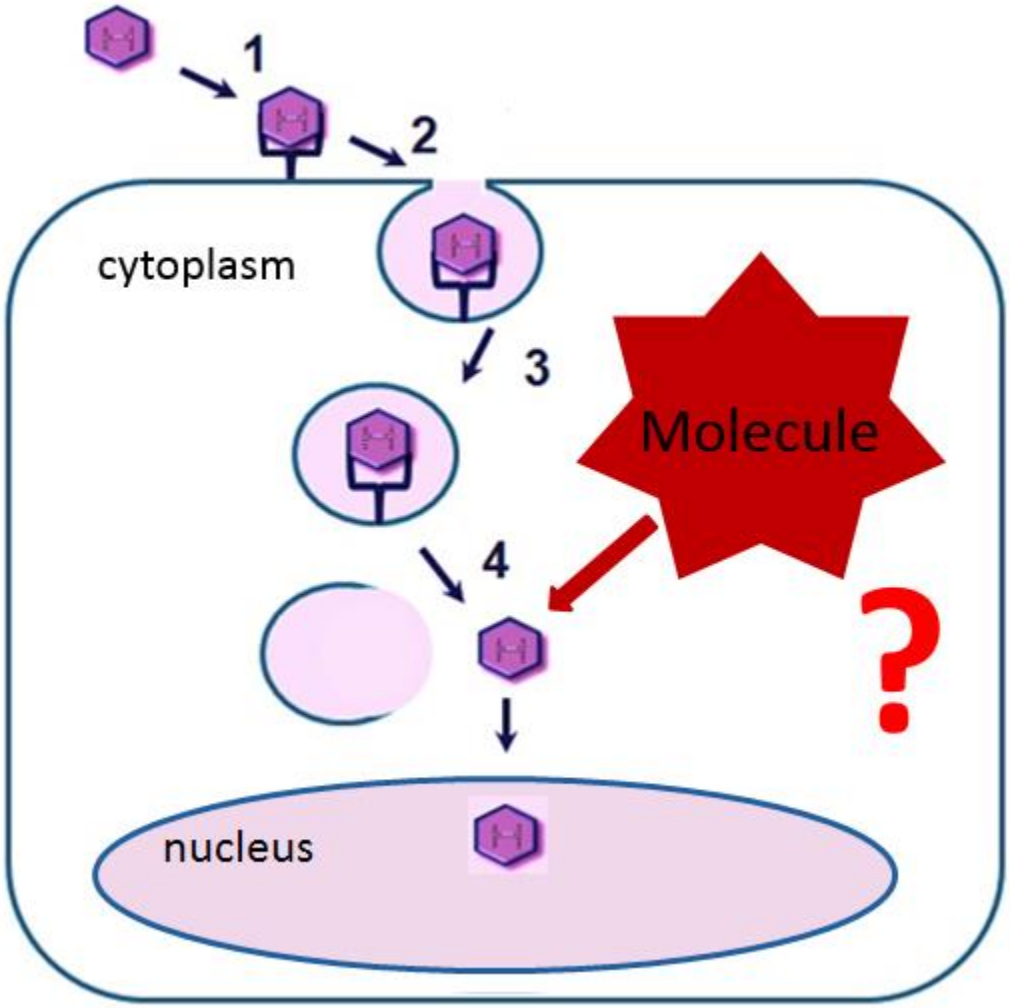


Illustration 12. The effect of different molecules on AAV genomic stability in the cytoplasm.

The second aim of this project is to screen different molecules both known and unknown enhancers/inhibitors that could affect AAV trafficking through the cytoplasm using our dual reporter system.

CHAPTER 2

MATERIALS AND METHODS

In vitro experiments

Cell lines

Cell lines HeLaS3 and Hek293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (purchased from ThermoFisher Scientific catalog number: 11965-126) supplemented with 10% fetal bovine serum broth (purchased from Sigma catalog number: 12003C) and with 1% Penicillin-Streptomycin (purchased from ThermoFisher Scientific catalog number: 15140-122). GM16095 cells (a human fibroblast cell line from the Coriell Institute) were cultured in minimum essential media (purchased from ThermoFisher Scientific catalog number: 11095-072) with 10% fetal bovine serum with 1% Penicillin-Streptomycin. Cell line Hek293T7 (expressing T7 RNA polymerase) were cultured in DMEM supplemented with 10% fetal bovine serum, and 500μg of G418 (purchased from sigma catalog number: G1279-1G). HeLaS3 and Hek293 cells were purchased from the ATCC (American Type Culture Collection) and maintained in a humidified 37 °C incubator with 5% CO2 as described previously (84).

Construction of rAAV(ss) scAAV(ds) and Ad vector plasmids

PssAAV-CB-CL plasmid was constructed using pssAAV-CB-EGFP as a backbone digesting with BamHI. The insert was cloned in with the Cypridina luciferase gene from pSV40-Cluc (purchased from New England Biolabs) using BamHI and

SpeI. pssAAV-CB-CL-T7-CG(dual reporter) plasmid was constructed using pssAAV-CB-CL as the backbone and cutting with EcoRI and BamHI. The insert was cloned in by cutting with XbaI and BamHI into the T7-cytoGluc cassette from the pCI-cyto-Gluc (purchased from Genescript). pssAAV-CB-Gluc plasmid was constructed using pssAAV-CB-EGFP as a backbone digesting with BamHI. The insert was cloned in using BamHI site in the Gluc gene from the pCI-CMV-Gluc(ordered from Genescript). pdsAAV-CB-Gluc plasmid was constructed using pdsAAV-CB-EGFP as a backbone digesting with HincII. The insert was cloned in using the XbaI and BamHI site in the Gluc gene from the pCI-CMV-Gluc(purchased from Genescript). pdsAAV-T7-CytoGluc plasmid was constructed using pdsAAV-CB-EGFP as a backbone digesting with HindIII and MluI. The insert was cloned in by cutting with XbaI and BamHI in pCI-cyto-Gluc(purchased from Genescript). pdsAAV-CB-CytoGluc plasmid was constructed using pdsAAV-CB-EGFP as a backbone digesting with HincII and ClaI. The insert was cloned in by cutting with PciI in the pCI-cyto-Gluc(Genescript). AAV-LacZ, Ad-LacZ, and AAV-GFP plasmids were constructed as previously described (85). AAV-hAAT plasmid was constructed as previously described (84).

Ligation and transformation of luciferase plasmids

The plasmid backbones were digested overnight with previously listed enzyme and then dephosphorylated using the rapid Alkaline phosphatase protocol from Roche (catalog number: 04898133001). Inserts were digested and blunted with T4 DNA polymerase protocol (purchased from New England BioLabs (NEB) catalog number: M0203S) and 10mM dNTPs (purchased from New England BioLabs catalog number N0447S). DNA was run on a 0.8% agarose gel, size of interest/fragment was extracted and purified using

ThermoFisher Scientific GeneJet Gel extraction kit (catalog number: K0691). Next constructs were ligated together using the QuickLiagase kit and protocol from NEB (catalog number: E1201L). Constructs (10uL of ligation mix) were transformed into 50uL of DH5-α bacteria (purchased from ThermoFisher Scientific catalog number: 18265017) for 20 minutes on ice. Then samples were heat shocked for 40 seconds at 42℃ and cooled on ice for 5 minutes. Samples were given 200uL of LB broth (purchased from Sigma catalog number: L3022-6X1KG) and shaken at 37℃ for 1 hour. Then 200uL of each sample was plated on agarose plates with selection marker of Ampicillin (purchased from Sigma catalog number: A0166-100G). Plates were incubated overnight at 37℃. Colonies were inoculated in LB broth with 200mg/mL of Ampicillin.

AAV and Ad vector production

Hek293 cells were cultured in roller bottles and used to produce rAAV vectors in serotype 2 or 8 by triple plasmid transfection as described previously (86). Briefly, the cells were collected at 72 hours post-transfection and the rAAV vectors were then purified by two rounds of cesium chloride(purchased from sigma catalog number C4036-1KG) gradient ultracentrifugation. After extensive buffer exchange against phosphate-buffered saline with 5% D-sorbitol (purchased from sigma catalog number: S1876-10MG), the purified virus was stored at −80°C before use. Vector purity and genome titer were analyzed by silver staining (Pierce silver stain kit purchased from ThermoFisher Scientific catalog number: 24612), and real-time PCR described previously (86). Ad virus production was performed as previously described (87).

Establishment of Hek293 cell line expressing T7 RNA polymerase

The pCI-neo plasmid(purchased from promega catalog number: E184) and a plasmid containing the T7 RNA polymerase gene (kindly provided by Bernard Moss at the NIH) were transfected together into Hek293 cells using LipofectAmine (purchased from ThermoFisher Scientific catalog number:11668-019) to the manufacturer's instructions. 6 hours after transfection DMEM was changed to DMEM with 500µg G418 (purchased from sigma catalog number G1279-1G) for 48 hours. Single cell clones were selected and expanded for further experiments. Hek293 T7 positive cells were determined using a pRS134-T7-GFP (purchased from Addgene catalog number: 33130) plasmid transfection. The established cell clone was maintained in the G418 selection medium.

Gaussia luciferase(GLuc) assay

GLuc expression from the medium was determined by adding 1µl of 200 µg/ml coelenterazine substrate (purchased from Nanolight catalog number: 55779-48-1) to 50µL of sample. Detection was read on a POLARstar omega 385 bioluminescence plate reader.

RNA extraction

RNA was extracted from cells using 0.5 ml of TriReagent (purchased from sigma catalog number: T9424). Cells were incubated in TriReagent for 5 minutes RT and then 100µL of chloroform (purchased from sigma catalog number: 67-66-3) was added to each sample and then incubated at RT for 15 minutes. Next samples were centrifuged at 12,000g for 15 minutes at 4℃. The aqueous phase was transferred to a clean tube and 250µL of isopropanol (purchased from sigma catalog number: 67-66-0) was mixed into

each sample. The samples were incubated at -20°C for 30 minutes and then centrifuged at 12,000g for 8 minutes at 4°C. Isopropanol was removed and the RNA pellet was washed in 75% Ethanol (purchased from Pharmco-AAPER catalog number: 111ACS200). The samples were then centrifuged at 7,500g for 5 minutes at 4°C. Ethanol was removed and pellet was air dried for 30 minutes. Pellet was dissolved in 25µL of Sterile water (purchased from Fisher catalog number: 7732-18-5). Following RNA isolation, DNAse Treatment and removal was performed and followed the protocol from Ambion (catalog number: AM1906). After 2 µg of total RNA was reverse transcribed following the protocol from the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems(catalog number: 4368814). Two microliters of DNA was subjected to PCR amplification using the protocol from Taqman Gene expression Master Mix kit from Applied Biosystems (catalog number: 4369016). Taqman primers were specifically designed to the Cypridina luciferase (catalog number: 133527449) and Gaussia luciferase (catalog number 133527453) genes from IDT. Also purchased from IDT was a GAPDH control gene (133527457). Calculations were converted from CT values to fold change using excel.

Detection of rAAV genomes in both the cytoplasm and the nucleus using VVT7.

Hek293 cells were infected with ssAAV2-CB-CL-T7-CG dual reporter vector at an MOI of 10, and dsAAV-CB-GFP(dsEgfp) at an MOI of 10 as a control, and VVT7 (kindly provided by Bernard Moss at the NIH) at an MOI of 1. Luciferase expression was detected 48hours post-infection.

Verification of T7-CytoGLuc construct in 293 and 293T7 cells.

Hek293 (control cell line) and 293T7 cells were transfected with these plasmids: pdsAAV-CB-GLuc, pdsAAV-T7-Gluc, pdsAAV-CB-CytoGluc, and pdsAAV-CB-GFP for 16 hours using polyjet reagent protocol (purchased from SignaGen Laboratories catalog number: SL100688). Polyjet protocol as follows: for each well, 0.5 ml of DEMM was added 30 minutes before transfection. For each well, .5 µg of DNA into 25 µl of serum-free DMEM was diluted. Gently mixtures were pipetted up and down briefly. For each well, 1.5 µl of PolyJet™ reagent into 25 µl of serum-free DMEM with High Glucose was diluted. Gently mixtures were pipetted up and down 3~4 times to mix. Next, the diluted PolyJet™ reagent was added immediately to the diluted DNA solution all at once. The cells were incubated for ~15 minutes at room temperature to allow PolyJet™/DNA complexes to form. Lastly, the 50 µl PolyJet™/ DNA mixture was added drop-wise onto the medium in each well and the mixture was homogenized by gently swirling the plate. Removal of PolyJet™/DNA complex-containing medium and replacement with fresh media was performed 16 hours post-incubation. Media was harvested and luciferase expression was detected 48 hours post-incubation.

LacZ staining

HelaS3 cells were pretreated with Cidofovir (purchased from Cayman Chemical company catalog number: 113852-37-2) or Ganciclovir (was purchased from cayman chemicals catalog number*: 82410-32-0)* at 100µg/mL or 500µg/mL of each drug or *Phosphate-buffered saline* (PBS) (purchased from Corning catalog number: 21-031-CV) for 16 hours. The cells were washed twice and infected with Adenovirus (lacZ) at an

MOI of 0.5 or Adeno-associated virus(LacZ) at an MOI of 10. LacZ stain was performed 24hrs post-infection at 37℃. Media was discarded and cells were fixed for 5 minutes at 4℃ with fixing solution. Fixing solution is 37% Formaldehyde (purchased from sigma catalog number: 50000) and 25% Glutaraldehyde (purchased from sigma catalog number: 111-30-8) in 1 mL of PBS. The cells were then washed 3x with cold PBS and then stained at 37℃ with staining solution. Staining solution is 40mg/mL X-gal (purchased from sigma catalog number: R0401 in *Dimethyl sulfoxide* (DMSO) (purchased from sigma catalog number: 67-68-5), 1M MgCl2 (purchased from sigma catalog number: 7791-18-6), Potassium ferricyanide (purchased from sigma catalog number: 13746-66-2), and Potassium Ferro cyanide (purchased from sigma catalog number:14459-95-1) in 1mL of PBS. LacZ stain visualization was performed 24 hours post-infection.

CDV luciferase drug studies

Cells were pretreated with indicated concentrations of CDV or Mock treatment (PBS) for 16 hours. Cells were washed twice to remove drug then infected with rAAV2 or rAAV8 luciferase vector at an indicated MOI. Luciferase expression was detected at indicated time points using the BioLux Cypridina Luciferase Assay Kit protocol (purchased from NEB catalog number: E3309L).

CDV GFP experiments

Cells were pretreated with indicated concentrations of CDV or Mock treatment(PBS) for 16 hours. Cells were washed twice to remove drug then infected with rAAV2 or rAAV8 GFP reporter vector at an indicated MOI. GFP visualization was performed 24 or 48 hours post-infection.

CDV hAAT ELISA experiments

Cells were pretreated with indicated concentrations of CDV or Mock treatment (PBS) for 16 hours. Cells were washed twice to remove drug then infected with rAAV2 or rAAV8 hAAT reporter vector at an indicated MOI. hAAT expression was assayed by harvesting media either 24 or 48 hours after infection. Protocol for ELISA Assay for hAAT is as follows: plates were coated with hAAT antibody coating (purchased from sigma catalog number: A0409) at 1 1:1000 in coating buffer. Coating Buffer recipe is 5.3g of Na_2CO_3 (purchased from sigma catalog number: 497-19-8), 4.2g of $NaHCO_3$ (purchased from sigma catalog number: 144-55-8), 1g sodium azide (purchased from sigma catalog number: 26628-22-8) in 1L of distilled H_2O. The solution was adjusted to a pH of 9.6. 100uL of antibody in coating buffer was added to each well and incubated at 4˚C overnight. Next the plate was blocked with 300uL per well of 3% BSA (Bovine Serum Albumin). 3% BSA is 1.5g BSA (Purchased from sigma catalog number: 9048-46-8) into 50mL Phosphate Buffered Saline (PBS) solution with the detergent Tween® 20 (PBST) (purchased from cell signaling technology catalog number: 9809) at room temperature (RT) for 1 hour. Following the plate was washed 3x with PBST. Samples of media (200uL) were added as well as a hAAT standard (1000ng/mL). A1-antitrypsin Human AAT proteinase inhibitor (purchased from US Biological catalog number: A2298-29P) was serial diluted and used as a standard to compare with the samples. Next, the plate was washed 3x with PBST. Detecting Antibody (HRP conjugated, antibody purchased from US biological catalog number: A2298-28A) was used in a 1:5000 dilution. 100ul per well was added to the plate and incubated at RT for 1 hour. The plate was washed 3x with PBST. Then 100uL per well of the TMB microwell peroxidase

substrate system kit from KPL (catalog number: 50-76-00) was added. 0.5M *Sulfuric acid* (purchased from sigma catalog number: 7664-93-9) was added to stop the reaction at 50uL per well. The plate was read at 450nm using a Molecular Devices brand ThermoFisher Scientificmax microplate reader.

Time course of AAV-mediated transduction experiments

HelaS3 Cells were pretreated with increasing concentrations of CDV or GCV for 16 hours. Cells were washed to remove drug then infected with rAAV vector at an MOI of 10. Luciferase expression was detected indicated time points.

CDV optimal pretreatment experiment

HelaS3 Cells were pretreated with 100µg/mL of CDV or PBS for 16 hours before infection. Cells were infected with rAAV2-Luciferase at an MOI of 10. Four or eight hours post-infection HelaS3 Cells were treated with 100µg/mL of CDV or PBS. Media was harvested 48 hours post-infection. Luciferase expression was detected at indicated time points.

HIRT extraction

HIRT extraction buffer: Tris (pH 8.0) 1M to a final concentration of 10mM (purchased from ThermoFisher Scientific catalog number: 15568-025), EDTA (0.5M) to a final concentration of 10mM (purchased from Sigma catalog number: 60-00-4). SDS (sodium dodecyl sulfate) (10%) to a final concentration of 1% (purchased from Sigma catalog number:151-21-3), Proteinase K (20mg/ml) (purchased from sigma catalog number: 3115887001), and RNase A (20mg/ml) to a final concentration of 50ug/ml (purchased from ThermoFisher Scientific catalog number:12091-021). Cells were re-suspended in

850µL of HIRT extraction buffer. Then 250µl of 5M NaCl (purchased from sigma catalog number: 7647-14-5) was added to each tube and incubated at 4°C overnight. Samples were centrifuged for 45 minutes at 4°C at 14,000 RPMs. The supernatant was collected. Extraction was performed by taking the top clear layer twice after mixing with pheno-chloroform(purchased from Sigma catalog number: P3803-400mL). For each extraction, tubes were mixed up and down until milky. Next, samples were set on ice for 5 minutes and centrifuged at 4°C at 14,000 RPMs for 5 minutes. The top clear layer was extracted twice carefully. After the third extraction, twice the volume of 100% ethanol (purchased from Pharmco-AAPER catalog number: 111ACS200) was added. Samples were mixed gently and placed on ice for at least 30 minutes or at -80°C overnight. Next, samples were centrifuged at 4°C at 14,000 RPMs for 15 minutes. Supernatant was removed and pellet was washed with 70% ethanol, approximately 500mL. The samples were air dried for 2 minutes and 50µl of TE buffer (purchased from ThermoFisher Scientific catalog number: V6231) was added to allow DNA to dissolve completely.

Southern blotting hybridization

Alkaline agarose gel electrophoresis was used to analyze the DNA of the AAV genome as described previously (88). Briefly, HIRT DNA was extracted and 10µg of HIRT extracted DNA was resolved on a 1% alkaline agarose gel for 75 minutes at 120Volts. Gel was next washed in 0.25M HCl (purchased from Sigma catalog number: 7647-01-0) for 30 minutes. Next gel was washed in 0.4M NaOH (purchased from Sigma catalog number: 1310-73-2) for 20 minutes. Gel was transferred to a membrane(purchased from GE healthcare catalog number: RPN303B) overnight using 0.4M NaOH, two glass containers and 6 sheets of Whatman paper/filter paper(purchased from BioRad catalog

number: 1703965). Southern Blot sandwich consists of in order: three Whatman papers, membrane, gel, three more Whatman papers in one glass container. All items are saturated in 0.4M NaOH. The second glass container contains 1 liter of 0.4M with a Whatman paper connecting the two glass containers to allow the gel to transfer to the membrane overnight. The membrane is washed twice in in 2xSSC (purchased from Promega catalog number: V4261) and air dried completely overnight. After gel transfer, the membrane was hybridized with 32P-labeled probe (purchased from Perkin Elmer Catalog number: BLU513H500UC) that was produced using a fragment specific to the Cypridina Luciferase or Gaussia Luciferase portion of each vector. The purified fragment was labeled by a [α-32P] dCTP using the protocol from Prime-IT II Random Primer Labeling Kit (purchased from Aligent Technologies catalog number: 41106000). The blot was hybridized with 20µL of probe in Southern Blot oven at 65℃ overnight. The membrane was analyzed by X-ray autoradiography (purchased from Santa Cruz Biotechnology catalog number: sc-201697) overnight.

Real-time polymerase chain reaction (PCR)

10µg of each sample after HIRT extraction was treated with 1µL of DNaseI (purchased from sigma catalog number: 4716728001) and 0.5µg/mL of proteinase K (purchased from sigma catalog number: 3115887001). All qPCRs were carried out in an Eppendorf Mastercycler ep realplex machine using the Fast SYBR Green Master Mix (purchased from ThermoFisher Scientific catalog number: 4385612) and realplex software. In a 20 µl reaction system, the final concentrations of reagents were 0.4 µ*M* of each primer, 5 m*M* $MgCl_2$, 10 µl template, and 1× Master Mix. The PCR protocol was as follows: 1 cycle of 20 sec at 95°C followed by 40 cycles of 3 sec at 95°C and 30 sec at 60°C. A

melting curve analysis was added after PCR. SYBR Green primers were designed for the Cypridina Luciferase (forward primer: TACTGACTACAGCCATCCTACC and reverse primer: CAGGCTCCTTCAGCATCAAA), as well as cytochrome C gene as a control (forward primer: GAGTTCAAGAAAGAATGCTACAT and reverse primer: GTAAGGACAGTCCTGGCAATGAAC).

Viral Entry Experiment:

HelaS3 cells were pretreated with CDV (100µg/mL) or PBS for 16hrs. Next the cells were washed. Then infected with rAAV2-CB-Cluc at an MOI of 10 for 2 hours then washed to remove unattached virus. Cells were harvested right after wash (0 hour) and 2 hours post-wash. DNA was extracted using HIRT Extraction protocol. Southern blot analysis to the luciferase gene probe was performed as stated previously in this section.

CDV pretreatment on scAAV vectors

Cells were pretreated with 5-500µg/mL of CDV or Mock treatment (PBS) for 16 hours. Cells were washed twice to remove drug then infected with scAAV2 luciferase vector at an MOI of 10. Luciferase expression was detected at 48 hours post-infection.

Cell Fractionation Protocol

Cells were treated with 100µg/mL of CDV or PBS and infected with a rAAV2-Luciferase vector at an MOI of 10. Cells were then washed in PBS. The cells were gently resuspended in in 1000uL of cold hypotonic buffer. Hypotonic buffer consists of 10mM HEPES (pH7.9) (purchased from sigma catalog number: 7365-45-9), 1.5Mm MgCl2 (purchased from sigma catalog number: 7791-18-6), 10mM KCL (purchased from sigma

catalog number:7447-40-7), 0.5mM DTT (purchased from sigma catalog number: 3483-12-3), and 0.5mM PMSF (purchased from sigma catalog number:329-98-6). Samples were incubated on ice for 15 minutes. Next, 10uL of 10% NP40 (purchased from ThermoFisher Scientific catalog number: 28324) was added to each tube, and was vortexed for ~ 30 seconds. Then samples were centrifuged for 8 minutes at 500rpm 4℃. The supernant (which contains the cytoplasmic fraction) is separated out into a new tube and put on ice. The pellet (which contains the nuclear fraction) was resuspended with 1mL of hypotonic buffer. DNA was precipitated with 300µL 5M NaCl overnight at 4℃. Next HIRT extraction was performed as stated earlier.

Comparative drug studies with Hydroxyurea and Etoposide

Hydroxyurea (catalog number: 127-07-1) and Etoposide (33419-42-0) were purchased from Sigma and diluted in DMSO. Cells were pretreated with increasing concentrations of CDV (10µg/mL, 50µg/mL, or 100µg/mL) PBS, HU (40mM), ETO (10µM), in combination of CDV with HU, or in combination of CDV with ETO for 16 hours. Cells were washed twice to remove drug then infected with rAAV2 luciferase vector at an MOI of 10. Luciferase expression was detected 48 hours post-infection.

Comparative drug studies with MG132

MG132 were purchased from Sigma (catalog number C2211). Cells were pretreated increasing concentrations of CDV (10µg/mL, 50µg/mL, or 100µg/mL), PBS for 16 hours. Two hours prior to infection MG132 (20µM) was added to cells alone or in cells already pretreated with CDV. Cells were incubated two hours with drugs then cells were

washed twice to remove drug then infected with rAAV2 luciferase vector at an MOI of 10. Luciferase expression was detected 48 hours post-infection.

Statistical analyses

Statistical analysis was determined using a 2-tailed student *t* test. The differences were considered significant when p was<0.05

Ganciclovir drug studies

Cells were pretreated with 5-500 µg/mL of Ganciclovir (was purchased from Cayman chemicals catalog number*: 82410-32-0)* or Mock treatment (DMSO) for 16 hours. Cells were washed twice to remove drug then infected with rAAV2 or scAAV2 luciferase vector at an MOI of 10. Luciferase expression was detected 48 hours post-infection.

Nuclear Entry Experiment:

HelaS3 cells were pretreated with CDV (100ug/mL) or PBS for 16hrs. Next the cells were washed. Then infected in a small plate (6cm dish) with rAAV2-CB-Cluc at an MOI of 10 for 2 hours then washed to remove unattached virus. Cells were collected at this time and marked as 0hr time point. Cells were washed and harvested at 4 and 24 hours post-infection wash. Cells were then fractionated and then HIRT extracted as listed previously. Southern blot analysis was performed to compare AAV DNA status between cytoplasm and nucleus as previously listed. Bands of rAAV were quantified using ImageJ software to a 500pg control of luciferase probe.

Cytoplasmic and nuclear drug studies

N-acetyl-L-leucyl-L-leucyl-L-norleucinal, (LLnL) was purchased from Sigma (catalog number: 110044-82-1). Cells were pretreated with CDV (100µg/mL), Hydroxyurea (1mM), LLnL (5uM) or Mock treatment (DMSO) for 16 hours. Cells were washed twice to remove drug then infected with rAAV2 dual luciferase vector at an MOI of 10. Luciferase expression was detected at indicated time points post-infection.

Cytoplasmic and nuclear CDV experiment

Cells were pretreated with CDV (100µg/mL), or Mock treatment (PBS) for 16 hours. Cells were washed twice to remove drug then infected with rAAV2 dual luciferase vector at an MOI of 10. Luciferase expression was detected at indicated time points post-infection.

Cytoplasmic and nuclear BafilomycinA1 experiment

BafilomycinA1 (was purchased from Sigma catalog number: 88899-55-2). Cells were pretreated with 0-500nM concentrations of BafilomycinA1, or Mock treatment (DMSO) for 2 hours prior to rAAV infection. Cells were then infected with rAAV2 dual luciferase vector at an MOI of 10. Cells were incubated with drug and rAAV for two hours then washed twice to remove drug. Luciferase expression was detected at 24 and 48 hours post-infection.

Drug transcriptome analysis

HelaS3 cells were pretreated with PBS or 100µg/mL CDV. Cells were collected in 1ml cold PBS and centrifuged at 2000rpm for 5min at 4^{o}C. The cell pellets were stored at -

80^{o}C immediately. RNA was extracted using the RNAeasy Mini Kit (purchased from Qiagen catalog number 74104). These samples were sent out for transcriptome analysis. Data was analyzed using a 1.5 fold cut off via Ingenuity software. The significance values for the canonical pathways and diseases & function pathways are calculated by Fisher's exact test right-tailed. The significance indicates the probability of association of molecules from our dataset with the canonical pathway or diseases & function pathway by random chance alone.

Lipofectamine 2000 protocol for siRNA

ATRIP siRNA was purchased from SANTA CRUZ Biotechnology (catalog number sc-44801). For 24 well plate, each sample was aliquoted 50uL per tube of Opti-MEM media (purchased from ThermoFisher Scientific catalog number: 51985091). 3uL of LipofectAmine (purchased from ThermoFisher Scientific catalog number:11668-019) reagent was added into 50µL Opti-MEM medium in a separate tube. Next siATRIP RNA (25pmol, 50pmol, and 100pmol) was added into 50µL. The lipofectAmine mix was combined with each siATRIP RNA tube. The mixture was incubated for 5 minutes at RT. The siATRIP RNA/ lipofectAmine mix was added directly to wells in a drop by drop manner and incubated on cells for 6 hours.

In vivo experiments

Mouse strain

Exon 16 FVIII knockout Balb-c HA mice were obtained from Dr. Haig Kazazian (University of Pennsylvania, Philadelphia, PA). All mice were housed in a specific pathogen-free environment with a normal diet. All surgical procedures involving mice

were in accordance with institutional guidelines under approved protocols at the Temple University. Mice were anesthetized using Isoflurane (provided by the Temple animal facility induction 3-5%, maintenance 1-3%). Mice were monitored every five minutes by absence of limb withdrawal from toe pinch. At the termination of this experiment, mice were scarified by asphyxiation from gas cylinder(via inhalation from carbon dioxide gas cylinder) followed by cervical dislocation.

AAV vector injections

6-8 week old BalbC/HA mice were put above a heat lamp (35℃ for 10 mins) to increase blood flow in tail veins. Immediately prior to use, AAV vector was diluted in 0.9% NaCl (purchased from sigma catalog number: 7647-14-5) solution to a total volume. A volume of 200 µl was injected into the tail vein of each mouse. Total vector injection was 1 x 10^{11} viral particles/mouse. Syringes were pre-coated with 1:10,000 of pluronic F68 (purchased from ThermoFisher Scientific catalog number: 24040-032) to prohibit AAV sticking to the plastic sides.

CVD preliminary animal experiment

Human Light chain (LC) vector at $1x10^{11}$ viral particles was injected into a 6-8-week old HA mouse via tail vein. Doses of 100mg/kg(high dose) of CDV or 30 mg/kg(low dose) of CDV or PBS were injected subcutaneously twice two days apart prior to rAAV injection and then injected once a week for 4 weeks post-infection. Mouse plasma samples after vector administration were harvested by retro-orbital bleeding at regular intervals using sodium citrate (purchased from sigma catalog number: 6132-04-3) as an anticoagulant at a final concentration of 0.38% (wt/vol). The blood samples were then centrifuged at 4 °C for

10 minutes at 10,000 rpm in a microcentrifuge. The collected plasma samples were stored at -80°C for ELISA assay.

Enzyme linked immuno assay (ELISA)

An enzyme linked immuno assay (ELISA) was used to measure the amount of human LC antigen found in a sample. Plates are coated with human light chain antigen purchased from Green Mountain Antibodies (catalog number: GMA-8018) in coating buffer overnight at 4° C. The next day the coating buffer was removed and 3% BSA in PBST was added for 1 hour to block. After 1 hour the wells are washed with PBST three times. Samples were added in a dilution of 1/20 and incubated at RT for 1 hour. Next, wells were washed with PBST three times. Secondary antibody to light chain (purchased from Green Mountain Antibodies catalog number: GMA-8022) was diluted in PBST at a 1:2000 dilution and added to the plate. After 1 hour the antibody was removed and the wells were washed 3x with PBST. Horseradish peroxidase (purchased from sigma catalog number: 9003-99-0) was diluted in PBST at a ratio of 1:10,000 and added to wells. After 1 hour the wells were washed 3x with PBST. Wells were developed using a KLP brand TMB microwell peroxidase substrate system(as stated before) and the plate was read at 450 nm using a Molecular Devices brand ThermoFisher Scientificmax microplate reader. Kogenate (purchased from Bayer healthcare) was serial diluted 1:2 and used as a standard to compare with the samples.

CHAPTER 3

RESULTS

rAAV genomes can be detected in the cytoplasm using the novel dual reporter trafficking system

Developing a system to detect rAAV genomes in the cytoplasm

The first major aim of this project is to design and implement a novel dual reporter system that will show the status of AAV vectors in both the cytoplasm and the nucleus **(Ill. 9)**. Our new system is comprised of a single-stranded AAV reporter vector containing two different types of luciferases: CLuc and GLuc. *CLuc gene* is placed under the control of a nuclear promoter and therefore it is expressed only in the nucleus. The second, *Gaussia* Luciferase, is modified and called cytoGLuc. The cytoGLuc is under the control of the T7 promoter, which is a cytoplasmic promoter that is activated by T7 RNA polymerase. The cytoGLuc was designed with two introns in the gene **(Ill. 10)**. GLuc expression will be detected when AAV is in the cytoplasm in the presence of T7 RNA polymerase. However, inside the nucleus the introns of CytoGLuc will be spliced out thus making the gene non-functional. This design ensures there should be no GLuc expression coming from the nucleus. Both of these luciferase proteins are secreted. In preliminary studies, Vaccinia virus carrying the T7 RNA polymerase (VV T7) was employed to provide T7 RNA polymerase to activate the cytoplasmic T7 promoter. VV is a large DNA virus that replicates only in the cytoplasm. Hek293 cells were infected with ssAAV2-CB-CL-T7-CG (dual reporter vector), dsAAV-CB-GFP (dsEgfp), in conjunction with VVT7 or left

uninfected. DsEgfp and Media (no infection) were utilized as negative controls. The dual reporter vector (ssAAV2-CB-CL-T7-CG) in conjunction with VVT7 indicated that AAV is present in both the cytoplasm and nucleus 48 hours post-infection **(Fig. 1)**. There was a noticeable decrease in nuclear transgene expression when cells were co-infected with the dual reporter vector and VVT7 compared to only dual reporter vector suggesting VVT7 is toxic to cells within our system. VVT7 was determined not to be an optimal choice for T7 RNA polymerase expression.

Next we sought to transfect a plasmid carrying the T7 RNA polymerase gene (pAAV-CB-T7RP) to compare to VVT7 infection and a control plasmid with no T7 RNA polymerase (pCI-neo). The results demonstrate that we can detect AAV in the cytoplasm **(Fig 2)** by transfecting pAAV-CB-T7RP without decreasing nuclear transduction **(Fig 3)**. AAV was detected in the cytoplasm with .2µL or 1µL of pAAV-CB-T7RP + VssAAV2-CB-CL-T7-CG) **(Fig 2)**. The highest AAV expression in the cytoplasm was observed in the presence of pCI-neo + VssAAV-CB-CL-T7-CG 1µL + VVT7 **(Fig 2)**. However, there was also a minimal decrease in AAV nuclear expression with pCI-neo + VssAAV-CB-CL-T7-CG 1µL + VVT7 compared to pCI-neo + VssAAV-CB-CL-T7-CG 1µL **(Fig 3)**. There was an increase observed in AAV expression with pAAV-CB-T7RP + dual reporter vector compared to pCI-neo + dual reporter vector in the nucleus **(Fig 3)**. To eliminate transfecting Hek293 cells, a Hek293 stable cell line expressing the T7 RNA polymerase was constructed. Clones were transfected with pRS314-T7-EGFP to verify T7 RNA polymerase production. In the presence of T7 RNA polymerase, pRS314-T7-GFP will show GFP expression.

FIGURE 1.

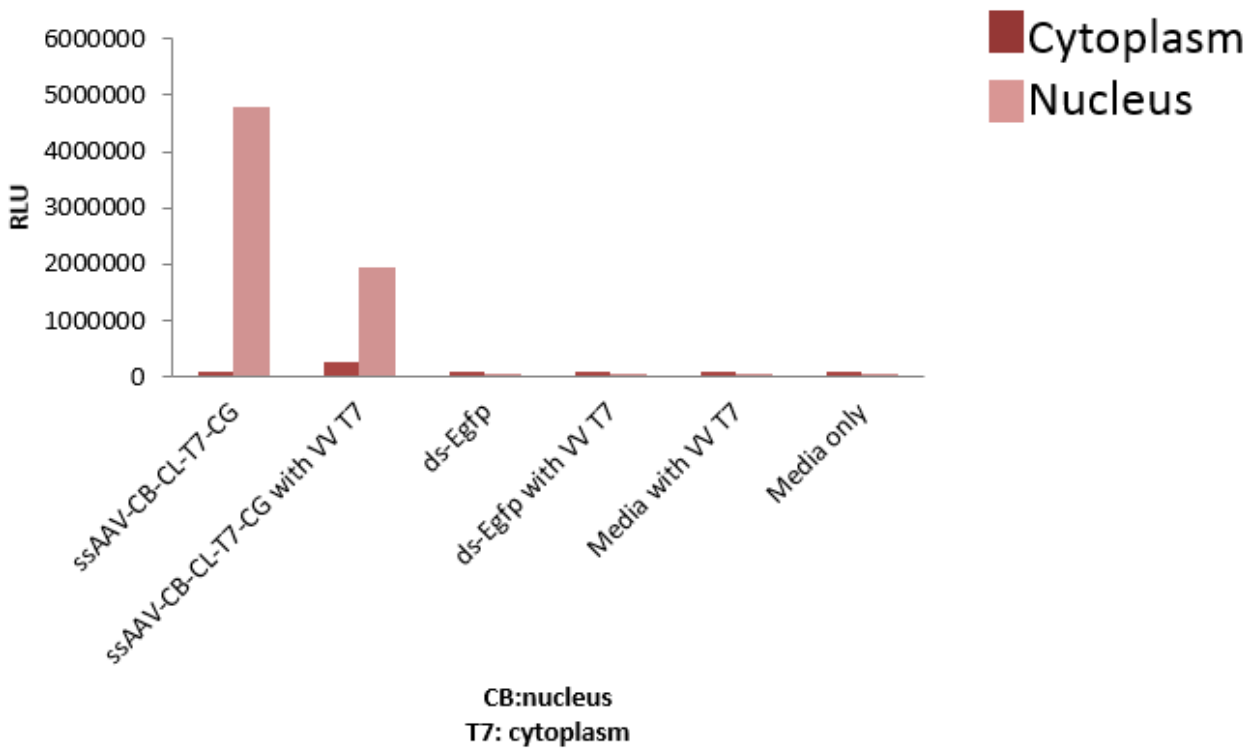


Figure 1. Detection of AAV in both the cytoplasm and the nucleus using dual reporter system and VVT7.

Hek293 cells were infected with ssAAV2-CB-CL-T7-CG (dual reporter vector), dsAAV-CB-GFP(dsEgfp), or Media (no AAV present) at an MOI of 10 alone or in conjunction with VVT7 at an MOI of 1. DsEgfp and Media were used as negative controls. Luciferase expression was detected using the enzymatic assay 48 hours post-infection in both compartments: nucleus(pink) and cytoplasm(red).

FIGURE 2.

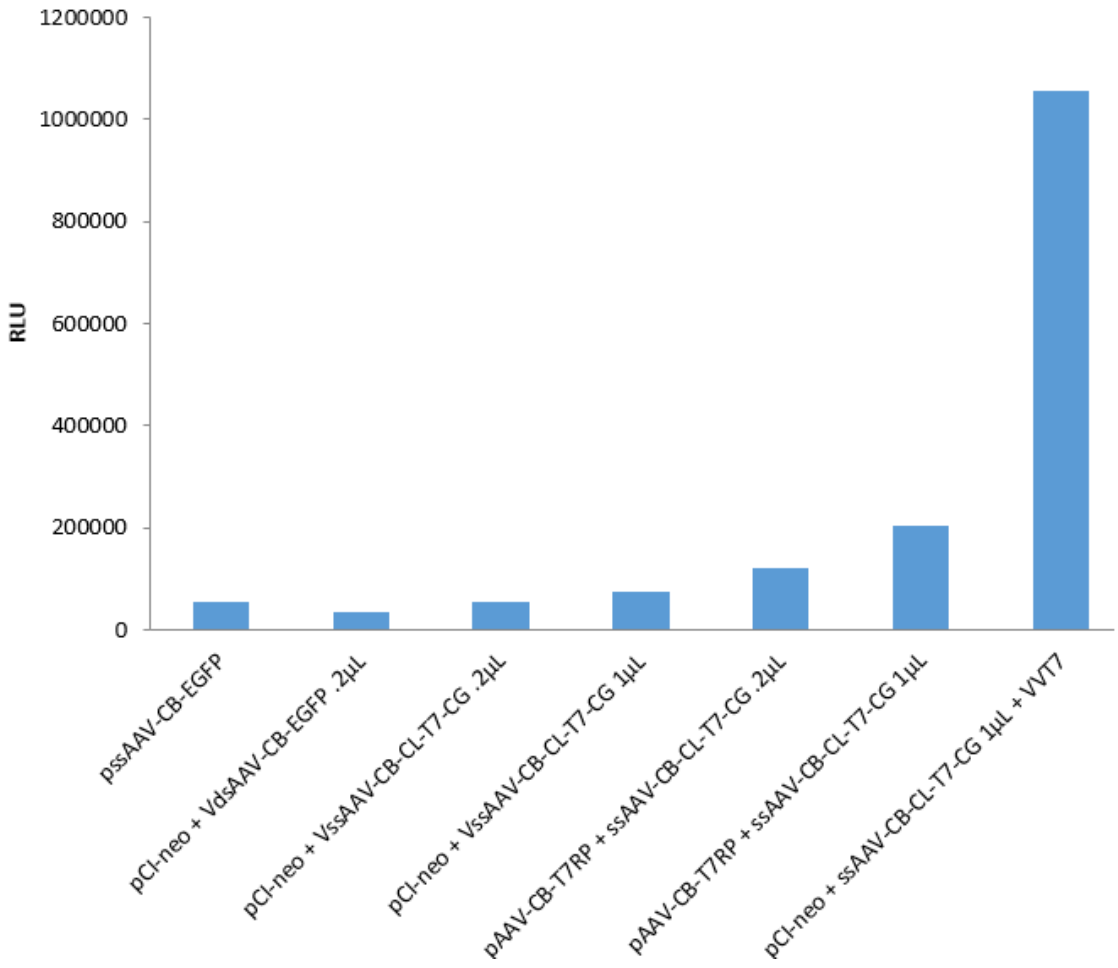


Figure 2. Comparing the amount of AAV present in the cytoplasm between a transfection of a plasmid carrying T7 RNA polymerase versus VVT7.

Hek293 cells were transfected with pCI-neo or pssAAV-CB-GFP (negative plasmid controls for T7 RNA polymerase production) or pAAV-CB-T7RP (plasmid carrying T7 RNA polymerase gene) for 16 hours. Cells were then infected with VssAAV2-CB-CL-T7-CG (dual reporter vector) at the volume indicated alone or in conjunction with VVT7 at an MOI of 1. Cells were also infected with VdsAAV-CB-GFP (negative luciferase control). Luciferase expression was detected using the enzymatic assay 48 hours post-infection.

FIGURE 3

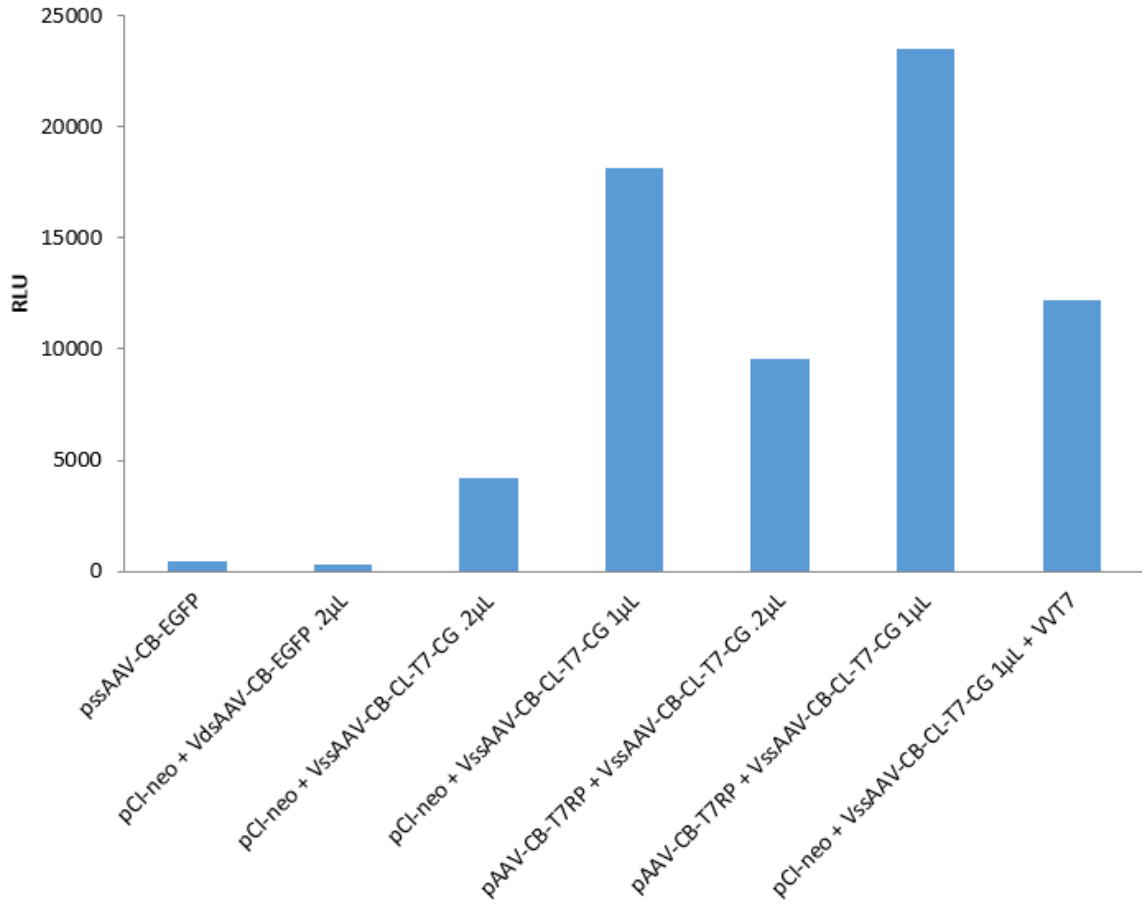


Figure 3. Comparing the amount of AAV present in the nucleus between a transfection of a plasmid carrying T7 RNA polymerase versus VVT7. Hek293 cells were transfected with pCI-neo or pssAAV-CB-GFP (negative plasmid controls for T7 RNA polymerase production) or pAAV-CB-T7RP (plasmid carrying T7 RNA polymerase gene) for 16 hours. Cells were then infected with VssAAV2-CB-CL-T7-CG (dual reporter vector) at the volume indicated alone or in conjunction with VVT7 at an MOI of 1. Cells were also infected with VdsAAV-CB-GFP (negative luciferase control). Luciferase expression was detected using the enzymatic assay 48 hours post-infection.

Over 50 clones were tested to find the clone with the highest expression of T7 RNA polymerase. Clone 17 exhibited the highest GFP expression, which was indicative of the highest T7 RNA polymerase expression, and hence was chosen for subsequent experiments and labelled 293T7 cell line **(Fig 4)**. To verify that the resulting clone, which was renamed 293T7, expresses T7 RNA polymerase a side-by-side comparison of 293T7 and Hek293 cells was performed **(Fig 5 & Fig 6)**. Cells were transfected with pRS314-T7-GFP to verify T7 RNA polymerase production. As a positive control, pRS314-T7-GFP + pAAV-CB-T7RP (expressing T7 RNA polymerase) were transfected together and displayed GFP expression in both cell lines **(Fig 5 & Fig 6)**. A non-transfected well was used as a negative control in both cases. The parental cell line (Hek293) did not express GFP with pRS314-T7-GFP transfection **(Fig 6)**. 293T7 cells expressed GFP when transfected with pRS314-T7-GFP confirming T7 RNA polymerase production **(Fig 5)**. To verify that the promoters and genes used in our dual reporter system are functional, several constructs were made: pdsAAV-CB-GLuc (a positive control for nuclear GLuc expression), pdsAAV-T7-Gluc (test the restriction of the T7 as a cytoplasmic promoter), pdsAAV-CB-CytoGLuc (test CytoGLuc is being spliced in the nucleus) and pdsAAV-CB-GFP(transfection control as well as an negative control for luciferase expression). Hek293 (control cell line) and 293T7 cells were transfected with these plasmids and luciferase expression was detected 48 hours post-transfection **(Fig 7)**. Our results demonstrate that GLuc expression is observed with pdsAAV-CB-GLuc (a positive control). We observed a reduction in luciferase expression with pdsAAV-T7-GLuc indicating the specificity of the T7 promoter for cytoplasmic expression. There was a reduction in luciferase expression with pdsAAV-CB-CytoGLuc showing that the CytoGLuc is spliced inside the nucleus when under the control of the

nuclear CB promoter. These results establish that when the T7 cytoplasmic promoter is combined with our CytoGluc gene, we created a strong cytoplasmic reporter construct for observing and quantifying AAV in the cytoplasm.

Detection of rAAV genomes in the cytoplasm

After establishing the functionality of our dual reporter construct, we infected 293T7 and Hek293 cells with our dual reporter vector and investigated AAV status in the cytoplasm 24 hours **(Fig 8A)** and 48 hours **(Fig 8B)** post-infection. We observed AAV transgene expression in 293T7 cells, but not in the control Hek 293 cells, indicating that AAV can be quantified in the cytoplasm. To validate the luciferase dual reporter system, we extracted RNA from cells and performed a PCR analysis, which indicated that AAV is present in the cytoplasm both 18 hours **(Fig 9A & 9B)** and 24 hours **(Fig 10A & 10B)** post-infection. Thus, our approach allowed us to demonstrate AAV in the cytoplasm **(Fig 8, Fig 9 & Fig 10)**.

FIGURE 4

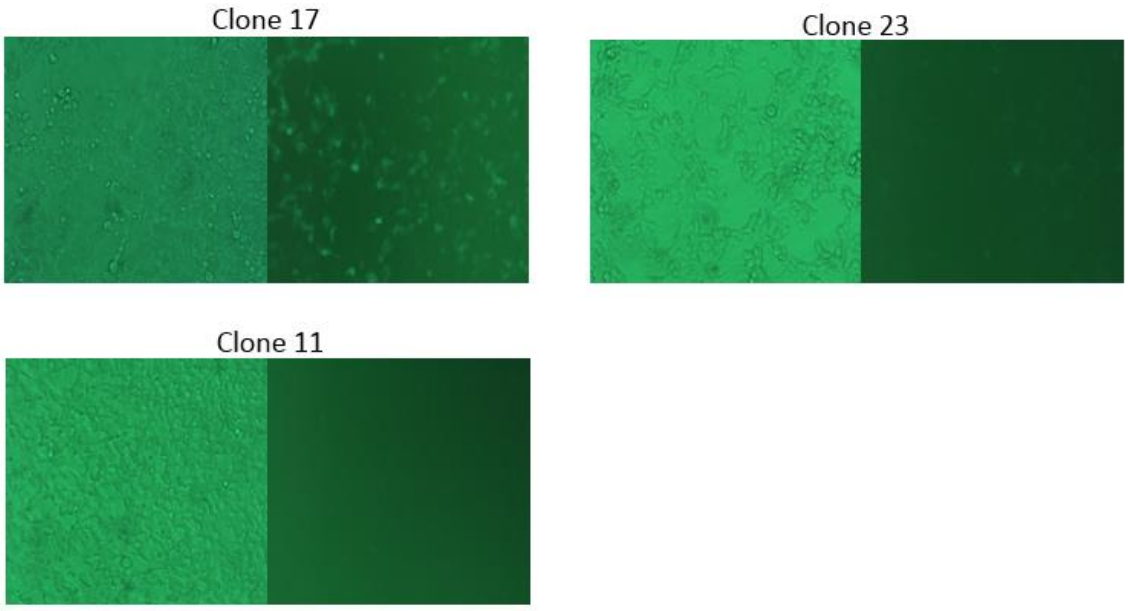


Figure 4. Clone 17 expresses T7 RNA polymerase.

Hek293 cells were transfected with pCI-neo and pAAV-CB-T7RP (plasmid carrying T7 RNA polymerase gene). Colonies were selected and grown in G418 media. Clones were screened by transfecting with pRS314-T7-EGFP to verify T7 RNA polymerase production by GFP expression.

FIGURE 5

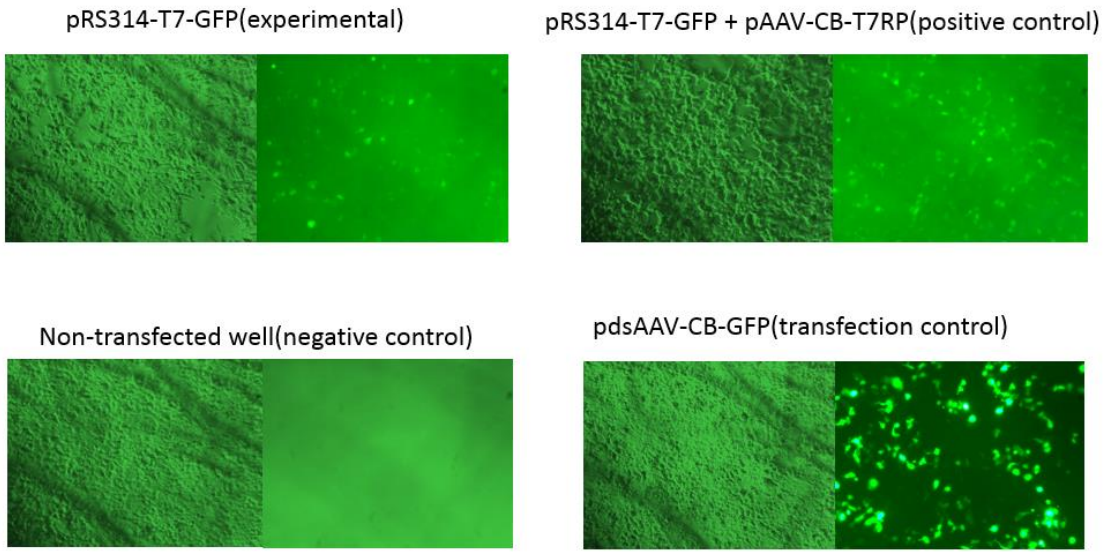


Figure 5. Verification of 293T7 cell line expressing T7 RNA polymerase.

293T7 (clone 17) cells were incubated with pRS314-T7-GFP to verify T7 RNA polymerase production or with pAAV-CB-T7RP + pRS314-T7-GFP as a positive control. pdsAAV-CB-GFP was used as an overall transfection efficiency control. A non-transfected well was used to display the lack of GFP. The cells were analyzed 24 hours post-transfection.

FIGURE 6

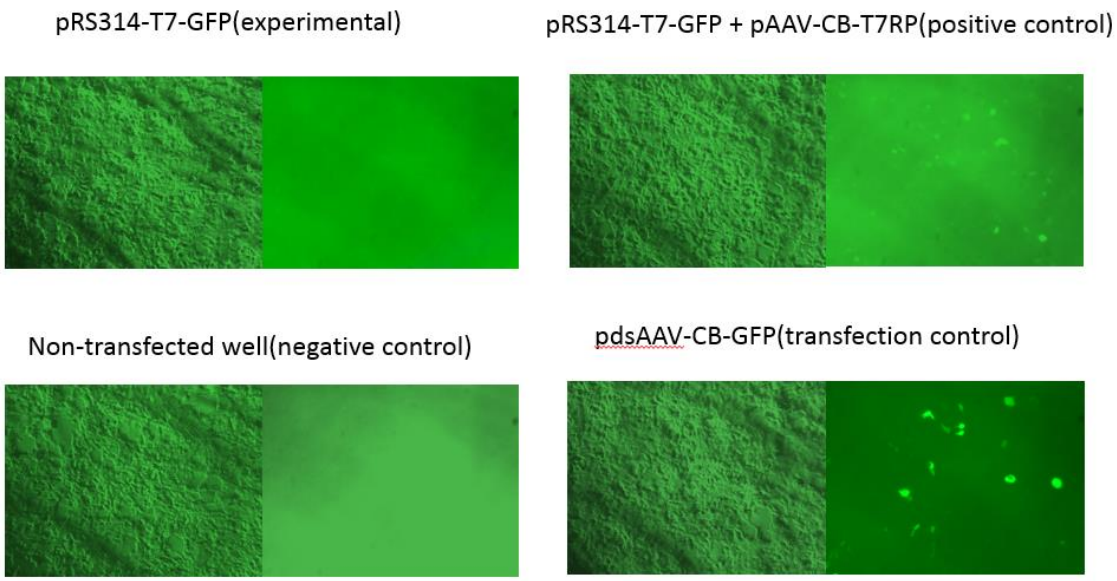


Figure 6. 293 cells do not express T7 RNA polymerase.

Hek293 (parental cell line) cells were incubated with pRS314-T7-GFP to verify no T7 RNA polymerase production or with pAAV-CB-T7RP + pRS314-T7-GFP as a positive control. pdsAAV-CB-GFP was used as an overall transfection efficiency control. . A non-transfected well was used to display the lack of GFP. The cells were analyzed 24 hours post-transfection.

FIGURE 7

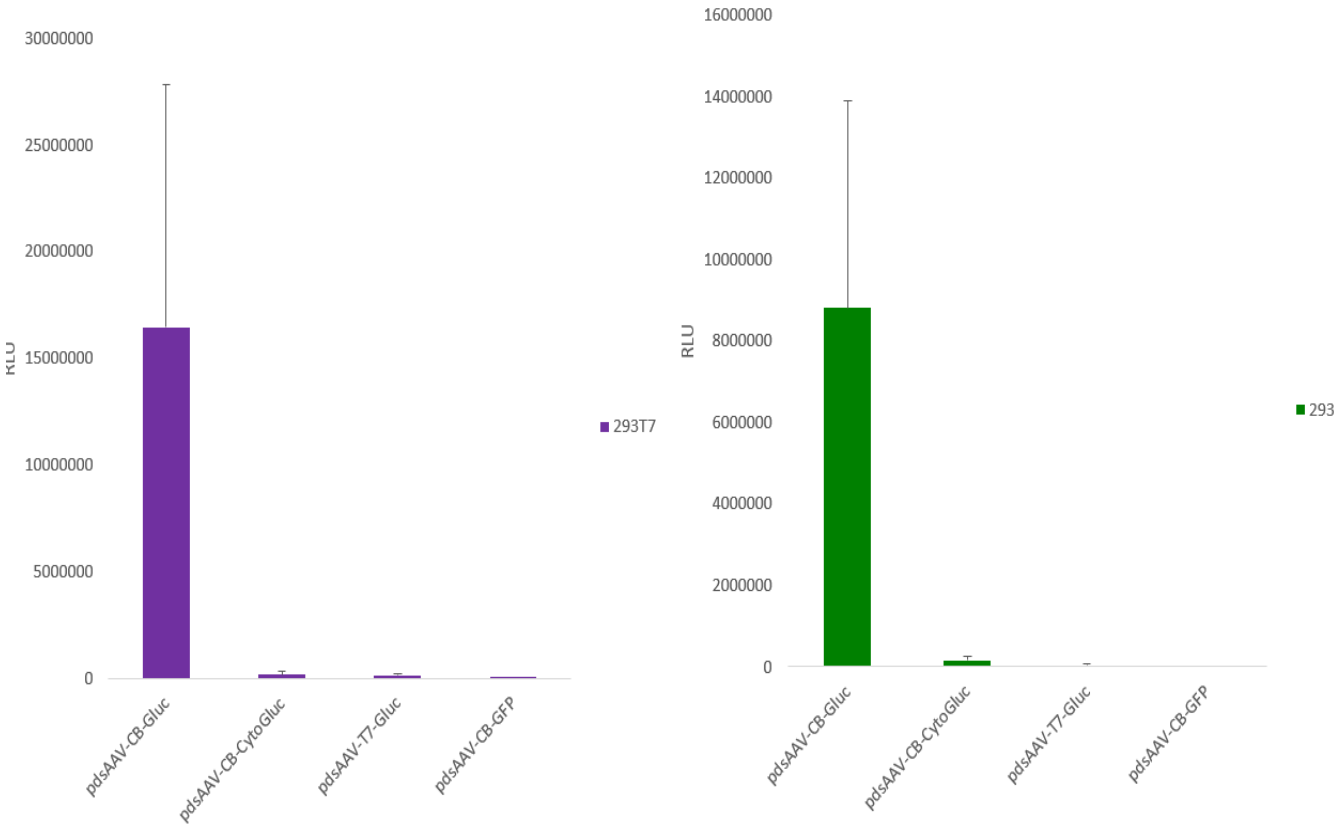
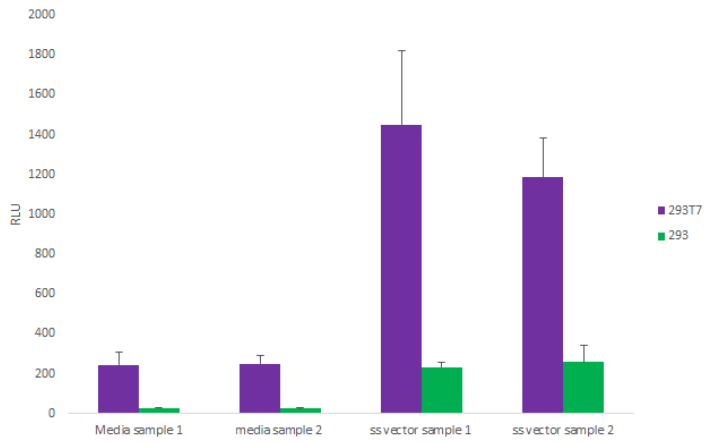


Figure 7. Verification T7-CytoGLuc construct in 293 and 293T7 cells

Hek293 (control cell line) in green and 293T7 cells in purple were incubated with: pdsAAV-CB-GLuc (a positive control for nuclear GLuc expression), pdsAAV-T7-Gluc (test the restriction of the T7 as a cytoplasmic promoter), pdsAAV-CB-CytoGluc pdsAAV-CB-CytoGLuc(test CytoGLuc is being spliced in the nucleus), and pdsAAV-CB-GFP(negative control for luciferase activity) for 16 hours. Media was harvested and luciferase expression was detected using the enzymatic assay 24 and 48 hours post-transfection. The mean and SD of three independent experiments is presented.

FIGURE 8

A



B

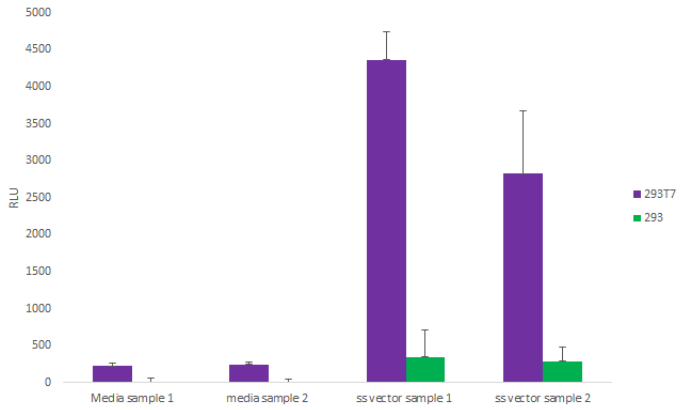
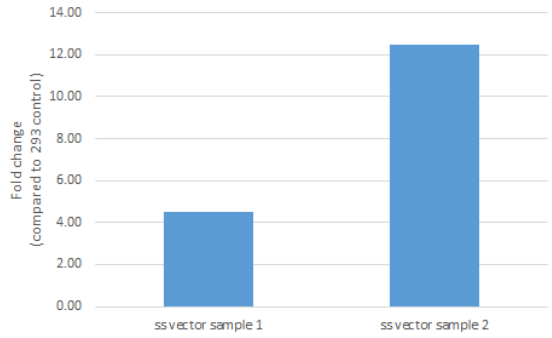


Figure 8. Detection of AAV genomes in the cytoplasm using the dual reporter system

Hek293T7 and Hek293 (control) cells were infected with dual reporter vector or left uninfected in duplicate wells labelled sample 1 and sample 2. Media was harvested and luciferase expression was detected using the enzymatic assay 24 and 48 hours post-infection. **(A) 24 hour time point (B) 48 hour time point.** The mean and SD of three independent experiments is presented.

FIGURE 9

A



B

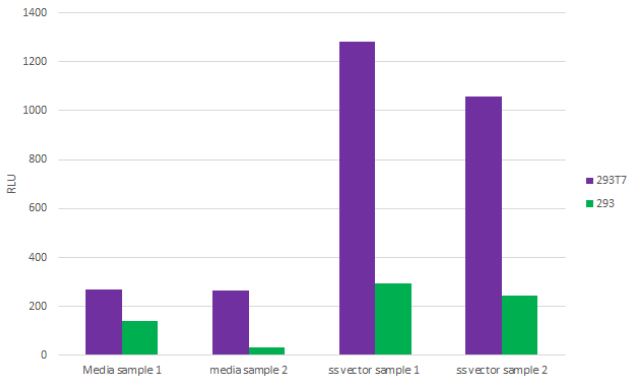
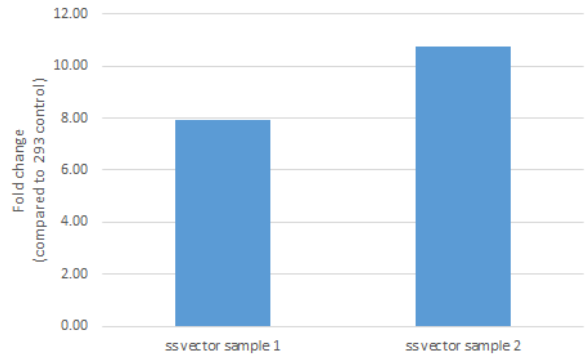


Figure 9. rAAV present in the Cytoplasm 18hr time point. Hek293T7 and Hek293(control) cells were infected with dual reporter vector or left uninfected in duplicate wells labelled sample 1 and sample 2. Media and cells were harvested 18 hours post-infection. **(A) PCR analysis** RNA was extracted and then made into cDNA for Real-time PCR analysis using specially designed Taqman probes for the luciferase genes as well as a GapDH control. Data is expressed as fold change in genomes present compared to 293 control cells. **(B) Luciferase expression** was detected using the enzymatic assay 18 hours post-infection.

FIGURE 10

A



B

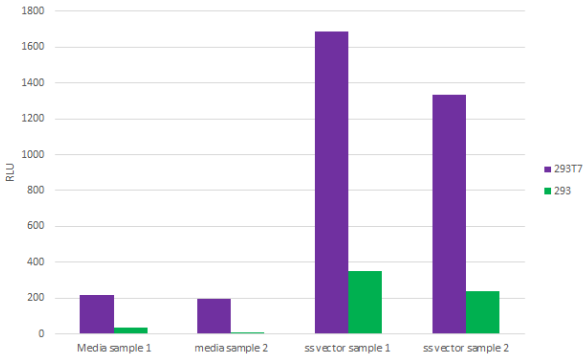


Figure 10. rAAV present in the Cytoplasm 24hr time point. Hek293T7 and Hek293(control) cells were infected with dual reporter vector or left uninfected in duplicate wells labelled sample 1 and sample 2. Media and cells were harvested 24 hours post-infection. **(A) PCR analysis** RNA was extracted and then made into cDNA for Real-time PCR analysis using specially designed Taqman probes for the luciferase genes as well as a GapDH control. Data is expressed as fold change in genomes present compared to 293 control cells. **(B) Luciferase expression** was detected using the enzymatic assay 24 hours post-infection.

Enhancement of rAAV transduction by antiviral drugs

Cidofovir (CDV)

After establishing that our dual reporter system is functional, we screened both known and unknown drugs/molecules to better understand AAV trafficking and overall vector transduction. We demonstrated that CDV, a previously known antiviral drug, enhances AAV transgene expression. CDV has been documented to inhibit the growth of viruses with DNA polymerases, such as Adenovirus and Herpes viruses, *in vitro* (69). Its active metabolite, Cidofovir diphosphate, hinders viral replication by selectively inhibiting viral DNA polymerases (69). To evaluate CDV's enhancement of AAV transgene expression, a side-by-side comparison of the effects of HeLaS3 pretreatment with CDV on Adenovirus expressing LacZ gene (Ad-LacZ) and Adeno-associated virus expressing LacZ gene (AAV-LacZ) was conducted **(Fig.11)**. Cells were pretreated with PBS, 100µg/mL CDV, or 500µg/mL CDV and then infected with Ad-LacZ or AAV-LacZ virus. Cells treated with PBS displayed the largest amount of Ad-LacZ expression as compared to 100µg/mL CDV or 500µg/mL CDV, indicating that CDV inhibits Ad-LacZ. As the concentration of CDV increased, the number of visible Ad-LacZ expressing cells decreased showing the effectiveness of CDV against larger DNA viruses possessing a polymerase, such as Adenovirus. However, the opposite effect was observed with AAV-LacZ infected cells when pretreated with CDV. There was an increase in the number of LacZ expressing cells when pretreated with both concentrations of CDV compared to PBS. These results demonstrated that CDV inhibits Adenovirus but enhances AAV transgene expression.

Figure 11.

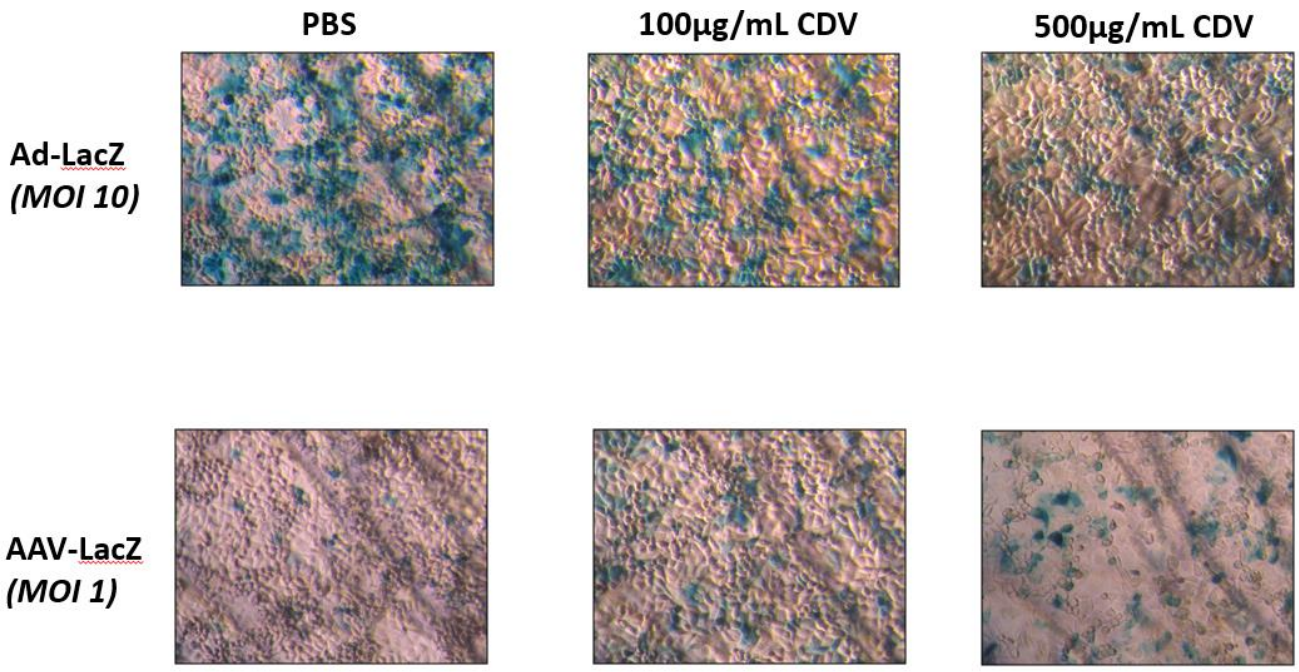


Figure 11. Cidofovir inhibits Adenovirus but enhances AAV.

HelaS3 cells were pretreated with PBS, 100µg/mL CDV, or 500µg/mL CDV for 16 hours. The cells were washed once and infected with Adenovirus (Ad-lacZ) at an MOI of 0.5 or Adeno-associated virus (AAV-LacZ) at an MOI of 10. LacZ stain was performed 24 hours post-infection. The following figure is a representative of 3 independent experiments.

When different cell types GM16095 (fibroblast cells) **(Fig 12A)**, Hek293 (kidney cells) **(Fig 12B)**, and HelaS3 (carcinoma cells) **(Fig 12C)** were pretreated with PBS or increasing doses of CDV for 16 hours no toxicity to any cell type was observed. Treatment of cells with CDV prior to rAAV2-luciferase infection led to a ~2- to 5-fold increase in luciferase expression compared to PBS treatment depending on cell type and concentration. These results indicate that CDV enhances AAV transduction of multiple cell types. To investigate if this enhancement is serotype dependent, GM16095 **(Fig 13A),** Hek293 **(Fig 13B)**, and HelaS3 **(Fig 13C)** cells were pretreated with increasing doses CDV for 16 hours and infected with a rAAV8-luciferase vectors. Treatment of cells with CDV prior to rAAV8-luciferase displayed an increase in luciferase expression compared to PBS treatment **(Fig 13)**. This result also suggests that CDV does not affect receptors for viral entry since these two serotypes have different cellular receptors. Next, the use of other reporter vectors was employed to demonstrate whether CDV can consistently enhance AAV transgene expression. HelaS3 **(Fig 14A)** and GM16095 **(Fig 14B)** cells were pretreated with CDV and infected with rAAV2-CB-LacZ. HelaS3 **(Fig 15A)** and GM16095 **(Fig 15B)** cells were pretreated with CDV and infected with rAAV2-CB-GFP. Both HelaS3 and GM16095 displayed increases in LacZ expression when pretreated with CDV compared to PBS **(Fig 15)**. HelaS3 **(Fig 16A)** and GM16095 **(Fig 16B)** cells were pretreated with CDV and infected with rAAV8-CB-GFP. HelaS3 and GM16095 demonstrated increases in GFP expression when pretreated with CDV compared to PBS **(Fig 16)**. HelaS3 **(Fig 17A)** cells were pretreated with CDV and infected with rAAV2-CB-hAAT and Hek293 **(Fig 17B)** cells were pretreated with CDV and infected with rAAV8-CB-hAAT. HelaS3 and Hek293 cells showed increases in hAAT levels when pretreated with CDV compared to PBS **(Fig**

15). There were increases in transgene expression with rAAV2-CB-LacZ, rAAV2-CB-GFP rAAV8-CB-GFP, rAAV2-CB-hAAT, and rAAV8-CB-hAAT vectors when cells were pretreated with CDV compared to PBS. Results from these experiments demonstrate that CDV enhances multiple AAV vectors. A time course of CDV pretreatment on HelaS3 cells was performed to determine if CDV could indeed sustain transgene levels *in vitro*. HelaS3 cells were pretreated with increasing concentrations of CDV for 16 hours. Cells were washed to remove drug and then infected with rAAV-luciferase vector at an MOI of 10. Luciferase expression was detected 24, 36, 48, and 72 hours post-infection. An increase in transduction with both rAAV2 serotype **(Fig 18A)** and rAAV8 serotype **(Fig 18B)** was observed at all time points. CDV enhanced transgene expression as long as 72 hours post-infection **(Fig 18)**. In order to determine if CDV is efficacious only with pretreatment, we examined whether CDV is effective at enhancing rAAV transgene expression when given before or after rAAV infection (**Fig 19)**. The treatment time appears to be of critical importance, treatment of HeLaS3 with 100µg/mL CDV prior to viral infection enhanced AAV transgene expression, while the same treatment after rAAV infection reduced the enhancement effect (**Fig 19)**. In order to confirm our previous *in vitro* results, both Southern blot analysis and detection of the amount of AAV genomes present using real-time quantification was performed to examine the effect of CDV on the AAV genome status. HelaS3 Cells were pretreated with 100µg/mL of CDV or PBS for 16 hours, then infected with rAAV2-luciferase vector at an MOI of 1 or 10. Cells were harvested 24 hours post-infection and DNA was extracted. Southern blot results demonstrate that CDV enhances viral accumulation of AAV **(Fig 20B)**.

FIGURE 12.

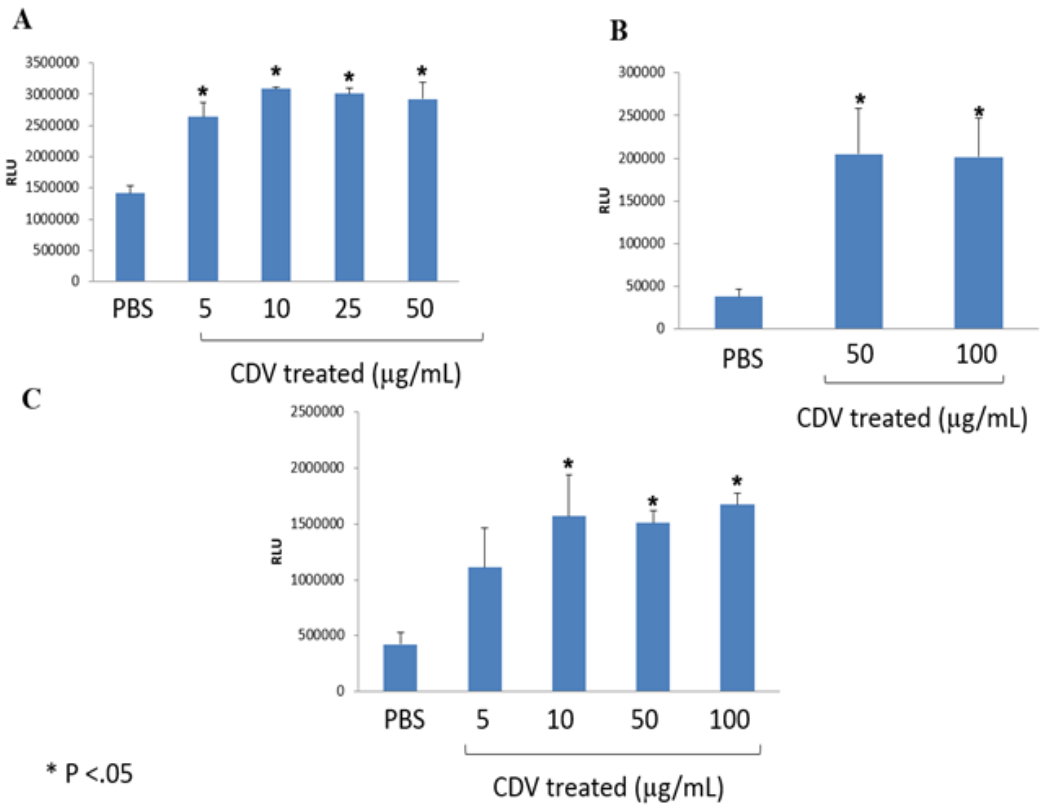


Figure 12. CDV pretreatment enhances rAAV2-luciferase transduction.

Cells were pretreated with PBS or with increasing concentrations of CDV for 16 hours. Cells were washed to remove drug then infected with rAAV2-CB-Cluc at an MOI of 10. Luciferase expression was detected using the enzymatic assay 48 hours post-infection. **(A) 16095 cells (B) Hek293 cells (C) HelaS3 cells.** The mean and SD of triplicate wells is presented. Statistical analysis is based on a 2-tailed-student's *t*-test.

FIGURE 13.

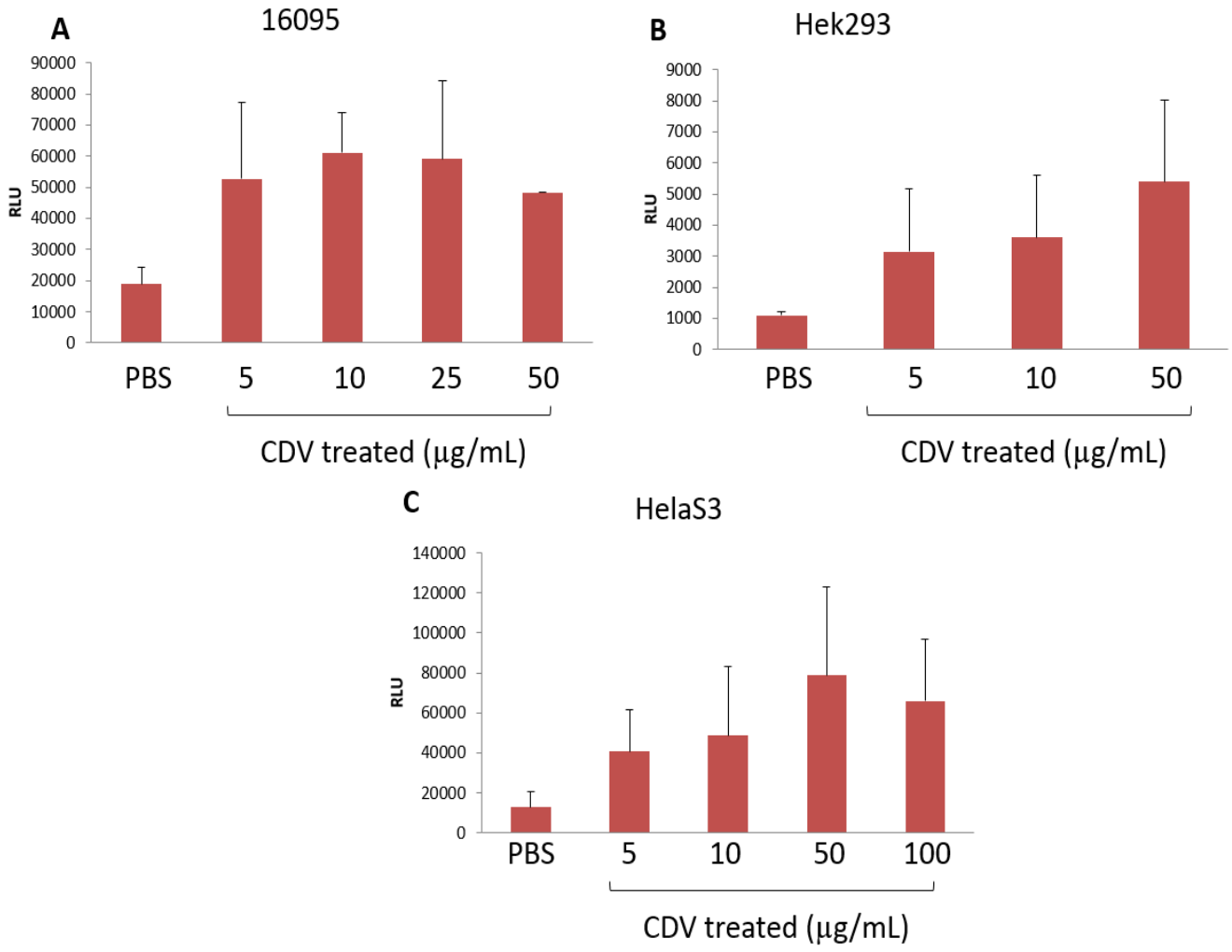


Figure 13. CDV pretreatment enhances rAAV8-luciferase transduction.

Cells were pretreated with PBS or with increasing concentrations of Cidofovir for 16 hours. Cells were washed to remove drug then infected with rAAV8-CB-Cluc at an MOI of 10. Luciferase expression was detected using the enzymatic assay 48 hours post-infection. **(A) 16095 cells (B) Hek293 cells (C) HelaS3 cells.** The mean and SD of triplicate wells is presented.

FIGURE 14.

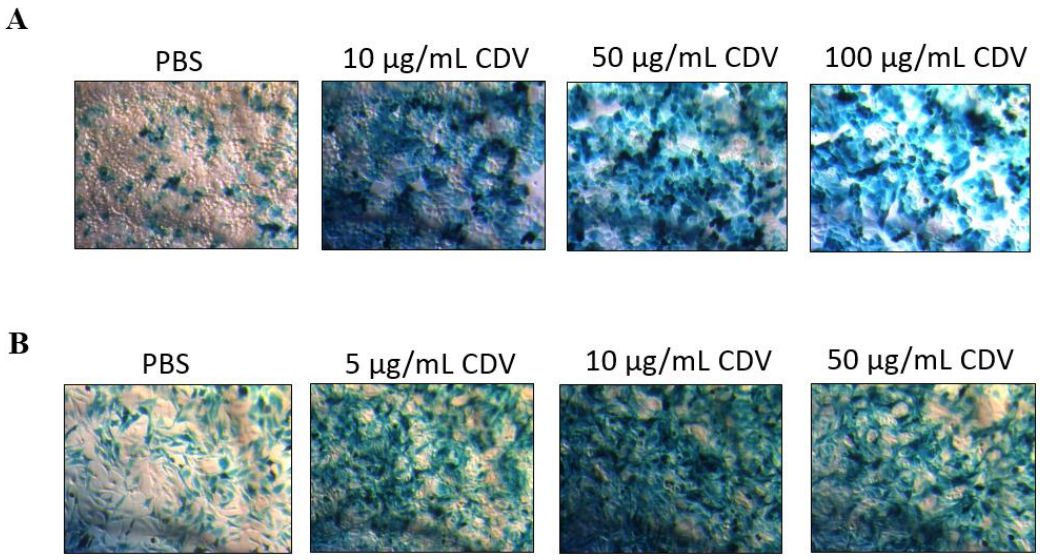
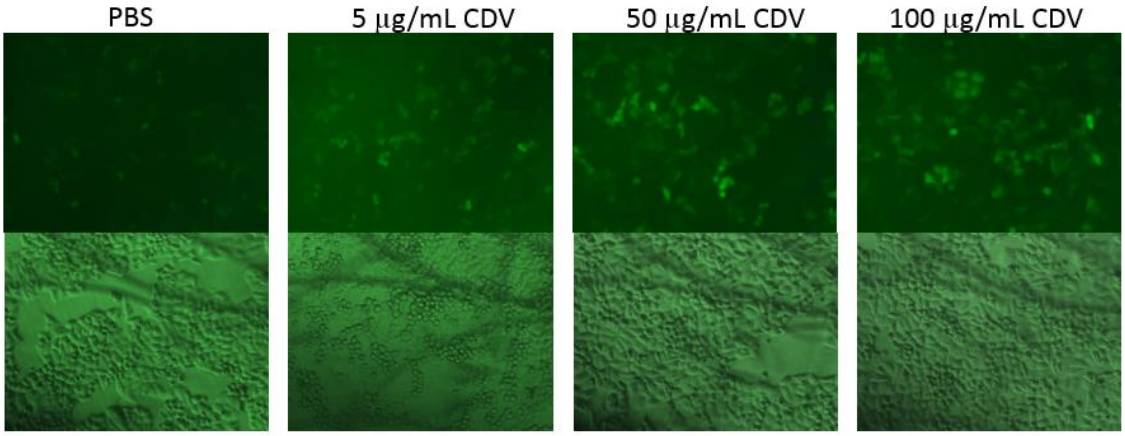


Figure 14. CDV pretreatment enhances rAAV2-LacZ transduction.

Cells were pretreated with PBS (Mock) or increasing concentrations of Cidofovir for 16 hours, washed and infected with AAV2 (LacZ) MOI of 10. LacZ stain was performed 24 hours post-infection: **(A) HelaS3 cells (B) GM16095 cells.**

FIGURE 15.

A



B

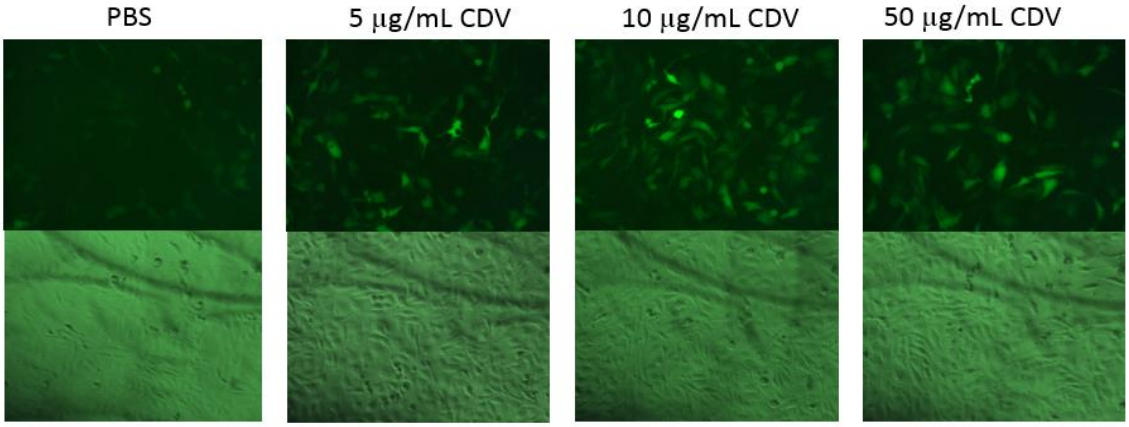
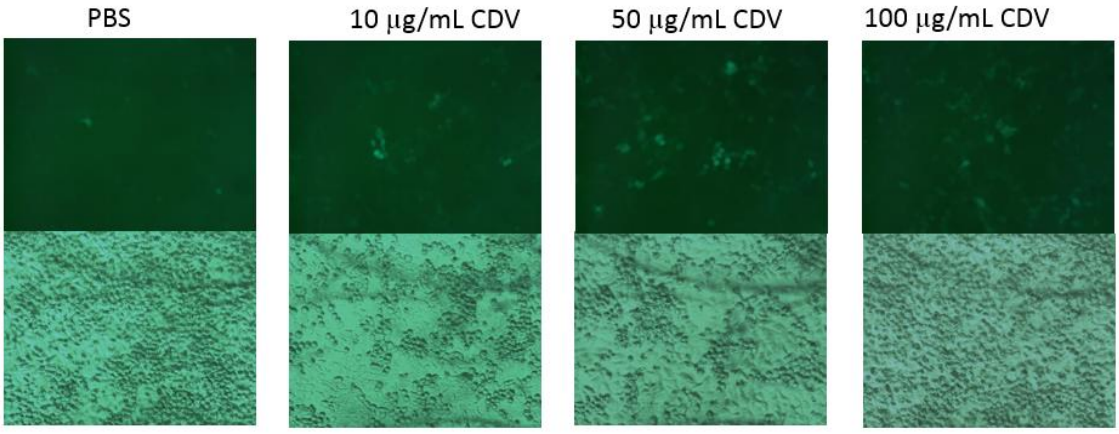


Figure 15. CDV pretreatment enhances rAAV2-GFP transduction.

Cells were pretreated with PBS (Mock) or with increasing concentrations of Cidofovir for 16 hours. Cells were washed to remove drug then infected with rAAV2-CB-GFP at an MOI of 10. Photos were taken 24 hours post-infection. **(A) HelaS3 cells (B) 16095 cells.** The following figure is a representative of 3 independent experiments.

FIGURE 16.

A



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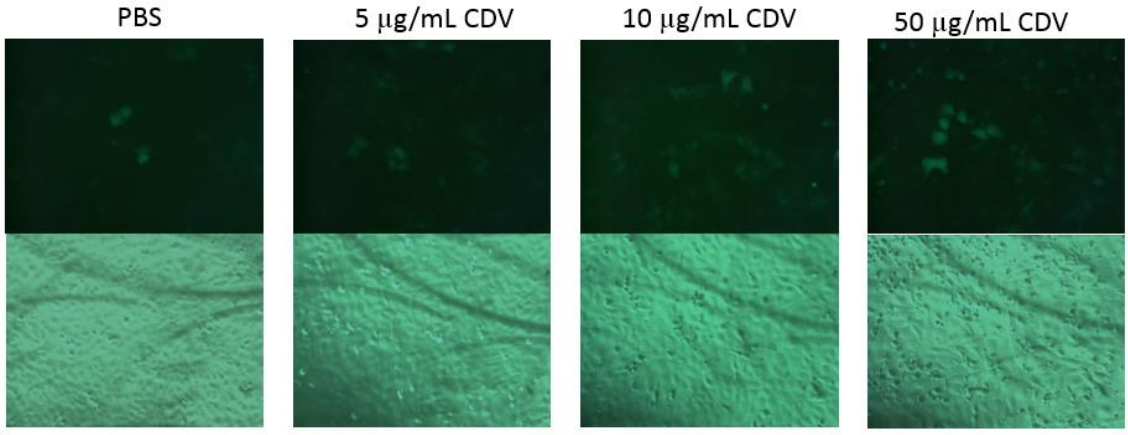
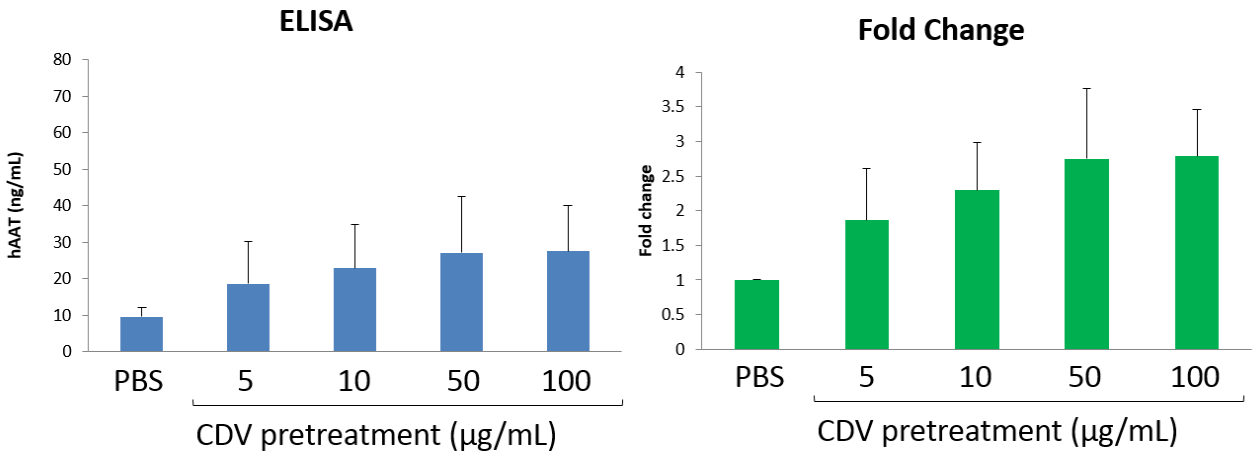


Figure 16. CDV pretreatment enhances rAAV8-GFP transduction. Cells were pretreated with PBS (Mock) or with increasing concentrations of Cidofovir for 16 hours. Cells were washed to remove drug then infected with rAAV8-CB-GFP at an MOI of 50. Photos were taken 24 hours post-infection. **(A) HelaS3 cells (B) 16095 cells** The following figure is a representative of 3 independent experiments.

FIGURE 17

A



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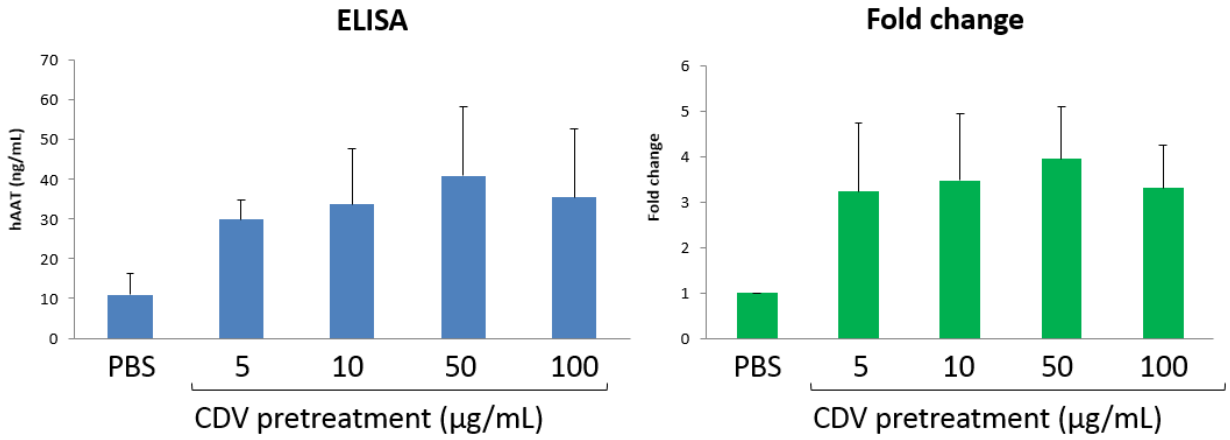
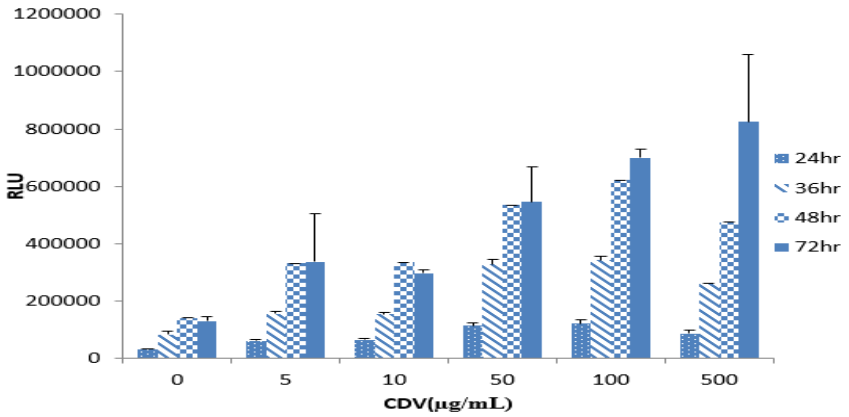


Figure 17. CDV pretreatment enhances rAAV-hAAT transduction. Cells were pretreated with PBS (Mock) with increasing concentrations of Cidofovir for 16 hours. Cells were washed to remove drug then infected with Media was harvested 24 hours post infection and ELISA was performed to measure hAAT levels. **(A) HelaS3 cells with rAAV8-CB-hAAT an MOI of 50. (B) Hek293 cells with rAA2-CB-hAAT an MOI of 10.** The mean and SD of three independent experiments is presented.

FIGURE 18.

A



B

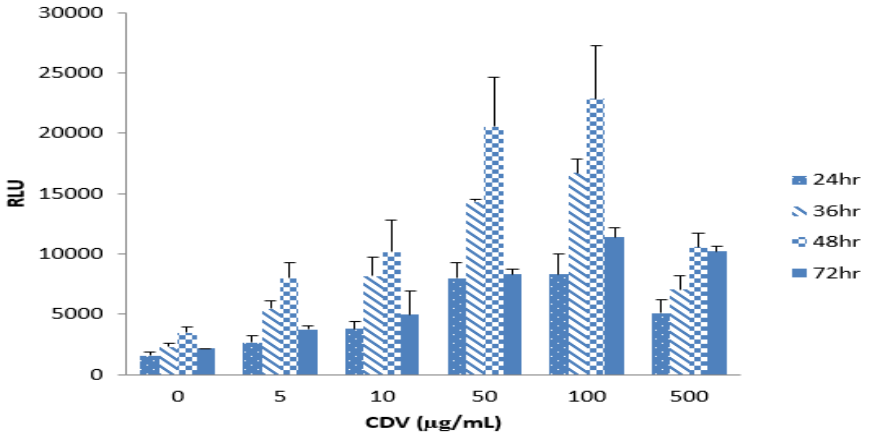


Figure 18. CDV pretreatment enhances rAAV-luciferase transduction as long as 72 hours post-infection. HelaS3 Cells were pretreated with PBS or with increasing concentrations of CDV for 16 hours. Cells were washed to remove drug then infected with rAAV-luciferase vector at an MOI of 10. Luciferase expression was detected using the enzymatic assay 24, 36, 48, and 72 hours post-infection. The mean and SD of duplicate wells is presented. **(A) rAAV2-CB-CL (B) rAAV8-CB-CL**

FIGURE 19.

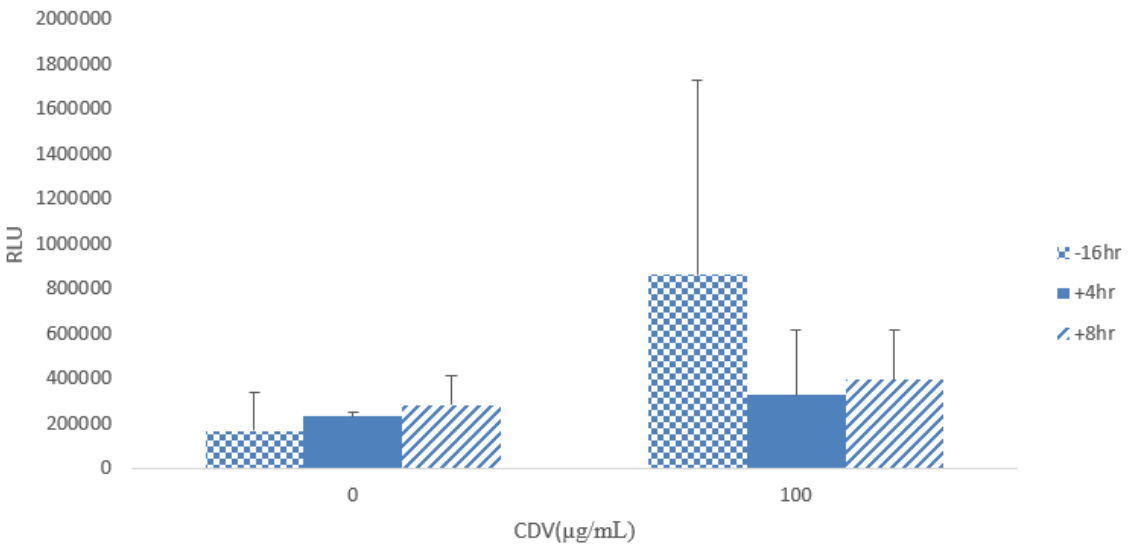


Figure 19. Cidofovir pretreatment is optimal for AAV enhancement.

HelaS3 Cells were pretreated with 100µg/mL of CDV or PBS for 16 hours before infection. Cells were infected with rAAV-Luciferase at an MOI of 10. 4 or 8 hours post-infection 100µg/mL of CDV or PBS was added to cells. Luciferase expression was detected using the enzymatic assay 48 hours post-infection. The mean and SD of three independent experiments is presented.

FIGURE 20.

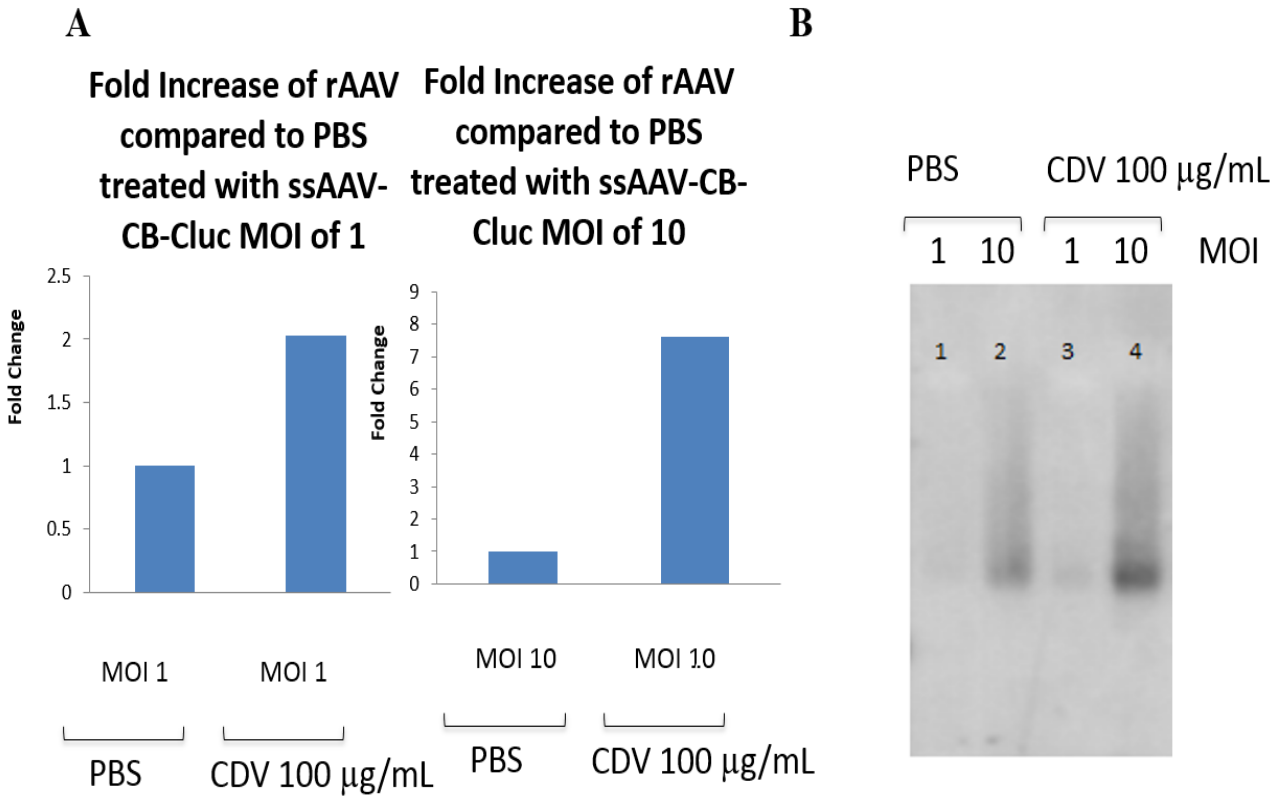


Figure 20. Viral Accumulation in HelaS3 cells after pretreatment of Cidofovir.

HelaS3 Cells were pretreated with 100µg/mL of CDV or PBS for 16 hours. Cells were washed to remove drug then infected with rAAV2-CB-Cluc at an MOI of 1 or 10. Cells were harvested and DNA was extracted 24hrs post-infection. **(A) Detection of the amount of AAV genomes** present using real-time quantification was performed to examine the effect of CDV on the AAV genome status. The ssAAV genome was normalized using the cytochrome B signal and is presented as fold change compared to PBS treatment. **(B) Southern blot analysis** Hybridization of low molecular weight (LMW) DNA against the luciferase gene probe was performed to detect AAV genomes.

Real-time quantification of the AAV genome was performed and indicated an enhancement in AAV levels with CDV pretreatment compared to PBS (**Fig 20A)**. Taken together these results demonstrate that CDV leads to an increase in viral accumulation inside cells (**Fig 20).** A similar experiment with increasing concentrations of CDV or Mock pretreatment on HelaS3 cells was conducted. Southern blot results demonstrated an increase of AAV in a dose-dependent manner **(Fig 21B)**. Real-time quantification demonstrated that CDV enhances viral accumulation at concentrations of 50µg/mL and 100µg/mL of CDV compared to PBS **(Fig 21A)**. To examine if CDV treatment can enhance other forms of AAV vectors, southern blot analysis was performed on DNA extracted from HelaS3 cells **(Fig 22)** and Hek293 cells **(Fig 23).** Southern blot analysis revealed that CDV pretreatment leads to the formation of both AAV monomers (linear replication form monomer) and AAV dimers (pair of linear monomers) **(Fig 22)**. Monomer and dimer formation was observed in CDV treatment compared to mock in HelaS3 cells **(Fig 22)**. Monomer formation was also seen in Hek293 cells **(Fig 23)** indicating that monomer formation can be seen in both Hek293 and HelaS3. An accumulation of ssDNA was observed with CDV treatment compared to Mock (PBS) **(Fig 22 & Fig 23)**. These results indicated CDV is enhancing AAV. To establish that CDV in combination with AAV causes an increase rAAV genomes, HelaS3 cells were pretreated with increasing concentrations of CDV or Mock and separated into two groups: non-infected or infected (rAAV at a MOI of 10). To demonstrate that the difference was not due to the total amount of DNA loaded, an ethidium bromide gel stain was performed **(Fig 24A)**. With pretreatment of CDV and infection with rAAV vectors, there is rAAV genome accumulation in dose dependent manner.

FIGURE 21.

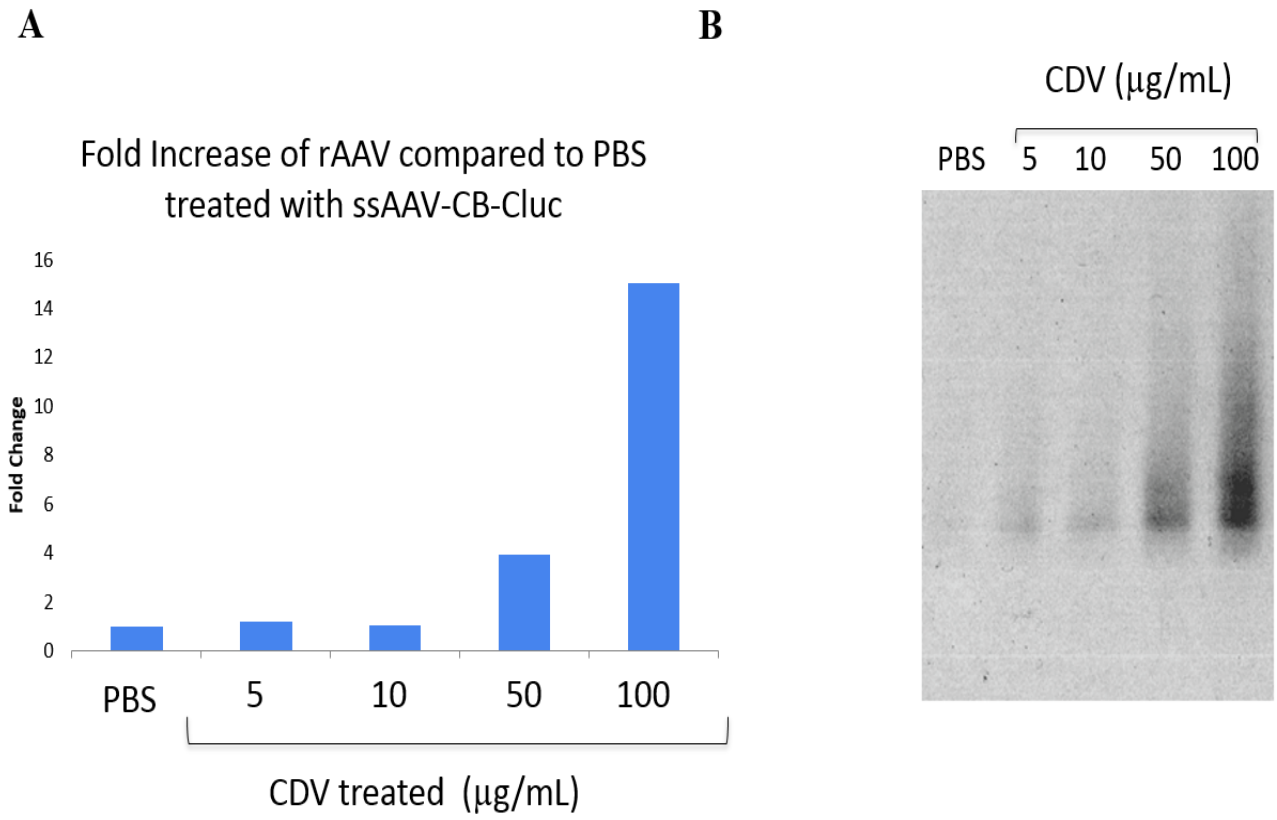


Figure 21. Viral Accumulation in HelaS3 cells after pretreatment of Cidofovir is dose-dependent.

HelaS3 Cells were pretreated with increasing concentrations of CDV or PBS for 16 hours. Cells were washed to remove drug then infected with rAAV2-CB-Cluc at an MOI of 10. Cells were harvested and DNA was extracted 24hrs post-infection. **(A) Detection of the amount of AAV genomes** present using real-time quantification was performed to examine the effect of CDV on the AAV genome status. The ssAAV genome was normalized using the cytochrome B signal and is presented as fold change compared to PBS treatment. **(B) Southern blot analysis** Hybridization of LMW DNA against the luciferase gene probe was performed to detect AAV genomes.

FIGURE 22.

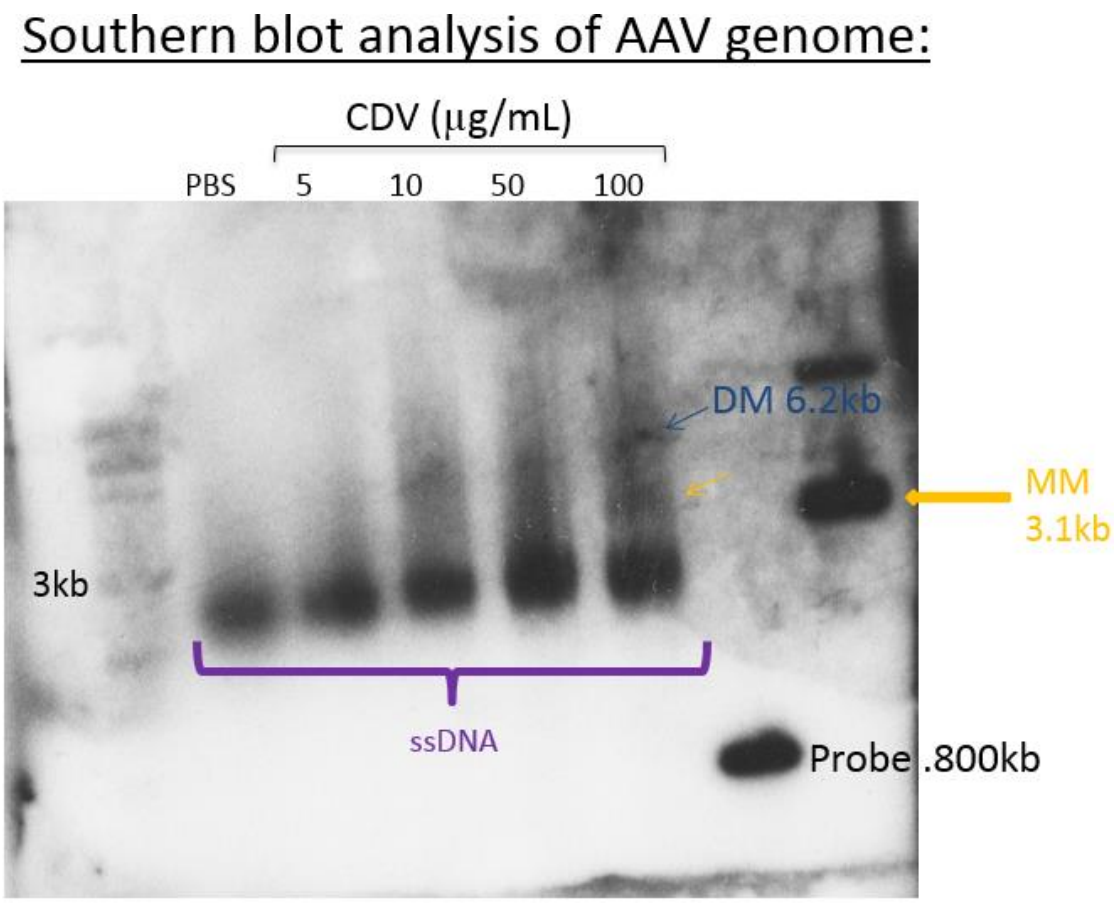


Figure 22. Formation of monomer and dimer with CDV treatment in HelaS3 cells.

HelaS3 cells were pretreated with increasing concentrations of CDV or Mock for 16 hours. Cells were washed to remove drug then infected with rAAV2-CB-Cluc at an MOI of 10. Cells were harvested and DNA was extracted 24 hours post-infection. Southern blot analysis of the hybridization of LMW DNA against the luciferase gene probe was performed to detect AAV genomes. An accumulation of ssDNA (purple), monomer formation(yellow arrow 3.1kb) and dimer formation(blue arrow 6.2kb) was observed with CDV treatment. The following figure is representative of 2 independent experiments.

FIGURE 23.

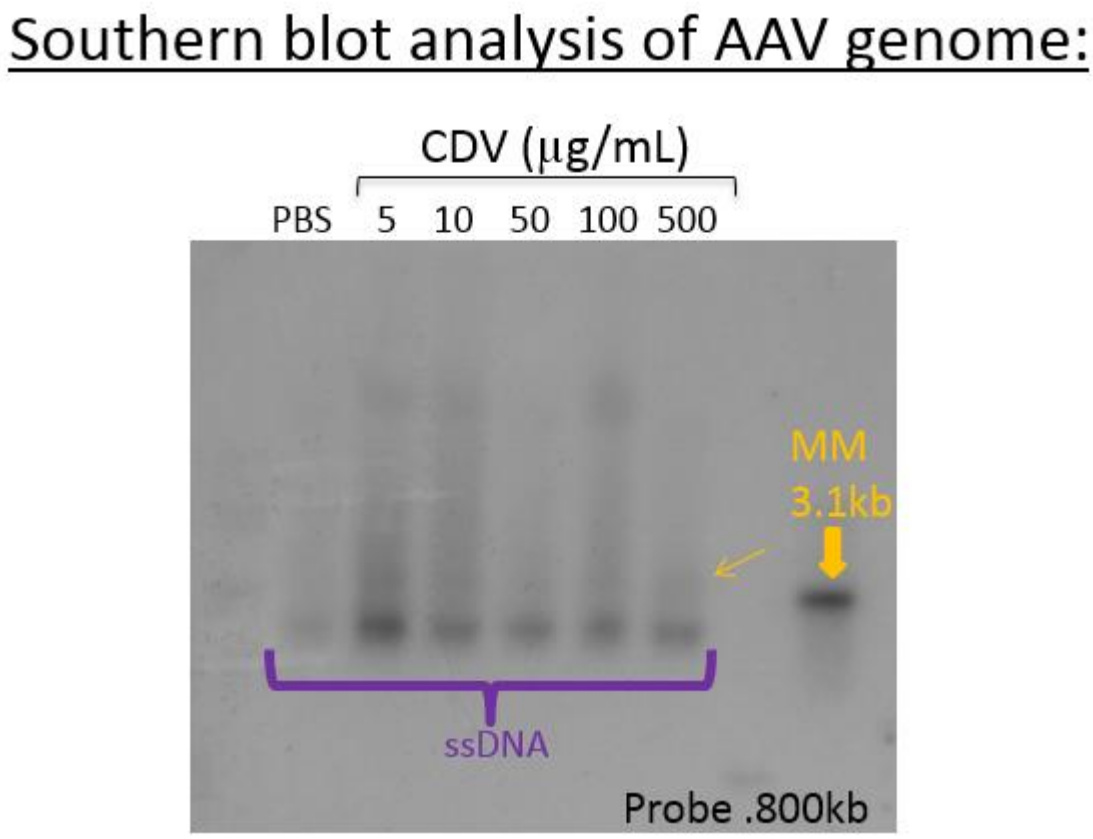


Figure 23. Formation of monomer with CDV treatment in Hek293 cells.

Hek293 cells were pretreated with increasing concentrations of CDV or Mock for 16 hours. Cells were washed to remove drug then infected with rAAV2-CB-Cluc at an MOI of 10. Cells were harvested and DNA was extracted 24 hours post-infection. Southern blot analysis of the hybridization of LMW DNA against the luciferase gene probe was performed to detect AAV genomes. An accumulation of ssDNA (purple) and monomer formation (yellow arrow 3.1kb) was observed with CDV treatment. The following figure is a representative of 2 independent experiments.

FIGURE 24.

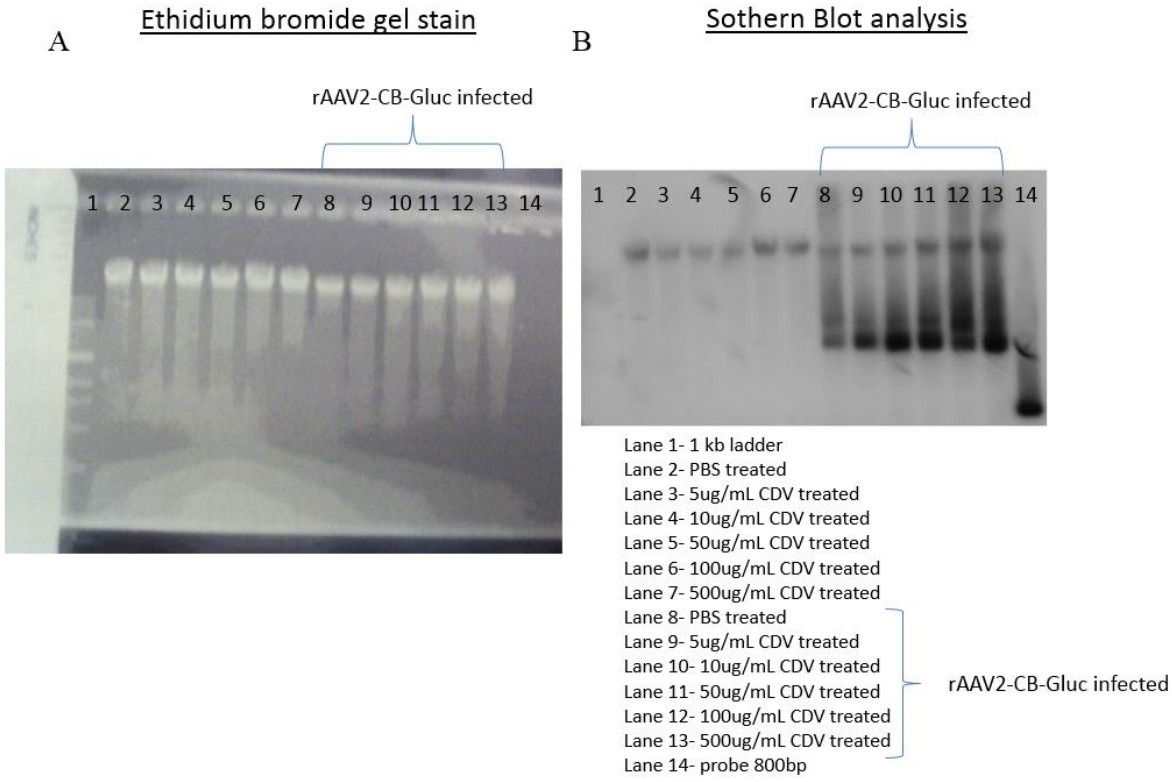


Figure 24. Viral Accumulation in HelaS3 cells after pretreatment of Cidofovir with loading control.

HelaS3 cells were pretreated with increasing concentrations of CDV or Mock for 16 hours. Cells were washed to remove drug then separated into two groups: non-infected and infected with rAAV2-CB-Cluc at an MOI of 10. Cells were harvested and DNA was extracted 24 hours post-infection. **(A) Ethidium bromide gel staining** No differences in DNA loading between samples. **(B) Southern blot analysis** Hybridization of LMW DNA against the luciferase gene probe was performed to detect AAV genomes. An accumulation of ssDNA was observed only with cells treated with CDV and infected with AAV. The following figure is a representative of 2 independent experiments.

As anticipated there is no rAAV genome present when non-infected cells are pretreated with CDV **(Fig 24B)**. To determine if CDV enhances viral entry into the cell, HelaS3 Cells were pretreated with 100μg/mL of Cidofovir or PBS (mock treatment) for 16 hours, then infected with rAAV2-luciferase vector. Cells were incubated at 37° C for two hours to allow viral entry, then cells were washed to remove any unattached virus. Cells were harvested immediately after wash and 2 hours after wash. Southern blot analysis demonstrates that there is no enhancement effect seen 2 or 4 hours after viral entry suggesting that CDV enhances transduction downstream of viral entry **(Fig 25)**. ScAAV vectors are more efficient at transduction than rAAV vectors because scAAV vectors bypass the conversion from a single-stranded to a double-stranded form (52). However, the decreased packaging capacity as well as increased immune response to these vectors hinder their efficacy. We investigated if CDV can also enhance scAAV vectors. Hek293 cells were pretreated with increasing doses of CDV for 16 hours then infected with scAAV2-CB-GL **(Fig 26A)** or with scAAV8-CB-GL **(Fig 26B)**. Unlike the observed increased in rAAV transgene expression, scAAV transgene expression was not enhanced with CDV pretreatment. This same no enhancement result was seen in HelaS3 cells pretreated with CDV and infected with a scAAV2-CB-GL **(Fig 26C)** or a scAAV8-CB-GL **(Fig 26D)**. No enhancement of scAAV with CDV pretreatment was confirmed by southern blot analysis. HelaS3 cells pretreated with CDV and infected with a scAAV2-CB-G. DNA was extracted 24 hours post-infection and demonstrated no difference in the amount of AAV present in cells between mock and CDV treated cells with scAAV vectors **(Fig 27)**. To further evaluate CDV enchantment of rAAV vectors, drug studies comparing CDV with known rAAV enhancing drugs was performed.

FIGURE 25.

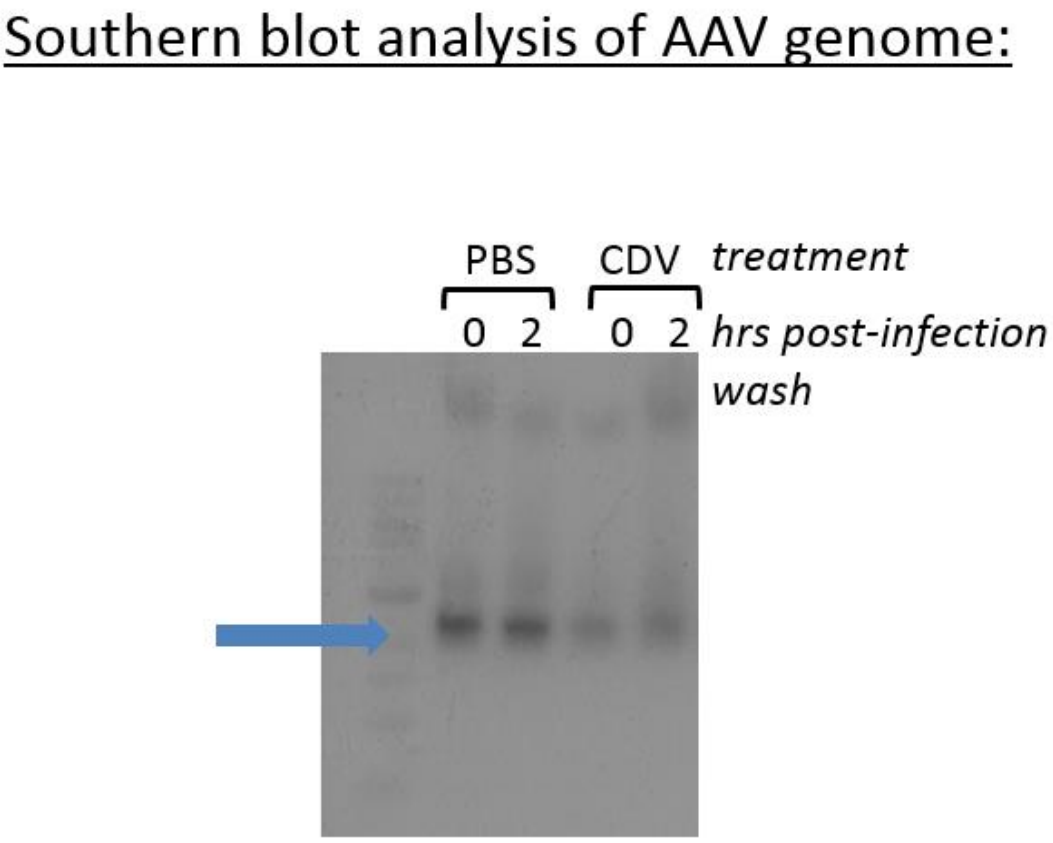


Figure 25. CDV does not enhance viral entry.

HelaS3 cells were pretreated with PBS or 100µg/mL CDV for 16 hours. Cells were infected with rAAV2-luciferse virus MOI of 10 for 2 hours. Cells were washed and harvested at the time of wash (0hr). 2 hours post-wash cells were harvested (2hr). DNA was extracted. Hybridization of LMW DNA against the luciferase gene probe was performed to detect AAV genomes. There was no enhancement of AAV after CDV pretreatment 0 or 2 hours post-infection. The following figure is a representative of 2 independent experiments.

FIGURE 26.

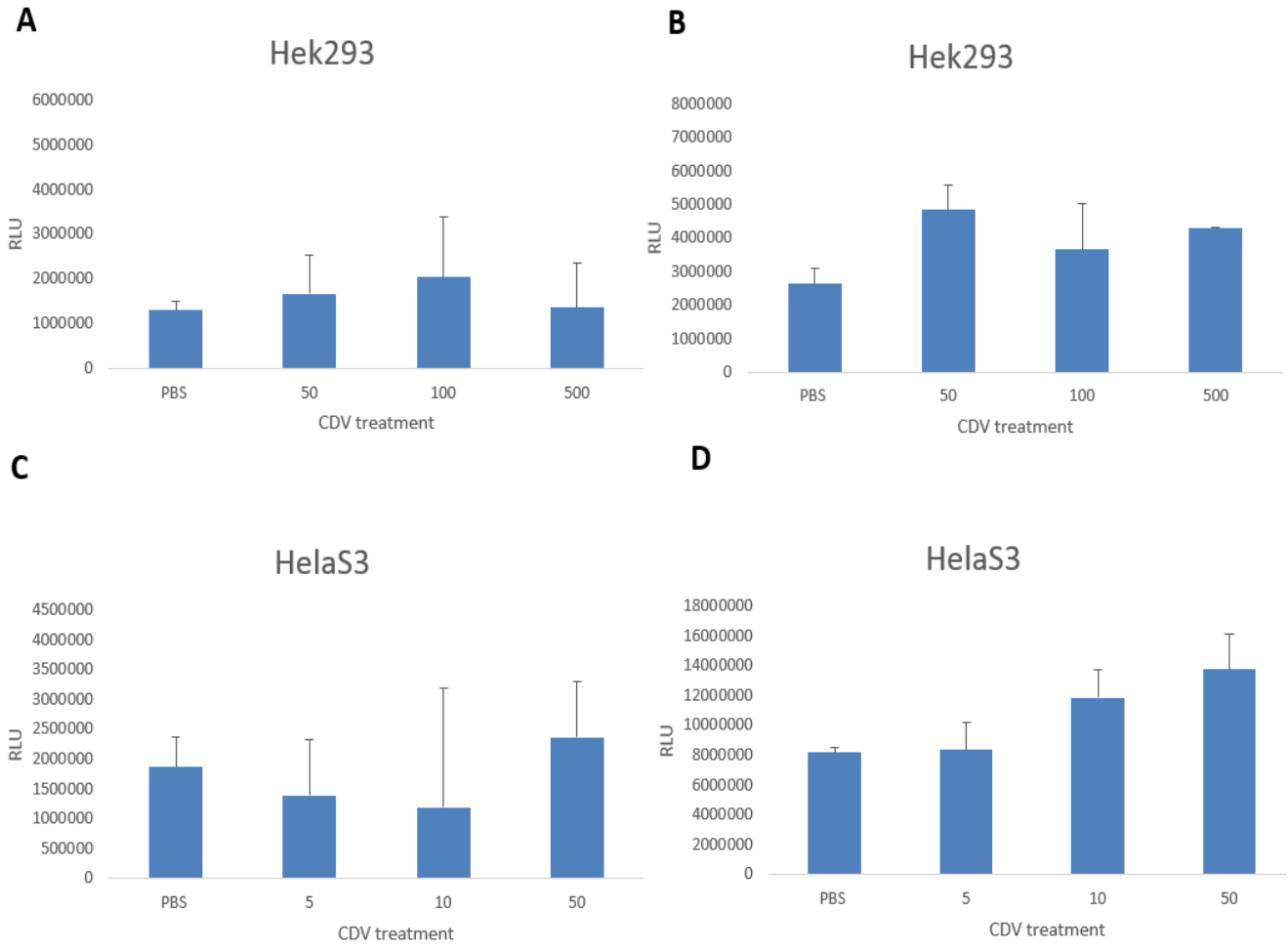


FIG 26: CDV pretreatment has no enhancement effect on scAAV vectors

Cells were pretreated with increasing concentrations of CDV for 16 hours. Cells were washed to remove drug then infected with scAAV-luciferase vectors at an MOI of 10. Luciferase expression was detected using the enzymatic assay 48 hours post-infection. **(A) Hek293 cells with scAAV2-CB-GL (B) Hek293 cells with scAAV8-CB-GL (C) HelaS3 cells with scAAV2-CB-GL (D) HelaS3 cells with scAAV8-CB-GL**. The mean and SD of two independent experiments is presented.

FIGURE 27.

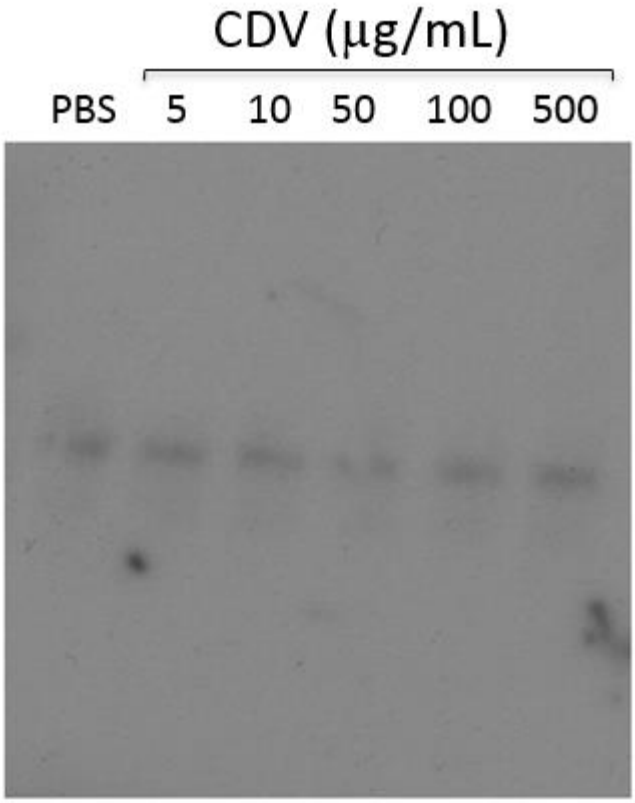


FIG 27: CDV pretreatment has no enhancement effect on scAAV genomes

HelaS3 Cells were pretreated with increasing concentrations of Cidofovir for 16 hours. Cells were washed to remove drug then infected with scAAV2-CB-Cluc at an MOI of 10. Cells were washed to remove drug then infected with scAAV2-CB-Gluc at an MOI of 10. Cells were harvested 24hrs after infection. DNA was extracted using HIRT Extraction protocol. Hybridizing LMW DNA against the luciferase gene probe. The following figure is a representative of 2 independent experiments.

MG132 is a peptide aldehyde that effectively blocks the proteolytic activity of the 26S proteasome complex (63). Degradation of AAV in the proteasome may occur before AAV can complete infection thus hindering the virus' efficiency. HelaS3 cells were pretreated PBS (mock treatment), 10µg/mL CDV, 50µg/mL CDV, 100µg/mL CDV or 20µM MG132 alone or in combination. Cells were washed to remove drugs then infected with scAAV2-CB-Cluc at an MOI of 10. Results of MG132 and CDV treatment demonstrated that at the highest concentration of both drugs combined there was a statistically significant increase in expression compared to each of the highest concentrations drugs alone **(Fig 28)**. Our data suggested that CDV and MG132 enhance through similar pathways since there was an increase in transgene expression when both drugs were combined. If the drugs worked through similar pathways, we would have expected to see no increase in transgene expression when the two drugs were combined, since there would be a redundancy in the pathway being upregulated. Another known rAAV enhancing drug we compared to CDV was ETO, inhibitor of DNA topoisomerase II (58). HelaS3 cells were pretreated with increasing concentrations of CDV, PBS, or ETO alone or in combination. Cells were washed to remove drugs then infected with scAAV2-CB-Cluc at an MOI of 10. Results demonstrated a slight enhancement effect on rAAV transgene expression in the presence of both CDV and ETO treatment on cells **(Fig 29)**. The results suggested that these two drugs act through separate pathways to enhance rAAV vectors. We ascertained if CDV can enhance through DNA damage pathways like other known rAAV enhancing drugs possibly like HU. HelaS3 cells were pretreated with increasing concentrations of CDV, PBS, or HU alone or in combination. Cells were washed to remove drugs then infected with scAAV2-CB-Cluc at an MOI of 10.

FIGURE 28.

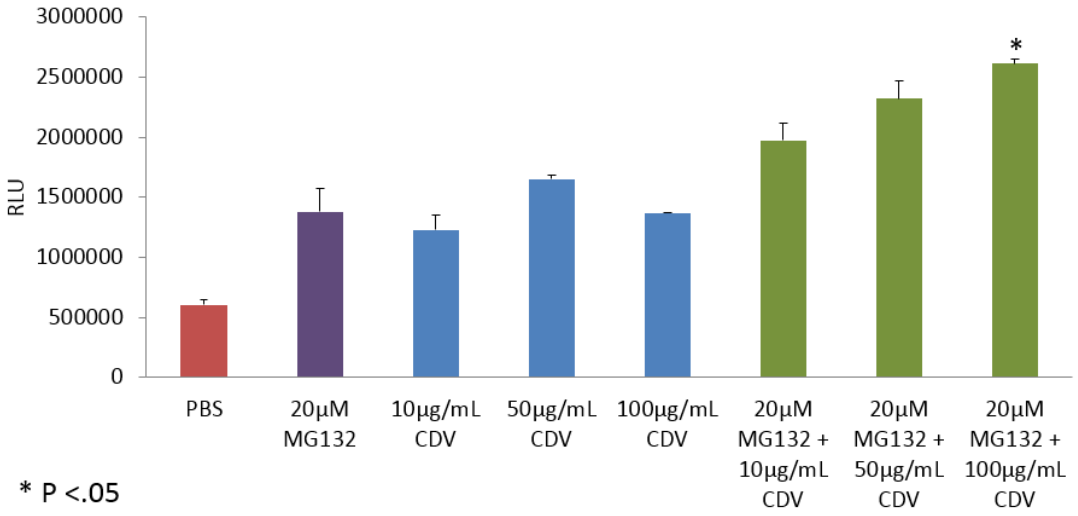


FIG 28: CDV and MG132 enhance through alternative pathways.

Cells were pretreated with increasing concentrations of CDV (10µg/mL, 50µg/mL, or 100µg/mL) PBS, or MG132 (20µM) or in combination of CDV with MG132. Cells were washed to remove drugs then infected with scAAV2-CB-Cluc at an MOI of 10. Luciferase expression was detected using the enzymatic assay 48 hours post-infection. The mean and SD of duplicate wells is presented. Statistical analysis is based on a 2-tailed-student's *t*-test.

FIGURE 29.

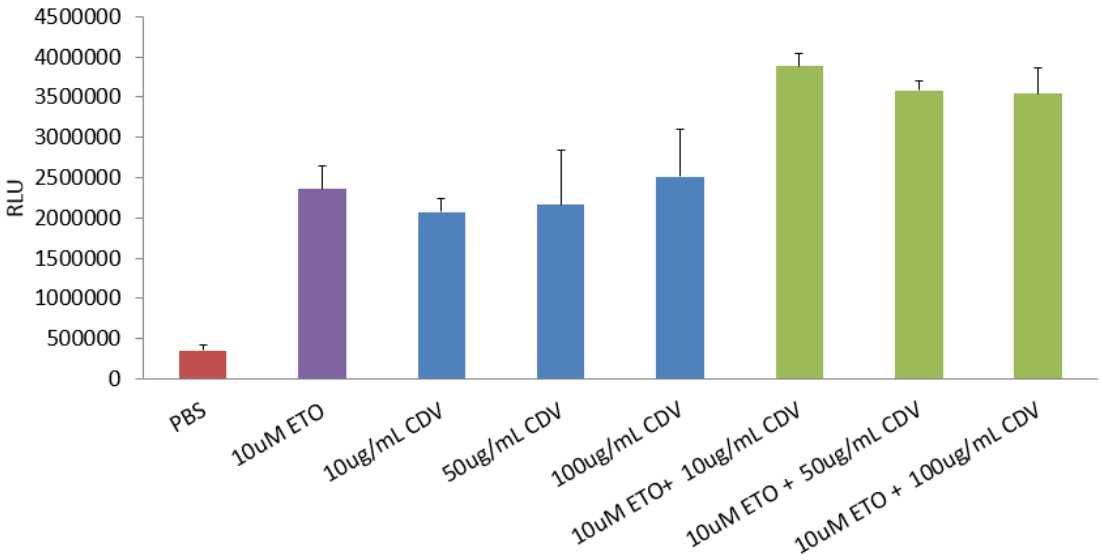


FIG 29: CDV and ETO enhance through alternative pathways.

Cells were pretreated with increasing concentrations of CDV (10µg/mL, 50µg/mL, or 100µg/mL) PBS, or ETO (10µM) or in combination of CDV with ETO. Cells were washed to remove drugs then infected with scAAV2-CB-Cluc at an MOI of 10. Luciferase expression was detected using the enzymatic assay 48 hours post-infection. The mean and SD of duplicate wells is presented.

Results demonstrated no additive enhancement on rAAV transgene expression in the presence of both CDV and HU **(Fig 30)**. It is known that HU can enhance rAAV vectors through multiple pathways. For example, HU has been postulated to enhance through increasing nuclear translocation as well as aiding in conversion to the transcriptionally active double-stranded form (57) (58) (62) (66) (67). We did not see an increase in transgene when both drugs were present in HelaS3 cells suggesting that HU and CDV enhance through similar pathways **(Fig 30)**. The results propose that CDV could enhance the same or similar pathway as HU to enhance AAV transgene expression. Investigation of transcriptome analysis of HelaS3 cells pretreated with CDV or PBS was examined to explore if any of the common DNA sensing and DNA damage genes previously known to be associated with AAV in the literature were upregulated (27) (57) (66). In this study we choose to focus on pathways that have already been associated with AAV transduction like DNA sensing and DNA damage, endosomal formation, and viral trafficking due to our results from the comparative drug study. Also CDV had similar activity to arrest cells in S phase like previous reporter AAV enhancing drugs (58) (59) (73) (74). We did not find upregulation in genes we focused on related to DNA damage response or p53 with our transcriptome analysis of CDV treated cells suggesting CDV does not enhance through these pathways **(Fig 31)**. However, there was a minimal increase with the ATRIP (ATR interacting protein) gene. The ATRIP protein binds to single-stranded DNA coated with replication protein A. Since rAAV is a single-stranded virus, we investigated if this protein could play a role in CDV enhancement of rAAV vectors. If ATRIP is important in CDV enhancement of rAAV transgene expression then a decrease in RLU readings with siRNA treatment would be expected.

FIGURE 30.

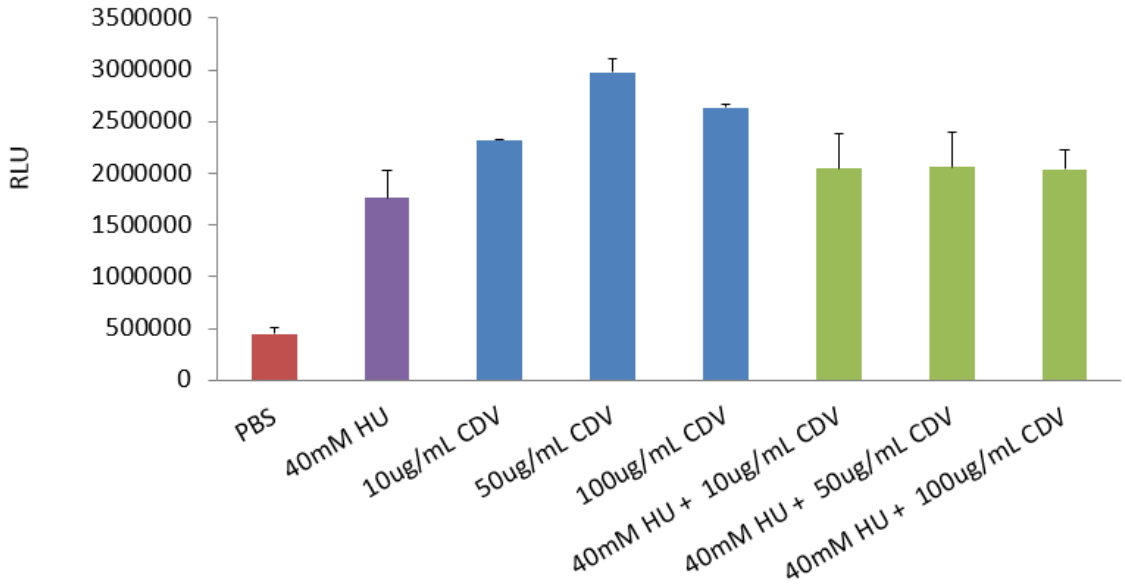


FIG 30. CDV and HU enhance through similar pathways.

Cells were pretreated with increasing concentrations of CDV (10µg/mL, 50µg/mL, or 100µg/mL) PBS, or HU (40mM) or in combination of CDV with HU. Cells were washed to remove drugs then infected with scAAV2-CB-Cluc at an MOI of 10. Luciferase expression was detected using the enzymatic assay 48 hours post-infection. The mean and SD of duplicate wells is presented.

FIGURE 31.

Gene	Function	Fold change
Rad50	MRN complex proteins	0.914611
Nibrin	MRN complex proteins	1.238233
Mre11	MRN complex proteins	1.020403
ATRIP	Protein binds to single-stranded DNA coated with replication protein A.	1.613513
Rad9	Protein is found to possess 3' to 5' exonuclease activity, which may contribute to its role in sensing and repairing DNA damage	1.275014
TOPBP1	Plays a role in the rescue of stalled replication fork	1.074268
ATM	Recruited and activated by DNA double-strand breaks	1.010706
ATR	Protein kinase that is involved in sensing DNA damage and activating the DNA damage checkpoint, leading to cell cycle arrest	1.272469

Figure 31. DNA damage related genes are not upregulated after CDV pretreatment.

HelaS3 cells were pretreated with PBS or 100ug/mL CDV. Cells were collected in 1ml cold PBS and centrifuged at 2000rpm for 5min at 4^{o}C. The cell pellets were stored at -80^{o}C immediately. RNAeasy Mini Kit (Qiagen) was used for RNA extraction. Transcriptome analysis was conducted.

However, no differences were observed between siRNA ATRIP treatments suggesting it does not play a major role in CDV enhancement of rAAV transgene expression **(Fig 32)**. Next to ascertain if CDV can enhance not only *in vitro* but in an *in vivo* setting, a preliminary animal study was conducted. CDV at 100mg/kg(high dose) or 30 mg/kg(low dose) or PBS was injected subcutaneously twice two days apart prior to rAAV injection and then injected once a week for 4 weeks post-infection. Human Light chain(LC) vector at $1x10^{11}$ viral particles was injected into a 6-8-week old BalbC/HA mice via tail vein. Results demonstrated an increase in LC levels with a dose of 30mg/kg in mice compared to PBS treated group as long as 14 weeks post vector injection. No increases in LC levels were observed at the higher dose of the treated group suggesting the lower dose may be optimal for future studies **(Fig 33)**. The results from the preliminary animal study demonstrated CDV enhances rAAV vectors *in vivo*.

Ganciclovir (GCV)

To expand on that idea of CDV enhancement, the investigation of a similar an antiviral drug within the same class as CDV was examined. Ganciclovir (GCV) was studied to determine its effect on rAAV transduction. Following similar experiments as CDV, a side by side comparison of GCV pretreatment on HelaS3 cells was conducted **(Fig 34)**. HelaS3 cells pretreated with H20 (Mock) or 100µg/mL GCV or 500 µg/mL GCV for 16 hours and then infected with followed by infection with Ad-LacZ or AAV-LacZ. HelaS3 cells pretreated with PBS Ad-LacZ displayed the largest amount of Ad-LacZ expressing cells. As the concentration of GCV increased the number of visible Ad-LacZ expressing cells decreased showing the effectiveness of GCV against larger DNA viruses like Adenovirus.

FIGURE 32.

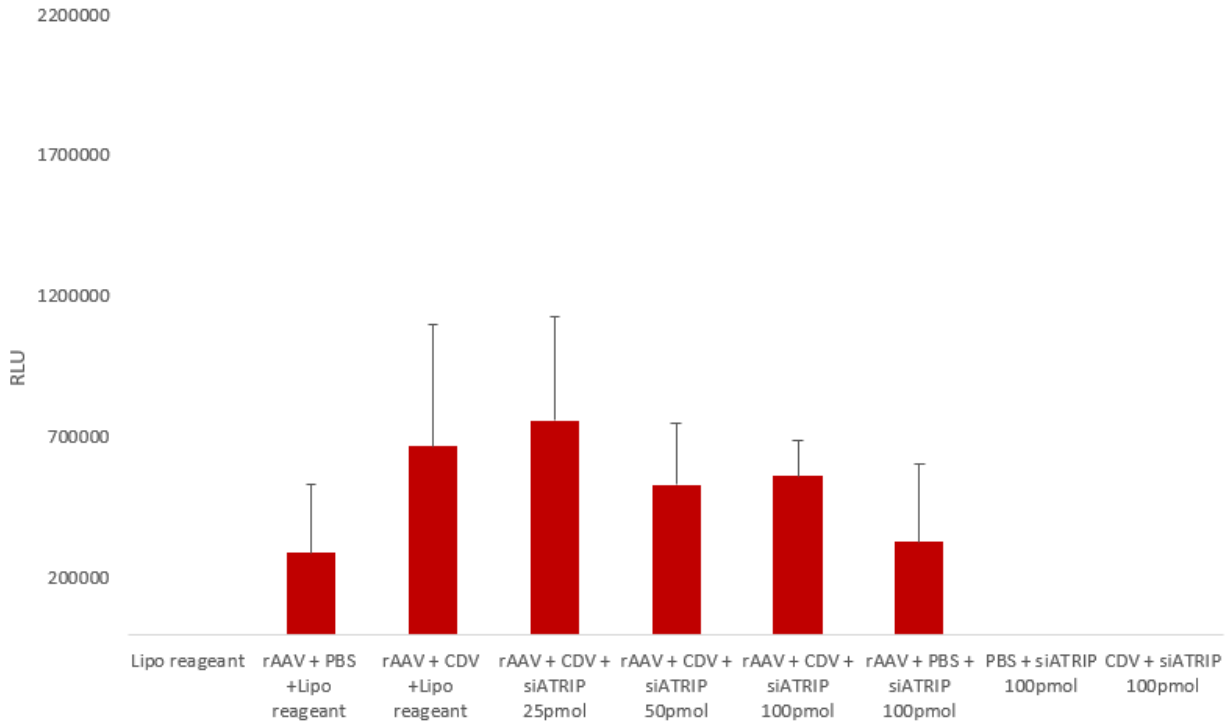


Figure 32. ATRIP does not play a major role in CDV enhancement of rAAV

HelaS3 cells were transfected with siATRIP RNA (25pmol, 50pmol, and 100pmol) for 6 hours. Cells were then treated with CDV or PBS for 16 hours. Cells were washed and infected with a rAAV2-CB-CL vector at an MOI of 10. Luciferase expression was detected using the enzymatic assay 48 hours post-infection. The mean and SD of three independent experiments is presented.

FIGURE 33.

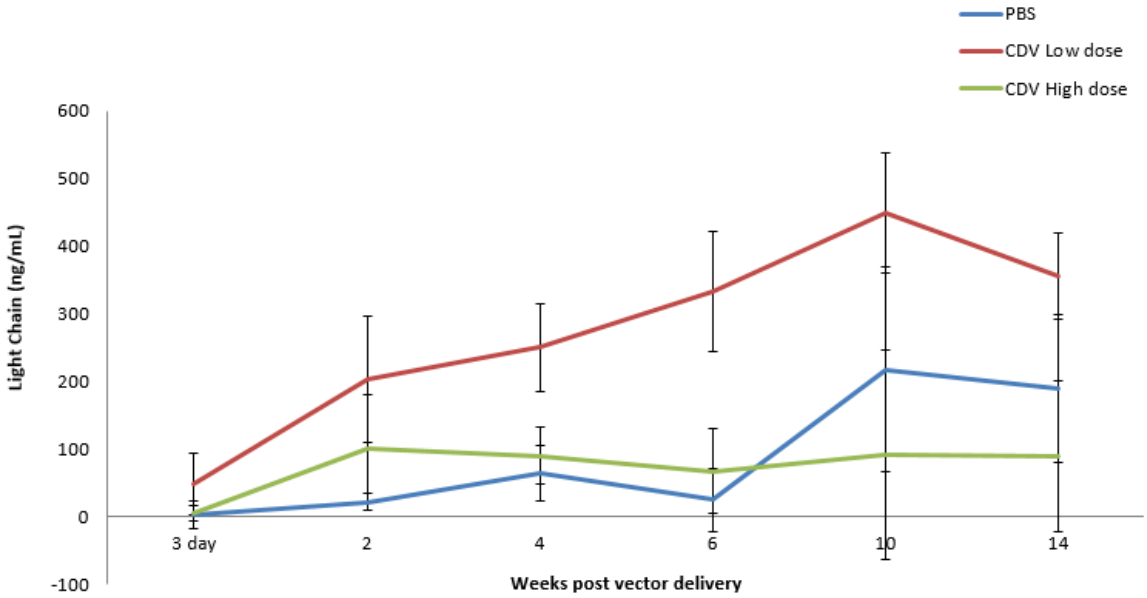


Figure 33. CDV enhances rAAV vectors *in vivo*.

Male Balbc-HA mice (n=4) were treated with PBS, Low Dose of CDV (30mg/kg), or High Dose of CDV (100mg/kg). Mice were injected with rAAV8-Human factor 8 Light Chain vector at a dose of 0.67x10^11 particles. Plasma samples were taken at indicated time points post vector delivery. ELISA assay was used to measure LC antigen level.

FIGURE 34.

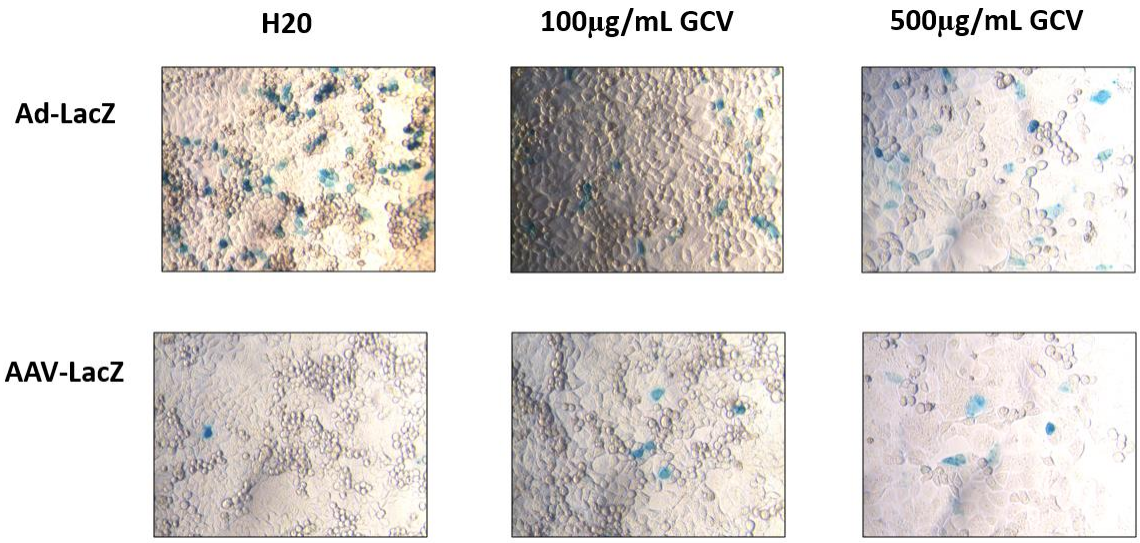


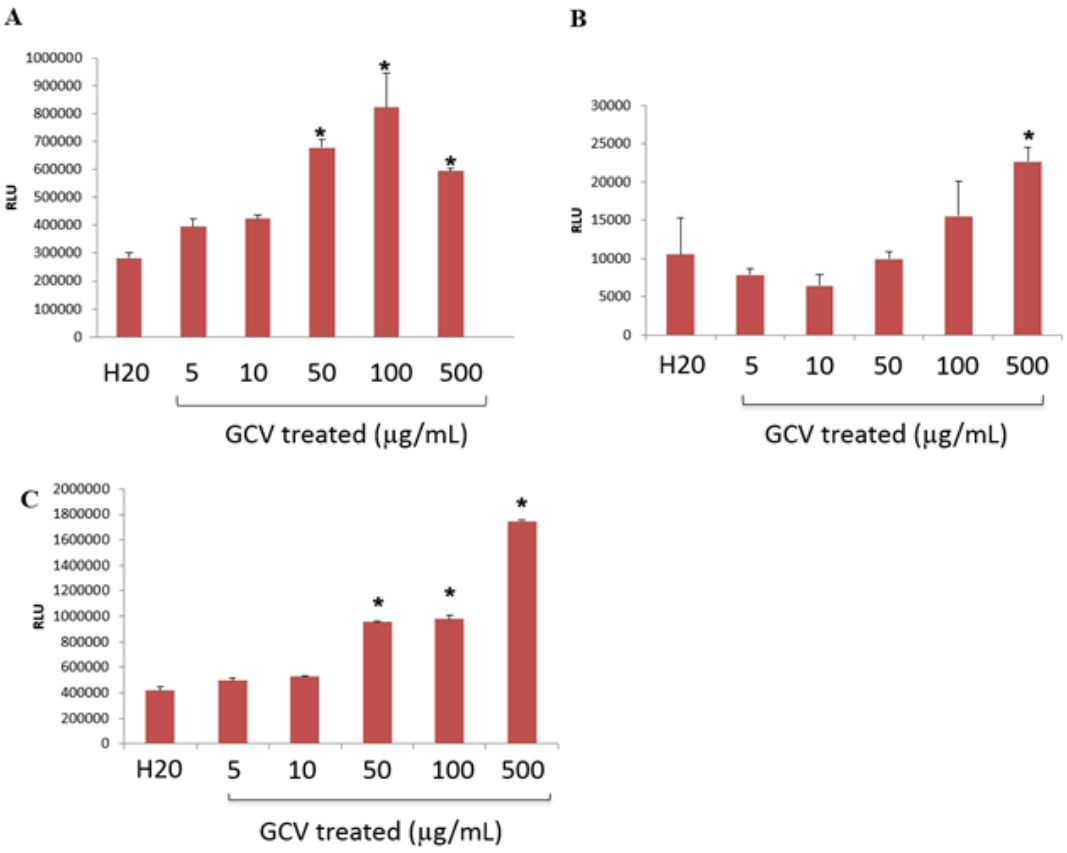
Figure 34. GCV inhibits Adenovirus but enhances AAV.

HelaS3 cells were pretreated with H20(Mock) or 100µg/mL GCV or 500 µg/mL GCV for 16 hours. The cells were washed once and then infected with Adenovirus(Ad-lacZ) at an MOI of 0.5 or Adeno-associated virus(AAV-LacZ) at an MOI of 10. LacZ stain was performed 24 hours post-infection. The following figure is a representative of 3 independent experiments.

However, the opposite effect was observed with AAV-LacZ infected cells pretreated with GCV. There was an increase in the number of AAV-LacZ expressing cells when pretreated with GCV compared to mock treated cells. These results demonstrated that GCV like CDV inhibits Adenovirus but enhances AAV transgene expression. To confirm the LacZ results, GCV enhancement experiments were conducted with rAAV2-luciferase vectors on different cell types GM16095 **(Fig 35A)**, Hek293 **(Fig 35B)**, and HelaS3 **(Fig 35C),** cells. Cells were pretreated with increasing doses of GCV or Mock for 16 hours without observing toxicity to the various cell types and then infected with a rAAV2-lucierfase vector. Treatment of cells with GCV demonstrated about a 4-fold increase in luciferase levels compared to Mock treatment **(Fig 35)**.

Investigation if GCV can enhance scAAV vectors was conducted next. GM16095, Hek293, and HelaS3 cells were pretreated with increasing concentrations of GCV for 16 hours and infected with a scAAV2-lucierfase vector. GCV did not enhance transgene expression of scAAV vectors on GM16095 **(Fig 36A)**, Hek293 **(Fig 36B)**, and HelaS3 cells **(Fig 36C).** A time course of GCV pretreatment on HelaS3 cells was performed to determine if GCV can sustain transgene levels *in vitro*. HelaS3 Cells were pretreated with increasing concentrations of GCV for 16 hours. Cells were washed to remove drug then infected with rAAV2-luciferase vector at an MOI of 10. Luciferase expression was detected 12, 24, 36, 48, and 72 hours post-infection. An increase in transduction with both rAAV2 was observed at all time point **(Fig 37)**. GCV enhanced transgene expression as long as 72 hours post-infection **(Fig 37)**. The detection of transgene expression as early as 12 hours post-infection was observed **(Fig 37)**. These experiments establish that there is also an increase in rAAV transgene expression *in vitro* with GCV similar to CDV.

FIGURE 35.



*P<.05

Figure 35. GCV pretreatment enhances AAV2-luciferase transduction.

Cells were pretreated with increasing concentrations of GCV for 16 hours. Cells were washed to remove drug then infected with rAAV2-CB-Cluc at an MOI of 10. Luciferase expression was detected using the enzymatic assay 48 hours post-infection. **(A) 16095 cells (B) Hek293 cells (C) HelaS3 cells.** The mean and SD of triplicate wells is presented. Statistical analysis is based on a 2-tailed-student's *t*-test.

FIGURE 36.

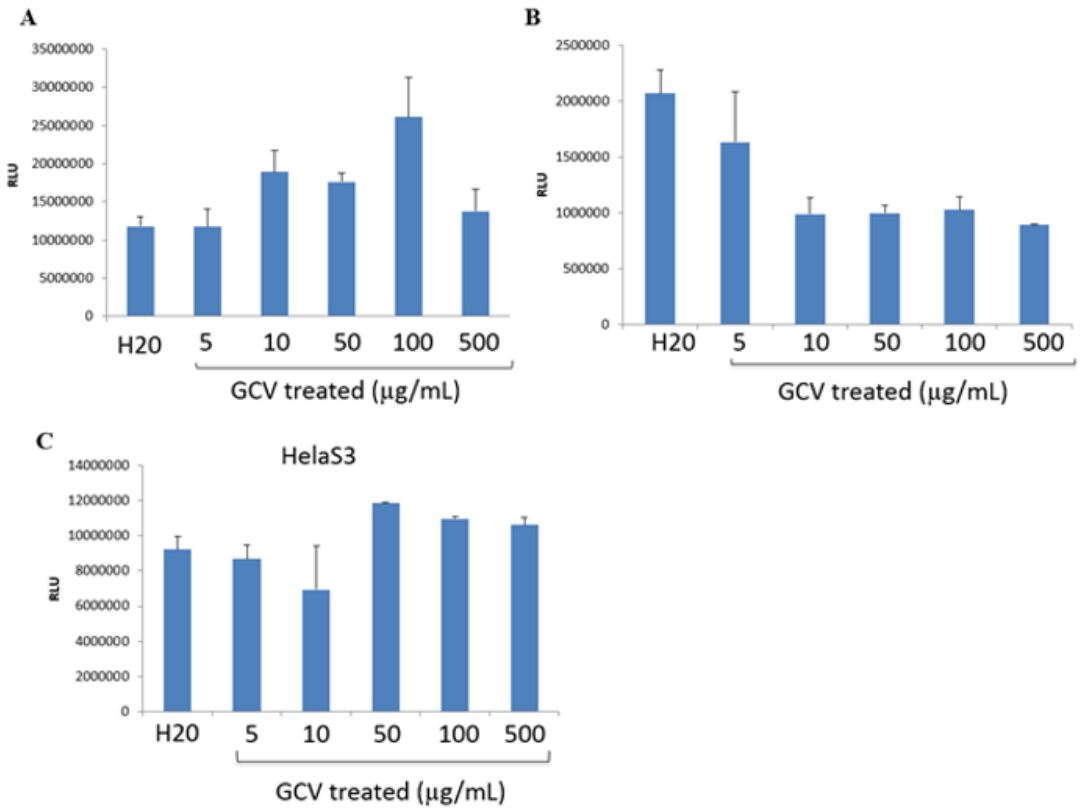


Figure 36. GCV pretreatment has no enhancement effect on scAAV vectors

Cells were pretreated with increasing concentrations of GCV for 16 hours. Cells were washed to remove drug then infected with scAAV2-CB-Cluc at an MOI of 10. Luciferase expression was detected using the enzymatic assay 48 hours post-infection. **(A) 16095 cells (B) Hek293 cells (C) HelaS3 cells.** The mean and SD of triplicate wells is presented.

FIGURE 37.

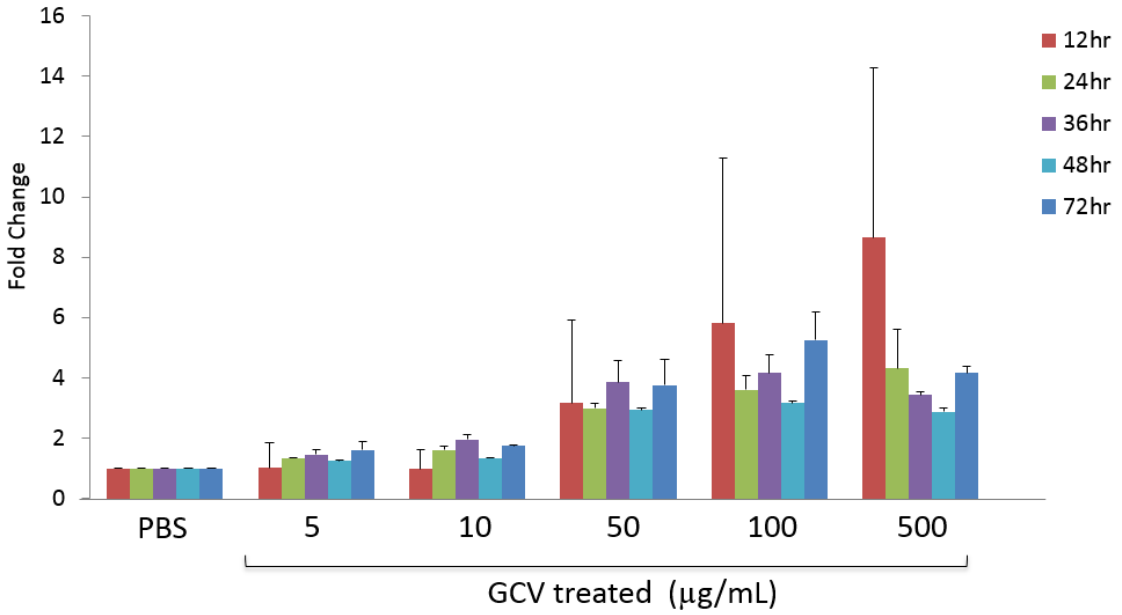


Figure 37. GCV pretreatment enhances rAAV-luciferase transduction as long as 72 hours post-infection.

HelaS3 cells were pretreated with increasing concentrations of GCV for 16 hours. Cells were washed to remove drug then infected with rAAV2-CB-CL vector at an MOI of 10. Luciferase expression was detected using the enzymatic assay 12, 24, 36, 48 and 72 hours post-infection. The mean and SD of duplicate wells is presented.

Taken together the combined data of both CDV and GCV demonstrates a new class of rAAV enhancing drugs.

The relationship between rAAV genomes in the cytoplasm and the nucleus

Cidofovir pretreatment enhances viral accumulation of rAAV vectors in both the cytoplasm and nucleus 24 hours post-infection.

Our project has designed and utilized a dual luciferase reporter to track rAAV vectors as they traffic through the cytoplasm into the nucleus **(Fig 1).** This rAAV dual reporter vector contains a modified Gaussia (GLuc) luciferase gene that is only functional in the cytoplasm (CytoGLuc) which is under the control of the T7 promoter, a cytoplasmic promoter, which must be activated by T7 RNA polymerase along with the CLuc gene under the control of the nuclear CB promoter. Utilizing this dual reporter system, the AAV status in the cytoplasm and nucleus was determined using different drug treatments. HelaS3 cells were pretreated with DMSO, HU (1mM)**,** LLnL (5µM), and CDV (100µg/mL) for 16 hours, then washed and infected with the dual reporter vector **(Fig 38)**. There was enhancement with CDV in cytoplasm and nucleus 24 and 48 hours post infection compared the control (DMSO). Enhancement was the highest in both the cytoplasm and nucleus with CDV at the 48 hour time point. This result suggested that CDV can enhance rAAV transgene expression in both cellular compartments. There was observed increased in rAAV transgene expression with HU treatment in the nucleus as predicted from previous literature. There was a minimal increase in both the cytoplasm and nucleus with HU

FIGURE 38.

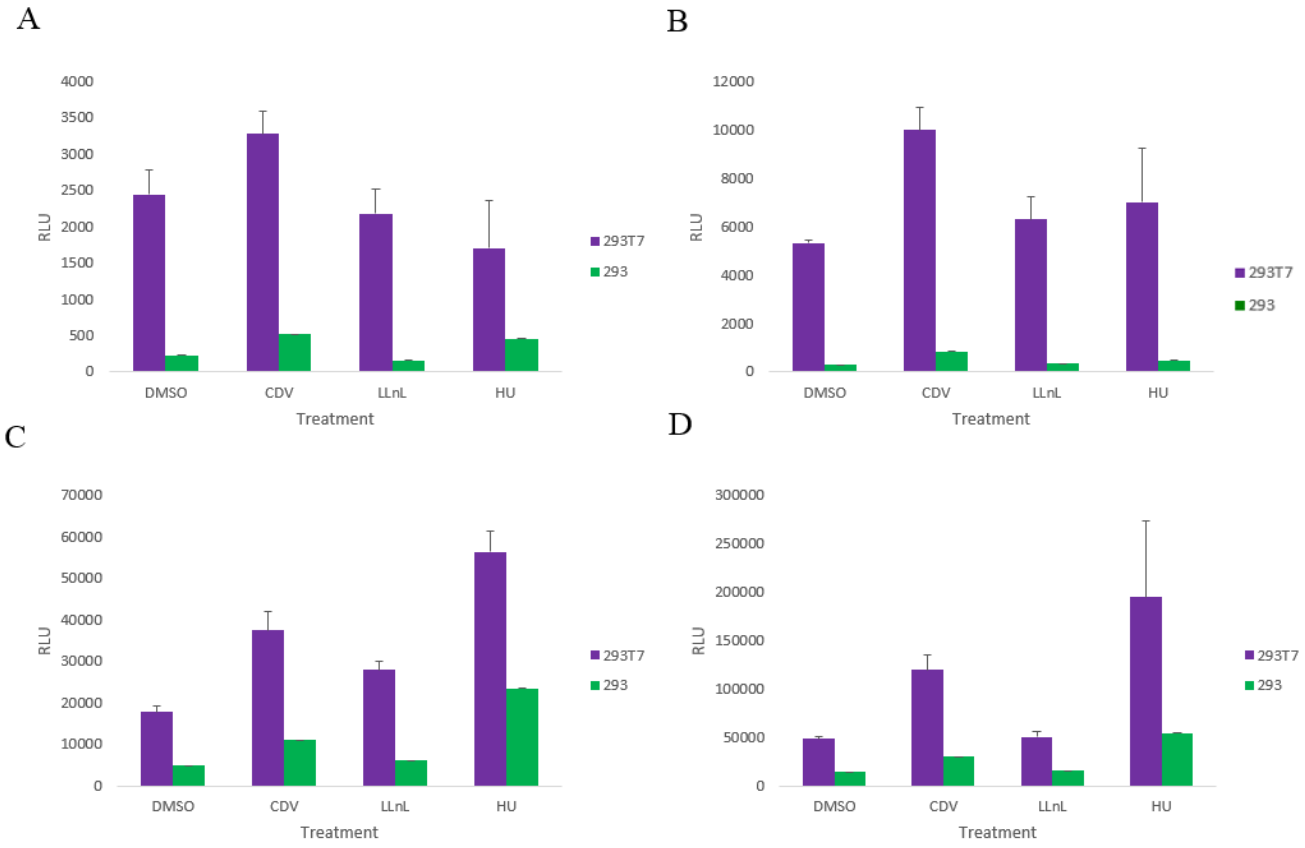


Figure 38. CDV enhances AAV transgene expression in both the cytoplasm and nucleus 24 and 48 hours post-infection.

HelaS3 cells were pretreated with HU**,** LLnL, and CDV for 16 hours, then washed and infected with the dual reporter vector Cidofovir (100ug/mL), Hydroxyurea(1mM), LLnL (5uM) or Mock treatment (DMSO) for 16 hours. Cells were washed twice to remove drug then infected with the dual reporter vector at an MOI of 10. Luciferase expression was detected using the enzymatic assay 24 and 48 hours post-infection. The mean and SD of duplicate wells is presented. **(A) Cytoplasm 24 hour. (B) Cytoplasm 48 hour. (C) Nucleus 24 hour. (D) Nucleus 48 hour.**

treatment at 48 hours suggesting an increase in both compartments; however, it was not two-fold like CDV. There was not a substantial increase observed with LLnL. Further investigation of CDV alone using this dual reporter system demonstrated that results of enhancement both in the cytoplasmic and nuclear compartments are repeatable **(Fig 39)**. To determine if CDV enhances nuclear entry of the virus, southern blot analysis of DNA taken from both the cytoplasmic and nuclear fractions was performed **(Fig 40A)**. Image J software analysis was used to quantify AAV in samples **(Fig 40B)**. The results demonstrated that there is an increase in viral accumulation in both the cytoplasm and nucleus 24 hours post-infection wash. This result suggests that CDV may enhance rAAV vectors as they traffic through the cytoplasm since we observed an increase in both cellular compartments. The CDV data suggested that there is a positive correlation with enhancement in both the cytoplasm and nucleus.

Bafilomycin A1 treatment decreases rAAV transgene expression in both the cytoplasm and nucleus 24 and 48 hours post-infection.

The dual reporter system was used to understand how Bafilomycin A1, which inhibits endosomal acidification and negatively impacts rAAV transduction, could impact the cytoplasmic and nuclear correlation. We investigated if using a known rAAV inhibitor in our dual reporter system would result in a negative correlation between the cytoplasm and nucleus. Hek293T7 cells were pretreated with different concentrations of BafilomycinA1, or Mock treatment (DMSO) for 2 hours prior to rAAV infection. Cells were then infected with rAAV2 dual luciferase vector at an MOI of 10. Cells were incubated with drug and rAAV for two hours then washed twice to remove drug. Luciferase expression was detected 24 and 48 hours post-infection.

FIGURE 39.

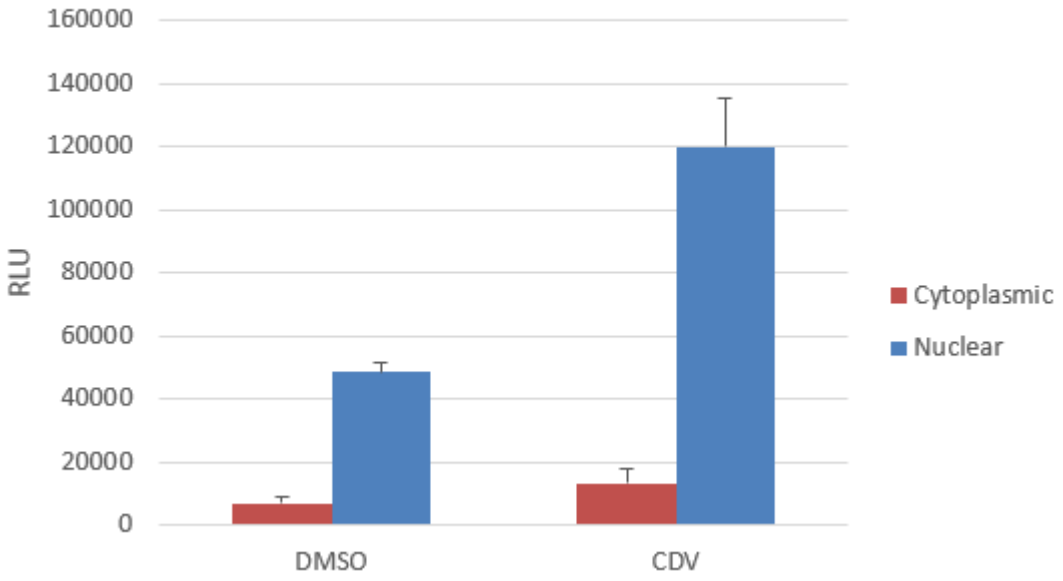
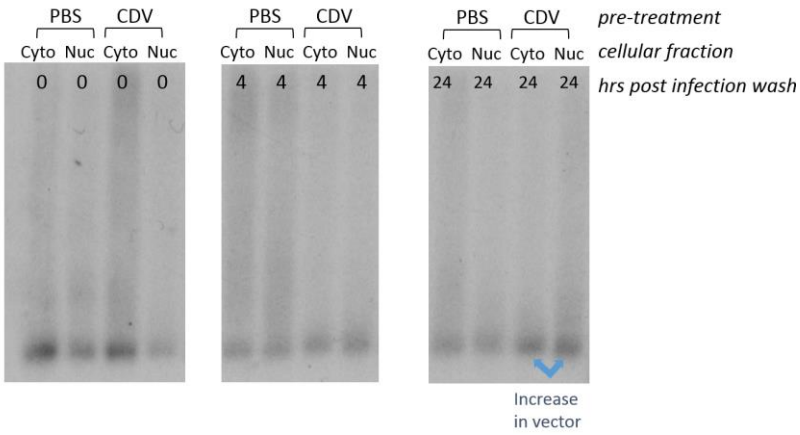


Figure 39. CDV pretreatment enhances rAAV transgene expression in both the cytoplasm and nucleus 24 hours post-infection.

293T7 cells were pretreated with DMSO or CDV (100µg/mL) for 16 hours. Cells were then washed and infected with the dual reporter vector at an MOI of 10. Luciferase expression was detected using the enzymatic assay 24 hours post-infection. The mean and SD of three independent experiments is presented.

FIGURE 40.

A



B

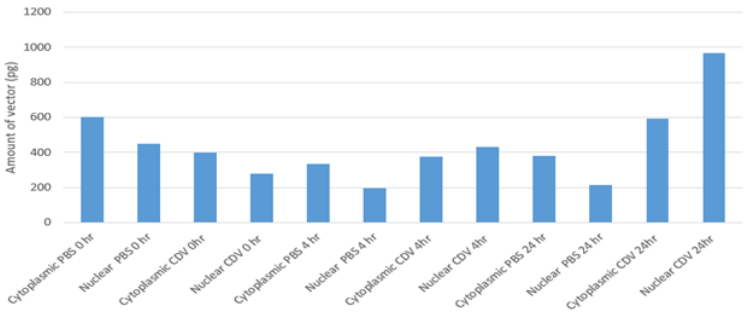


Figure 40. CDV enhances nuclear entry of AAV.

HelaS3 cells were pretreated with CDV (100ug/mL) or PBS for 16hrs. Next the cells were washed and infected with rAAV2-CB-Cluc at an MOI of 10 for 2 hours then washed to remove unattached virus. Cells were collected at this time and marked as 0hr time point. Cells were washed and harvested at the 24 hours after infection. (**A) Southern blot (B) ImageJ analysis of Southern blot intensity** Amount of vector present per sample based on 500pg loading control (not shown).

FIGURE 41.

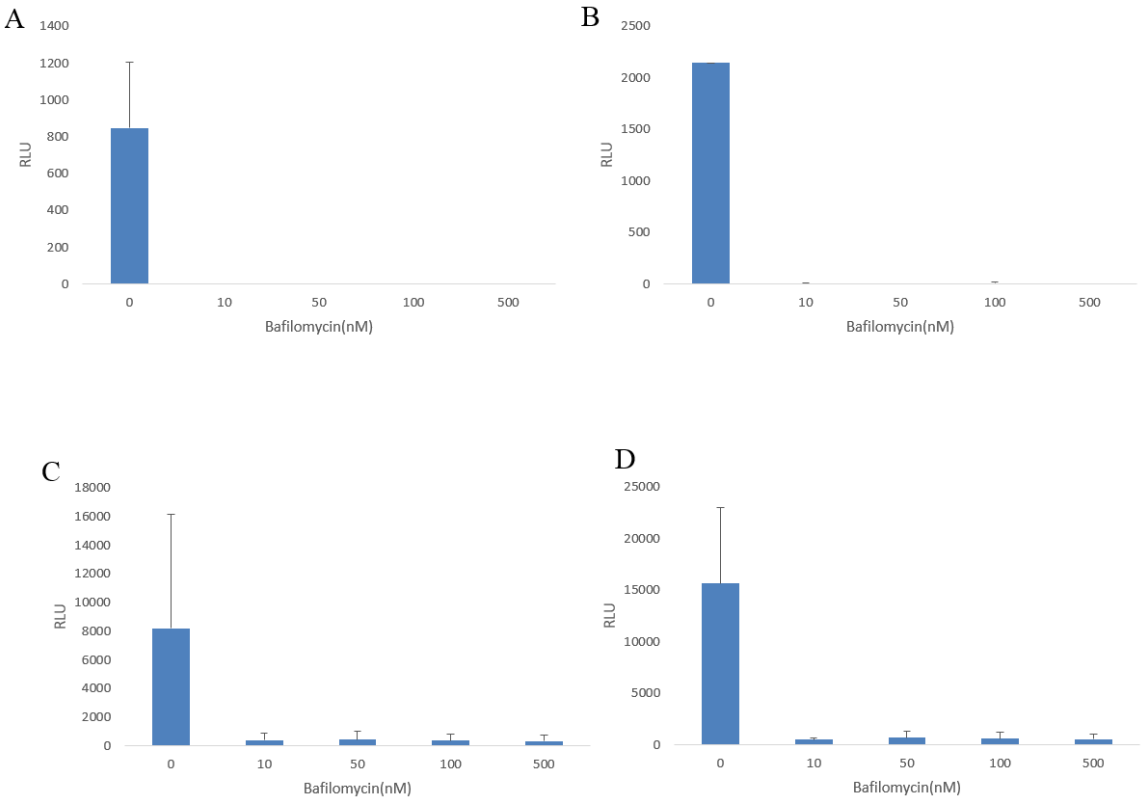


Figure 41. Bafilomycin A1 treatment decreases rAAV transgene expression in both the cytoplasm and nucleus 24 and 48 hours post-infection.

Hek293T7 cells were pretreated with different concentrations of BafilomycinA1, or Mock treatment (DMSO) for 2 hours prior to rAAV infection. Cells were then infected with rAAV2 dual luciferase vector at an indicated MOI of 10. Cells were incubated with drug and rAAV for two hours then washed twice to remove drug. Luciferase expression was detected using the enzymatic assay 24 and 48 hours post-infection **(A) Cytoplasm 24 hour. (B) Cytoplasm 48 hour. (C) Nucleus 24 hour. (D) Nucleus 48 hour.** The mean and SD of three independent experiments is presented.

The decrease in transgene expression as a result of Bafilomycin A1 was seen both in in the cytoplasm at 24 hours **(Fig 41A)** and 48 hours **(Fig 41B)** as well as in the nucleus at 24 hours **(Fig 41C)** and 48 hours **(Fig 41D)** indicating that the status of AAV in the cytoplasm could be a predictor to nuclear transduction.

Cidofovir pretreatment upregulates genes involved in endosomal processing and motility.

Next to identify what cellular changes occur as a result of pretreatment with CDV, RNA was extracted from HelaS3 cells treated with CDV (100µg/mL) or PBS and transcriptome analysis was conducted. The transcriptome data was analyzed using Ingenuity software and a 1.5 fold increase threshold was applied for all group analyses. The ingenuity knowledge base is a data repository that organizes biological interactions and functional annotations created from millions of individually modeled relationships between proteins, genes, complexes, cells, tissues, drugs, and diseases. These modeled relationships, pathways, or findings are manually reviewed for accuracy. The Ingenuity knowledge base enables access to relevant and substantiated knowledge from primary literature, as well as public and third-party databases like Entrez Gene, RefSeq, and Gene Ontology. These group databases are generated prior to data input and are based on the literature. The significance values for the group analyses are calculated by Fisher's exact test right-tailed. The significance indicates the probability of association of molecules from each group analysis by chance alone. Thousands of group database analyses were investigated and analyzed; however, we choose to focus only on two group analyses, canonical group analysis and diseases & function group analysis, for the relevance to this

study. Within each group database molecular relationships are defined as pathways. Pathways are defined as cluster of genes whose products in some way interact with each other in a certain group of functions. These pathways include and are not limited to surface molecules, miRNA and transcription factors, which may be causing observed gene expression changes. Also pathways contain information on phosphorylation cascades, protein-protein or protein-promoter interaction networks, and chemical/drug effects on proteins. The Ingenuity software analyzed over 600 known pathways (defined as metabolic and signaling pathways) within their canonical group database. Canonical group analysis evaluates 302 different metabolic pathways. These metabolic pathways include and are not limited to biosynthesis, degradation, and oxidation pathways. Canonical group analysis also evaluates 354 different signaling pathways. These signaling pathways include and are not limited to apoptosis, thrombin, iNOS, and integrin pathways. We chose to show only the top five pathways up-regulated within the canonical group. From these five pathways up-regulated, we also chose to concentrate on the pathway dealing with mechanism of viral exit from host cells, because of our finding showing the increases of AAV expression in the cytoplasm and nucleus (**Fig 42)**. We speculated that CDV could enhance trafficking of AAV thus leading to an increase in AAV transgene expression. The second group analysis we chose to highlight was the disease and function group. We chose to show only the top five pathways unregulated within the diseases & function group **(Fig 43).** Disease and function group analysis predicts downstream effects on biological and disease processes. For example, this group analysis could show the genes in the particular analysis that have a causal or correlative relationship with the disease or function and indicate how they might increase or decrease

the disease or function. Also the disease and function analysis displays molecules associated with a disease or toxicity phenotype. We focused on the cellular movement pathway, which belongs to the diseases & function group **(Fig 43).** We speculated that CDV could enhance trafficking and choose to focus on motility genes like those associated with cellular movement. The up-regulation of the mechanism of viral exit from host cells and the cellular movement pathways reinforced the idea that CDV enhances viral trafficking since the protein products of these gene clusters are located in the cytoplasm. If CDV enhances AAV trafficking then we speculated that genes involved in movement through the cell would be upregulated to facilitate this process. We examined more closely the upregulated genes in these two groups with Ingenuity software. There was an increase observed in several genes of interest including those involved in the endosomal processing. Our results show an upregulation of Annexin A2 oligonucleotides as well as other proteins involved in endosomal formation and acidification **(Fig 44A & 44B)**. DJ439575, DJ439583, DJ439557 and DJ439566 are Annexin A2 oligonucleotides. These are important for cell sorting within the endosome. VPS25 functions in sorting of ubiquitinated membrane proteins during endocytosis. VPS4A is involved in late steps of the endosomal formation. ACTA1 and ACTA2 are involved in various types of cell motility. SH3GLB2 is involved in endocytosis pathway. CHMP6 also functions in endosomal sorting complex required for transport. CHMP2B is a component of the heteromeric ESCRT-III complex (Endosomal Sorting Complex Required for Transport III) that functions in the recycling or degradation of cell surface receptors.

FIGURE 42.

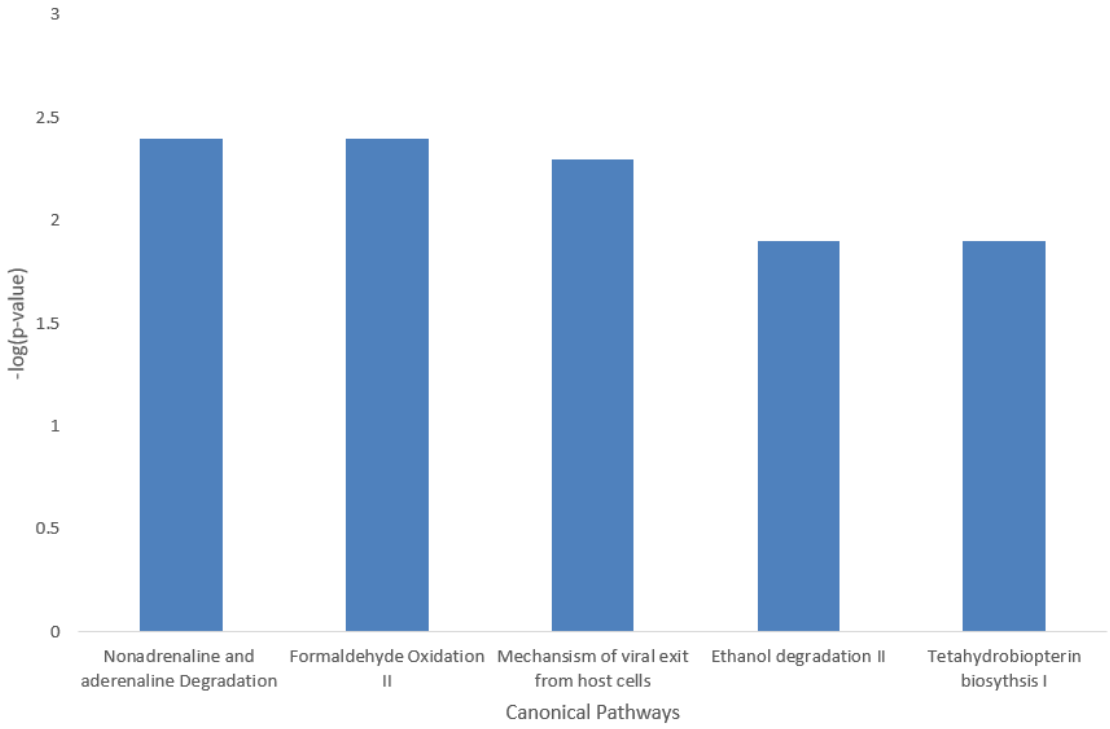


Figure 42. Mechanisms of Viral Exit from host cells are up-regulated in HelaS3 cells after CDV treatment.

HelaS3 cells treated with CDV (100µg/mL) or PBS and transcriptome analysis was conducted. Cells were collected in 1ml cold PBS and centrifuged at 2000rpm for 5min at 4ºC. RNA was extracted and a transcriptome analysis was conducted. Canonical pathway analysis was performed and the data was analyzed using a 1.5 fold cut off via Ingenuity software.

FIGURE 43.

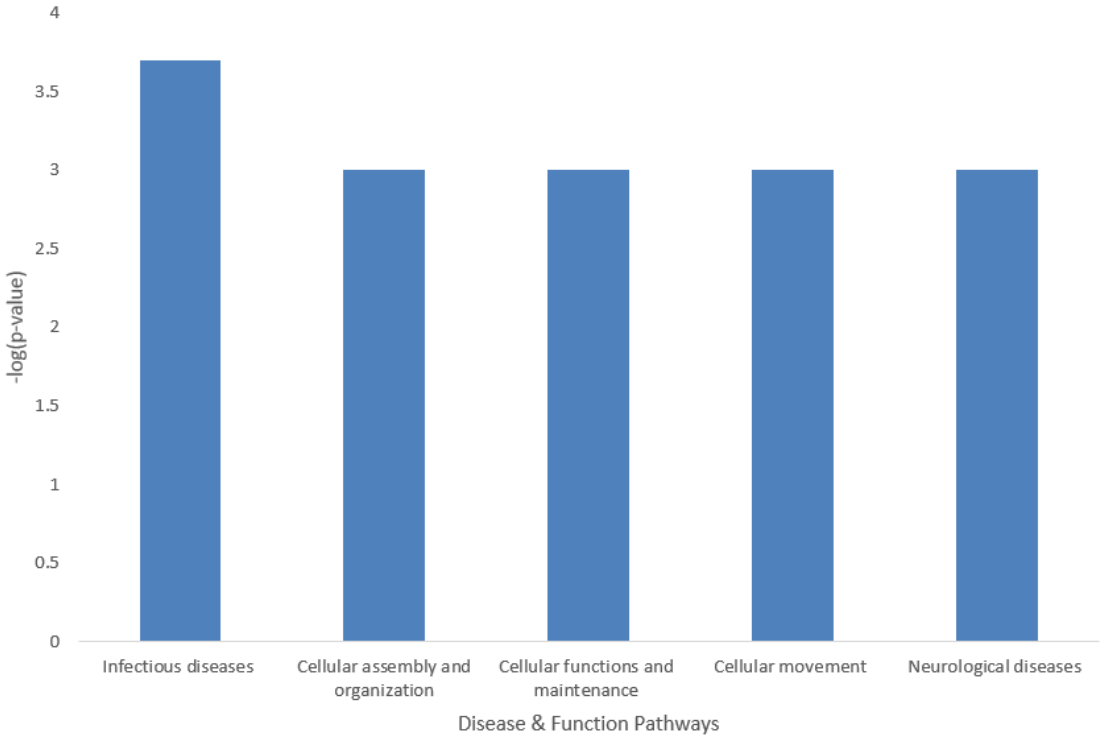
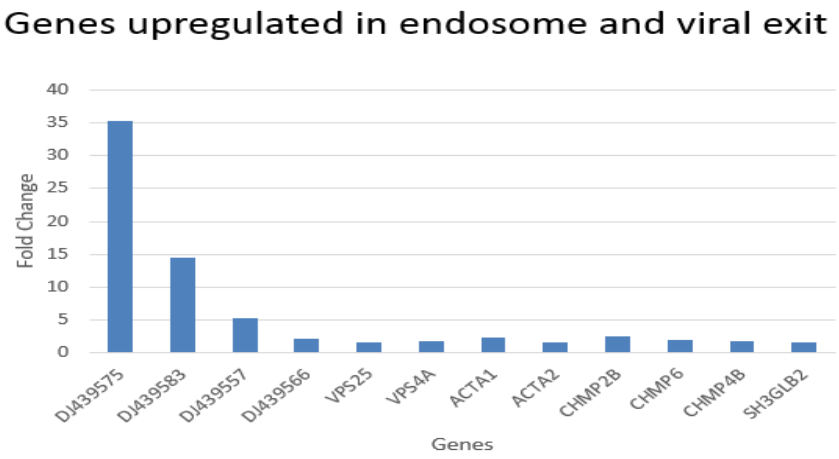


Figure 43. Cellular movement is up-regulated in HelaS3 cells after CDV treatment.

HelaS3 cells treated with CDV (100µg/mL) or PBS and transcriptome analysis was conducted. Cells were collected in 1ml cold PBS and centrifuged at 2000rpm for 5min at 4ºC. RNA was extracted and a transcriptome analysis was conducted. Disease & function pathway analysis was performed and the data was analyzed using a 1.5 fold cut off via Ingenuity software.

FIGURE 44.

A



B

Gene	Function
DJ439575,DJ439583, DJ439557,DJ439566	Annexin A2 protein has been proposed to function inside the cell in sorting of endosomes
VPS25	is a subunit of the endosomal sorting complex required for transport II (ESCRT-II). It functions in sorting of ubiquitinated membrane proteins during endocytosis
VPS4A	involved in late steps of the endosomal multivesicular bodies (MVB) pathway
ACTA1	belongs to the actin family of proteins, which are highly conserved proteins that play a role in cell motility, structure and integrity
ACTA2	belongs to the actin family of proteins and is involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells.
CHMP2B	encodes a component of the heteromeric ESCRT-III complex (Endosomal Sorting Complex Required for Transport III) that functions in the recycling or degradation of cell surface receptors
CHMP4B	is part of the endosomal sorting complex required for transport (ESCRT) complex III (ESCRT-III), which functions in the sorting of endocytosed cell-surface receptors into multivesicular endosomes
SH3GLB2	related to pathways involving endocytosis
CHMP6	member of the chromatin-modifying protein/charged multivesicular body protein family. Proteins in this family are part of the ESCRT-III (endosomal sorting complex required for transport III) which degrades surface receptors, and in biosynthesis of endosomes

Figure 44. Genes of interest upregulated in HelaS3 cells after CDV treatment.

HelaS3 cells were pretreated with PBS or 100ug/mL CDV. Cells were collected in 1ml cold PBS and centrifuged at 2000rpm for 5min at 4^{o}C. RNA was extracted and samples were sent out for transcriptome analysis. Data was analyzed using a 1.5 fold cut off via Ingenuity software. **(A) Fold change compared to PBS (B) Function of each gene.**

CHMP4B is part of the endosomal sorting complex required for transport (ESCRT) complex III (ESCRT-III), which functions in the sorting of endocytosed cell-surface receptors into multivesicular endosomes. There is only a modest increase with some of the cellular movement genes (1.5 to 2.5-fold increase). This could be accounted by the fact that CDV enhances AAV transgene expression in vitro ~2- to 5-fold **(Fig 12).** Also, validation of the importance of these genes associated with CDV needs to be confirmed. As stated previously, AAV vectors require endosomal acidification and early endosomal escape for efficient AAV vector transduction (30). We postulate that CDV upregulation of these genes contribute to the enhancement effect on rAAV transduction since these genes are important for AAV trafficking. These genes include Actin and endosomal genes. These molecules are involved in and associated with endosomal sorting complexes as well as required for transport inside the cell. This finding along with our southern blot result supports our theory that CDV enhances rAAV trafficking since there was an observed enhancement of rAAV vectors in both the cytoplasm and nucleus 24 hours post-infection following CDV pretreatment. Also utilizing our dual luciferase reporter system, there was an increase in transgene expression present with CDV pretreatment compared to PBS in both the cytoplasm and nucleus 48 hours post-infection suggesting that CDV enhances AAV trafficking.

CHAPTER 4

DISCUSSION

Our hypothesis was a large amount of AAV lost during the intracellular trafficking of the virus can hinder the efficiency of AAV transduction. Multiple studies illustrated that AAV trafficking is a rate-limiting step for transduction (2) (8) (14) (36) (37) (47) (68). Previous findings establish that impaired trafficking from the cytoplasm into the nucleus is a very important problem in efficient AAV transduction in the context of gene therapy. To date, there is still little known about the cellular mechanisms controlling the trafficking of the vector from the cytoplasm into the nucleus as well as what causes the degradation of these vectors as they traffic (2) (8) (14) (36) (47) (61). We hypothesized that a large majority of the vectors are lost during cellular trafficking of the virus thus ultimately hindering AAV transduction (14). Understanding this particular area of AAV biology can improve the efficacy of this vector for the treatment of monogenetic diseases targeted by gene therapy.

The first goal of this project was to develop a novel dual reporter system to detect AAV trafficking. This new system detects AAV in both the cytoplasmic and nuclear compartments of the cell. Our project sought to approach this problem by designing and implementing a novel dual reporter system to observe how the transgene expression in the cytoplasm relates to overall vector transduction by utilizing luciferase genes that are secreted by two different promoters. Grasping how AAV's status in the cytoplasm directly or indirectly relates to overall nuclear transduction gives understanding and ways to

improve trafficking of these vectors for clinical use. Many other studies have utilized alternative methods to track AAV vectors as they move within the cell for example GFP-fusion rAAV vectors, siRNA, or drugs. These methods seek to understand AAV trafficking patterns in order to pin point steps of importance for AAV infection whether they be enhancement or inhibition steps (14) (36) (37) (39) (68). Our dual reporter system is much easier to use compared to previous approaches, since media is harvested at any time point and two simple luciferase assays are conducted to determine the effect of a molecule or drug on rAAV trafficking. Another question our study sought to answer was: is it possible to detect and quantify AAV vectors as they move through the cytoplasm into the nucleus using our system. We demonstrated using our dual reporter system that it is possible to detect AAV in the cytoplasm **(Fig 1, Fig 2, and Fig 8)**. There have been previous studies using fluorescent AAV to observe AAV trafficking patterns (13) (28) (36) (32) (37) (43) (47); however, we wanted to go one step further in our study and be able to easily and quickly quantify AAV in both the nucleus and cytoplasm. The dual reporter system is easier to use compared to alternative methods like laser scanning microscopy capturing confocal images to track one molecule of rAAV trafficking through the cell. Sonntag *et al* used AAV vectors with various mutations to understand trafficking; however, the process of designing and manufacturing multiple mutant AAV vectors can be extensive and lengthy (40). The dual reporter system allows for rapid detection of AAV in both cellular compartments using luciferase assays with only one AAV reporter vector. Our system permits the researcher to screen multiple drugs/molecules at once instead of designing a different system or assay for each drug or molecule. This system was designed to specifically indicate whether or not the AAV genome is exposed before reaching the nucleus, where there is a great

potential for DNA degradation. The dual reporter system indicated that AAV vectors can be detected in the cytoplasm **(Fig 1, Fig 2, and Fig 8)**. Pertaining to the first goal of this project we have demonstrated that the novel dual reporter system is functional **(Fig 7)** and that it can detect rAAV transgene expression in the cytoplasm **(Fig 8)**. The results from the dual reporter system indicate that AAV is present in the cytoplasmic compartment at both 24 and 48 hours post-infection **(Fig 8)**. These dual reporter system results were confirmed using PCR analysis specifying not only that our dual reporter system is functional, but again demonstrating detection of AAV in the cytoplasm **(Fig 9 and Fig 10)**. The amount of vector detected in the cytoplasm was low, but taking into consideration that the cytoplasm may be the area of major loss of AAV vectors we expected that result. A low amount of AAV detected in the cytoplasm compels us to determine how to improve transduction at this step. The enhancement of the AAV amount in the cytoplasmic compartment would lead to the corresponding enhancement in overall nuclear transduction of AAV.

Originally we employed VVT7; however, the use of VVT7 added complexity to the dual reporter system, since VVT7, a second virus, is very toxic. There was an observed decrease in luciferase detection with ssAAV-CB-CL-T7-CG reporter used in conjunction with VVT7 as compared to ssAAV-CB-CL-T7-CG alone **(Fig 2)**. The infection of cells with VVT7 was an extra step in the overall procedure, and we preferred to exclude this step. To eliminate the need for VVT7 in the dual reporter system, we used a plasmid containing the T7 RNA polymerase **(Fig 2)**. However, the use of transfection and then treatment with chemical compounds along with AAV infection seemed to add an extra step **(Fig 2 & Fig 3)**. Furthermore, transfecting a T7 RNA plasmid for each experiment would

not give consistent T7 RNA polymerase production among studies. Therefore, we constructed a stable Hek293 cell that constitutively expresses T7 RNA polymerase. This cell line eliminates the need for transfection of T7 RNA polymerase in order to use the novel dual reporter system **(Fig 4)**.

Another unique feature of our dual reporter system is that it includes a cytoplasmic reporter. There are very few known cytoplasmic promoters. Our system utilized the T7 promoter, which must be activated by T7 RNA polymerase. As an extra added precaution to the cytoplasmic reporter cassette, the special *Gaussia l*uciferase (cytoGLuc) gene was generated to ensure this reporter cassette is solely cytoplasmic **(Ill. 11)**. The cytoGLuc was designed with two introns in the gene. The cytoGLuc gene has the same amino acid sequence as the original GLuc gene; however, small variations in the three letter genetic code sets up splice sites to eliminate the possibility of producing a functional mRNA from the CytoGluc gene in the nucleus. When our reporter vector is in the cytoplasm along with T7 RNA polymerase, the T7 promoter is activated and GLuc expression in the cytoplasm is detected. However, inside the nucleus the introns are spliced out; thus making the gene non-functional. This was confirmed by the minimal readings with the constructs of pdsAAV-T7-GLuc and pdsAAV-CB-CytoGLuc **(Fig 7)**. By pairing different luciferase genes with our two different promoters, we observed very little luciferase expression from the pdsAAV-CB-CytoGLuc construct demonstrating that the CytoGLuc is not functional in the nucleus. The pdsAAV-T7-Gluc construct verified restriction of the T7 promoter to the cytoplasm when paired with a functional GLuc gene. Our results indicate that joining the cytoGLuc gene with the cytoplasmic T7 promoter **(Fig 7)** makes for an efficient cytoplasmic reporter cassette. The combination of the nuclear cassette (CB-CL) with the

cytoplasmic cassette (T7-CytoGLuc) is the first vector in AAV biology that allows for the detection of AAV in two different cellular compartments.

The second goal of this project was to screen, using the dual reporter system, compounds for their ability to enhance rAAV vector transduction by stabilizing AAV genome as the virus traffics through the cell. After establishing the novel dual reporter system, we examined the effects of different molecules or drugs on the cellular trafficking of AAV from the cytoplasm to the nucleus. The reporter system gave us insight on the trafficking patterns of the virus during a critical step in transduction: movement in the cytoplasm. We demonstrated how useful this dual reporter vector system is as a tool in screening drugs to observe their effect on rAAV trafficking by discovering a new class of rAAV enhancing drugs, antiviral drugs. Results from the dual reporter system indicated that CDV enhanced AAV transduction in both the cytoplasm and nucleus **(Fig 38)**. This result allows us to demonstrate a link between the AAV status in the cytoplasm and the nucleus. We hypothesize that the AAV level in the cytoplasm could be a predictor for nuclear transduction.

The intracellular trafficking of AAV is very inefficient and leads to a large amount of vector being degraded thus leading to a decrease in overall transduction. The mechanisms that cause AAV degradation as it traffics are not fully understood and remain to be elucidated. The combined use of rAAV vectors with a drug may rescue some vectors from degradation, thus enhancing overall vector transduction. One of the major rate-limiting steps in rAAV-mediated gene expression is the movement of the virus in the endosomal compartments from the cytoplasm into the nucleus (4) (13). The results from the luciferase assays and genomic Southern blot analyses specify that CDV treatment may

increase the amount of AAV not only in the nucleus, but also in the cytoplasm **(Fig 38, Fig 39, and Fig 40)**.

Using multiple AAV reporter vectors, this project demonstrated that CDV can consistently enhance rAAV vectors *in vitro* **(Fig 14- Fig 17)**. Also the observation that CDV pretreatment enhanced two different serotypes, AAV2 and AAV8, indicates that this drug does not affect the receptors mediating the entry of the virus into the cell, since both serotypes were enhanced **(Fig 12 & Fig 13)**. However, further studies with other AAV sero-types should be performed to strengthen that CDV enhances AAV in a sero-type independent manner. Southern blot analysis of cells pretreated with CDV and then infected with rAAV revealed no difference in the amount of vector present between 0 and 2 hours post-infection, again proposing that CDV does not enhance viral entry **(Fig 25)**. These results indicate that CDV enhances rAAV transduction downstream of viral entry. The experiment carried out to determine if CDV pretreatment is necessary for rAAV enhancement shows that CDV could potentially create a favorable cellular trafficking environment for the vector because the drug must be given before AAV infection and not after **(Fig 19)**. Using Ingenuity software analysis, we choose to focus on upregulation in endosomal genes, because we observed the enhancement in the cytoplasmic compartment using the dual reporter system. The correlation of an increase in endosomal genes as well as the increase in AAV in the cytoplasm after CDV pretreatment allows us to speculate that CDV provides AAV with a more suitable trafficking environment with less endosomal degradation. This favorable trafficking environment leads to an increase in overall transduction.

Multiple papers have identified CDV as a drug that can interfere with DNA synthesis most likely by promoting the stalling of the DNA replication fork (69) (71) (72). The incorporation of CDV into cellular DNA activates DNA-damage response pathways due to an increase in DNA breaks (69) (71) (76). It is widely known in the field of AAV biology that induction of DNA damage causes an increase in AAV transduction (57) (66). We investigated if the increase in transgene expression in CDV-treated versus PBS-treated cells observed could be explained by the conversion of ssDNA to dsDNA due to the activation of DNA damage pathways (27) (66). Our results obtained in the experiments with scAAV vectors also demonstrated no enhancement, thus suggesting that CDV could enhance AAV's conversion to the double-strand form, since no enhancement was observed with the double-stranded scAAV vectors **(Fig 26 & Fig 27)**. Considering these scAAV results, we cannot rule out the possibility of CDV enhancing AAV transduction at more than one step during infection since the enhancement effect was observed with only rAAV and not scAAV. This result also allows us to speculate that rAAV and scAAV vectors differ in their trafficking patterns or cellular processing in the cytoplasm. We did observe monomer and dimer formation with CDV treatment compared to PBS **(Fig 22 & Fig 23)** and we speculate that this is due to CDV increasing the overall amount of AAV in the cell thus allowing more AAV to become double-stranded.

CDV has also been attributed to accumulation of cells in S-phase as well as induction of p53 both of which have been associated with increasing rAAV transduction *in vitro* (71) (76) (77) (89). The MRN complex proteins, Mre11, Rad50, and Nbs1, play an important role in the initial processing of double-strand DNA breaks prior to repair by homologous recombination or non-homologous end joining (57) (66) (90). It has been

suggested in AAV literature that these proteins may play a role in aiding AAV in conversion from a single strand to a double strand capable of expressing an encoded gene (57). We choose to focus on these important molecules during our transcriptome analysis and did not find an upregulation in genes related to DNA damage response or p53 with our transcriptome analysis of CDV treated cells proposing CDV does not enhance through these DNA damage pathways **(Fig 31)**. Of interest, ATRIP binding to single-stranded DNA coated with replication protein A was slightly upregulated. Taking into consideration that AAV is a single-stranded virus, the investigation if ATRIP could play a role in CDV enhancement of rAAV vector was undertaken **(Fig 32)**. There were no observed differences between siRNA ATRIP treatments compared to the treatment with a control plasmid. We speculated that ATRIP may not play a major role in CDV enhancement of rAAV transgene expression or that ATRIP exerts an additive effect with other molecules to enhance AAV. Further investigation would be needed to test these hypotheses. We choose to concentrate on other data from the transcriptome analysis like endosomal genes and viral exit genes since these pathways were found within the cytoplasm and the project's scope was concentrated within the cytoplasmic compartment.

There have been multiple studies investigating the effect of various DNA synthesis inhibitors, Hydroxyurea, Etoposide, and aphidocholin, on rAAV vector transduction (38) (52) (53). To evaluate CDV enchantment of rAAV vectors, studies comparing CDV with known rAAV enhancing drugs were performed. Our results from the combined treatment of MG132 and CDV demonstrated that at the highest concentration of both drugs there was a statistically significant increase in AAV expression compared to each of the highest drug concentrations alone **(Fig 28)**. It has been speculated that MG132 aids in AAV

accumulation in the nucleus by blocking the proteolytic activity of the 26S proteasome complex thus allowing less virus to get degraded. It is known that HU can enhance transduction with rAAV vectors through multiple pathways for example through an increase in nuclear translocation as well as by aiding in conversion of ssAAV to the transcriptionally active dsAAV form (52) (53) (57) (68). We choose to direct our transcriptome analysis of cells pretreated with CDV or Mock treatment to common pathways or genes previously known to be associated with AAV transduction in the literature. In this study we concentrated on pathways that have already been associated with AAV transduction like DNA sensing and DNA damage, endosomal formation, and viral trafficking. We speculated that these pathways may be more related to CDV enhancement of AAV because of the association of CDV incorporating itself into DNA as well as the trafficking results we observed using our dual reporter system. This study did not find upregulation in any genes related to DNA damage response or p53 in CDV treated cells indicating that CDV does not enhance AAV transduction through these pathways **(Fig 31)**. Taken together, we speculate that CDV enhances AAV transduction through a pathway similar to that utilized by HU in the context of increasing trafficking of the virus, but not via DNA damage pathways, since we did not observe a marked increase in any of the important factors within these DNA pathways (68).

Another known rAAV enhancing drug we compared to CDV was ETO. ETO is an inhibitor of DNA topoisomerase II. Combination of both drugs seemed to slightly enhance transgene expression compared to each drug alone suggesting that the two drugs act through separate pathways to enhance rAAV vectors **(Fig 29)**. It has been speculated that ETO enhances rAAV by aiding in conversion of second-strand synthesis (58). Referring

again to the transcriptome analysis of cells pretreated with CDV or PBS, there was not an upregulation of DNA damage genes reinforcing the notion that CDV and ETO enhance AAV through alternative pathways **(Fig 31)**.

A large majority of these previously listed drugs enhance rAAV transduction *in vitro*; however, the improved transduction efficiencies seen in cells is not observed in animals. CDV is a better alternative drug choice compared to already known rAAV enhancing drugs because it is already approved by the FDA and be used in clinical trials (69) (74) (77). CDV displays less toxicity compared to other rAAV enhancing drugs like hydroxyurea or etoposide (69) (73). CDV does exhibit renal toxicity. The nephrotoxicity associated with CDV is facilitated by a faster uptake of the molecule at the basolateral membrane of the kidney proximal tubular cells than the efflux of CDV into the urine (75). This effect causes an accumulation of CDV in the renal tubular cells (75). The accumulation in the renal tubular cells can be prevented by co-administration of probenecid, intravenous hydration, and an infrequent treatment schedule of CDV. Probenecid is an inhibitor of anion transport that interferes with the transporter-mediated tubular uptake of CDV and is concurrently given to patients with CDV treatment (75).

Proteasome inhibitors (PI) have been shown in the literature to increase AAV infection efficiency in a cell-type and serotype-specific manner. Although the exact mechanism of rAAV enhancement by PIs remains unknown, it has been postulated that PIs aid in one or more of the following events: blocking degradation of capsids in the cytoplasm, blocking degradation of capsids in the nucleus, or indirectly improving AAV genome stability (14) (63) (64). A major drawback to using PIs is toxicity and multiple administrations required. The PI, bortezomib, is generally given clinically in several

courses, each treatment consisting of 2 weeks of twice-weekly administrations. The side effects of this PI can be very severe to the patient and are peripheral neuropathy, cytopenias, thrombocytopenia, neutropenia (18) (22) (23). This demonstrates again that CDV is a much safer alternative when compared to drugs already in clinical trials, such as bortezomib. In humans, CDV is administered as a 1 hour intravenous infusion with saline hydration and concomitant oral probenecid to ameliorate the renal toxicity. This treatment regimen is a less toxic and infrequent treatment option than those typical for the current rAAV-enhancing drugs like proteasome inhibitors (69) (74). Another alternative to circumvent the renal toxicity issue of CDV is to use its derivatives like alkoxyalkyl esters of cidofovir or brincidofovir (91). These derivatives do not have the associated renal toxicity and can be given orally (91). Esterification of CDV with an alkoxyalkyl group enables drug adsorption in the gastrointestinal tract, a valuable feature for future patient treatment (75). Also, modified esters of CDV are in development that could potentially have enhancement profiles for AAV similar to that of brincidofovir; however, further testing of these compounds within *in vitro* and *in vivo* settings with AAV would be needed to validate this speculation. CDV metabolites have long half-lives ranging from 15 to 65 hours, thus allowing for the long lasting effects of this drug, which can been seen in our *in vitro* and *in vivo* results with CDV **(Fig 18 and Fig 33).** Enhancement of rAAV transgene levels compared to mock was observed 72 hours post-infection **(Fig 18).** In preliminary animal studies, an increase in hLC levels induced by CDV at a dose of 30mg/kg as compared to PBS was detected as long as 14 weeks post vector delivery **(Fig 33)**. The highest dose given in clinical trials with CDV was 10mg/kg (68) (73); although a study in rats did use a dosage of 100mg/kg suggesting higher doses can be used in animal models

(92). Studies optimizing the drug regimen and determining if a lower dose can be administered with enhancement effects still maintained is an area of investigation (92). Previous studies have cited CDV as a carcinogen in rats, and this is important to consider for dosing and overall outcome for future animal experiments (73) (74) (92). It is a very interesting concept using a marketed antiviral drug to enhance a virus' infection. Although CDV is known to inhibit large DNA viruses possessing polymerases, in the context of this project it has been established that CDV can enhance AAV, a small DNA virus lacking a polymerase. This is the first time the class of antiviral drugs to which both CDV and GCV belong is shown to enhance AAV vector transduction. CDV is an optimal drug choice to be given in conjunction with AAV, because we show the enhancement of both *in vitro* and *in vivo* expression of AAV **(Fig 12, Fig 13, & Fig 33)**.

This project demonstrated that CDV, a marketed antiviral drug, enhances rAAV transduction. We investigated if another marketed antiviral drugs that belong to the same class as CDV can enhance rAAV transduction. The effect of GCV on rAAV transduction was examined. Our studies found that there is also an increase in transgene expression *in vitro* with GCV treatment similar to CDV **(Fig 34, Fig 35, & Fig 37)**. Our data proposes that these two antiviral drugs, CDV and GCV, may enhance AAV transduction through a common mechanism since an increase in AAV transgene levels was seen with both drugs using rAAV vectors. Unlike CDV, GCV needs to be phosphorylated by viral enzymes, not cellular ones (79). This information allows us to speculate that the mechanism of enhancement observed by these antiviral drugs is due to a function other than inhibiting DNA incorporation. This notion is consistent with the idea that CDV and GCV have alternative drug uses other than their previously known viral inhibition. Our results

demonstrate that both GCV and CDV enhance AAV transduction. However, in order to confirm if both drugs function in a similar way for enhancing AAV further *in vitro* and *in vivo* testing would be required. It would be interesting to see if GCV can also enhance rAAV transgene expression in animals as well as to determine if GCV and CDV enhance similar pathways like endosomal processing and cellular movement genes.

Using RNA sequencing and Ingenuity software analysis of cells pretreated with CDV, we focused our attention on several different clusters of genes upregulated **(Fig 42 & Fig 43)**. Noradrenaline and adrenaline degradation pathways were upregulated **(Fig 42)**. These pathways are involved in the body's central response to the stress reaction. Another pathway upregulated was the Formaldehyde oxidation II (glutathione-dependent) which is an important necessity for most life forms, since organisms require detoxification of the highly toxic formaldehyde **(Fig 42)**. The RNA sequencing results show an upregulations in many pathways. Due to multiple previous AAV studies linking the vector with endosomal processing and cellular movement genes, we focused our attention on those pathways for understanding CDV enhancement of rAAV transduction in the cytoplasm.

The RNA sequencing results show an upregulation in several Annexin A2 oligoes as well as other genes involved in endosomal formation and acidification reinforcing the findings from our dual reporter system in the cytoplasm **(Fig 44)**. Sanlioglu *et al* demonstrated in an earlier study that inhibiting rAAV endocytosis reduces rAAV transgene expression (25). The study also found that cytoskeletal movement elements like actin are important for trafficking of the AAV into the nucleus. Sonntag *et al* also confirmed the necessity of endosomal processing of rAAV vectors by showing that AAV injected into the cytoplasm of a cell or nucleus results in decreased transduction levels (40). Again our

RNA sequencing results support these findings by demonstrating an upregulation in several genes involved in endosomal formation and acidification along with cellular movement actin genes (ACTA1 and ACTA2) **(Fig 44)**.

Two different Ingenuity software analyses showed an upregulation in cellular movement pathways allowing us to speculate that CDV enhances AAV transduction at the cellular trafficking step. Also cellular assembly and organization was upregulated with CDV treatment compared to mock suggesting that these genes can contribute to CDV creating a favorable trafficking environment for rAAV infection **(Fig 43)**. Of note, both infectious diseases as well as cancer pathways were upregulated during CDV pretreatment. This is in agreement with previous data with CDV, since this drug is associated with antiviral activity as well as cancer in the literature **(Fig 43)**. Within the scope of this study we concentrated on endosomal and trafficking genes in the cytoplasm for CDV enhancement. Our study speculates that CDV mediated upregulation of endosomal processing genes and genes associated with viral exit, such as actin, contribute to the enhancement effect seen with AAV transduction. The genes we found upregulated are important for AAV trafficking and efficient transduction of the virus.

The understanding of AAV trafficking is an important area of research for improvement of AAV vectors. The importance of acidification in the endosomes during rAAV trafficking has been established (4) (13) (30) (37) (40) (41). An earlier paper by Bartelett *et al* has highlighted that AAV vectors escape from endosomes and that an acidic environment is needed for proper infection of this virus (30). It is generally believed that AAV2 escapes the early or late endosomes before it makes its way into the nucleus. The study utilized Bafilomycin A_1, a potent inhibitor of the vacuolar H^+-ATPase responsible

for acidification of endosomal vesicles. Bartelett's study found that bafilomycin A_1 blocked AAV infection of HeLa cells supporting the requirement of endosomal acidification and early endosomal escape for efficient AAV infection (30). The results from our study support Bartelett's findings **(Fig 41)**; in addition we demonstrate an important link of AAV levels between the cytoplasm and nucleus affecting overall transduction **(Fig 38- Fig 41)** (30). Examining the CDV data, we observed a positive correlation between AAV levels in the cytoplasm and AAV levels in the nucleus **(Fig 38- Fig 40).** CDV pretreatment both in the context of the dual luciferase reporter system as well as Southern blot analysis displayed a correlation between the genome status between these two compartments and the drug **(Fig 38- Fig 40)**. There were differences in the amount of AAV present in the between PBS and CDV at earlier time points **(Fig 40)**; however, we choose to focus on the differences at 24 hours post-infection due to similar results seen with the dual reporter system at 24 hours **(Fig 39)**. Bafilomycin A_1 in the context of assessing the relationship between the genome status of AAV in the cytoplasm and nuclear transduction was performed. The results obtained using this drug reinforced our idea that there is a direct link between AAV in the cytoplasm and nucleus since there was an observed decrease in both cytoplasmic and nuclear readings with drug treatment **(Fig 41)**. The CDV and Bafilomycin A_1 studies show a direct relationship between the cytoplasmic AAV status and nuclear transduction. The results from our studies lead us to speculate that the AAV status is the cytoplasm is a predictor of nuclear transduction. This speculation signifies the importance of AAV trafficking through the cytoplasm as well as how it can ultimately affect AAV vectors in clinical trials.

The theory that AAV's status in the cytoplasm is a predictor for nuclear transduction, is emphasized with ingenuity software analysis. We observed genes upregulated in the cytoplasm, such as those associated with endosomal formation and cellular movement. The results of experiments with bafilomycin also showed a negative correlation between AAV levels in the cytoplasm and in the nucleus. When AAV in the cytoplasm is disturbed using bafilomycin, there is a decrease in nuclear transduction. This reinforces the direct link between AAV present in the cytoplasm and nuclear transduction of the virus. There was also a minimal increase observed with HU treatment at 48 hours emphasizing direct correlation between the two compartments; however, it was not two-fold like CDV or as drastic difference as bafilomycin. Establishing a link between AAV status in the cytoplasm and nuclear transduction highlights how significant trafficking of rAAV is for efficient transduction. The expansion of this dual reporter system in further pursuing the relationship between AAV status in the cytoplasm and overall transduction efficiency may reveal more pathways important for efficient trafficking of AAV vectors.

In conclusion, this study has developed and implemented a novel dual reporter system to detect and screen the effect of different drugs on the cellular trafficking of AAV from the cytoplasm into the nucleus. Using our novel dual reporter system, a new class of rAAV enhancing drugs was identified. The ability of CDV to enhance rAAV transgene expression with two different serotypes demonstrates the utility of this drug in improving gene transfer for future studies. Our study may eventually lead the development of novel strategies that would further advance the use of AAV vectors to treat genetic disorders.

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