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E1B-19k as novel target against human adenovirus infections
Essay

Master's Thesis

Master of Science Medical Pharmaceutical Sciences
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Contents

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Abstract

Human adenoviruses are a diverse group of pathogens that cause a wide range of illnesses. One key aspect in the virus is the E1B-19k protein, which plays a crucial role in viral replication and host immune evasion. The protein does this by inhibiting apoptosis via binding to the pro-apoptotic factors BAK, BAX and BIK from the Bcl-2 family. Additionally, the protein presents caspase activation by interacting with dead domains (DDs) in TNFR1, Fas and TRAIL. Fas-mediated apoptosis involves FADD and procaspase 8; E1B-19k blocks this apoptotic pathway and thereby enhancing survival of the virus and replication in the host cells. A correct understanding of these mechanisms is crucial in the development of targeted antiviral strategies. This essay focuses on the role of E1B-19k in replication of human adenoviruses and its potential as a target for antiviral strategies. Current antivirals for human adenoviruses are discussed, together with proposals of novel strategies using E1B-19k as a target.

1 Introduction

1.1 Human Adenoviruses

The human adenoviruses, or Mastadenoviruses, (HAdVs) were first derived via isolation from human adenoids (Rowe et al., 1953). These viruses, which lack an envelope and are double-stranded DNA viruses, have been characterized as pathogens capable of infecting various human tissues and causing various diseases (Horwitz, 1990). Especially children, the elderly and individuals with a compromised immune system are susceptible to the disease. Currently, over 100 adenoviruses are known of which 51 serotypes are human adenoviruses which are further classified into seven species (*human adenovirus A to G*), however species F and G are often combined into F (Kajon et al., 2019; Wirth and Ylä-Herttuala, 2014). Classification is based on biological properties, DNA sequence homology, oncogenicity in rodents and tropism (Sharma et al., 2009; Burrell et al., 2016). Table 1 shows the different subgroups of the human adenovirus, together with their site of infection and oncogenic potentials (Roelvink et al., 1998; Ison, 2006).

Table 1: Summary of Species and Their Oncogenicity

Species	Site of infection	Oncogenicity in rodents
A	RT, UT, GIT	High
B	RT, eye, UT, GIT	Moderate
C	RT, UT, GIT	Low
D	Eye, GIT	Low
E	Eye, GIT	Low
F+G	GIT	Unknown

Abbreviations: RT: respiratory tract, UT: urinary tract, GIT: gastrointestinal tract

Respiratory diseases range from mild cold-like symptoms to severe pneumonia. Urinary tract infections mainly result in cystitis (Murphy et al., 1993; Klein et al., 2015). Gastrointestinal diseases include gastroenteritis, mainly in young children (Eifan et al., 2023). Conjunctivitis is a common and highly contagious indication of the infection in the ocular region (Hoffman, 2020). In rare cases, an infection with the human adenovirus leads to neurological diseases, such as encephalitis, acute disseminated encephalomyelitis (ADEM) and meningitis (Schwartz et al., 2019).
Besides the diseases that are associated with adenoviruses, they have gained significant attention in scientific research due to their potential as gene carriers in gene therapy and as vectors in cancer treatment (Breyer et al., 2001).

1.2 Antiviral drug development

The year 1963 marked a milestone in the antiviral drug discovery: the first drug for the treatment of viral infections, idoxuridine, was approved. To this day idoxuridine is still used in the treatment of keratitis, a form of herpes. Since the approval of idoxuridine in 1963, only 90 new antivirals have been approved, of which more than 30 are to fight AIDS, however effective treatments for a wide range of viral infections are still lacking (De Clercq and Li, 2016). Vaccines are proven to be effective in eradicating a significant amount of viral diseases, such as measles, polio and smallpox, however, for

most respiratory infections vaccines are not effective. There are two options for targets in the antiviral drug design: either by targeting the viral proteins (such as E1B-19k and gp120) or cellular proteins (such as CD4 and CCR5). Targeting viral proteins is usually more specific, but yields a higher risk of developing resistance. Targeting cellular proteins on the other hand, have a broader range, but could be more toxic (De Clercq, 2002). Designing antivirals is a multidisciplinary, difficult and costly process that will take years until the drug is approved and brought to the market. The process of drug discovery and development can be divided into five phases: two preclinical phases and 3 clinical phases. The preclinical phases consist of the drug discovery and the preclinical development. The clinical phase consists of phases I-III. Antiviral drug discovery starts with understanding the virus's life cycle and pathogenesis, followed by target identification for drug action. When the target is found, drug candidates are tested via high-throughput screening for interaction with the found target. Once the lead compound is identified, the compound is optimized to improve its efficacy, stability and potency and to reduce potential side effects. During the preclinical development, the pharmacokinetic properties of the compound are analyzed and the optimal dosage is determined in rodents and non-rodents. The preclinical phase consists mainly of short-term toxicology studies. The first clinical phase takes place in healthy male volunteers to test for drug safety, pharmacokinetics and side effects at different doses. The starting dose in mg/kg in human trials, according to the FDA, is calculated via $\frac{NOAEL}{safetyfactor \cdot converstionfactor}$. The safety factor is usually 10; the conversion factor is based on the body surface area and differs between species (FDA, 2005). In phase II the drug candidate is tested on a few hundred patients in a double-blind randomized controlled trial with a place to assess efficacy and dosage. In phase III the drug candidate is labeled and given out to up to 5.000 patients in a large-scale controlled clinical trial, which requires close cooperation and coordination with the Independent Ethics Committee and Institutional Review Board (FDA, 1996). When the drug is approved, the companies should monitor drug safety and usage in post-marketing surveillance to detect rare side effects, drug interactions and the use in a non-controlled environment.

2 Background

2.1 Structure of human adenoviruses

Although different subgroups of the human adenovirus exist, they all share similar structural characteristics. The complete infectious particle, the virion, is an icosahedral-shaped protein shell surrounding a protein core. The overall structure of this capsid is between 65 and 80 nm in diameter and is composed of 252 capsomeres (Boulanger et al., 1977). This capsid consists mainly of fibers (trimer), penton bases and hexons (Horne et al., 1959; van Oostrum and Burnett, 1985). These fibers, which are sticking out from the capsid, play a crucial role in the attachment of the virus to their host cell (Stasiak and Stehle, 2020). Figure 1 shows the structure of the Mastadenovirus. The pseudo triangular number (Pseudo T) of 25 indicates that each icosahedral asymmetric unit (encircled in red) consists of 25 proteins.

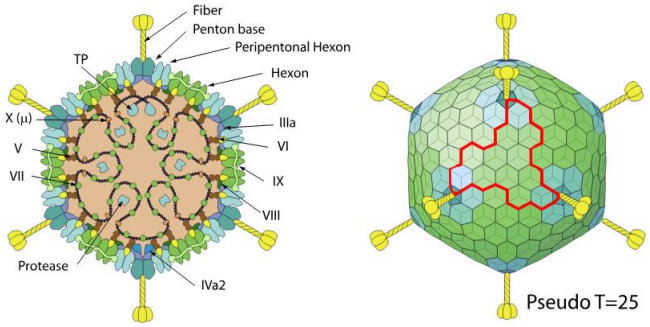

Figure 1: Structure of the Mastadenovirus. Adapted from (Hulo et al., 2011)

Figure 2 shows the crystalline structure of the adenovirus fiber with its key elements. The trimer is divided into the tail with the N-terminus where it extends from each penton base on the capsid of the virus, the shaft and the C-terminal knob via which the virus binds to the host cell. The fiber has two main well-understood functions, namely the interaction with the penton base and with the host cell receptor (Chroboczek et al., 1995). The fiber shaft is a rod-like structure essential for the effective binding of the virus to the host cells (Wu et al., 2003). The length of the shaft in human adenoviruses is related to the number of β-repeats and varies greatly between different serotypes with the shortest shaft consisting of 6 repeats (HAd3) and the longest having 21.5 repeats (HAd3) (Green et al., 1983; Signäs et al., 1985).

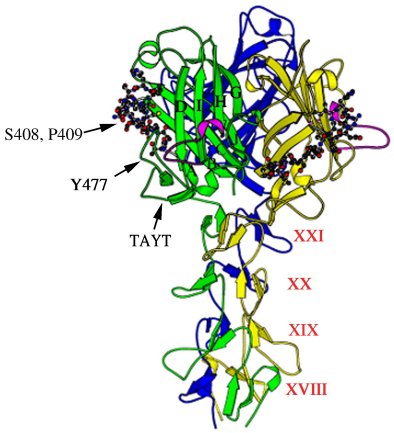

Figure 2: Crystal structure of HAdV2. Adapted from (NIC, 2005)

The hexon trimer consists of a triad of two similar β-barrels (Rux and Burnett, 2004). The penton base is a pentamer of protein III that contains the Arg-Gly-Asp (RGD) sequence that triggers endocytosis of the virus into the host cell (Zubieta et al., 2006).

2.2 Human adenovirus's genome and genes

The genome of the human adenovirus is a linear, double-stranded DNA with a length between 30 and 36 kbp with inverted terminal repetitions with both 5'-ends connected to a terminal protein (TP)

(LEP, 2008; Hoeben and Uil, 2013). The genome consists of six early units (E1A, E1B, E2A, E2B, E3, E4) and the delayed early units (IX, IVa2). These eight transcription units are dependent on RNA polymerase II for transcription. Also, the Major Late transcription unit is present on the genome and encodes for multiple proteins via alternate splicing and differential polyadenylation (Charman et al., 2019). Figure 3 shows the genome of the HAd2 with its genes, based on the time of their expression. On the left are the early and the intermediate genes, followed by the late genes (L1-L5) that are part of the Major Late transcription unit. Each gene has a distinct function in viral replication, such as assembly and generation of the surface proteins of the virion, genome replication and translation and host modulation. During the initial phases of infection, when the virus enters the nucleus of the host cell, the early genes are maximally expressed, indicating their role in orchestrating the first steps in viral replication. These first steps include the blocking of host cell immune responses to IFNs and stress-induced apoptosis (WOL, 1999).

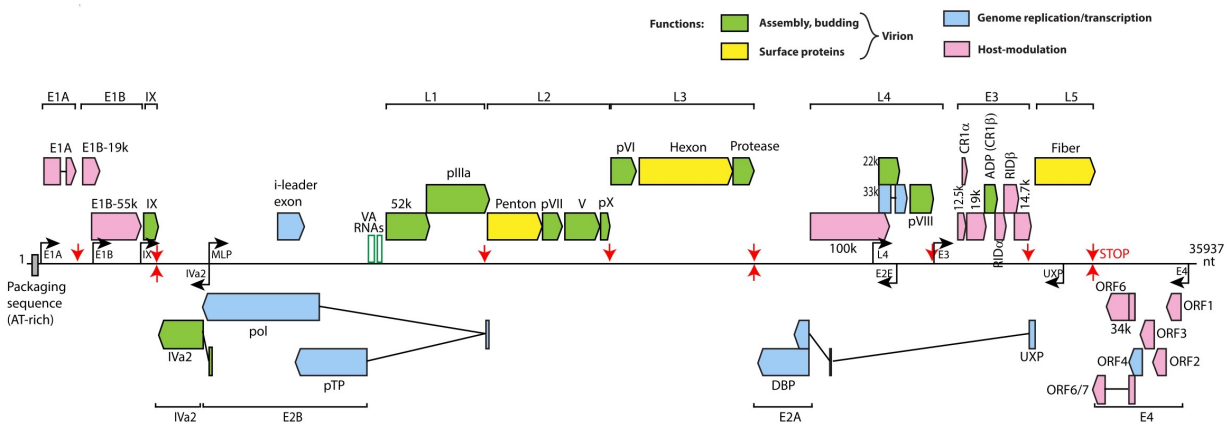

Figure 3: Human adenovirus C serotype 2 genome. Adapted from (Hulo et al., 2011)
The diagram shows the presence of the early, intermediate and late genes and the presence of the packaging sequence. The bent arrows indicate the presence of the promoters, the red arrows indicate the stop signs, the 35937nt at the end indicates the number of nucleotides/base pairs on the strand.

The intermediate genes IX and IVa2 are thought to play important roles in packaging the viral genome into the protein core. Studies have shown that IVa2 is involved in the encapsidation process, together with the L1 and L4 proteins, via binding specifically to the viral genome's packaging sequence (Christensen et al., 2008). The interaction with L4 is needed for genome packaging itself (Ahi et al., 2015), while the interaction with the L1 induces the ability of the genome to be encapsidated and virus replication (Perez-Romero et al., 2006). During the late phase of transcription, there is an increase in expression in the late transcriptional units L1 to L5. To these transcriptional units belong certain encapsidation and surface proteins, as seen in figure 3. The late phase starts approximately 12h after infection and its main function is synthesizing the gene products that are related to the formation and assembly of the capsid proteins (Crisostomo et al., 2019).

2.3 E1B-19k in adenoviruses

The gene of interest in our study is the E1B-19k, which is one of the early genes, contains 175 amino acids and is predominantly present in the nuclear envelope and lamina of the infected and transformed cells (Tarodi et al., 1993; Consortium, 2022). The 19k part refers to the molecular mass in Da. The

E1A region can immortalize cells on its own, although it needs the E1B region for a complete transformation (Tarodi et al., 1993). The E1B-19k is a functional analogue of the Bcl2 protein, which is a protein that regulates apoptosis of cells via either inducing or inhibiting apoptosis by interacting with the tumor suppressor gene p53 (Levine and Oren, 2009).

The E1A unit can induce apoptosis via the degradation of MCL-1 (anti-apoptotic protein), which causes the release of BAK (pro-apoptotic protein from the BAK-MCL-1 complex), but its effect is counteracted by the E1B-19K protein (Boyd et al., 1994; Cuconati et al., 2003). Figure 4 shows the pathway via which E1B-19k counteracts the E1A-induced apoptosis.

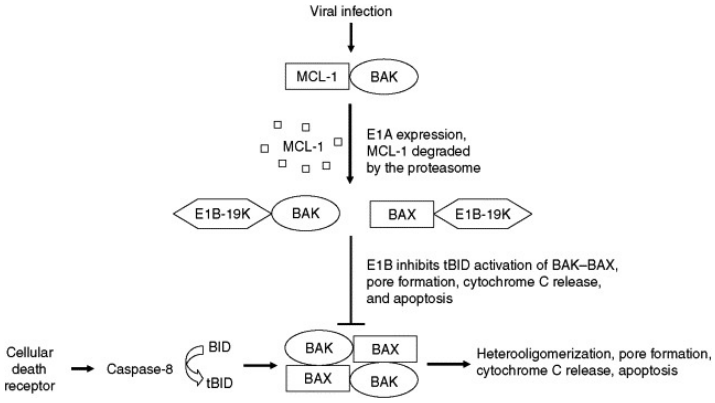

Figure 4: Apoptotic pathway of E1B-19k. From (Turnell, 2008)

E1B-19k counteracts the E1A-induced apoptosis via binding to the BAK and BAX to block the death cell receptor (Cuconati et al., 2002).

2.4 Replication

Human adenovirus replication is an efficient process that produces one million copies of its viral DNA within 40h (Hoeben and Uil, 2013). The coxsackievirus and adenovirus receptor (CAR) are present in different mammalian tissues and epithelial cells. On the epithelial cells, the receptor acts as a cell adhesion molecule (CAM) (Wehbi et al., 2020). Upon entry of the adenovirus into the nucleus of the host cell, the DNA replication of the virus is initiated. First, the early genes are expressed that are responsible for producing components for the viral DNA replication system. This machinery is assembled around the genomes, leading to replication. Replication is initiated at position 4 of the DNA template where a trinucleotide sequence is formed by attaching a pre-TP (explained later) to the DNA to create an intermediate called pTP-CAT (King and Van der Vliet, 1994). As viral replication advances, the initial complexes break down, leading to an increased polymerization rate. Signals for this breakdown are the dissociation of NFI and Oct-1 (see 2.4.1). During packaging of the genome inside the preformed capsids, the virus uses a motor protein that is driven by ATP hydrolysis. This motor protein is able to effectively compress the genetic material into the limited space of the capsid during the genome packaging stage (Sun et al., 2010). When these new virions are formed in the nucleus, they are released from the cell through lysis.

2.4.1 Early genes

The early genes encode for the DNA replication machinery that consists, among others, of the preterminal protein (pTP) and DNA polymerase (Pol) which are both encoded by E2B and the DNA-binding protein (DBP) that is encoded by E2A. The DNA replication pre-initiation complex consists of a heterodimer of pTP and Pol, and DBP. pTP contains the amino acid Ser580, which is essential for the covalently binding of dCMP by Pol (Smart and Stillman, 1982) which acts as the first nucleotide in the newly formed DNA strands. Also, host proteins are necessary for initiating replication: NFI and Oct-1. NFI and Oct-1 both increase the stability of the pre-initiation complex by binding as a dimer to a recognition sequence 5'-TGGATTGAAGCCAA-3' and an octamer motif next to the NF1 site, respectively (Mul et al., 1990; Mul and Van der Vliet, 1992; Van Leeuwen et al., 1997).

2.4.2 Intermediate genes

IX and IVa2 are proteins that play a role in the assembly of the virion, but are also essential in facilitating the transcription of the late genes. The sequence coding for the protein IX is located between the early genes E1B open reading frame and its polyadenylation sequence (Parks, 2005). Previous studies have demonstrated the role of IX in thermally stabilizing the virion (Colby and Shenk, 1981; Dmitriev et al., 2002; Vellinga et al., 2005) and its role in transcriptional activity through its C-terminal leucine repeats (Rosa-Calatrava et al., 2001). The intermediate protein IVa2 is strongly associated with the packaging of the genome. Although the viral protein attaches to several A repeats within the genome, previous studies have shown a stronger affinity of the protein to the sequences that are related to packaging (Zhang and Imperiale, 2003). The protein initiates genome recognition and recruitment of the late 22k protein to the packaging genome by being the first factor to bind to this packaging sequence (Ostapchuk et al., 2005).

2.4.3 Late genes

The late genes consist mainly of the MLTU and are expressed after the onset of replication. The late genes encode for the proteins that play a role in the assembly and encapsidation of the viral genome (Babich et al., 1980). The primary transcript of the MLTU can become polyadenylated at 5 different positions, upon which five different mRNA families (L1-L5) can be produced. Using alternative splicing the MLTU can produce at least 20 different mRNAs (Biasiotto and Akusjärvi, 2015). As seen in figure 3, the MLTU contains 5 different L-families with each their own set of proteins that are involved in the synthesis of structural proteins: fibers, hexons, penton bases and the stabilizing and assembling proteins IIIa, VI, V and VII.

2.5 Interactions between E1B-19k and host cell proteins

The adenovirus E1B-19k protein has been identified as a factor in the modulation of certain mechanisms within the host cell, including altering the host cell's immune responses and its replication. Understanding these interactions is crucial in the development of therapeutic strategies, such as in identifying targets for therapeutic intervention, modulating the immune responses of the host cell against the virus and in prevention of the virus-induced diseases.

2.5.1 Evasion of host apoptosis

Viral replication is essential in the proliferation of viruses. For DNA viruses with a large and complex genome, host cell apoptosis before or during the viral replication is unwanted, causing the virus to come up with mechanisms to inhibit host cell apoptosis. As mentioned in section 2.3, the human adenovirus inhibits host cell apoptosis by interacting with the Bcl-2 family. The E1b-19k protein has two known mechanisms via which it can inhibit apoptosis: via binding to pro-apoptotic factors from the Bcl-2 family and thereby inhibiting apoptosis in the host cell and via inhibiting caspase activation (White, 1998). Due to the homology between E1B-19k and Bcl-2 (both containing the BH1 and BH3 domain (Chiou et al., 1994)) it allows for pro-apoptotic proteins that were first thought to only bind to specific sequences of the Bcl-2 also bind to these homologous sequences of E1B-19k (Han et al., 1996). These pro-apoptotic proteins are BAX, BAK and BIK. BAX and BAK are activated upon receiving an apoptotic stimulus, which causes them to oligomerize

at the outer membrane of mitochondria, promoting permeabilization of the membrane which allows the release of pro-apoptotic factors that activate the caspase cascade (Wold et al., 1999a; Peña-Blanco and García-Sáez, 2018). This activation leads to cell death within minutes (Tait and Green, 2010). Figure 5 is a visual representation of how inhibiting BAX and BAK on the outer membrane of the mitochondrion leads to an inhibited release of cytochrome C (Cyt C), which inhibits the activation of caspase. BIK only has the BH3 domain, in contrast to the Bcl-2 homologues that also contain the BH1 domain. BIK on its own is not able to induce apoptosis; the protein needs active BAX to induce cell death. In cells that lack BAX, but BAK is present, apoptosis cannot be induced upon introducing BIK (Gillissen et al., 2003).

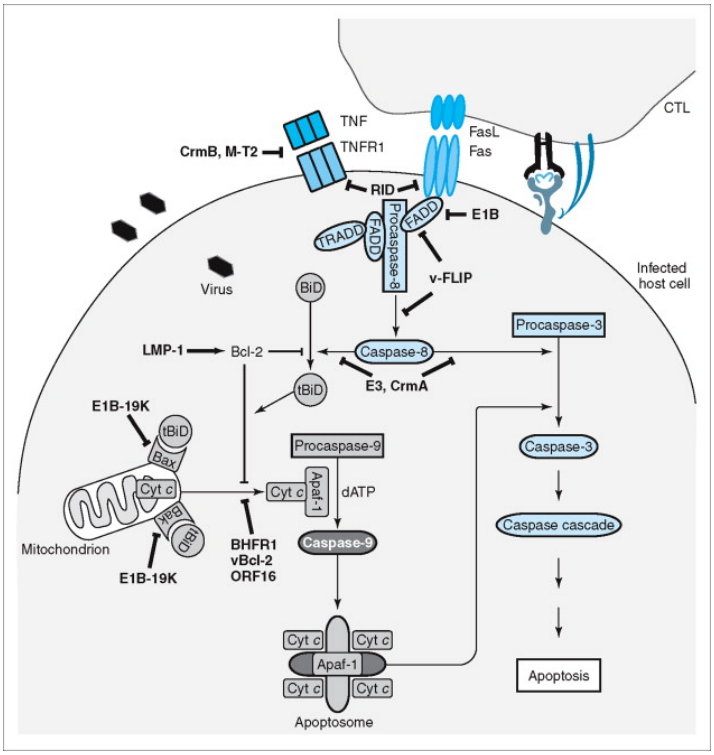

Figure 5: Viral evasion of host cell apoptosis. From (Mak and Saunders, 2006).

Overview of the apoptotic pathways in cells. E1B-19k can evade host cell apoptosis via blocking the release of cytochrome c and via preventing caspase activation by blocking the formation of procaspase 8

Another way E1B-19k can inhibit apoptosis of the host cell is via preventing caspase from becoming activated by disrupting the interaction between caspases and adaptor proteins (White, 1998). The E1B-19k protein targets death domains (DDs) in TNFR1, Fas and TRAIL that are present in most cells. The ligands for these domains are present on and secreted by cytotoxic T lymphocytes (CTLs) and monocytes (Mariani and Krammer, 1998). Upon binding of the ligand with the DD, the receptors oligomerize, together with proteins, form the death-inducing signaling complex (DICS), which is essential in Fas-mediated apoptosis (Ashkenazi and Dixit, 1998; Li et al., 2013). Fas connects with the Fas-associated death domain (FADD) through the DD. FADD consists of a death effector domain (DED) that interacts with procaspase 8, which can initiate an apoptotic signal (Wold et al., 1999b). This mechanism is also shown on figure 5.

3 Antivirals against human adenovirus

The search for more effective antivirals has been an ongoing quest in the medical and scientific community. Human adenoviruses are capable of infecting different parts of the human body: from the eyes and the airways to the intestines and nervous system. Despite adenovirus being a common infection, the treatment options are limited. For the majority of individuals, an infection with the adenovirus is not life-threatening, but for immunocompromised patients, this infection could be life-threatening (Liu et al., 2023). Most treatment options are supportive and are based on relieving the symptoms and supporting the body's immune response. However, for immunocompromised individuals, such as the elderly and those who underwent organ transplantations, there is a need for specific antiviral therapies.

3.1 Current antiviral agents

Currently, there are no drugs on the market that are specific for adenoviruses. However, there are two antivirals on the market that have been shown to be effective in human adenoviruses: cidofovir and ribavirin. Cidofovir is nowadays primarily used in the treatment of cytomegalovirus. Ribavirin is mainly used as a treatment for human orthopneumovirus, hemorrhagic fevers and hepatitis C, although ribavirin cannot be used as a monotherapy in chronic hepatitis C infections (Zydus Pharmaceuticals, 2022).

3.1.1 Cidofovir

Cidofovir (figure 6) was originally used as a treatment for cytomegalovirus retinitis infections in AIDS patients, although it can also be used as a treatment for forms of the human papillomavirus, polyomavirus, herpes simplex and the poxvirus (Lea and Bryson, 1996). It is a monophosphate analogue of the nucleotide cytosine and inhibits viral DNA synthesis (Dodge et al., 2021). However, its use is limited due to the nephrotoxicity (Lalezari, 1997). Cidofovir is thought to act through two different mechanisms: by chain termination of the viral strand and by inhibiting viral DNA polymerase (Clercq and Neyts, 2009b; Chamberlain et al., 2019). Unlike most antivirals, cidofovir does not depend on the action of viral kinases to become phosphorylated in order to become active. Also, cidofovir has shown resistance to phosphatases, which is of great importance in its antiviral activity, since compounds need to stay phosphorylated to remain effective against viruses (Xiong et al., 1996).

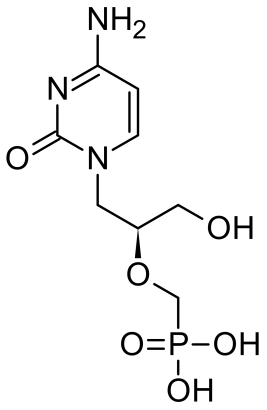

Figure 6: Structure of Cidofovir

The first mode via which cidofovir has its antiviral effect is via chain termination. After the intracellular release of the compound, it will compete with the natural building blocks of the host cell's DNA in order to incorporate itself into the replicating viral DNA strand. Cidofovir has certain active metabolites (PMEApp and PMPApp) that act as the chain terminators (Clercq and Neyts, 2009a). The active metabolite mimics dCTP, which is a building block of DNA. Viral DNA polymerase incorporates the active metabolite into the chain. However, the incorporation of only one active metabolite is not sufficient for an effective termination of the elongation of the viral DNA strand (Xiong et al., 1997). Figure 7 illustrates the antiviral mechanism of cidofovir, depicting how the compound interferes with the replication processes of viruses. Since cidofovir has shown some nephrotoxicity, cidofovir must

be administered together with intravenous saline hydration and probenecid to reduce the severity of nephrotoxic events and to reduce the renal tubular secretion of the compound respectively (Lea and Bryson, 1996).

The second mechanism via which cidofovir has its antiviral effects is via directly inhibiting DNA polymerase, independent from the incorporation of cidofovir's metabolites on the replicating strand. Little is known about this mode of action of directly inhibiting DNA polymerase using cidofovir. However, it is expected that the active metabolite of cidofovir itself binds to the DNA polymerase, most likely to the dNTP site as a competitive inhibitor of dNTP: a substrate for DNA synthesis (Chamberlain et al., 2019; Liu and Großhans, 2019).

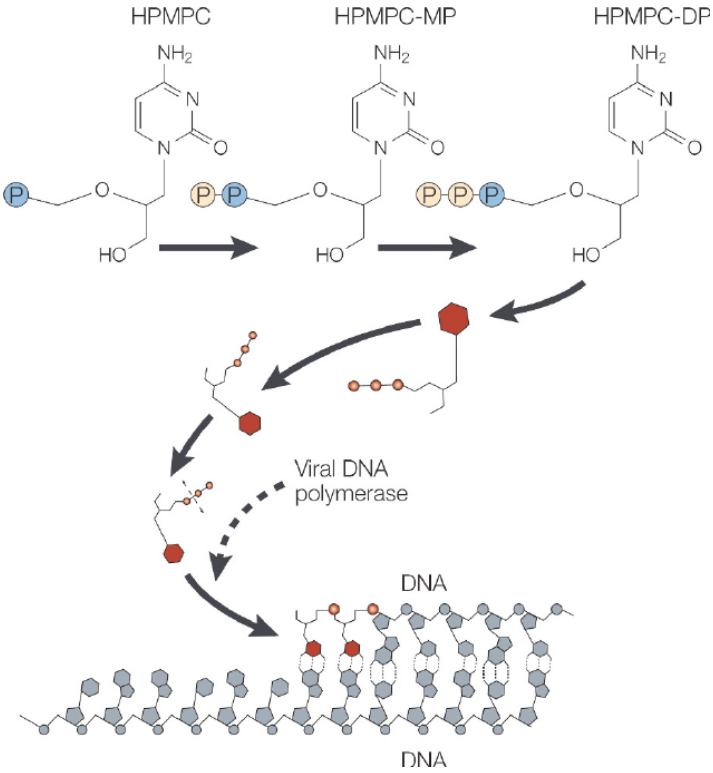

Figure 7: Cidofovir's antiviral mechanism of action. From (Clercq and Neyts, 2009a).
After phosphorylation of the compound, two units of cidofovir's active metabolite incorporate themselves into the replicating viral DNA strand, acting as a chain terminator to block further replication of the viral DNA strand.

3.1.2 Ribavirin

The second antiviral agent used in the treatment of adenoviruses is ribavirin (figure 8). Ribavirin was first synthesized in 1972 by Sidwell and colleagues and was active against 16 different DNA and RNA viruses *in vitro* (Sidwell et al., 1972). Differently from cidofovir, ribavirin is mainly used as a treatment for RSV infections, Lassa fever and the Hantavirus. Together with other antivirals, it can be used in the treatment of hepatitis C. Ribavirin was found to be a guanosine analogue through X-ray crystallography (Prusiner and Sundaralingam, 1973) and is thought to exert its antiviral effects via several direct and indirect mechanisms.

The direct mechanisms include the inhibition of viral polymerases, the suppression of RNA capping activity and by elevating mutation rates through the integration of ribavirin into the newly synthesized genomes. The indirect mechanisms include an immunomodulating effect that maintains the antiviral Th1 response and the reduction of GTP levels via inhibition of inosine monophosphate dehydrogenase (IMPDH) (Graci and Cameron, 2006). The latter mechanism is thought to be of importance in the antiviral properties of the drug, since is indirectly inhibits RNA and DNA synthesis. Ribavarin converts inosine monophosphate into xanthosine monophosphate, which is eventually metabolized into GTP and dGTP. These two molecules serve as precursors in the synthesis of RNA and DNA. GTP also plays an important role in energy storage, in translation itself by ribosomes and glycoproteins and in cell signaling (PAR, 1995; Graci and Cameron, 2006). The second indirect antiviral mechanism of ribavirin is through phenotype switching of Th1 to Th2 (Hultgren et al., 1998), Th1 responses are mainly associated with IL-2, gamma-interferon and TNFα expression. Th2 responses enhance humoral immu-

nity and are characterized by the expression of IL-4, IL-5 and IL10 (Mosmann and Coffman, 1989). Ribavirin can directly inhibit replication via interfering with viral polymerases. Ribavirin's primary metabolite is RTP, which interacts with viral polymerases and thereby inhibits nucleic acid synthesis (Graci and Cameron, 2006). RTP can be incorporated into the viral DNA to form a template that pairs with equal efficiency to cytidine triphosphate and uridine triphosphate. This leads to an increase in G→A and A→G transitions within the viral genetic material and thereby enhancing gene mutations (Te et al., 2007). The last proposed mechanism of ribavirin's antiviral mechanism is the posttranslational capping at the 5' end of the viral mRNA. This process involved the addition of a guanine pyrophosphate cap to the 5' end of the translated mRNA by the enzyme mRNA guanylyltransferase. This cap is crucial for protecting the mRNA from being broken down by enzymes in the host and for facilitating its translation. When the guanosine is substituted with ribavirin at this 5' end, the molecule will interfere with the methylation phase and thereby disrupting mRNA translation (Te et al., 2007). Ribavirin will thus block the RNA capping at the 5' end.

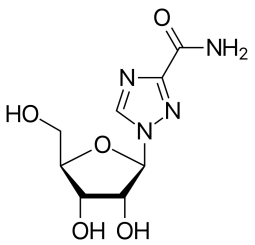

Figure 8: Structure of ribavirin.

4 Future research

4.1 Novel treatment options targeting E1B-19k

As mentioned earlier, the E1B-19k protein plays a crucial role in adenovirus pathogenesis by primarily inhibiting apoptosis in host cells, allowing the virus to replicate without being interrupted by the host cell's antiviral mechanisms. By targeting the E1B-19k protein, the apoptotic pathway could be restored, leading to a reduction in viral replication and elimination of infected cells. Also, targeting solely E1B-19k, offers a certain degree of specificity in antiviral treatments, since treatment options targeting this protein would mainly affect the infected cells and thereby sparing the non-infected cells. This specificity leads to a reduction of side effects. Currently. there are no specific treatment options that directly target the E1B-19k gene. However, using known techniques such as antisense oligonucleotides, CRISPR/Cas9 gene editing, RNA interference and zinc finger nucleases could offer pathways for specifically targeting the E1B-19k gene.

4.1.1 Antisense oligonucleotides

Antisense oligonucleotides (ASOs, figure 10) are short-stranded ($\pm$ 20 nucleotides) deoxyribonucleotide analogues that bind to its complementary mRNA (Chan et al., 2006). The length of the ASOs are crucial: it must be long enough to ensure specificity to its target mRNA and to form a stable DNA/RNA hybrid. However, increasing the length of the strand increases the probability of non-specific binding to other sequences, leading to unintended effects (Spurgers et al., 2008). The main mechanism of action involves specific base-pairing with the target mRNA, leading to RNA degradation by RNase H1 (Cerritelli and Crouch, 2009). This RNase H1 recognizes and hydrolyses the RNA/DNA duplex that is formed by the ASO and the target mRNA (Dias and Stein, 2002). This results in a decrease in protein levels in the cell.

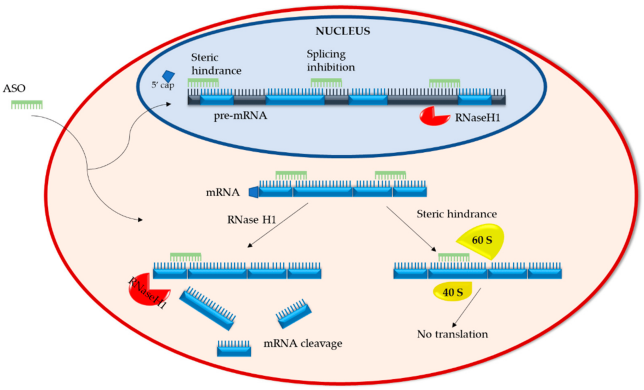

Figure 9: Mechanisms of action of antisense oligonucleotide. From (Gagliardi and Ashizawa, 2021).
ASOs interfere with mRNA in two ways: via (i) facilitating mRNA cleavage via RNase H and (ii) via obstructing key processing events of the translation initiation complex.

In addition to inducing RNA degradation, ASOs can also inhibit translation via steric hindrance (Dias et al., 1999). Certain ASOs operate by binding to the target RNA, leading to an inhibition in translation, rather rather than inducing degradation. This arrest in translation is achieved by preventing the target mRNA from binding to the 40S and the 60S ribosomal subunits (Bennett and Swayze, 2010). These ASOs bind to the translation initiation region of the target mRNA.

However, the use of ASOs is not without difficulties. The main issue with the use of the ASOs *in vivo* is the instability due to rapid degradation by nucleases (Maier et al., 1995). To overcome this instability, ASOs

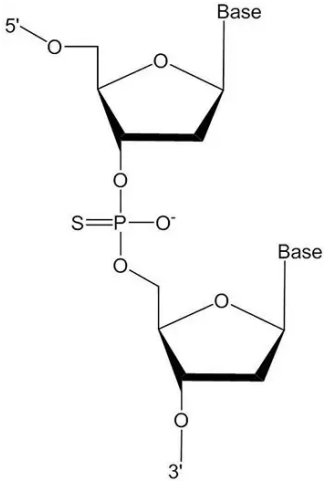

Figure 10: Antisense oligonucleotide structure. From Sigma-Aldrich

undergo chemical enhancements to improve their stability and introduce other desirable properties, such as the placement of a phosphorothioate at the 3' end, rather than a phosphodiester.

Modifications can significantly improve the ASO's stability, extend its presence in the bloodstream and enhance tissue retention, however, these modifications could lead to a reduced affinity to their target mRNA and internalization to cells may not be efficient (Stein and Krieg, 1994). Also, modified ASOs tend to bind non-specifically to proteins in the bloodstream and within cells, potentially reducing their effectiveness and leading to side effects such as triggering the immune system.

To further improve ASO's stability, modifications at the 2' position of the ribose ring have been developed. Figure 11 shows this modification at the 2' position. The addition of the 2'-O-(2-methoxy)ethyl (2-Ome) group increases the binding ability of the ASO to the target mRNA, makes it more resistant against degradation and avoids RNase H1-induced cleavage (Prakash and Bhat, 2007).

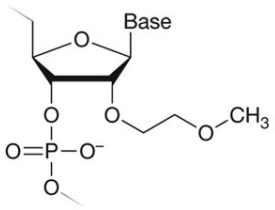

Figure 11: 2'-O-(2-methoxy)ethyl modification. Adapted from (Rinaldi and Wood, 2018)

A potential new approach for treating human adenoviruses involves adapting ASOs to target the splicing process. By focusing on ASO's splicing modulation, targeting the E1B-19k locus on the viral RNA, removing this part could be feasible. These splicing modulators interfere with the process of splicing during gene expression, rather than during translation. Splicing can occur as exon skipping, where ASOs bind to the pre-mRNA splicing machinery, to skip mutated or target exons to eventually produce the wanted proteins (Wahl et al., 2009) Using splice-modulating oligonucleotides may be effective in precisely removing the E1B-19k portion from the viral RNA.

4.1.2 CRISPR/Cas9 gene editing

CRISPR/Cas9 is a genome editing system that was originally part of the defense system of archaea and bacteria against phages, utilizing RNA to guide the Cas9 nuclease to a specific sequence (Shmakov et al., 2015). It is used to insert, modify, or remove parts of the DNA in living cells. The mechanism of action of CRISPR/Cas9 consists of three steps: target sequence recognition, DNA cleavage and DNA repair (Ming et al., 2016). For the CRISPR/Cas9 system to work properly, two essential components are needed: guide RNA (gRNA) and Cas9 proteins. Initially, the gRNA locates and binds to the complementary DNA sequence within the gene of interest. Following this, the gRNA guides the Cas9 enzyme to the target gene to create double-stranded breaks in the DNA. These breaks are later repaired using non-homologous end joining or homology-directed repair. In the case of targeting E1B-19k, a specific gRNA should be engineered that is complementary to the E1B-19k sequence.

4.1.3 RNA interference

RNA interference (RNAi) is a biological process of gene silencing that involves the use of small interfering RNAs (siRNAs) to specifically target and degrade mRNA molecules thereby inhibiting the expression of the targeted protein and controlling gene activity, posttranslationally (Almeida and Allshire, 2005; Rao et al., 2009; Moore et al., 2010). This method could be a promising strategy for the treatment of (severe) human adenovirus cases by targeting the E1B-19k protein.

This sequence-specific mRNA degradation is achieved through an enzymatic process that uses the cell's RNA-induced silencing complex (RISC) (Zhang, 2013). This targeted approach can disrupt the virus's ability to prevent apoptosis in infected cells, leading, again, to a reduction of the virus in

patients.

The process of RNA interference (figure 12) can be divided into two steps: the RNAi initiating step and the effector step. The initial step involves the binding of RNA nucleases to a larger double-stranded RNS (dsRNA) molecule, followed by its cleavage into siRNA. These siRNAs consist of two strands: the sense and the antisense strand. While siRNAs can be naturally generated from the longer dsRNA precursors through the action of the RNAse III family enzyme Dicer, they can also be artificially synthesized (Zhang et al., 2004; Moore et al., 2010). The effector step's goal is the degradation of mRNA. In this step, the siRNA strands associate with an RNAi-specific protein complex, leading to the assembly of RISC. In the presence of ATP, this complex is thought to undergo activation, leading to the unwinding of the siRNA. This unwinding leads to the antisense strand becoming exposed and generating an active complex (Zamore et al., 2000; Nykänen et al., 2001).
However, another mechanism leading to siRNA unwinding has been identified more recently. Rather than ATP being the driving force behind siRNA unwinding, several studies have shown that Argonaute 2 (AGO2) is responsible for the siRNA unwinding (Agrawal et al., 2003; Okamura et al., 2004).
Upon incorporation of the siRNA into RISC, the siRNA engages with the Argonaute 2 component of the complex. This interaction triggers the unwinding of the siRNA duplex and leads to the degradation of the sense strand. The remaining antisense strand then directs the RISC to the complementary target mRNA, inducing mRNA cleavage (Agrawal et al., 2003). This strand eventually leads to mRNA degradation.

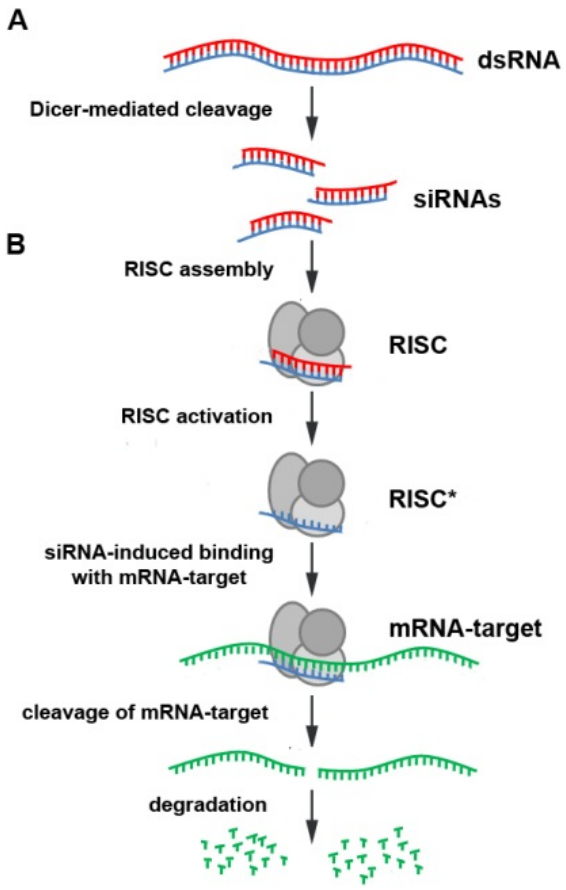

Figure 12: Mechanisms of action of RNA interference. From (Petrova et al., 2013)
(A) initiation phase and (B) the effector phase

4.1.4 Transcription activator-like effector nucleases and Zinc finger nucleases

The fourth method that could be used to target the E1B-19k gene, is using transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs) (figure 13). These proteins are work by combining the DNA-binding properties of zinc fingers and TALE with the DNA-cleaving capability of the Fok1 nuclease (Li et al., 1992; Boch et al., 2009). The zinc finger domains and TALEs are engineered to specifically recognize and bind to a certain DNA sequence.

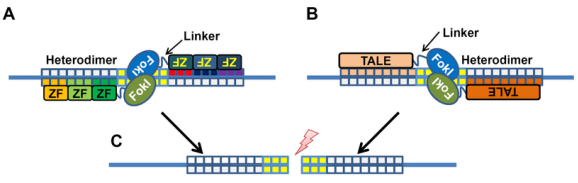

Figure 13: Mode of action of ZFNs (A) and TALEN (B). Adapted from (Sung et al., 2012)

For an effective cleavage, two units should be present:
both recognizing opposing sequences. The DNA-recognizing units and the Fok1 are connected with a linker. After the Fok1s have caused a double-strand break, this can be repaired through non-homologous end joining (NHEJ) and homologous recombination (HR) (Moore and Haber, 1996; Thompson and Schild, 2001). To specifically target the E1B-19k gene using these techniques, the zinc finger domains or the TALE should be designed to detect the E1B-19k gene sequence.

4.2 Challenges in targeting E1B-19k

Targeting the E1B-19k gene in HAdVs is complex due to viral resistance. Mutations in the viral genome, as a response to the treatment, can lead to changes in the E1B-19k gene sequence, reducing the effectiveness of the therapies since those are targeted to their original sequence. These mutations can significantly hinder the long-term success of gene-targeting treatment options. Another concern is the safety, due to potential off-target effects and immune responses. Possible off-target effects include unintended interactions with similar sequences in the host genome, leading to genetic modification in healthy cells, which could disrupt normal cellular functions. Immune responses against the treatment could lead to inflammation and off-target effects.

4.3 Designing E1B-19k-specific RNA interference treatment

Targeting and degrading the E1B-19k protein using RNAi can be achieved by designing siRNAs that specifically target the mRNA encoding the E1B-19k protein. To design the siRNA strands, the E1B-19k mRNA sequence must be known in order to identify suitable binding regions. These regions should be accessible for RISC to bind to and highly specific for the E1B-19k sequence to avoid unwanted effects, as described generally by Svoboda (2007). Once the target mRNA sequence is identified, the siRNA duplexes can be synthesized. The sense strand is identical to the E1B-19k protein mRNA sequence and the antisense strand will be complementary to the mRNA strand. The antisense strand will eventually guide the RISC to the target. To increase the stability and reduce degradation by nucleases, incorporation of the previously mentioned 2-Ome should be considered. After synthesis of the siRNA duplex, they need to be delivered to the cells. This can be done using various methods ranging from electroporation to nanoparticles and viral vectors (Arabsolghar and Rasti, 2012; Setten et al., 2019). The choice of the delivery system is dependent on several factors including the application, target cells and scalability. Nanoparticles offer the greatest potential, due to reduced immune responses in comparison to viral vectors and the since *in vivo* application is possible.

4.4 Novel theoretical approaches

One novel approach to treating human adenovirus infections could be by designing molecular machines that dismantle viral particles. This potential treatment option involves the use of nanotechnology to create nanoscale devices or materials that can recognize viral or non-endogenous structures. These devices trap and dismantle the pathogen before it can interact with the human cells. Dismantling and inactivation of the virus can be done using known strategies, such as DNAse I and micrococcal nuclease for genome breakdown and changes in pH and temperature for denaturation of the genetic material (Vermes and Haanen, 1994). Once the viral particle is recognized and trapped by the nanodevice, degradation can be initiated inside this machine via DNAse I or micrococcal nucleases, which are both non-specific nucleases. Another way is to increase the temperature inside the device by using magnetic nanoparticles and an alternating magnetic field (AMF) (Yu et al., 2022). Since DNA

denaturates at temperatures over 42°C, this could be a possible way to dismantle the virus. Research has shown that pH levels also affect the stability and infectivity of viruses, however, the pH level at which a virus becomes ineffective differs greatly between viruses, ranging from extremely acidic to highly alkaline environments (Salo and Cliver, 1976). The major advantages and disadvantages of the proposed novel treatment options are summarized in table 2.

Table 2: Advantages and disadvantages of proposed theoretical treatment options

Method	Advantages	Disadvantages
Nucleases	High specificity and efficiency	Risk of side effects; Technical complexity
AMF	Controlled activation and localization	Risk of damaging surrounding tissue
pH modification	Simple biochemical approach	Risk of host cell damage; High variability among viruses

4.5 Translation into clinical applications

Targeting the E1B-19k protein has clinical relevance primarily in the context of cancer therapy and in treating adenovirus infections in immunocompromised patients. Many types of cancer cells evade apoptosis, which allows the cells to proliferate uncontrolled. Since E1B-19k inhibits apoptosis in the host cell, targeting this gene could help restore the apoptotic pathway in cancerous cells, associated with adenovirus infections. Also, targeting E1B-19k may be beneficial in developing therapies for other diseases where host cell apoptosis plays a crucial role, such as in certain neurodegenerative disorders where improper apoptosis leads to neuronal death. To translate E1B-19k targeting into clinical application, a thorough understanding of the mechanism of action of apoptosis inhibition is required to develop effective treatment options.

5 Conclusion

The E1B-19k protein of the HAdV plays an important role in the viral life cycle of the virus, particularly in its ability to evade host apoptosis. This evasion is the key to the successful replication of the virus within the host. Targeting this gene could thus offer a great potential treatment option for those infected with the virus. Currently, only two antivirals for HAdVs are on the market: cidofovir and ribavirin. However, with the use of modern gene editing techniques, such as ASOs, CRISPR/Cas9, RNAi, TALENs and ZFNs, targeting the E1B-19k gene for a more effective treatment is within reach. However, using gene editing could lead to viral resistance. A novel, theoretical, method could be using nanodevices to induce degradation of the viral DNA or reducing the infectivity of the virus by denaturing the DNA with the use of locally increased temperatures and pH levels.

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