Early host cell interactions and antivirals against ocular adenoviruses.

Rickard Storm
En liten bok till mina VÄNNER, som står mig nära!
En liten bok till LINDA, min fantastiska flickvän!
En liten bok till min FAMILJ, som alltid funnits där!
En liten bok till min MAMMA, som förevigt är saknad!
En liten bok som BEVISAR vad jag kan göra med ert stöd!
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Abstract

Viruses are common causative agents of ocular infection among humans. Epidemic keratoconjunctivitis (EKC) is a severe and contagious ocular disease with reported outbreaks worldwide. It is estimated that this disease affects 20-40 million individuals every year, which leads to huge socioeconomic costs for the affected countries. EKC is endemic in Japan with half a million to one million cases every year. EKC is characterized by keratitis and conjunctivitis but is also associated with pain, edema, lacrimation, and decreased vision that can prolong for months after the infection and in rare cases years. This disease is caused by human adenoviruses (HAdVs), which belong to the family of Adenoviridae. Currently, there is no available treatment against EKC.

EKC is mainly caused by HAdV-8, HAdV-19, HAdV-37, HAdV-53, HAdV-54, and HAdV-56, which belong to species D HAdVs. HAdV-8, HAdV-19 and HAdV-37 have previously been shown to use sialic acid (SA)-containing glycans as cellular receptors to bind to and infect human corneal epithelial (HCE) cells. To characterize the receptor in more detail, we performed a glycan array, which included SA-containing glycans. A branched hexasaccharide terminating with SA in each arm was identified as a candidate receptor. This glycan corresponds to the glycan motif found on a ganglioside, GD1a. By performing a series of biological and biochemical experiments we confirmed the function of the GD1a glycan as a cellular receptor for EKC-causing HAdVs. However, the glycan used as a receptor was linked to plasma membrane protein(s) through O-glycosidic bonds, rather than to a lipid (as in the ganglioside). X-ray crystallography analysis showed that the two terminal SA:s interacted with two of the three previously identified SA-binding sites on the knob domain of the HAdV-37 capsid protein known as the fiber.

Based on the structural features of the GD1a:HAdV-37 knob interaction, we assumed that a three-armed molecule with each arm terminating with SA
would be an efficient inhibitor. Such molecules were designed, synthesized and found to efficiently prevent HAdV-37 binding to and infection of corneal cells. These results indicate that trisialic acids-containing compounds may be used for treatment of EKC.

After binding to its primary receptor, most HAdVs have been shown to interact with αVβ3 and αVβ5 integrins to enter human cells. This interaction occurs through the RGD (arginine-alanine-aspartic acid) motif in the capsid protein known as the penton base. However, it was not clear if corneal epithelial cells express αVβ3 and αVβ5 integrins. Thus, to better understand additional early steps of infection by EKC-causing HAdVs, we performed binding and infection competition experiments using human corneal epithelial cells and siRNA, integrin specific antibodies, peptides and RGD-containing ligands indicating that α3, αV, β1 affected HAdV-37 infection of but not binding to HCE cells. We could also see that HAdV-37 co-localize with α3 and αV at after entry into HCE cells. In situ histochemistry confirmed that the expression of α3 and αV in human corneal tissue. Overall, our results suggest that αV and α3 integrins are important for HAdV-37 infection of corneal cells.

Altogether, these results provide further insight into the biology of HAdVs and open up for development of novel antiviral drugs.
Summary in Swedish-
Populärvetenskaplig sammanfattning
på Svenska

Virus är en vanlig orsak till olika humana ögonsjukdomar. En sådan virusorsakad ögonsjukdom heter epidemisk keratokonjunktivit (EKC). EKC är en allvarlig och smittsam sjukdom med rapporterade sjukdomsfall och utbrott världen över. Uppskattningsvis drabbas mellan 20-40 miljoner individer av denna sjukdom varje år, vilket leder till stora samhällsekonomiska förluster hos de drabbade länderna. EKC väller stora problem i Japan med en halv- till en miljon rapporterade fall varje år. EKC kännetecknas av olika symtom så som inflammation av hornhinna och bindhinna tillsammans med värk, ödem, rinnande ögon och nedsatt syn som kan pågå i månader eller år efter insjuknandet. EKC orsakas av flera olika typer av adenovirus (HAdVs) som tillhör familjen *Adenoviridae*. Det finns idag inga tillgängliga läkemedel för att behandla EKC.

Baserat på hur GD1a glykanen interagerade med HAdV-37 knoppen, föddes tanken att kunna blockera/hämma denna interaktion, genom att tillverka molekyler med tre eller flera avslutande SA. En sådan molekyl skulle kunna interagera med alla 3 SA-inbindningsställen i varje fiberknopp. En molekyl med tre SA (tri-sialylerad molekyl) visade sig vara bäst på att hämma HAdV-37 bindning och infektion av ögonceller. Denna molekyl förädlades genom att skapa olika analoger som visade sig vara mer än 1000 gånger mer effektivt att hämma HAdV-37 bindning och infektion till ögonceller än vad GD1a-kolhydraten var. Våra resultat visar att tri-sialylerad molekyl är en lovande läkemedelskandidat för att i framtiden kunna behandla EKC.

De flesta HAdVs använder sig av integrinerna αVβ3 och αVβ5 för transport in i cellen. Dock uttrycks inte αVβ3 och αVβ5 integriner på ögonens hornhinna, som är den vävnad som EKC-orsakande HAdVs infekterar. Genom att behandla humana hornhinneepitel-cellers (HCE-celler) med olika komponenter kunde vi konkludera att integrinerna, α3 och αV är viktiga för att infektion av EKC-orsakande HAdVs.

Genom detta arbete har jag identifierat tidiga cellulära interaktionspartners, och där igenom ökat förståelsen för hur HAdVs orsakar EKC, samt utvecklat och utvärderat nya läkemedelkandidater med förmåga att hindra EKC-orsakande HAdVs för att binda till och infektera ögonceller.
List of papers


3. Triazole Linker-Based Trivalent Sialic Acid Inhibitors of Adenovirus Type 37 Infection of Human Corneal Epithelial Cells. (Submitted). Rémi Caraballo, Michael Saleeb, Johannes Bauer, Antonio-Manuel Liaci, Naresh Chandra, Rickard J Storm, Lars Frängsmyr, Weixing Qian, Thilo Stehle, Niklas Arnberg, and Mikael Elofsson.


Publication not included in this thesis:

<table>
<thead>
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<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Aa:s</td>
<td>Amino acids</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenovirus death protein</td>
</tr>
<tr>
<td>AdPol</td>
<td>Adenovirus polymerase</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ALA</td>
<td>Alanine</td>
</tr>
<tr>
<td>ARD</td>
<td>Acute respiratory disease</td>
</tr>
<tr>
<td>CAR</td>
<td>Coxsackievirus and adenovirus receptor</td>
</tr>
<tr>
<td>CCP</td>
<td>Complement control proteins</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CFG</td>
<td>Consortium for Functional Glycomics</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>DBP</td>
<td>DNA binding protein</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSG-2</td>
<td>Desmoglein-2</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra cellular matrix</td>
</tr>
<tr>
<td>EKC</td>
<td>Epidemic keratoconjunctivitis</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FII</td>
<td>Factor II; prothrombin</td>
</tr>
<tr>
<td>FVII</td>
<td>Factor VII; proconvertin</td>
</tr>
<tr>
<td>FXI</td>
<td>Factor IX; Christmas factor</td>
</tr>
<tr>
<td>FX</td>
<td>Factor X; Stuart-Power factor</td>
</tr>
<tr>
<td>GLA</td>
<td>γ-carboxyl glutamic acid</td>
</tr>
<tr>
<td>GAGs</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>GBM</td>
<td>Glomerular basement membrane</td>
</tr>
<tr>
<td>GFOGER</td>
<td>Glycine-phenylalanine-hydroxyproline-glycine-glutamic acid-arginine</td>
</tr>
<tr>
<td>GON</td>
<td>Group of nine</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HAdV</td>
<td>Human adenovirus</td>
</tr>
<tr>
<td>HCE</td>
<td>Human corneal epithelial</td>
</tr>
<tr>
<td>HVR</td>
<td>Hypervariable regions</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>HSGAGs</td>
<td>Heparan sulphate glycosoaminoglycans</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulphate</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycans</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Inhibitory concentration 50%</td>
</tr>
<tr>
<td>ICTV</td>
<td>International committee of taxonomy of viruses</td>
</tr>
<tr>
<td>IGDD</td>
<td>Isoleucine-glycine-aspartic acid-aspartic acid</td>
</tr>
<tr>
<td>ILK</td>
<td>Integrin linked kinase</td>
</tr>
<tr>
<td>ITRs</td>
<td>Inverted terminal repeats</td>
</tr>
<tr>
<td>LDV</td>
<td>Leucine-aspartic acid-valine</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MIDAS</td>
<td>Metal-ion-dependent adhesion site</td>
</tr>
<tr>
<td>MLP</td>
<td>Major late promotor</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear export signal</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>NPC</td>
<td>Nuclear pore complex</td>
</tr>
<tr>
<td>PC</td>
<td>Protein C</td>
</tr>
<tr>
<td>PCF</td>
<td>Pharyngoconjunctival fever</td>
</tr>
<tr>
<td>PDZ</td>
<td>Postsynaptic density 95, Discs large, Zonula occludens-1</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinases</td>
</tr>
<tr>
<td>Pro</td>
<td>Proline</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine binding</td>
</tr>
<tr>
<td>PtDs</td>
<td>Dodecahedral particles</td>
</tr>
<tr>
<td>RCA</td>
<td>Regulators of complement activation</td>
</tr>
<tr>
<td>RGAD</td>
<td>Arginine-Glycine-Alanine-Aspartic acid</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-Glycine-Aspartic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SA</td>
<td>Sialic acid</td>
</tr>
<tr>
<td>SCR</td>
<td>Short consensus repeat</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinases</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>STD NMR</td>
<td>Saturation transfer difference Nuclear magnetic resonance</td>
</tr>
<tr>
<td>STP</td>
<td>Serine, threonin, proline</td>
</tr>
<tr>
<td>TP</td>
<td>Terminal protein</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
</tbody>
</table>

x
Aims of the thesis

**Overall aim:** The overall aim of this work was to better understand the early host-cell interactions of EKC-causing adenoviruses.

**Aim 1:** Identify and characterize the cellular receptor(s) used by EKC-causing adenovirus.

**Aim 2:** Evaluate the inhibitory effect of tri- and tetravalent sialic acid-containing compounds on HAdV-37 binding to and infection of human corneal epithelial cells.

**Aim 3:** Identify and characterize tissue specific expression and function of integrins during HAdV-37 infection of ocular cells.
Introduction:

History

Searching for the causative agent of an acute respiratory infection in 1953, Rowe et al. isolated human adenoviruses (HAdVs) from adenoid tissue. [1]. Shortly after this discovery, Hilleman and Werner identified HAdV as the causative agent of a respiratory disease affecting military recruits [2]. HAdVs can also infect eyes, gastrointestinal tract, urinary tract, and liver [3]. Some HAdVs can also cause a severe and contagious ocular disease called epidemic keratoconjunctivitis (EKC). Referring to EKC as keratitis punctate superficialis in 1889 Austrian doctors were first to clinically describe EKC [4]. Soon EKC was described in case reports from other countries all over the world such as United Kingdom, Japan and the USA [5-7]. Affecting 226 people the first reported outbreak occurred in Bombay (today Mumbai), India in 1901 [8]. Between 1900 and the 1930s, EKC was primarily described in the Indian subcontinent. During the 1930s the first outbreak in the USA was reported at a hospital in California [9]. In 1941, a shipyard in Hawaii reported a large outbreak of EKC (10,000 cases) [10]. As similar outbreaks were reported at other US shipyards the disease was quickly termed “shipyard eye” [11, 12]. After being called by several names (e.g superficial punctate keratitis, keratitis subepithelialis, keratitis nummularis and keratitis disciformis [4-9, 13]), in 1942 the disease was given its current name, epidemic keratoconjunctivitis [14]. In 1955, a first causative agent of EKC was isolated and named human adenovirus type 8 (HAdV-8) [15]. Over the years, more HAdV types have been isolated from EKC patients: HAdV-19 (1959) [16], HAdV-37 (1976) [17], HAdV-53 (during 1980s) [18], HAdV-54 (2000) [19], and HAdV-56 (2008) [20]. Historically, of these six types HAdV-8, HAdV-19 and HAdV-37 have been considered to be major causative agents of EKC.

In addition to being identified as a human pathogen, HAdVs have been used to investigate many scientific questions, such as viral and cellular gene
expression and regulation, DNA replication, cell cycle control and cellular growth regulation. To date, the most significant scientific contribution by HAdV-related research was the discovery of messenger RNA (mRNA) splicing by Sharp and Roberts in 1977 [21, 22]. For this discovery of they received the Nobel Prize in physiology/medicine in 1993. Today HAdVs are frequently used as vectors in gene therapy, cancer therapy and for vaccine development [23]. In the future HAdVs may also be important tools for treatment of cancer, genetic diseases, and prevention of other infectious diseases such as the ongoing development of a vaccine against the ebola virus [24].

**Taxonomy and clinical /pathological aspects**

**Taxonomy**

According to the International Committee of Taxonomy of Viruses (ICTV) [25], the family *Adenoviridae* consists of five genera: mastadenovirus (AdV isolated from mammals), aviadenvirus (birds), atadenoviruses (reptiles and birds), ichtaadenovirus (fish), and siadenovirus (frog). The 56 HAdV types belong to the genus mastadenovirus and include seven species (A-G) with species D representing the majority of EKC-causing HAdVs (Table 1). Historically, the classification of HAdVs is based on their ability to agglutinate erythrocytes, oncogenicity in rodents, DNA homology and tropism. For example species A HAdVs are characterized by high oncogenicity in newborn hamsters as compared to species B, C and D [26]. HAdVs are also classified into serotypes based on resistance to neutralization by antisera against already known HAdV types. The serotypes are placed into different species depending on features such as: A) lack of cross-neutralization, and B) if the phylogenetic distance is more than 15% [25]. These methods have been used to classify HAdV-8, HAdV-19, and HAdV-37 as individual serotypes. However, during the last few years three additional EKC-causing HAdVs have been detected: HAdV-53, HAdV-54 and HAdV-56 [18-20, 27]. HAdV-53 is serologically related to HAdV-22 but the
fiber share similarities with HAdV-8 [19]. Similar to HAdV-8, HAdV-54 is neutralized by HAdV-8 specific antibodies [27]. HAdV-56 shows serological similarities with four other HAdVs (HAdV-9, HAdV-15, HAdV-26 and HAdV-29) [20]. HAdV-53, HAdV-54 and HAdV-56 have not been classified based on serology, but instead they are characterized based on whole genome sequencing and bioinformatic analysis and are referred to as genotypes or types [28]; However no consensus has emerged on how to classify new types beyond HAdV-51 [28, 29].

Clinical and pathological aspects

HAdVs resist physical and chemical agents, and can remain infectious at room temperature up to three weeks [3]. Due to their environmental resistance HAdVs can be spread in many different ways such as person-to-person, through water, or surfaces such as door knobs and equipment [3]. Although HAdV infections can be asymptomatic, they are also common causative agents of respiratory, ocular and gastrointestinal diseases (Table 1) [3]. It is estimated that HAdVs cause about 3% of all infections among civilian populations and 7% if only febrile illnesses are calculated [30]. HAdVs have also been reported to cause disease in urinary bladder, the liver, and in rare cases pancreas [31] and heart [3]. HAdV-1, HAdV-2, HAdV-5, and HAdV-6 (species C) are common causative agents of respiratory disease in children but rarely in adults. Respiratory diseases in adults are more often caused by HAdV-3 and HAdV-7 (species B) [3]. In children younger than five 5 years old, HAdVs account for 5 % of all upper respiratory tract infections [32], such as acute respiratory disease (ARD), a disease, which is also common among military recruits as the result of crowded living conditions and strenuous training [33]. ARD are mostly caused by HAdV-4 and HAdV-7 and in rare cases by HAdV-3. In addition HAdV-3, HAdV-7 and HAdV-14 are the causative agents of a disease with respiratory symptoms accompanied with conjunctivitis referred as pharyngoconjunctival fever (PCF) [3].
Many HAdV types are associated with diarrhea and have been isolated from stool samples, with HAdV-40 and HAdV-41 as the most common types associated with gastrointestinal disease [34-37]. In children, HAdV-40 and HAdV-41 are common causes of diarrhea (8.0% of all cases Sweden [38], 6.9% in USA [39], 1.5% in Brazil [40] and 10.8% in Shenzhen in China [41]).

Table 1. Classification and tropism of human adenoviruses.

<table>
<thead>
<tr>
<th>Species</th>
<th>Type</th>
<th>Tropism</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>12, 18, 31</td>
<td>Intestine</td>
</tr>
<tr>
<td>B1:</td>
<td>3, 7, 16, 21</td>
<td>Respiratory tract, eye</td>
</tr>
<tr>
<td>B2:</td>
<td>11, 14, 34, 35, 50, 55</td>
<td>Respiratory tract, eye, urinary tract</td>
</tr>
<tr>
<td>C</td>
<td>1, 2, 5, 6</td>
<td>Respiratory, lymphoid</td>
</tr>
<tr>
<td>D</td>
<td>8, 9, 10, 13, 15, 17, 19, 20, 22-30, 33, 36-39, 42-49, 51, 53, 54, 56</td>
<td>Eye, intestine</td>
</tr>
<tr>
<td>E</td>
<td>4</td>
<td>Respiratory tract, eye</td>
</tr>
<tr>
<td>F</td>
<td>40, 41</td>
<td>Intestine</td>
</tr>
<tr>
<td>G</td>
<td>52</td>
<td>Intestine</td>
</tr>
</tbody>
</table>

In otherwise healthy individuals, HAdV infections are mostly mild but in immunocompromised patients, they can cause severe and even lethal diseases. HAdVs are frequently isolated in immunocompromised patients, where the infections last longer and are more severe, and result in more death [42]. Here, HAdVs from species A, B and C are the most commonly isolated. Types of all the HAdVs, species A HAdV-31 causes most deaths [43]. HAdV-35 has been shown to cause fatal cases among pediatric patients [44]. Strikingly, several new types belonging to species D HAdVs have been isolated from AIDS patients (HAdV-42 to HAdV-47) [45, 46].
Epidemic keratoconjunctivitis (EKC)

EKC is a severe and contagious ocular disease, with symptoms such as tearing, redness, foreign body sensation, photophobia, lid swelling, follicular hypertrophy, edema, hyperemia, small petechial hemorrhages, and decreased vision [47-49]. In more severe cases, symptoms include formation of pseudomembranes, iritis, and in rare cases increased intraocular pressure, [50] and subconjunctival hemorrhages [51]. EKC is often bilateral, i.e. the infection spread from one eye to the other, but in 25% of the cases EKC is unilateral (only affecting one eye) [52]. Typically, the infection in the first eye lasts for approximately 14 days and in total the acute disease is cleared in 18 to 21 days after onset.

The clinical patterns of EKC with viral replication in cornea are well documented [48, 49] (Figure 1a-c).

**Figure 1a.** The first symptoms appears three to four days post infection and includes follicular conjunctivitis, diffuse punctate keratitis, and foreign body sensation [53].
Figure 1b. After one week, fluorescein staining can reveal small irregular lesions on corneal epithelium. These irregularities/lesions cause other symptoms such as irritation, tearing and photophobia, which are present through the second week of infection [53].

Figure 1c. During the second and third week, immune cells invade the affected area and form subepithelial infiltrates, which are hallmarks of EKC. These infiltrates appear as greyish-white dot and are visible with the naked eye when illuminated. The infiltrates decrease vision, but usually disappear after a few weeks or months. Some patients may experience decreased vision for as long for several years [48].
Six HAdVs cause the more common, severe form of EKC: HAdV-8, HAdV-19, HAdV-37, HAdV-53, HAdV-54, HAdV-56. The most frequently detected viruses vary from year to year. Historically, the most common types are HAdV-8, HAdV-19, and HAdV-37. HAdV-4 can also cause a milder form of EKC [47, 54]. The other HAdVs that cause EKC are HAdV-53, HAdV-54, and HAdV-56; these HAdVs are recently identified as additional EKC-causing viruses and share homology with other HAdVs [18-20, 27]. Outbreaks of EKC have been reported from around the world such as USA [55], Germany [56, 57] and Japan [18-20, 27, 54]. In these countries different HAdV types cause EKC. In a report from USA, HAdV-8 and HAdV-19 was described as the most common causes of EKC during 2008-2010 [55], whereas in Germany the numbers of cases have increased by 250% between 2008 and 2010, caused mainly by HAdV-8 and HAdV-37 [57]. In Japan, multiple outbreaks have been reported describing HAdV-4, HAdV-19 and HAdV-37 as causative agents [18] and 500,000 to one million cases are reported every year [54, 58]. Outbreaks of EKC often start in eye clinics or similar facilities or in schools or military bases [47]. Although these large numbers of cases lead to significant socioeconomic losses and suffering [54], no antiviral treatments for EKC are available.

**Corneal structure and anatomy**

The cornea has three main functions: as barrier and protection for the eye, for filtration of specific ultraviolet wavelengths, and for refraction. The cornea is a transparent tissue, which is important for maximization of the refraction of light. To achieve this transparency, the cornea has no blood vessels. The cornea consists of five layers: epithelium, Bowman’s layer, stroma, Descemet’s membrane, and endothelium (Figure 2).
**Figure 2.** The cornea with its five layers: epithelium, Bowman’s layer, stroma, Descemet’s membrane, and endothelium, these layers function as a barrier, filter, and for refraction of light. The illustration was reprinted with permission from the publisher [59].

The epithelium consists of 5-7 cell layers and, together with the film of tears, helps to protect the eye. It is also responsible for absorption of oxygen and nutrients. Three different cell types make up the epithelium: a monolayer of basal cells (closest to Bowman’s layer), wing cells, and squamous/superficial cells (the outermost cell layer closest to the tear film) [60, 61]. The squamous/superficial cells that are in contact with the tear film have microvilli, which increase the surface area and give a closer contact with tears [61, 62]. The basal cells are the only dividing cells, which lose their dividing capability as they are moving towards the tear film [61, 63]. The basal cells are attached to Bowman’s layer, which is mainly composed of collagen. The stroma accounts for almost 90% of the thickness of the cornea and is mainly composed of water, collagen, keratinocytes/fibroblasts, and even glycosaminoglycans and proteoglycans [60]. The stroma contributes to strength and elasticity, and regulates transport of nutrients and other components between stroma and endothelial cells, which could be compared with a circulation system. Descemet’s membrane is a thin and strong tissue mainly consisting of collagen, and is located under the stroma. This
membrane functions as a barrier, to protect the cornea from injuries. The Descemet's membrane is responsible for transportation of nutrients and maintenance of optimal hydration to prevent corneal edema [62]. The inner layer of the cornea is the endothelium, which regulates transport of fluids and other components such as leukocytes.

**Structure and function of adenovirus capsid proteins**

HAdVs are noneveloped, double-stranded DNA viruses with a protecting icosahedral capsid. The capsid is approximately 90 nm in diameter, has a mass of 150 MDa and is exclusively composed of proteins. Eleven structural proteins, called II-XII according to their molecular weight, have been identified using SDS-PAGE analysis [64]. The 12 vertices are equipped with protruding fiber proteins [65] (Figure 3).
Figure 3. HAdV structure with major and minor structural capsid proteins and the core proteins. The illustration was reprinted with permission from the publisher [66].

**Function of major capsid proteins**

**Hexon protein (pII)**

Consisting of over 900 amino acids (aa:s), the hexon the most abundant (240 trimers) and the largest capsid protein. A monomeric hexon consists of two antiparallel β-barrels, stabilized by an internal loop and three protruding loops located on the external side (Figure 4a) [67]. Based on the location in
the capsid, the hexon is divided into four groups dependent on function and position: H1, H2, H3, and H4, [68]. H1 interacts with the penton base at the capsid vertices. The capsid has 60 H1 units, which corresponds to one H1/penton base monomer. The H2, H3 and H4 are clustered together and form a unit called group of nine (GON). At the top of the hexon surface there are nine hypervariable regions (Figure 4a). These hypervariable regions are shaped as α-helixes/jelly rolls [69] and constitute type-specific epitopes for neutralizing antibodies that are unique for each serotype [67, 70, 71]. Hexons can interact with coagulation factor IX or X, thus enhancing the ability of HAdVs to infect of hepatocytes or other cells [72-74].

**Penton base (pIII)**

The penton base, a homopentameric protein, is located at the base of each 12 vertice (Figure 3 and 4). The penton base consists of 572 aa:s (the size differs from type to type) that forms two domains, a lower and an upper domain. The lower domain has a jelly roll form, generated by two to four stranded anti-parallel β-sheets that form a β-barrel. The upper domain has two insertions that change the folding compared to the lower domain. One of these insertions contains the RGD (Arg-Gly-Asp) loop (Figure 4b), which is involved in internalization of the virus into the cell through interactions with cellular integrins [75] and helps the virus to escape from the endosome [76]. The RGD-motif is shared by all HAdVs except HAdV-40 and HAdV-41 [77]. Instead this two viruses has other motifs in this loop HAdV-40 have RGAD (Arg-Gly-Ala-Asp) and HAdV-41 have IGDD (Ile-Gly-Asp-Asp), which may result in usage of alternative internalization pathways than HAdVs that use RGD [78]. Together with fibers, penton base protein can form a smaller structure called dodecahedrons, which can interact with a number of cellular proteins such as Desmoglein-2 [79] and ubiquitin ligases (WWP1, WWP2 and AIP4) [80]. These ligases interact with the penton base xPPxY motifs; the mechanisms and importance of these interactions are not fully understood. The penton base has a pore in the center of the complex, which
is where the interaction with the N-terminal domain of the protruding fiber binds [81].

![Diagram showing the structure of major capsid proteins and fiber protein (pIV)]

**Figure 4.** Structure of the major capsid proteins. **a)** Position of seven of the nine hypervariable loops in the HAdV-5 hexon. **b)** A cryo-EM image of HAdV-5 penton base with its five monomers and the location of the RGD-loop. **c)** Composition of the HAdV-35 fibers with three monomers visualized in green, red and blue. **d)** HAdV-2 fiber in atomic structure with positions of receptor binding sites for CAR, CD46 and heparan sulfate. The illustration was reprinted from with permission from the publisher [66].

**Fiber protein (pIV)**

The fiber, is a trimeric protein anchored to the capsid via the conserved N-terminal tail FNPVYPY [81-83]. The structure of the fiber can be divided into three regions: i) the N-terminal tail, ii) the central shaft and iii) the terminal knob domain. The number of aa:s in the fiber differs from type to type as the
fiber is constructed from different number of repeats, ranging from six repeats in HAdV-3 [84] to 23 repeats in HAdV-12 [66]. These repeats consist each of 15-20 aa:s (Figure 4c). HAdV-40, HAdV-41, and HAdV-52 express two different fibers, one short and one long, with different receptor specificities [85-87]. Species C HAdVs have a KKTK motif, which can bind to heparan sulfate glycoaminoglycans (HSGAGs). This interaction can mediate CAR-independent virion binding to cells [88, 89]. The C-terminus consists of about 180 aa:s of the fiber and forms a globular head, also called the knob. The most well studied HAdV receptors are CAR and CD46. These receptors interact with the fiber through a number of loops DG, HI and AB, which are located on the side of the knob (Figure 4d) [90, 91]. Structural studies of CD46:HAdV-11 knob complexes revealed that interactions with the knob induces a fundamental conformational change of CD46 [92]. This change has not been observed for CAR. EKC-causing HAdVs bind to sialic acid (SA), and the interaction occurs at three SA-binding sites located at the top of the knob [93]

**Function of minor capsid proteins**

This group of proteins is less studied compared to the major capsid proteins. Their main function is to support assembly and the stability of the capsid by acting as cement.

**Protein IIIa (pIIIa)**

The exact function of pIIIa is not known. However, the protein consists of 570 aa:s and has a of molecular weight of 65,5 kDa. pIIIa contains many helices, at both the N-terminal and C-terminal domain of the protein [69]. The protein contains multiple phosphorylation sites that are phosphorylated early during infection [94-96]. It is not fully understood how these phosphorylations affect HAdV life cycle. Located on the inside of the capsid, pIIIa seems to act as a stabilizer by interacting with the penton base, the
hexon and pVIII [97]. The N-terminal domain of pIIIa have been reported to support viral genome packaging [98].

**Protein VI (pVI)**

pVI is involved in many events during the HAdV infection. pVI is located on the internal side of the capsid and consists of 206 aa:s resulting in a molecular weight of 22 kDa. The exact position within the capsid is not known, but it has been suggested to interact with peripentalon hexons based on x-ray crystallography [99, 100]. This location was recently confirmed, and the form of pVI was shown to be a propetide [101]. However, cryo-EM studies suggest that the pVI is located in the central cavities of the hexon protein [102, 103]. pVI is active during virus escape from the endosome, by interacting with the endosome membrane through its N-terminal amphipathic helix [104-106]. After escaping from the endosome, the virion moves toward the nucleus along the microtubule network help of the PPxY motif [107]. This motif is also able to activate gene expression [108]. The C-terminal domain interacts with importin α/β, resulting in entry into the nucleus [109]. pVI contains two nuclear-locations signals (NLS) and two nuclear export signals (NES), which are proteolytically removed during maturation [109, 110].

**Protein VIII (pVIII)**

Very little is known about the function of pVIII (molecular weight 25 kDa). Mutation of pVIII results in a thermolabile phenotype, suggesting that pVIII contributes to capsid stability [111]. Studies of pVIII in porcine adenovirus type 3 show that pVIII interacts with the packaging protein IVa2, suggesting an involvement in genome packaging [112]. Both cryo-EM and crystallographic atomic structure investigations have confirmed that the position of pVIII is at the internal side of the capsid [97, 102]. pVIII exists in two independent monomers; one interacts with pIIIa and hexon and one with pIX and hexon [113].
Protein IX (pIX)

With a molecular weight of 14,3 kDa and consisting of 139 aa:s, pIX is the smallest of the minor capsid proteins and is only found in mastadenovirus genus [114]. pIX interacts with hexons, and is located in the cavities formed between the peaks of hexons [99, 100]. The monomeric structure of pIX is α-helical, which forms trimers with its leucine-zipper domain. The trimeric structure has a supercoiled structure with three extended arms [64, 114]. pIX interacts with hexons, and has been called the capsid cement because it stabilizes the capsid [115]. HAdV-5 that lacks pIX, still propagates in same way as wild type viruses but cannot form GONs properly and is heat-sensitive [116, 117]. Studies reveal that viruses that express the trimeric N-domain of pIX are heat-stable [114]. One study suggests that pIX may be involved in modulation of virus tropism or interferes with immune responses [118]. pIX seems to be important for viral entry as well as transport via the microtubule network [119, 120]. pIX also act, as a transcriptional activator of major late genes [121, 122]. Some studies support and some oppose the idea that pIX are involved in packaging of the HAdV genome [123, 124].

Adenovirus core proteins and non-structural proteins

Unlike bacteriophages most HAdVs do not have a well-ordered symmetry of its DNA [125, 126]. The HAdV genome consists of linear double-stranded DNA of approximately 36 kb. The core consists of with five proteins: terminal protein (TP), pV, pVII, µ and pIVa2. TP binds to the 5' end of the viral DNA and helps initiate viral replication [127, 128]. With about 800 copies/virion, pVII is the most abundant protein in the virion. Together with protein µ, pVII is responsible for packaging of the genome into nucleosome-like structures [129-131]. Some studies suggest that pVII is involved in virus transport into the nucleus due to the NLS-containing regions found in the protein [132]. pV has only been found in mastadenoviruses, and connects the core to the capsid by interacting with pVI, pVII, and/or DNA [133, 134].
As with pVII and γ, pIVa2 can interact with DNA, but pIVa2 is sequence specific in its binding to DNA. In addition, pIVa2 is also involved in activation of the major late promoter and is responsible for the specific packaging of DNA [135].

The virion contains about 30 different non-structural proteins. The function of all these proteins are not well described but most of them, have catalytic or regulatory responsibilities [25]. As these proteins are expressed in low copy numbers, their functions in the virion are difficult to study. Of these 30 different non-structural proteins only two have been studied in detail: DNA binding protein (DBP) and viral protease. DBP binds to single-stranded DNA, protects it from nuclease digestion [136], and destabilizes the double helix during the DNA replication [137]. DBP includes two domains [138-140]: the N-terminal region contains NLS motif, for transportation into the nucleus [141] and the highly conserved C-terminal binds to DNA and is involved in DNA replication [142]. The HAdV protease is the only known protease to require DNA for maximum activity [143]. The protease is an endopeptidase, an enzyme that plays a vital role in capsid assembly of infectious particles [144]. Each virion contains 10-30 individual proteases [145] and protease mutants inhibit virions from establishing an infection [146]. Most of the minor capsid and the core proteins are cleaved by the protease during viral maturation.
Adenovirus infection and life cycle

Attachment

The primary attachment between virus and host cell is the start of the viral infection and life cycle. Attachment occurs through interactions with one or more cellular receptors. HAdV binds to and interacts with several membrane-bound or soluble proteins, producing a way for the virus to enter the host cell. The receptors usage depends on HAdV types and species (Figure 5, Table 2).

Figure 5. Illustration of the cellular receptors used by different HAdV species. The illustration was reprinted with permission from publisher [147].
Table 2. Identified cellular receptor for human adenoviruses.

<table>
<thead>
<tr>
<th>Species</th>
<th>Type</th>
<th>Receptors</th>
</tr>
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<tbody>
<tr>
<td><strong>A</strong></td>
<td>12, 18, 31</td>
<td>CAR [90]</td>
</tr>
</tbody>
</table>
| **B1:** | 3, 7, 16, 21 | Desmoglein 2 [79]  
CD80, CD86 [148]  
CD46 [149]  
Heparan sulfate [150] |
| **B2:** | 1, 14, 34, 35, 50 | CD46 [149, 151]  
Desmoglein 2 [79]  
CD80, CD86 [148] |
| **C**   | 1, 2, 5, 6 | CAR [90, 152]  
Heparan sulfate [88]  
VCAM-1 [153]  
MHC-1 [154]  
Scavenger receptor A-II [155] |
| **D**   | 8, 9, 10, 13, 15, 17, 19, 20, 22-30, 33, 36-39, 42-49, 51, 53, 54. | CAR [90]  
Sialic acid [156]  
GD1a glycan [157]  
CD46 [158] |
| **E**   | 4 | CAR [90] |
| **F**   | 40, 41 | CAR [90] |
| **G**   | 52 | Sialic acid [87] |
Coxsackievirus and adenovirus receptor, CAR

CAR is a type 1 transmembrane protein belonging to the immunoglobulin superfamily. CAR belongs to a subgroup of proteins that share a common structure in the extracellular domain with one V (variable) and one C (conserved) Ig-loop, a transmembrane domain, and a cytoplasmic C-terminal tail [159] (Figure 6). At least two isoforms exist, CAREx7 and CAREx8. These two isoforms differ in their intracellular C-terminal domain, which differs in both human- and mouse-CAR [160, 161]. The extracellular domain consists of two immunoglobulin-like domains called D1 and D2 (Figure 6).

**Figure 6.** Structure of CAR with its two extracellular domains consists of two immunoglobulin-like domains (D1 and D2), a transmembrane domain and a cytoplasmic, C-terminal domain. The illustration was reprinted with permission from the publisher [162].

CAR functions as a tight junction protein, where the cell-to-cell contact is generated by homo-dimer interaction between the two D1 domains [162].
Within the tight junction CAR is associated with zonula occludens-1 (ZO-1), a protein that functions as a linker and a communicator between the cells [159, 163-165]. The cytoplasmic tail of CAR contains PDZ (Postsynaptic density 95; Discs large, Zonula occludens-1,) binding motifs and is a protein-protein interaction site. CAR has been shown to interact with MUPP1, MAGI-1b, PICK1 and PSD-95 through this domain [166, 167]. The functions of these interactions are not totally clear, but they may be involved in localization of CAR [167, 168].

CAR is named on the basis of its function as a cellular receptor for species B coxsackieviruses and species C adenoviruses [152]. CAR functions as a cellular receptor in vitro for multiple HAdVs, but not for species B HAdVs [90]. The affinity of the CAR:HAdV knob interaction has been determined by surface plasmon resonance (SPR) to be on a low nM level [169]. However, it is not clear whether CAR is a receptor for HAdVs in vivo, as the protein is not available/expressed at the apical side of polarized cell [159]. In addition, the expression of CAR within human tissues does not correlate with HAdVs tropism. HAdVs that bind to CAR causes respiratory tract, ocular and gastrointestinal tract infections. CAR is expressed in high levels in tissues such as testis, prostate and heart and is expressed in low levels in kidney, lungs and leukocytes. However, one splice variant of CAR, CAR_{Ex8} is expressed on the apical side on polarized cells and has been proposed to function as an apical receptor [170]. The tissue distribution of CAR_{Ex8} is not clear.

Another, interesting aspect is that soluble HAdV fibers disrupt CAR-CAR interactions [171]. This disruption triggers HAdV-5 uptake/entry in neurons and neuronal cells [172], and initiates cell signaling through phosphatidylinositol 3-kinase (PI3K) [173]. Knob-CAR interaction can also generate an inflammatory response in human respiratory cells [174]. CAR is also a regulator of expression and activation of integrins, a transmembrane receptor that functions as co-receptor for many HAdVs [175, 176]. CAR is also important during the early development of the heart [177, 178].
CD46

CD46 belongs to the family of regulators of complement activation (RCA). CD46 is a type I transmembrane glycoprotein, a protein that is expressed in most tissues. At least four isoforms are generated by alternative gene splicing (Figure 7). The extracellular structure of CD46 consists of four domains (1-4), referred to as complement control proteins (CCPs) or short consensus repeats (SCR). The region closest to the membrane is the STP (serine-threonine-proline) rich domain, which contains high amounts of O-linked glycans. The size of this region varies depending on the type of splicing variant (BC or C). As CAR, CD46 also has a transmembrane domain and cytoplasmic domain. The cytoplasmic domain has two splice variants Cyt1 and Cyt2; these variants contain different motifs involved in signaling and downstream functions (Figure 7).

CD46 regulates the activation of the complement system by serving as a cofactor for the serine protease factor I, inactivating the ability of C3b and C4b to bind to host cells [179, 180]. CD46 regulates cytoskeleton formation and is involved in epithelial barrier maintenance [181]. CD46 is also been associated with different cellular proteins such as β1-integrins [182, 183], tetraspans [182, 183] and Drosophila tumor suppressor (DLG4) [184, 185]. In addition to importance of complement regulation CD46 also functions as cellular receptor for species B HAdV, and may also be a receptor for species D HAdV-37 [158]. Unlike CAR, CD46 is expressed on the apical surface of epithelial cells [186]. Structural studies show that HAdV-11 interact with the membrane distal CCP1-2/SCR1-2 of CD46 (Figure 7). This study also shows that the interaction generated a conformational change of CD46 [92]. The affinity of species B HAdV:CD46 interaction ranges from nM to mM depending on the HAdV type [187-189]. In addition to HAdVs, CD46 is a cellular receptor for many other pathogens, such as measles virus, bovine viral diarrhea virus, human herpes virus -6, Streptococcus spp., and Neisseria spp. [190-194].
Figure 7. The four most common splice variants of CD46. These variants differ in the STP domain (with or without B segment) or in the cytoplasmic tail (Cyt1 or Cyt2). The two cytoplasmic tails contain different motifs that are involved in different cellular events. The illustration was reprinted with permission from the publisher [195].

Desmoglein 2

Desmoglein-2 (DSG-2) belongs to the cadherin family, which are calcium-binding, transmembrane glycoproteins. As CAR, DSGs function as tight junction proteins, but is also an important component of desmosomes. Desmosomes provide cell-to-cell adhesion through binding between DSG and DSG, DSG and desmocollin, or desmocollin and desmocollin. [196]. DSG and desmocollin are anchored to the cellular filaments through the
cytoplasmic proteins desmoplakin I and II [197] and plakoglobins [198]. DSG-2 is found in almost all human tissue [199].

DSG-2 has been shown to function as a cellular receptor for species B HAdVs; HAdV-3, HAdV-7, HAdV-11, and HAdV-14 [79]. Species B HAdVs can be divided into three groups depending on which receptor they use: (1) HAdV-16, HAdV-21, HAdV-35, and HAdV-50, which almost exclusively use CD46 as a cellular receptor; (2) HAdV-3, HAdV-7, and HAdV-14, which use the DSG-2 as cellular receptor; and (3) HAdV-11, which uses CD46 when it is available but uses DSG-2 as cellular receptor if CD46 is not available. However, a recent study has shown that HAdV-3 and HAdV-7 use CD46 as a cellular receptor instead of DSG-2, using an avidity mechanism [200]. This suggests that these viruses can use different receptors depending on whether CD46 and DSG-2 are expressed and available at the cell surface. More studies are needed to better understand the receptor interactions of species B HAdVs.

Species B HAdVs are well known for their overproduction of incomplete particles called dodecahedral particles (PtDs). PtDs consist of fibers and penton base proteins (but no DNA) and are able to interact with DSG-2, which leads to breaking up of tight junctions. This overproduction of PtDs may be a way for the virions to spread from cell to cell or within the tissue [201].

**Sialic acid/GD1a glycan**

Sialic acid (SA), also known as N-acetyl-neuraminic acid (Neu5Ac) was first discovered 70 years ago by Gunnar Blix and Ernst Klenk [202]. SA has a backbone structure consisting of nine carbons, which differs from other monosaccharaides with five or six carbons and an acidic α-keto sugar [203]. The SA family is large a family of monosaccharaides consisting of more than 50 different analogues (Figure 8) [203, 204]. Each tissue and species expresses its own specific modification and linkage of SA [205]. SA forms
(via carbon 2) glycosidic bonds with carbon 3 or 6 of the neighboring galactose or with carbon 8 of a neighboring SA. SA is common at the terminal position of glycan structures on glycoproteins or gangliosides [205]. Three analogues of SA are more common in animals than other SA:s: Neu5Ac, N-glycolyneuraminic acid (Neu5Gc), and N-acetyl-9-0-acetyleneuraminic acid (Neu5,9Ac₂). Neu5Ac is ubiquitous (i.e. found in all species), while Neu5Gc and Neu5,9Ac₂ are frequently expressed but not in all species [205]. The different analogues have been suggested to be involved in the conformation of membrane proteins such as ion channels and hormone receptors [206]. The main function of SA is to act as a recognition or protection site. Removal of SA from serum leads to uptake of glycoproteins by hepatocytes [207]. Red blood cells with reduced membrane expression of SA (due to age or to the influence of microorganism-encoded sialidase/neuraminidase) are degraded by binding to galactose-specific receptors of phagocytes [208]. Cancer cells overexpress SA to protect them from cellular defense mechanisms, resulting in increased the degree of malignancy [209].
The chemical structure of Neu5Ac/sialic acid. A number of different side chains can be added at the different carbon positions of Neu5Ac. The illustration was reprinted with permission from the publisher [210].

The EKC-causing HAdV-8, HAdV-19, and HAdV-37, use α2,3-linked SA/Neu5Ac as a cellular receptor [156]. This interaction is charge dependent, and involves positively charged fiber knob (pKₐ = 9.0–9.1) and the negatively charged SA (pKₐ = 2.6) [211]. The interaction occurs at three SA-binding sites, which are located on the top of the trimeric knob [93]. We recently identified a branched, di-sialylated glycan motif mimicking the motif present in the GD1a ganglioside as a cellular receptor for HAdV-8, HAdV-19, and HAdV-37. (This is further discussed in the Results and Discussion sections of Paper I). SA/Neu5Ac is also a receptor for many other viruses and bacteria, such as influenza A virus [212, 213], polyomaviruses JC and BC [214, 215], Haemophilus influenza [216], and Helicobacter pylori [217]. Influenza C virus and bovine coronaviruses have been shown to bind to Neu5Gc and 9-O-acetylated SA respectively [218, 219].
Heparan sulfate (HS)

Heparan sulfate is a form of sulfated polysaccharides that belongs to the glycosaminoglycan (GAG) family. GAGs can be found on proteoglycans such as syndecan 1-4, glypican 1-6, betaglycan, perlecan, and serglycin [220] with one or more GAGs attached. GAGs consist of different structures, which form linear chains by polymerization of disaccharide building blocks. Compared to other sugar residues, GAGs are much larger, with approximately 80 residues as compared to 10-12 residues in typical N-glycans. Heparan sulfate proteoglycans (HSPGs) play an important role in many cellular processes (summarized in Figure 9). GAGs are important components of the extracellular matrix (ECM) and constitute an adhesion surface for ECM proteins such as fibronectin, laminin, and collagen [221]. In the cornea, keratan sulfate is responsible for maintenance of the space between collagen type I molecules in the stroma, which creates transparency under which light can pass through without any scatter [222]. HSPG are also important in attachment and spread of fibroblasts and in attraction of leukocytes. Binding of fibronectin to HSPGs induces the formation of focal adhesion and stress fibers [223, 224]. Syndecan 1 and syndecan 4 can also regulate activation of αvβ3 and αvβ5 integrins by interacting with the extracellular domain of the β subunit [225-227]. On the luminal surface of endothelial cells HSPGs also interact with L-selectin on circulating leukocytes [228]. HSPGs are also involved in blood coagulation [229] and modulation of growth factor activities [230]. Adult kidneys contain of proteoglycans such as argin, perlecan, and collagen XVIII, which make up a filtration barrier in the glomerular basement membrane (GBM) [231]. Here, acidic GAGs on the proteoglycans prevent negatively charged substances from passing through the GBM through charge dependent repulsion [232]. Finally, HSPGs can also modulate lipid metabolism by acting as a receptor for lipases in the liver [233].
**Figure 9.** Summary of cellular functions and mechanisms of HSPG, such as (a) receptor or co-receptor, (b) crosstalk between cells and adhesion, (c) transcellular transport of chemokines, (d) chemokine presentation, (e) proteolytic removal leading to shedding of proteoglycans and their core proteins, (f) heparianse cleavage, (g) endocytosis of ligands, (h) lysosomal degradation of ligands, (i) cell adhesion, (j) interaction with the cytoskeleton, (k) organization of the ECM and formation of physiological barriers, (l) storage of growth factors, (m) packaging into secretory granules, and (n) presence in the nucleus, with unknown function and location. The illustration was reprinted with permission from the publisher [234].

HSPGs are commonly used as receptors by many pathogens, or they are involved in the infection cycle. HSPGs have been shown to interact with HAdV-2 and HAdV-5 and to act as possible co-receptors for HAdV-3 and HAdV-35 [88, 150]. The interaction between HSPG and HAdV-5 occurs through a KKTK motif located on the fiber shaft [88, 89]. HAdV-3 and HAdV-35 interact with HSPGs through different capsid proteins. HAdV-3 interacts with HSPGs through the knob, but the nature of the interaction between
HAdV-35 and HSPG is unclear [150]. There have also been reports of interaction between dodecahedral HAdV-3 and HSPGs [235, 236]. Recently, mouse adenovirus type 1 (MAV-1) was shown to interact with HSPGs [237]. Other pathogens that have been shown to interact with HSPG are *Borrelia burgdorferi, Chlamydia trachomatis, cytomegalovirus (CMV), dengue virus, herpes simplex virus (HSV), and HIV* [238-246].

**Coagulation factors**

There are five vitamin K-dependent coagulation factors: prothrombin (factor II), proconvertin (factor VII), Christmas factor (factor IX), Stuart-Power factor (factor X), and protein C (PC), which are synthesized in the liver and secreted into the circulation in inactive form. These proteins contain multiple sites for serine proteases, which cleave the factors to an active conformation. Except for vitamin K, these factors require Ca²⁺, phospholipids, and cofactors for their biological activity [247]. They are involved in the coagulation cascade and fibrinolysis pathways together with other blood components. The vitamin K-dependent coagulation factors require a posttranslational modification that is dependent on the cofactor, vitamin K. This modification consists of addition of a carboxyl group to the 10-12 glutamic acid residues, forming an γ-carboxyl glutamic acid (GLA) domain [248-250]. This domain has been reported (for factor IX (FIX), factor X (FX), and PC) to interact with membrane surface substrates in a Ca²⁺ dependent manner [251-254].

In 1995, intravenously added HAdV-5 was found at high titers in rat liver [255]. Recently, it was found that coagulation factors enhance HAdV-5 infection for liver cell line HepG2 [256]. Further studies confirmed that it was FX that was responsible for transduction of liver cells when HAdV-5 was administrated intravenously to mice or used to infect different cell lines [257]. The ability to bind to FX differs between HAdV species. All members of species C bind FX whereas species D members do not [258]. The GLA domain of FX binds to HAdV-5 through the HVR5 or HVR7 regions of the hexon [72, 258-260]. The HAdV-5:FX complex appears to interact with N-
linked and O-linked sulfate groups of HS side chains [261]. Other studies have shown that in complex with FX and FIX, HAdV-5 binds to cellular HS [74]. In addition, it was later shown that species A types HAdV-18 and HAdV-31 but not HAdV-12 interacted with FIX. Interaction with FIX enhanced binding of the virus to lung and gut epithelial cells [73]. HAdVs are common as vectors in cancer and gene therapy, and the interaction with FX cause problems in delivering the vector to the desired organ when it is injected intravenously. The interaction with FX can be inhibited by changing the HVR5 or HVR7 regions to those of non-binding HAdV types [258]. These types of modifications of HVR abolish HAdV interactions with FX in vitro and detarget HAdV from the liver in vivo [259, 262]. In the absence of FX, HAdV-5 is neutralized by IgM antibodies and activates the complement system [263]. Coagulation factors have also been shown to affect the infection cycles of other viruses such as herpes simplex type 1 (HSV-1) and adeno-associated virus type 2 (AAV-2) [264, 265].

Integrins

Integrins are membrane-bound non-covalently linked heterodimeric glycoproteins containing two subunits α and β. In humans, 18 α subunits and 8 β subunits have been identified, which form 24 unique heterodimeric complexes (Figure 10a). Integrins are expressed at different levels in all tissues, for example in corneal epithelium α2, α3, α6, αV, β1, and β4 are expressed [266]. Integrins consist of an extracellular domain, a single transmembrane domain, and a shorter cytoplasmic tail (Figure 10b). The extracellular domain of the α subunit consists of a seven-bladed β-propeller domain, which can bind Ca²⁺, in turn affecting ligand binding [267, 268]. Nine of the 18 α subunits (αD, αE, αL, αM, αX, α1, α2, α10, and α11) have an insert in the β-propeller of almost 200 aa:s also called the I or A domain [269]. This I/A domain is located on the apical side of the α subunit [270]. All β subunits contain an I domain, which is located near the β-propeller of the alpha subunit [271]. The two I domains of the α and β subunits are involved in ligand interaction, which is coordinated through Mg²⁺ (α
subunit) and/or Ca\textsuperscript{2+} (β subunit) at a site called the metal-ion-dependent adhesion site (MIDAS) [268, 272].

**Figure 10.** a) The 24 unique heterodimeric integrin complexes that have been detected in humans. The illustration was reprinted with permission from the publisher [273] b) The dimeric integrins can either be in an active state or an inactive state. They are activated by ligand interaction and/or internal activation. The illustration was reprinted with permission from the publisher [274].

Integrins have been shown to interact with multiple cellular ligands and to function as signal transmitters between the ECM and the cell. Integrins interact with ECM proteins through aa-motifs such as RGD, LDV (leucine-aspartic acid-valine), and GFOGER (Glycine-phenylalanine-hydroxyproline-glycine-glutamic acid-arginine) [275-279]. Many ECM proteins such as fibronectin, vitronectin, and fibrinogen contain RGD motifs and have been shown to interact with the following integrins: αVβ1, αVβ3, αVβ5, αVβ6, αVβ8, α5β1, α8β1, and αIIbβ3 [280]. VCAM-1 and MAdCAM-1 contain LDV motifs [281] and interact with αLβ2, αXβ2, αMβ2, αDβ2, α4β1, α4β7, α9β1 and αEβ7 [280]. In addition, α1β1, α2β1, α10β1, and α11β1 are known to interact with laminin and/or collagen through the GFOGER motif [280]. Structural studies of the interaction between α2 and the GFOGER peptide
showed that the glutamic acid (E) was of importance for the interaction [282]. Other laminin-interacting integrins are α3β1, α6β1, and α7β1 [280]. Ligand interactions generate an intracellular signal through the cytoplasmic tail of the β subunit. The cytoplasmic tail of β integrins contains NPxY and NxxY motifs, which can be bound by proteins with phosphotyrosine binding (PTB) domains. Talin and Kindling are two proteins with PTB domains and they are involved in intracellular activation of integrins [283-285]. The cytoplasmic tail of the β subunit is also involved in formation of adhesomes, which function as sensors that are involved in cell migration, differentiation, proliferation, and viability. The cytoplasmic tails of the α and β subunit have been shown to interact with focal adhesion kinase (FAK), Src family kinases (SFKs), paxillin, and integrin-linked kinase (ILK) [286, 287]. Interactions with FAK and SFKs control localization and activation of several GTPases, such as RhoA, cdc42, and Rac-1 [288, 289] and outside-in signaling [290].

HAdVs have been shown to interact with several integrins: αVβ1, αVβ3, αVβ5, αMβ2, and α3β1 [75, 291-295]. Several HAdV types have been shown to interact with integrins, but HAdV-2 and HAdV-5 are the most well studied [75]. Integrins function as co-receptors for HAdVs; after binding to a primary, cellular receptor, HAdVs interacts with integrins through its RGD motif on the penton base [75, 291, 293]. HAdV-40 and HAdV-41 lack the RGD motif, which results in a slower internalization in some cells [77, 81]. Integrins are involved in HAdV internalization and endosomal escape [75, 76]. However, αMβ2 is the only integrin that has been shown to function a primary attachment receptor on monocytes [294]. The interaction of HAdV-5 with integrin αVβ5 leads to integrin clustering and activation of multiple signaling pathways such as phosphatidylinositol-3-OH, extracellular regulated kinase 1/2, and p38 MAPK [296-299]. Cryo-EM studies of the interaction between HAdV-12 and αVβ5 revealed that one pentameric HAdV-12 penton base could interact with maximum of four integrins [300]. A recent study of the interaction between the penton base of HAdV-9 and integrin αVβ3 revealed that the interaction could occur in several different orientations. This study also showed that the affinity of this interaction was
Integrins are involved in internalization of other viruses, such as CMV (αVβ3), Kaposi’s sarcoma-associated herpes virus (α3β1), hantavirus (β3), and reovirus (β1) [302-305].

**Other adenovirus receptors**

**Scavenger receptor AII:** This molecule has been identified as a cellular receptor during HAdV-5 infection of Kupffer cells. The interaction between HAdV-5 and scavenger receptor AII was confirmed both *in vitro* and *in vivo* [155].

**CD80/CD86:** These proteins are mainly expressed on dendritic cells and have been identified as receptors for species B HAdVs [148]. However, many proteins have been identified as receptor candidates for species B HAdVs, and more studies are needed to determine the roles of CD80 and CD86 relative to those of other receptor candidates.

**Major histocompatibility complex-I α2/MHC-I α2:** This molecule is broadly expressed in human tissues. HAdV-5 binds better to MHC-I α2-transfected cells than to non-expressing cells [154, 306], and MHC-I α2 serves as a cellular receptor in absence of CAR [307].

**Internalization and trafficking**

After HAdV attachment and interaction with cellular receptors and coreceptors, different receptor-mediated endocytic pathways are triggered in a HAdV type and cell type dependent manner. HAdV-3 and HAdV-35 use macropinocytosis to enter HeLa (epithelial) cells [308, 309]. HAdV-2 and HAdV-5 have been shown to use clathrin-mediated endocytosis to enter epithelial cells [310-313]. Within 3-15 minutes after interaction with the cell, HAdV-2 can be seen in endosome [146, 311]. However, HAdV-5 has also been shown to use caveolae to enter plasmocytes [314]. HAdV-37 enters corneal fibroblasts (keratinocytes) through a lipid raft-dependent caveolin-1
mechanism [315]. HAdV escape from the endosome involves many different signaling pathways, proteins and conditions. Virions escaping to the cytosol require acidic pH [316, 317]. low pH changes the conformation of the viral capsid proteins, releasing partially disabled HAdV particles [317, 318]. Also, interactions with integrin αvβ5 are involved in the endosomal escape by triggering different signaling pathways, such as the phosphoinositide-3-OH kinase and Rho GTPase pathways [297, 319, 320]. The amphipathic helix of capsid protein VI (pVI) has also been suggested to be involved in escape from the endosome [104-106]. The escaped virion then interacts with dynein, which transports the virion to the nucleus by way of microtubule network [321, 322]. PVI helps the virion to move towards the nucleus along the microtubule networks through its PPxY motif [107]. At the nucleus, the HAdV DNA is delivered through the nuclear pore complex (NPC) in an active process. The delivery through NPC is dependent on importin alpha, which is also known to interact with pVI [109, 323]. Some other, cellular proteins have been suggested to be involved in NPC binding: nuclear pore CAN/Nup214, the chaperone, heat shock protein Hsc70, and histone H1 [321, 324, 325]. Internalization and transport of HAdV-2 and HAdV-5 to the nucleus is summarized in the (Figure 11).

Figure 11. Internalization and intracellular transportation of HAdV-2 and HAdV-5 from the membrane to the nuclear pore. The illustration was reprinted with permission from the publisher [326].
Genome structure

The HAdV genome consists of linear double-stranded DNA, 34-36 kbp in size, which can encode approximately 40 different proteins (Figure 12). The ends of the genome have 36-200 bp of inverted terminal repeats (ITRs), and each 5' end has a terminal protein (TP) bound to it [134]. These repeats function as origins of DNA replication [327]. The genome organization is conserved among the genera, and it involves three transcription units: the early transcription unit (E1A, E1B, E2, E3, and E4), the delayed-early transcription unit (IX, IVa2, and E2 late), and the late transcription unit, which give five families of late mRNA (L1-L5) [134, 327, 328]. All transcription units are transcribed by cellular RNA polymerase II except for one or two (depending on the species and type) virus-associated RNAs, which are transcribed by RNA polymerase III [329].

![Figure 12. Positions and directions of HAdVs genome with transcription units. The illustration was reprinted with permission from the publisher [330].](image-url)
Transcription unit function and HAdV replication

**The early transcription unit:** This unit has three main tasks: (i) to induce the host cell to enter the S-phase of the cell cycle, (ii) to modulate the cellular antiviral response, and (iii) to synthesize viral proteins needed for viral DNA replication [327]. E1A proteins bind to cell cycle regulators such as Rb-family proteins that activate the cell to enter the S-phase [331, 332]. The two E1A proteins function as transactivators, which means that they can activate both viral and cellular genes [331]. E1B encodes two proteins, E1B-55K and E1B-19K, which block cellular apoptosis but by different mechanism: E1B-55K through a p53-dependent pathway and E1B-19K through a p53-independent pathway [333, 334]. E2 encodes three proteins that are involved in viral replication: adenovirus polymerase (AdPol), DBP, and TP. E3 proteins are modulators of the host immune response. A recent study showed that the E3/49K protein of species D HAdVs suppresses leukocyte activation and effector functions [335]. The E4 region proteins are involved in many different functions such as transcriptional and translational regulation, nuclear export of viral mRNA, and inhibition of apoptosis [327].

**DNA replication:** This step starts 6-8 hours after infection (in HeLa cells). Replication starts at the first 50 bp of the ITRs, where a pre-terminal protein (pTP) binds and functions as a primer. The AdPol binds to pTP and forms a heterodimeric complex [336-338]. The AdPol functions both as a 5’ to 3’ DNA polymerase and as a 3’ to 5’ exonuclease, which enables proofreading during polymerization [339, 340]. The cellular transcription factors NFI and Oct-1 are recruited to this heterodimeric complex through DBP [341, 342]. These five proteins function as a pre-initiation complex, which starts the replication. Shortly after the start of replication, the AdPol separates from the pTP and chain elongation continues with AdPol, DBP, and cellular topoisomerase I [343, 344].
The delayed-early transcription unit: This unit consists of two genes encoding protein IX (pIX) and IVa2 (pIVa2), which are expressed immediately after initiation of DNA replication. pIX is a capsid protein that also activates transcription, but it does not have any specificity for the major late promoter (MLP) [327]. pIVa2, however, has dual functions as an activator of transcription from the MLP and is involved in packaging of the genome into the capsid [327]. These two proteins are expressed a little later than the early genes, and they are therefore called delayed early genes.

Late transcript unit: This unit encodes the capsid proteins, which are expressed after the onset of DNA replication. The expression is controlled by the MLP, and it encodes a single primary mRNA of 28 kbp, which is spliced into at least 20 distinct mRNAs. These mRNAs are grouped into 5 families L1-L5 [327]. The late transcription unit encodes the capsid proteins, which are required for assembly of new virions.

Assembly and release

The capsid proteins are assembled in the cytoplasm, where the hexon trimerizes with the help of the L4-encoded protein 100K [345]. The 100K protein have two functions; facilitate folding of the hexon monomer and support assembly of hexon trimers [346]. The formation of the penton occurs more slowly resulting in complex formation of penton base and fiber protein [347, 348]. Hexon and penton are then transported into the nucleus for further assembly. The import of hexon to the nucleus is mediated by pVI [109], but it is not known (as to the best of my knowledge) if pVI or other proteins also assist in import of pentons. Packaging of the genome into the capsid involves three proteins: IVa2, L1 52/55 proteins and L4 22kd protein [327, 349, 350]. Capsid and core proteins pVI, pVII, pVIII, µ, and TP are cleaved by the viral protease (an L3-encoded 23kd protein), which complete the assembly of an infectious virus particle [351]. The release of the virions is facilitated by the E3 11,6 kd protein also known as adenovirus death protein (ADP). The expression levels of ADP may determine if there will be a lytic of
persistent infection [352]. Excess of fibers interferes with intercellular
dimerization of CAR resulting in facilitated intercellular virion transport
[171].

**Treatment and vaccines**

Most HAdV infections are often asymptomatic or at least self-limited in
healthy individuals. Thus, the need to develop of antivirals against HAdVs
has not been of great interest. However, there are exceptions, as HAdVs
cause severe disease in immunocompromised patients, in patients with EKC,
and in military recruits with ARD [3]. The growing number of
immunocompromised patients due to AIDS and due to transplantations has
led to renewed interest in developing anti-HAdV drugs [58, 353, 354].

Cidofovir is a compound that mimics the nucleoside of cytosine, and it
thereby inhibits viral DNA polymerization [355, 356]. It is already approved
as a treatment for CMV retinitis in AIDS patients in the USA and the EU
[357]. Cidofovir has been evaluated as a treatment for EKC in many studies,
with different results. By using New Zealand white rabbits as a model for
ocular infections its effect has been studied on HAdVs from species C
(HAdV-1, HAdV-5 and HAdV-6) [358]. Unfortunately, this animal model
cannot be infected by EKC-causing HAdVs (HAdV-8, HAdV-19, and HAdV-
37), which make it difficult to evaluate new drug candidates *in vivo* against
these types. Unpublished data from a large study on humans in the USA
showed positive results from using cidofovir as a treatment for EKC.
Treatment of EKC with cidofovir both cleared the infection and prevented
spread to the other eye [58]. However, recent studies from Hawaii and
Europe have shown that cidofovir treatment may be associated with rare
cases of lachrymal canalicular blockage [58]. Cidofovir also appears to limit
to some extent systemic HAdV-related complications, and reduce the load of
HAdV DNA in blood of adult and pediatric bone marrow transplant patients
[359-362].
Another drug that has been tested against systemic HAdV infection is ribavirin. Ribavirin affects many different cellular and viral processes, and has been approved for systemic treatment of hepatitis C virus infection in combination of interferon α-2b. Ribavirin has been both successful [363-366] and unsuccessful [359, 367-369] as a treatment for systemic HAdV infection.

Zalcitabine is a nucleoside analog that is used for treatment for retroviruses infection. It has been shown to inhibit HAdV-2 infection both *in vitro* and *in vivo*, in pneumonia model [370].

The sulfated sialyl lipid NMSO3 has been shown to have antiviral activity against HAdVs [371]. It appears to inhibit viral absorption and has been shown to be effective against HAdV-2, HAdV-4, HAdV-8, and HAdV-37. NMSO3 is negatively charged, which may explain the inhibition of HAdV-8 and HAdV-37, which use SA as a cellular receptor. However, the inhibition mechanism of NMSO3 for HAdV-2 and HAdV-4 is not clear [371]. The main advantage of NMSO3 is that is does not show any cellular toxicity.

N-chlorotaurine is an antioxidant, which is produced by activated human granulocytes and monocytes and exhibit anti-inflammatory properties [372]. This molecule has been shown to inhibit HAdV-3, HAdV-4, HAdV-8, HAdV-19 and HAdV-37 infection of A549 cells [373]. N-chlorotaurine has been shown to be safe for topical administration to eyes of humans and rabbits, and to the ears of guinea-pig [374, 375]. It was found to efficiently abolish clinical signs of viral conjunctivitis in a small clinical trial in Austria [376]. This antiseptic-like compound may also be a promising drug against bacterial conjunctivitis [58].

The non-nucleoside and cobalt chelator compound doxovir has been shown to have activity against HAdVs. The exact mechanism of how this compound works is not fully understood. Doxovir was found to inhibit HAdV-5
conjunctivitis in a rabbit model. It was also active against HSV-1 infection in a rabbit keratitis model [377].

Large, multivalent SA-containing compounds inhibit infection of EKC-causing HAdVs in vitro [378-380]. These compounds/molecules inhibit EKC-causing HAdVs from binding to and infection of human corneal epithelial cells at µM concentrations. The mechanism of action is by aggregation of virions, thus preventing them from infecting other cells [378-380].

A high-throughput screening of a library of compounds and a series of optimization steps resulted in two antivirals called benzavir-1 and benzavir-2 [381-383]. By some unknown mechanism, these molecules appear to affect the viral DNA replication of different species of HAdV and HSV-1 and -2 [381-383].
Results and discussion

Paper I

The GD1α glycan is a cellular receptor for adenoviruses causing epidemic keratoconjunctivitis.


EKC is a contagious ocular disease that is mainly caused by three species-D HAdVs: HAdV-8, HAdV-19 and HAdV-37. These three HAdVs have been shown to use SA as a cellular receptor. To identify the SA-containing glycan structure(s) that function as cellular receptors for EKC-causing HAdVs, glycan array was performed by Consortium for Functional Glycomics (CFG). The HAdV-37 knob recognized among other glycans a disialylated glycan that is normally found on ganglioside GD1α. Soluble GD1α glycans and GD1α glycan-specific antibodies (clone EM9) inhibited HAdV-37 from binding to and infecting HCE cells. To further investigate whether the GD1α ganglioside itself functioned as cellular receptor, a set of HAdV-37 virion competition binding experiments were performed using i) SA-deficient Lec2 cells and Lec2 cells containing GD1α ganglioside, ii) GD1α-containing liposomes, and iii) P4, resulting in deficient de novo ganglioside biosynthesis. Surprisingly, neither of these treatments inhibited HAdV-37 binding as compared to control samples. However, the results from three other experiments suggested that the receptor used by HAdV-37 is rather constituted by a protein that carry glycan motifs identical to or resembling the glycan in the GD1α ganglioside: i) protease treatment of HCE cells prior to virion binding, ii), pretreatment of HCE cells (prior to virion binding) with benzyl-α-GalNAc, which inhibits de novo protein O-glycosylation via serine and threonine, and iii) an HAdV-37 knob overlay protein blotting experiment, which showed that the knob can interact with a number of plasma membrane proteins purified from HCE cells. We could not see any effect on
HAdV-37 binding to or infection of HCE cells, pre-treated with N-glycosidase F (removing N-glycans). From these results, we concluded that the GD1α glycan is used as receptor and is attached to a cell membrane protein through an O-glycosidic bond. Next, computer-based modeling was done, suggesting that the two terminal SAs of GD1α dock into two out three identical pockets in the trimeric HAdV-37 knobs. This was confirmed by saturation transfer difference (STD) NMR and also by X-ray crystallography. Structural data pointed out three residues to be of more important in the interaction: tyrosine (Tyr) 312, proline (Pro) 317, and lysine (Lys) 345, which was in agreement with previous structural studies of HAdV-37 knob and sialyllactose [93]. Lys345 was suggested to be critical for interaction with GD1α glycan by in silico substitution of Lys345 by alanine (Ala) and flow cytometry studies with wild type HAdV-37 knob and a Lys345Ala mutant knob confirmed that Lys345 is important for sialic acid-dependent binding. Surface plasmon resonance (SPR) was used to analyze the affinity between HAdV-37 knob and GD1α glycan. The obtained data suggested two different interactions: one with of lower affinity (265 μM) and one of relatively high affinity (19 μM). To confirm that all the EKC-causing HAdVs interact with GD1α glycans, fiber knobs from HAdV-5, HAdV-8, HAdV-9, HAdV-19, and HAdV-37, were preincubated with GD1α glycan, SA, or galactose before quantifying knob binding to HCE cells by means of flow cytometry. The GD1α glycan inhibited the binding of all knobs from species-D types but not of HAdV-5 (species C). In summary, we identified a glycan corresponding to the glycan motif of GD1α ganglioside to be a cellular receptor for EKC-causing HAdVs.

This article answered many of our questions regarding the interaction of EKC-causing HAdVs and corneal cells. However, there are questions that need to be answered to understand the HAdV-37:GD1α glycan interaction better. The first would be to determine whether the HAdV-37 knob can interact with other glycan structures, by using additional glycan arrays. Except for the glycan array we used with the help of the CFG other groups have developed similar but more powerful arrays. Blixt and co-workers have
developed a glycan array with 200 synthetic and natural glycans from glycoproteins and glycolipids [384]. Song and co-workers have developed a glycan array containing 77 sialylated glycans [385]. Another glycan array with a different set-up has helped to identify a receptor for B-lymphotropic polyoma virus and a co-receptor for serotype 1 reovirus [386, 387]. These glycan arrays have been tested with different viruses, lectins, and other carbohydrate ligands. If we had used these additional glycan arrays, we might have gained even better understanding of whether the GD1a glycan is a unique receptor for EKC-causing HAdVs.

We also found that the ganglioside itself did not function as a cellular receptor for EKC-causing HAdVs. However, we do not know yet whether the GD1a glycan can be found on glycoproteins through O-glycosidic bonds. Many groups are trying to identify glycans on glycoproteins. In one study, a highly glycosylated protein DMBT1 was found in human tears. By using LC-MS, they detected glycans that contained two terminal SAs but with different structure to the GD1a glycan [388]. This indicates that glycan structures similar to the GD1a glycan may also be found on proteins. In a recent paper, a method for identification of glycan structure and glycan sites was developed [389]. A protein family of particular interest may be mucins, which are rich in O-linked glycans and are abundant on ocular cell surfaces. Using this method (mass spectroscopy-based), it may be possible to determine if GD1a glycans or GD1a-like glycans are present on mucins, and to support the usage of GD1a glycans as receptors for EKC-causing HAdVs.

Another way to investigate the importance of glycans for EKC-causing HAdVs would be to knock down cellular glycosyltransferases. These knockdowns could be done either temporarily with siRNAs or by making stable cell lines with shRNA or CRISPR/Cas that do not express the specific glycosyltransferase. First, we would need to identify the glycosyltransferases that are unique to HCE cells or corneal tissue. Knockdown of unique glycosyltransferases could provide evidence of whether glycosylation is a tropism-dependent feature of EKC-causing HAdVs.
In this study, we used only corneal epithelial cells. EKC-causing HAdVs cause inflammation in both corneal and conjunctival tissue. It would therefore be interesting to determine whether GD1a glycans serve as receptors in conjunctival tissue as well. Here, human conjunctival HCO597 cells may be a good model cell line to study receptors used by EKC-causing HAdVs, since they can be grown in multilayers [390, 391].

We mutated Lys345 to Ala in the fiber knob of HAdV-37, which turned out to have a huge effect on the SA interaction. From this it would be of interest to make the same mutation in the whole virion. This because we cannot exclude the possibility that the results obtained may come from a conformation change in the HAdV-37 knob. Structural studies such as X-ray crystallography could be used to compare the conformations of the mutant knob and the wild type knob. Other aa:s are important for interaction with SA, Tyr312 and Pro317. It would also be interesting to determine what effect mutations of these aa:s would have on the interaction of SA. By mutating these aa:s in virions, we could find out whether these mutations are important for infection of HCE cells.

Finally, the GD1a could be a receptor candidate for several other species-D HAdVs. Flow cytometry analysis with fiber knobs from HAdV-5, HAdV-8, HAdV-9, HAdV-19, and HAdV-37 preincubated with GD1a revealed that non-EKC causing HAdV-9 may also use GD1a glycan as receptor. Since many species-D HAdV types have sequence homology with EKC-causing HAdVs, they may have the ability to bind SA or GD1a glycan. The GD1a glycan could therefore be a receptor for other species-D HAdVs.
Papers II and III

Paper II:

A potent trivalent sialic acid inhibitor of adenovirus type 37 infection of human corneal cells.
Spjut S, Qian W, Bauer J, Storm R, Frängsmyr L, Stehle T, Arnberg N, Elofsson M.

Paper III:

Triazole linker-based trivalent sialic acid inhibitors of adenovirus type 37 infection of human corneal epithelial cells.
Rémi Caraballo, Michael Saleeb, Johannes Bauer, Antonio-Manuel Liaci, Naresh Chandra, Rickard J Storm, Lars Frängsmyr, Weixing Qian, Thilo Stehle, Niklas Arnberg, and Mikael Elofsson (Submitted).

EKC is a contagious and severe ocular disease, which is estimated to cause 20-40 million cases every year worldwide. EKC is characterized by keratitis and conjunctivitis together with pain, tearing, edema, and reduced vision that may last for months or even years. Currently, there is no available treatment for EKC. Based on the interaction between EKC-causing HAdVs and GD1a glycans, compounds with three or more terminal SAs were designed that could function as antivirals for EKC.

In Paper II, three molecules were synthesized, all containing three components: terminal SA, linkers, and different center-molecules. This set-up generated thee molecules: two compounds with three terminal SAs (MEo322 and MEo323) and one compound with four terminal SAs (MEo324). The ability of these molecules to inhibit HAdV-37 binding to and infection of HCE cells were compared with SA and multivalent-SA, which are known to inhibit HAdV-37 binding to cells [378, 379] as controls. MEo322 proved to be the most efficient inhibitor of HAdV-37 binding to
HCE cells and was equally efficient as multivalent SA in inhibiting binding of HAdV-37 to HCE cells. In infection experiments ME0322 inhibited HAdV-37 infection of HCE cells, with an IC₅₀ of 0.38 µM, which is a lower concentration than any previously tested compound. The affinity between HAdV-37 knob and ME0322 was measured with SPR to be 14 µM, which is stronger than the affinity measured between GD1a glycan and HAdV-37 knob. The HAdV-37 knob:ME0322 interaction was also investigated with X-ray crystallography. The interaction studies showed that the three SAs of ME0322 interacted with the same aa:s as sialyllactose and GD1a.

In Paper III two new generations of trivalent-SA were synthesized. In the first generation the linker was exchange from that of ME0322 to a triazole ring, and synthesized with different lengths, 11a and 11b. The same linkers were used but with N-acyl-SA analogs resulting in compounds 12a and 12b. These four compounds were biologically evaluated in binding and infection experiments and compared with ME0322, SA, and GD1a glycan as controls. The results showed that 11a and 11b were the most efficient in inhibiting HAdV-37 binding and infection of HCE cells. 11b was the best inhibitor of HAdV-37 binding (IC₅₀ of 107 nM) and infection (IC₅₀ of 54 nM) as compared to ME0322 with an IC₅₀ of 3.2 µM in binding and of 408 nM in infection. However, these results did not give any conclusive information on the importance of the linker lengths, a and b. Based on these results, another generation was designed with the same linker and lengths as 11 but with the triazole ring moved closer to the SA. These were called compounds 17a and 17b. They were also evaluated in binding and infection experiments with ME0322 as control. Compound 17a proved to be even more efficient in inhibiting HAdV-37 binding and infection than the best compound in the first generation (11b). With an IC₅₀ of 1.4 nM in binding and 2.9 nM in infection it was more than 140 times more efficient than ME322 in inhibiting HAdV-37 binding to and infection of HCE. However, it is unclear why 17b showed lower efficiency in inhibiting HAdV-37 binding and infection of HCE cells than compounds 11a, 11b, and 17a. SPR experiments showed that the HAdV-37 knob bound with the strongest affinity to
compound 17a, with a $K_D$ of 9.5 µM. Structural studies of all compounds showed similar interactions to the compounds in paper II and in previous studies [93, 157]. From these studies we identified a new promising drug candidate for treatment of HAdV-37.

In these studies, SA-containing molecules were modified to optimize their inhibitory capacity. Compound 17a, which inhibited HAdV-37 binding and infection most efficiently, is a water-soluble molecule that could easily be added to non-toxic solutions for treatment of EKC.

In none of these studies we evaluated the inhibitory effect of trivalent-SAs on all six EKC-causing HAdVs. Today, we can express fiber knobs from all six EKC-causing HAdVs. By preincubating all six fiber knobs with trivalent-SAs before quantifying binding to HCE cells with flow cytometry, their inhibitory efficiency against all EKC-causing HAdVs could be tested. Hopefully, trivalent-SAs are as efficient attachment inhibitor against all EKC-causing HAdVs.

SA is a cellular receptor for many other viruses; one group of particular interest is influenza A virus. Influenza A virus also use SA as cellular receptors, which is mediated through the trimeric protein, hemagglutatinin (HA) [392]. Like HAdV-37, HAs have three apical, SA-binding sites, which could be reached by trivalent-SAs. The HA:trivalent-SA interaction could also be evaluated by SPR or ELISA, or for competition of virus binding and infection. A benefit of working with influenza A virus is that there are several available animal models, so if the trivalent-SA showed any effect/interaction with influenza A virus, there would be many animal models available to evaluate the in vivo effect of trivalent-SA. Today, there are no good animal models to study EKC-causing HAdVs infection or drug evaluation. There is one mouse model, C5BL/6J; here, the EKC-causing HAdVs are injected into the eye to study viral pathogenesis [393]. However, this model resembles the human conditions poorly since in this model the injected virions aggregate and are cleared relatively soon from the mouse stroma [394].
Other models of potential interest may be multi-cell layer structures formed by HCE and/or HCo597 cells [390, 391, 395]. Such systems have been used to study absorption ability of eye-specific drugs in ocular-like tissue [396, 397], and could be used to study toxicity of trivalent-SA.

**Paper IV**

**Involvement of corneal integrins during infection of human adenovirus type 37.**

Rickard J Storm, Lars Nygård Skalman, Lars Frängsmyr, Mona Lindström, Anandi Rajan, Richard Lundmark, Fatima Pedrosa Domellöf, and Niklas Arnberg *(Manuscript).*

All HAdVs except HAdV-40 and HAdV-41 have RGD motif in the penton base. This motif mediates interaction with cellular integrins for efficient entry and escape from the endosome. Integrins are heterodimeric proteins consisting of α- and β-subunits, which can form 24 unique complexes involved in many cellular events. In a previous ELISA experiment, HAdV-37 was shown to bind strongly to αVβ5, but according to the literature it is unclear whether αVβ5 is expressed in corneal epithelium. For this reason, we wanted to investigate which integrins are involved in HAdV-37 infection of, and binding to HCE cells. First, we characterized the expression of integrins in HCE cells with flow cytometry, and in human corneal sections with immunohistochemistry. From these experiments, we confirmed the result from a previous proteomic study [266] suggesting that HCE cells and corneal sections express α2, α3, α6, αV, β1, and β4. We could not find any expression of β3 or β5 suggesting that other integrins than αVβ3 or αVβ5 are involved in infection of corneal epithelium. To identify the specific integrin subunit(s) used for infection or binding, we used a number of Chinese hamster ovary cells (CHO) and human prostate cells (PC3N), transfected with cDNA:s encoding human integrins α2β1, α3, α5, αV (CHO), and α6 (PC3N). Two integrins, α3 and αV enhanced the infection by HAdV-37 but did not affect the binding by HAdV-37. These results were supported by data from
experiments where HCE cells were pretreated with anti integrin-specific antibodies. We also found that antibodies against the β1 subunit inhibited HAdV-37 infection of HCE cells. By pretreating HCE cells with integrin-specific siRNA, in combination with αV-specific antibodies, HAdV-37 infection of HCE cells was reduced significantly. Co-localization studies with Alexa Fluor-555 labeled HAdV-37 and antibody staining for α3 or αV integrins showed 20-40% virions co-localization with either α3 or αV at 30 min on ice or at 15 min or 30 min at 37°C. Preincubation of HCE cells with the integrin-binding, RGD-containing ligands vitronectin, fibronectin, and laminin 511 inhibited (vitronectin and laminin 511) or enhanced (fibronectin) HAdV-37 infection of and binding to HCE cells. To investigate the importance of the RGD motif further, we synthesized peptides that mimicked the RGD sequence in HAdV-37 penton base. As control peptides, we used the corresponding peptide where the RGD motif was exchanged for a triple Ala and a short peptide (GRGDSP) that has been shown to inhibit HAdV infection [75]. The RGD-containing peptides (but not the triple Ala control peptide) inhibited HAdV-37 infection of HCE cells by 50-65%. From this study, we concluded that α3 and αV integrins are important for HAdV-37 infection of, but not binding to HCE cells.

Our data suggested that HAdV-37 uses α3 and αV integrins to infect human corneal epithelial cells. However, the siRNA experiment should be performed in a different set-up. In this study, we performed knockdown in each well to simplify large screening. But it is more difficult to correlate the data with the controls (cell-based ELISA); the knockdown in one well may not be the same in another well. In a new experimental set-up, I want to perform the knockdown in 6-well plate instead of 96-well plates. The benefit of this approach is that all cells would be treated under same conditions. So when the siRNA-treated cells from 6-well plates are seeded out for analysis/infection, the knockdown control can be correlated to those cells that are infected by HAdV-37. Another benefit is that we could treat the cells for a longer time compared to the set-up in the manuscript, to optimize integrin knockdown.
As discussed in the manuscript, to fully understand the role of integrins during EKC infection, the integrin expression should be investigated in limbal cells and conjunctival cells. αVβ5 has been shown to interact strongly with HAdV-37 [293], but our data clearly showed that HCE cells and corneal tissues do not express αVβ5. Thus, it would be of interest to investigate the expression of αVβ5 in cells of limbal origin (primary human corneolimbal epithelial cells, HCEC) and cells of conjunctival origin (HC0597) [390, 398]. As shown in [398], αVβ5 integrins are expressed in HCEC cells at low levels, which might suggest different infection mechanism in limbal cells compare to corneal cells. However, this should to be tested before we can draw any conclusions.

As discussed in paper II and paper III, the HCE cells that we used can be grown in multilayer by air liquid interface. It would be interesting to see how integrin knockdowns interfere with HAdV-37 infection of multilayer HCE cells. These modifications of HCE cells could confirm the exact importance of α3 and αV integrins in HAdV-37 infection. However, since integrins are involved in cell-cell contacts, there is a risk that reduced integrin expression may interfere with the structural integrity of the multilayer.

In this study, we did not have the possibility to test the direct interaction between HAdV-37 penton bases and integrins. It would have been interesting to use soluble penton base protein to analyze the function of integrins in various assays. For example we could i) measure the affinity between integrins and the penton base in SPR or ELISA, ii) binding of soluble penton base proteins to HCE cells in the presences of antibodies or after pre-treatment with integrin siRNAs, or iii) compete with infection using soluble penton base proteins. This could give us valuable information about the virion:integrin interaction.
Concluding remarks

EKC is a severe and contagious ocular disease infecting 20-40 million individuals per year worldwide, and the disease is associated with huge socioeconomic costs. Due to the lack of efficient treatment against EKC, we started to search for cellular receptors used by EKC-causing HAdVs in order to identify new drug targets. We identified a glycan structure corresponding to the GD1a ganglioside, which interact with the knob domain of the viral fiber by its two terminal SAs. After a series of experiments we concluded that the ganglioside itself did not function as a cellular receptor, but the receptor is instead constituted by one or more membrane bound proteins carrying GD1a or very similar glycans via O-glycosidic bonds. SPR analysis demonstrated that the interaction between the knob and the glycan was a relatively strong protein-carbohydrate interaction ($K_D = 19 \mu M$). These findings enables the way for design and development of molecules with three or more terminal SAs, assumed to bind even stronger and prevent binding and infection even more efficient.

Guided by data from structure-function analysis, ten compounds with three or four terminal SAs were designed and evaluated in binding and infection experiments. Compound 17a efficiently inhibited HAdV-37 binding to (IC50 = 1.4 nM) and infection (IC50 = 2.9nM) of HCE, which was more than 1000 times more efficient than the GD1a glycan itself. Thus, these compounds show potential for topical treatment against EKC.

Finally, we investigated the importance of integrins during HAdV-37 binding to and infection of the HCE cells. First we established that the HCE cell line was a suitable model also for these studies, since this cell line exhibited similar integrin expression levels as primary corneal epithelial tissue. By a series of competition binding and infection experiments, including the usage of integrin-specific antibodies, ii) integrin-recognizing ligands, and iii) RGD-containing peptides in combination siRNA-based down regulation of
integrin expression and co-localization studies we suggested that $\alpha_3$ and $\alpha V$ integrins are important for HAdV-37 infection of HCE cells but not for binding to these.

In summary, I have identified an attachment receptor GD1a, and co-receptors (integrins) that are used by EKC-causing adenoviruses. These findings contribute with further insight into the biology of adenoviruses and also show that receptor-ligand interactions may be suitable targets for drug development such as optimization of trivalent-SA.
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