Human adenoviruses: new bioassays for antiviral screening and CD46 interaction

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To my family

Table of Contents

Abstract

Adenoviruses are common pathogens all over the world. The majority of the population has at some point been infected with an adenovirus. Although severe disease can occur in otherwise healthy individuals an adenovirus infection is most commonly self limited in these cases. For immunocompromised individuals however, adenoviruses can be life-threatening pathogens capable of causing disseminated disease and multiple organ failure. Still there is no approved drug specific for treatment of adenovirus infections. We have addressed this using a unique whole cell viral replication reporter gene assay to screen small organic molecules for anti-adenoviral effect. This RCAd11pGFP-vector based assay allowed screening without any preconceived idea of the mechanism for adenovirus inhibition. As a result of the screening campaign 2-[[2-(benzoylamino)benzoyl]amino]-benzoic acid turned out to be a potent inhibitor of adenoviral replication. To establish a structure-activity relationship a number of analogs were synthesized and evaluated for their anti-adenoviral effect. The carboxylic acid moiety of the molecule was important for efficient inhibition of adenovirus replication.

 There are 54 adenovirus types characterized today and these are divided into seven species, A-G. The receptors used by species B and other adenoviruses are not fully characterized. CD46 is a complement regulatory molecule suggested to be used by all species B types and some species D types but this is not established. We have designed a new bioassay for assessment of the interaction between adenoviruses and CD46 and investigated the CD46-binding capacity of adenovirus types indicated to interact with CD46. We concluded that Ad11p, Ad34, Ad35, and Ad50 clearly bind CD46 specifically, whereas Ad3p, Ad7p, Ad14, and Ad37 do not.

CD46 is expressed on all human nucleated cells and serves as a receptor for a number of different bacteria and viruses. Downregulation of CD46 on the cell surface occurs upon binding by some of these pathogens. We show that

early in infection Ad11p virions downregulate CD46 upon binding to a much higher extent than the complement regulatory molecules CD55 and CD59.

These findings may lead to a better understanding of the pathogenesis of adenoviruses in general and species B adenoviruses in particular and hopefully we have discovered a molecule that can be the basis for development of new anti-adenoviral drugs.

Summary in Swedish – Populärvetenskaplig sammanfattning på svenska

Adenovirus är en mycket vanlig orsak till infektion hos människa. Det finns mer än 50 olika typer av adenovirus som infekterar människa, dessa är indelade i sju grupper, A-G. En adenovirusinfektion kan vara symptomfri men drabbar oftast luftvägar, ögon och mage-tarm, infektion i urinvägar och tonsiller kan också förekomma. Infektionen går vanligen över utan problem men hos individer med nedsatt immunförsvar kan en adenovirusinfektion vara direkt livshotande. Trots detta är inte mekanismen för virus inträde i cellen helt förstådd och det finns inget läkemedel som är specifikt för behandling av adenovirusinfektioner. Detta är vad vi har fokuserat på.

Genom att använda ett modifierat virus som uttrycker ett fluorescerande protein, GFP, har vi studerat ett s.k. kemiskt bibliotek innehållande 9800 små organiska molekyler för att utvärdera deras eventuella antivirala effekt. På detta sätt har vi identifierat en molekyl, 2-[[2-(bensoylamino)bensoyl]amino]-bensoesyra, som i cellkultur visar tydliga antivirala egenskaper.

Vi har också på ett nytt sätt använt en etablerad metod för att studera vilka adenovirus i grupp B som binder till CD46. CD46 är ett protein som är en del av kroppens försvar mot infektioner. Det finns på ytan av alla celler utom röda blodkroppar och fungerar även som receptor för flera olika virus och bakterier. Vi kan konstatera att flera adenovirustyper i grupp B binder CD46 mycket starkt medan andra binder svagare och vissa inte alls.

List of papers

I. Adenovirus interactions with CD46 on transgenic mouse erythrocytes
Andersson E.K., Mei Y.F., Wadell G.
Virology 2010, 402 (1): 20-25.

II. Adenovirus 11p downregulates CD46 early in infection
Gustafsson, D., Andersson, E.K., Hu, Y.L., Lindman, K., Marttila, M., Strand, M., Wang, L., Mei, Y.F.
Virology 2010, 405 (2): 474-482.

III. Small molecule screening using a whole cell viral replication reporter gene assay identifies 2-[[2-(benzoylamino)benzoyl]amino]-benzoic acid as a novel anti-adenoviral compound
Andersson E.K, Strand M., Edlund K., Lindman K., Enquist P.A., Spjut S., Allard A., Elofsson M., Mei Y.F., Wadell G.
Antimicrobial Agents and Chemotherapy 2010, 54 (9): 3871-3877.

IV. Synthesis, biological evaluation, and structure-activity relationships of 2-[[2-benzoylamino)benzoyl]amino]-benzoic acid analogs as inhibitors of intracellular adenoviral replication.
Öberg, C.T., Andersson, E.K., Strand, M., Edlund, K., Tran, N.P.N., Mei, Y.F., Wadell, G., Elofsson, M.
Manuscript.

Abbreviations

aa	Aminoacid
Ad	Adenovirus
ADP	Adenovirus death protein
Adpol	Adenovirus DNA polymerase
AIDS	Acquired immune deficiency syndrome
CAR	Coxsackie and adenovirus receptor
CCPs	Complement control protein repeats
CCR5	Chemokine receptor
CMV	Cytomegalovirus
DBP	DNA binding protein
f	Fiber
fk	Fiber knob
GON	Group of nine
HA	Hemagglutination
HIV	Human immunodeficiency virus
HSPG	Heparan sulphate proteoglycans
HSV	Herpes simplex virus
ITR	Inverted terminal repeat
kDa	Kilodalton
MCP	Membrane cofactor protein
MLP	Major late promoter
MHC	Major histocompatibility complex
mRNA	Messenger RNA
NLS	Nuclear localization signal
NPC	Nuclear pore complex
NRTI	Nucleoside/nucleotide reverse transcriptase inhibitors
NNRTI	Non-nucleoside reverse transcriptase inhibitors
ORF	Open reading frame
Pb	Penton base
RCA	Replication competent adenovirus
RGD	Arginine-Glycine-Aspartic acid
RID	Receptor internalization and degradation complex
sBAR	Species B adenovirus receptor
sB2AR	Species B2 adenovirus receptor
SCR	Short consensur repeat
STP	Serine-Threonine-Proline
TP	Terminal protein
VA-RNA	Virus associated RNA
VZV	Varicella zoster virus

Introduction

History of adenoviruses

Adenoviruses were first isolated and cultured from 33 surgically removed adenoids by Rowe and colleagues in 1953 (Rowe & Huebner, 1953). About the same time a similar agent was isolated from military personnel with varieties of respiratory disease. This agent was at that time referred to as adenoid degenerating agent (A.D.) (Hilleman & Werner, 1954). In 1956 the name adenoviruses was proposed (Enders & Bell, 1956). Since then adenoviruses have been isolated from most human organ systems and also from virtually all vertebrates (Russell & Benkö, 1999; Wadell *et al.*, 1999).

Taxonomy and classification of adenoviruses

Adenoviruses have so far been isolated only from vertebrates and belong to the virus family *Adenoviridae*. This family is divided into four genera (and a fifth suggested genus) according to the International Committee on Taxonomy of Viruses (ICTV). The genera are *Mastadenovirus* infecting mainly mammals, *Aviadenovirus* infecting mainly birds and *Atadenovirus* and *Siadenovirus* with a broader host range. *Atadenovirus* are named after the high A+T content of their genomes and infect ruminant, avian, reptilian as well as marsupial hosts. *Siadenovirus* infect birds and frogs. The fifth suggested genus is *Ichtadenovirus* including adenoviruses isolated from fish (Benkö *et al.*, 2002; Kovács *et al.*, 2003). The human adenovirus types all belong to the *Mastadenovirus* genus and are grouped into seven species. Species designation is based on a number of characteristics including calculated phylogenetic distance, DNA hybridization, oncogenicity in rodents, host range, cross-neutralization, G+C contents of the genome and genetic organization of the E3 region among others (Benkö, 2005; Wadell *et al.*, 1980). Species B adenoviruses are further classified into two clusters, B1 and B2, based on their DNA restriction patterns (Wadell *et al.*, 1980). The 52 distinct types of adenoviruses, formerly referred to as serotypes, are

separated on the basis of genome analysis and of resistance to neutralization by heterologous hyperimmune sera with a ratio of homologous to heterologous neutralization greater than 1:16 (Wadell *et al.*, 1980).

Species A

Species A includes Ad12, Ad18, and Ad31.

Species B

Species B is further subdivided into species B1 and B2. Species B1 includes Ad3, Ad7, Ad16, Ad21, and Ad50. Species B2 includes Ad11, Ad14, Ad34, and Ad35.

Species C

Species C consists of Ad1, Ad2, Ad5, and Ad6.

Species D

Species D is the largest group of human adenoviruses containing 32 types. In this group Ad8-10, Ad13, Ad15, Ad17, Ad19-20, Ad22-30, Ad32-33, Ad36-39, Ad42-49, and Ad51 are included.

Species E

Species E contains only one human type, Ad4.

Species F

Species F includes the enteric adenovirus types Ad40 and Ad41.

Species G

Species G is the latest addition to the human adenoviruses and includes one single type, Ad52 (Jones *et al.*, 2007).

Additional adenovirus types, Ad53 and Ad54, causing epidemic keratoconjunctivitis have been identified and will in the near future most likely be established as species D types (Ishiko & Aoki, 2009; Ishiko *et al.*, 2008; Walsh *et al.*, 2009). A new species B type, Ad55, causing acute respiratory disease has recently also been suggested (Walsh *et al.*, 2010).

Biology

Structure of the virion

Adenoviruses are 70-90 nm in diameter, non-enveloped and exhibit icosahedral symmetry (Horne *et al.*, 1959). The virus particle consists of the outer protein capsid and the inner protein-DNA containing core structure. Around 13 % of the complete particle is constituted of dsDNA and most of the remaining part is protein. The majority of the mature virion proteins are numbered by Roman numerals according to their migration on SDS gels (Maizel *et al.*, 1968). Additional proteins are μ, the terminal protein and the viral protease.

The capsid is in principal composed of 240 trimeric hexon capsomeres (pII) that constitute the 20 triangular faces and 12 penton capsomers at each of the 12 vertices. Each penton consists of a pentameric base (pIII) with one or two protruding trimeric fiber proteins (pIV) non-covalently bound (Figure 1) (Ginsberg *et al.*, 1966; Kidd *et al.*, 1993; Schoehn *et al.*, 1996; van Oostrum & Burnett, 1985; Yeh *et al.*, 1994). It should be noted that most data stem from research on Ad2 and Ad5.
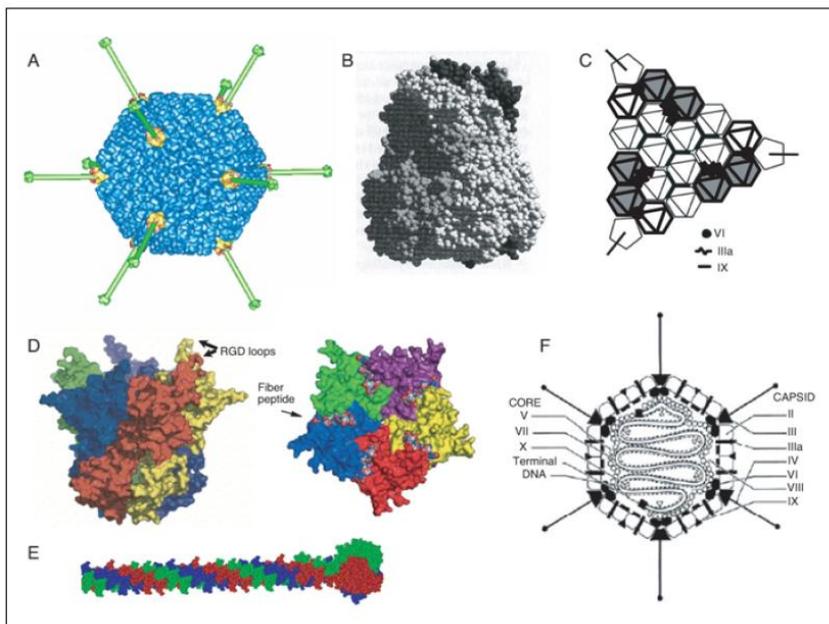
Figure 1. Structure of the Ad virion. A. Computer reconstruction of a cryo-microscopy image of Ad2. B. Model of the Ad5 hexon trimer. C. Model of the locations of polypeptides in one facet of the icosahedrons. D. Penton base as seen from the side (left) and from the top (right). E. Model of the Ad2 fiber shaft and knob. F. Schematic illustration of the Ad virion. Reproduced with permission from (Berk, 2007) Fields Virology 5th ed

Major structural proteins
Hexon (pII)

The hexon protein is the major component of the virus capsid, 720 hexon monomers form 240 homotrimers that constitutes the 20 triangular facets of the icosahedral capsid (Figure 1) (Ginsberg *et al.*, 1966; van Oostrum & Burnett, 1985). Each monomer is composed of two β-barrel domains and three extended loops. The six β-barrels of the trimer form a hexagonal base with a central cavity and the loops at the top form the outer surface of the virus particle. These hypervariable loops generate the type-specific antigenic epitopes (Athappily *et al.*, 1994; Rux & Burnett, 2000). The hexons associated with the pentons at each vertex are designated peripentonal

hexons, whereas the remaining hexons are termed 'groups of nine' (GONs) and make up the centre of the capsid facets (Burnett, 1985; van Oostrum *et al.*, 1987).

Penton base (pIII)

The penton base is a pentamer of pIII and lies at each of the 12 vertices of the virus capsid. The pentameric structure has a large central cavity where the trimeric fiber will bind (Stewart *et al.*, 1993; Zubieta *et al.*, 2005). Located on top of the pentamer subunits are loops containing the Arg-Gly-Asp (RGD) motif (Figure 1). The RGD motif mediates interaction with cell surface integrins during adenovirus internalization (Schoehn *et al.*, 1996; Wickham *et al.*, 1993). The RGD motif is conserved in all human adenovirus types with the exception of Ad40 and Ad41 (Albinsson & Kidd, 1999; Davison *et al.*, 1993).

Fiber (pIV)

The adenovirus fiber is a trimeric protein with an N-terminal tail, a central shaft and a C-terminal knob (Figure 1) (Green *et al.*, 1983). Crystallisation of the Ad2 fiber-penton base-complex has shown that a conserved amino acid sequence in the N-terminal tail of the fiber, FNPVYPY, is responsible for binding to the cavity of the penton base by salt bridges and hydrogen bonds (Zubieta *et al.*, 2005). The amino terminus of the fiber protein also encodes the nuclear localization signal (Hong & Engler, 1991).

The length of the fiber varies between adenovirus types due to different numbers of sequence repeats of 15-20 amino acids in the central fiber shaft. Ad3 has the shortest fiber with 6 repeats and Ad2 and Ad5 the longest with 22 repeats (Green *et al.*, 1983; Signäs *et al.*, 1985). There can be disruptions in the shaft repeats allowing hinge regions to be formed (Chroboczek *et al.*, 1995). The topology of the fiber shaft is a triple β-spiral fold of three intertwined polypeptide chains (van Raaij *et al.*, 1999b; Xia *et al.*, 1994). The most variable sequence of the fiber shaft is the exposed loop, in which 4, 5, or 12 residue turns have been observed (Chroboczek *et al.*, 1995).

The trimeric knob domain is responsible for the virus binding to the cellular receptor (Arnberg *et al.*, 2000; Bergelson *et al.*, 1997; Louis *et al.*, 1994; Mei & Wadell, 1996). Each knob monomer forms an eight-stranded anti-parallel β-sheet and contains a conserved trimerisation motif (Hong & Engler, 1996). Three monomers engage to form the propeller-like C-terminal knob domain (Figure 2) (van Raaij *et al.*, 1999a). Loops designated DG, HI, and AB are protruding from the knob (Xia *et al.*, 1994). For Ad12 it was shown that interaction with the cellular receptor CAR (coxsackievirus-adenovirus receptor) occurs via surface loops at the monomer interfaces (Bewley *et al.*, 1999). Crystallisation of Ad11p fiber knob with CD46 has revealed that this interaction also occurs via surface loops in the monomer interface areas on the side of the knob (Persson *et al.*, 2007). The sialic acid binding site of Ad19 and Ad37 is located on the very top of the fiber knob (Burmeister *et al.*, 2004).
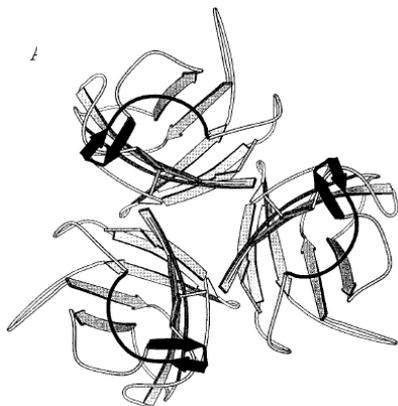

Figure 2. Top view of Ad5 fiber knob. From (Kirby *et al.*, 1999). Reproduced with permission from the American Society for Microbiology.

Minor structural proteins

pIIIa

The monomeric pIIIa is situated below the penton base at the interface of two neighbouring capsid facets where it interacts with the hexamers and is believed to stabilize the structure. It extends from the exterior to the interior of the capsid (Saban *et al.*, 2006; Stewart *et al.*, 1993). pIIIa is synthesized as a larger precursor protein of 67 kDa that during virion maturation is cleaved to a 63.5 kDa protein (Vellinga *et al.*, 2005). Features of unknown biological significance are established for pIIIa, including phosphorylation of at least part of the molecules and interaction with pVII as detected by co-immunoprecipitation (Boudin *et al.*, 1980; Stewart *et al.*, 1993). pIIIa also plays a role in the process of viral assembly and possibly in late viral protein synthesis (Boudin *et al.*, 1980; Molin *et al.*, 2002).

pVI

Mature pVI is 22 kDa and cleaved from a larger precursor protein. The protein is positioned on the inner surface of the capsid where it presumably connects two neighbouring hexons and may interact directly with the DNA (Figure 1) (Stewart *et al.*, 1993; Vellinga *et al.*, 2005). In the infection cycle pVI appears to aid escape of the viral particle from the endosome by inducing a pH-independent disruption of the endosomal membrane (Wiethoff *et al.*, 2005). pVI has another important function in the transport of hexons from the cytoplasm to the nucleus. The precursor of pVI links the hexon to the importin α/β-dependent nuclear import machinery. The nuclear localisation signals are located in a C-terminal segment of the precursor protein that is proteolytically cleaved during maturation (Wodrich *et al.*, 2003).

pVIII

pVIII is a dimeric protein that interacts with hexons of adjacent facets at its position at the inner surface of the triangular facets (Saban *et al.*, 2006; Stewart *et al.*, 1993). Like other adenovirus proteins it is synthesized as a

larger precursor and cleaved to the mature form. pVIII may be involved in the structural stability of the virion (Liu *et al.*, 1985).

pIX

The 14.3 kDa pIX is unlike the other minor capsid proteins unique to the *Mastadenovirus* genus (Vellinga *et al.*, 2005). In the centre of each capsid facet nine hexon capsomers form together with four trimers of pIX the so called GONs. In the current model of the adenovirus structure pIX is located between the hexons in the GON where it is believed to stabilize the capsid facets (Stewart *et al.*, 1993; van Oostrum & Burnett, 1985). This model is widely accepted but recent studies of capsid structure suggest alternative positions for pIX (Marsh *et al.*, 2006). Protein IX is not essential for propagation but the virus capsid is less thermostable for adenoviruses lacking pIX (Parks, 2005). The N-terminal domain of pIX is required for insertion into the virion capsid whereas the C-terminal domain is important for pIXs function as a transcriptional activator (Rosa-Calatrava *et al.*, 2001). Moreover, pIX is also involved in the formation of nuclear inclusion bodies in adenovirus infected cells (Rosa-Calatrava *et al.*, 2003).

The adenovirus core

The core of the adenovirus particle contains five known proteins and the approximately 36 kb linear double stranded viral DNA (Berk, 2007; van Oostrum & Burnett, 1985).

pV is like pIX unique to the *Mastadenovirus* genus and appears to form a layer around the DNA by non-specific association with the DNA and binding of penton base and pVI to connect the capsid to the core (Chatterjee *et al.*, 1985; Chatterjee *et al.*, 1986; Davison *et al.*, 2003). It has also been suggested that pV might be important for correct assembly of infectious viral particles (Ugai *et al.*, 2007).

pVII is a basic arginine-alanine rich protein and the most abundant core protein with more than 800 copies per virion (Black & Center, 1979). pVII has significant sequence analogy to histone H3 and a histone-like function wrapping the viral DNA around itself and thereby condensing it and silencing transcription (Cai & Weber, 1993; Johnson *et al.*, 2004; Newcomb *et al.*, 1984).

μ is a small basic DNA-binding protein that is involved in DNA condensation and charge neutralization (Anderson *et al.*, 1989; Keller *et al.*, 2001). Protein μ remains together with pVII associated to the viral genome during import at the nuclear pore complex (Chatterjee *et al.*, 1986).

pTP (precursor terminal protein) is a 80 kDa protein covalently attached to the 5´-ends of the viral DNA and is involved in the initiation of replication (Mysiak *et al.*, 2004; Rekosh *et al.*, 1977). During the late stage of virion maturation pTP is proteolytically cleaved by the adenoviral cysteine protease into the 55 kDa terminal protein (TP), which is still attached to the 5´-ends. The proteolytic processing is not required for viral DNA replication (Challberg & Kelly, 1981).

pIV2a interacts specifically with the DNA packaging signal and is responsible for the type specific DNA packaging into the capsid but also plays a role as a transcriptional activator of the major late promoter (Lutz & Kedinger, 1996; Zhang *et al.*, 2001).

Non-structural proteins (see also under Early transcription units)
About 30 non-structural adenoviral proteins have been described thus far. The precise function of many is still unknown but most are assigned catalytic and regulatory functions in the viral life cycle (San Martin & Burnett, 2003).

Viral life cycle

Adenoviral receptors

Most studies on the adenoviral life cycle have been performed on the species C types Ad2 or Ad5. Description of the internalization mechanism is based on these two types unless otherwise stated. Attachment of the fiber protein to a primary cellular receptor is the first step of internalization of the virus particle into the host cell (Philipson *et al.*, 1968).

CAR

The coxsackievirus-adenovirus receptor is common for coxsackie B viruses and some adenoviruses and was first described in 1997 (Bergelson *et al.*, 1997; Tomko *et al.*, 1997).

Representatives of all adenovirus species except species B have been shown to interact with soluble CAR and species C types use CAR as a functional receptor (Bergelson *et al.*, 1998; Roelvink *et al.*, 1998; Tomko *et al.*, 1997). CAR is a 46 kDa transmembrane protein belonging to the immunoglobulin superfamily. It has a 216-amino acid extracellular segment consisting of two immunoglobulin (Ig)-like domains (D1 and D2), a helical membrane-spanning domain and a 107-amino acid intracellular domain (Bergelson *et al.*, 1997; Coyne & Bergelson, 2005). Both adenoviruses and coxsackieviruses bind to the outermost extracellular D1 domain but recognize different specific sites (Bewley *et al.*, 1999; He *et al.*, 2001). CAR has been identified as a part of tight junctions and is expressed mainly on the basolateral surface of polarized cells (Cohen *et al.*, 2001; Pickles *et al.*, 2000). This position hides the molecule from virus approaching from the exposed apical side of the cell. It is not established how viruses can use the apparently inaccessible CAR as a functional receptor. One theory is that upon infection of the epithelial cells of the airways adenoviruses first infect specialized nonpolarized cells and then spread to neighbouring polarized cells or that CAR is made accessible by lesions in the epithelial layer (Meier & Greber, 2004; Walters *et al.*, 2002). It has also been suggested that adenoviruses

might use CAR in the escape phase as well as in the entry phase (Walters *et al.*, 2002).

CD46

Species B adenoviruses do not bind CAR like representatives of the other species. Instead CD46 has been identified as a receptor for some species B adenoviruses (Fleischli *et al.*, 2007; Gaggar *et al.*, 2003; Marttila *et al.*, 2005; Segerman *et al.*, 2003; Sirena *et al.*, 2004; Tuve *et al.*, 2006). CD46, also known as membrane co-factor protein (MCP), is expressed on all human cells except red blood cells (Riley-Vargas *et al.*, 2004). It is a transmembrane glycoprotein belonging to the regulators of complement activation (RCA) family of proteins (Liszewski *et al.*, 1991). The extracellular part of CD46 consists of four complement control protein repeats (CCPs)/short consensus repeat (SCR) domains and a heavily O-glycosylated serine/threonine/proline (STP) region closest to the plasma membrane (Figure 3). More than four STP regions and four cytoplasmic tails give rise to a number of different isoforms of CD46 ranging from about 50 kDa to 68 kDa in size (Post *et al.*, 1991; Seya *et al.*, 1999). The biological significance of the different isoforms of CD46 is not well understood but most common are the STP^{BC} and STP^C isoforms as well as the Cyt1 and Cyt2 isoforms of the cytoplasmic tail (Seya *et al.*, 1999). The natural CD46 ligands are C3b and C4b, and by acting as a co-factor for the serine protease factor I, CD46 mediates their breakdown and thereby protecting the host cell from homologous complement attack (Barilla-LaBarca *et al.*, 2002; Liszewski *et al.*, 1991; Oglesby *et al.*, 1992). CD46 has been suggested to link innate and adaptive immunity since ligand binding to CD46 affects the cellular immune functions such as cytokine, chemokine and nitric oxide production in T cells, B cells, macrophages and astrocytes as well as antigen presentation by MHC class I or II (Cardoso *et al.*, 1995; Karp *et al.*, 1996; Kemper *et al.*, 2003; Noe *et al.*, 1999; Rivailler *et al.*, 1998). In addition CD46 is also believed to play a part in reproduction since a tissue-specific variant is expressed on the inner acrosomal membrane of spermatozoa and placental trophoblasts and the expression of rodent CD46

is restricted to testis (Anderson *et al.*, 1993; Holmes *et al.*, 1992; Nomura *et al.*, 2001; Tsujimura *et al.*, 1998).
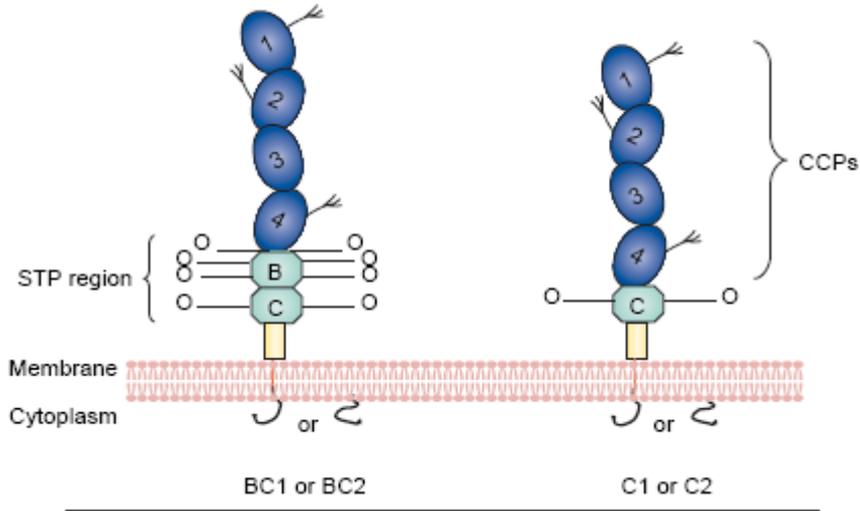

Figure 3. Schematic structure of human CD46. The CCP regions are *N*-glycosylated and the STP region is *O*-glycosylated. From (Riley-Vargas *et al.*, 2004). Reproduced with permission from Elsevier Ltd.

In addition to species B adenoviruses, CD46 is a receptor for a wide variety of pathogens including the Edmonston strain of measles virus (Dorig *et al.*, 1993), human herpes virus 6 (HHV6) (Santoro *et al.*, 1999), *Streptococcus pyogenes* (Okada *et al.*, 1995) and pathogenic *Neisseria* (*gonorrhoea* and *meningitidis*) (Källström *et al.*, 1997). The interaction with CD46 differs between the pathogens. Measles virus binds the SCR1 and SCR2 domains (Casasnovas *et al.*, 1999), HHV6 bind SCR2 and SCR3 (Greenstone *et al.*, 2002), *Streptococcus pyogenes* bind SCR3 and SCR4 (Giannakis *et al.*, 2002) and *Neisseria gonorrheae* bind SCR3 and the STP domain (Källström

et al., 2001). The Ad11p fiber knob has been crystallized in complex with CD46 and the two outermost SCR domains, SCR1 and SCR2, changes conformation as Ad11p-binding is mediated (Persson *et al.*, 2007). These two domains have also been shown to be used by Ad35 (Fleischli *et al.*, 2005).

CD46 can be internalized from the cell surface by at least two different pathways. The pathway via clathrin-coated pits seems to constitutively recycle CD46 from the cell surface to perinuclear multivesicular bodies. This pathway does not lead to downregulation of CD46 on the cell surface (Crimeen-Irwin *et al.*, 2003). Cross-linking of CD46 by for example multivalent antibodies at the cell surface leads to internalization via another pathway, through what appears to be macropinocytosis. Binding of measles virus, HHV6, *Neisseria* and Ad35 to CD46 results in downregulation of CD46 on the cell surface, albeit by different mechanisms (Crimeen-Irwin *et al.*, 2003; Gill *et al.*, 2003; Naniche *et al.*, 1993; Sakurai *et al.*, 2007; Santoro *et al.*, 1999; Schneider-Schaulies *et al.*, 1996). The implications of CD46 downregulation on infected cells are yet not well understood. In addition, the immunosuppressive effects of measles virus and HHV6 have partly been attributed to their interaction with CD46 (Marie *et al.*, 2001; Smith *et al.*, 2003).

Figure 4. Schematic illustration of the complement system. Activation of the complement cascade via the classical pathway (A), lectin pathway (B) or alternative pathway (C). From (Favoreel *et al.*, 2003). Reproduced with permission from author and publisher, Society for General Microbiology.

The role of CD46 in the complement system

The complement system is one of the branches of the immune response that when activated gives rise to a cascade of enzymatic reactions eventually leading to final destruction of the invading microorganism and the infected cell. There are three different ways for activation of the complement cascade, via the classical, lectin, or alternative pathway (Figure 4). The classical

pathway starts with binding of the complement protein C1q to antibody-antigen complexes or, in some cases, directly to the surface of the pathogen. The mannan-binding lectin (MBL) pathway is initiated by MBL binding to the pathogen. MBL is structurally similar to C1q and the lectin pathway is consequently very similar to the classical pathway. The third activation pathway, the alternative pathway, is a spontaneous process of hydrolysis of C3 in plasma and deposition of the product C3b on surfaces of host cells and pathogens. The complement cascade will proceed unless it is dowregulated by specific mechanisms. Activation via any of the pathways results in cleavage of C3 to C3b and subsequent cleavage of C5 which initiates the terminal pathway leading to formation of pores in lipid bilayers and eventually lysis. As the effects of complement activation are potentially dangerous for the host, this system is carefully regulated by the actions of complement-regulating proteins, among which CD46, CD55 and CD59 can be found. CD46 serves as a cofactor for the factor I-mediated cleavage of cell surface-bound C3b and C4b present on the same cell as CD46. Factor I is a constitutively active plasma protein but requires a substrate-binding cofactor protein such as CD46 to promote binding of factor I to the substrate (Favoreel *et al.*, 2003).

Species B receptors

Several molecules have been suggested to serve as cellular receptors for all or some of the species B adenovirus types, including CD46, CD80, CD86, and sialic acid (Gaggar *et al.*, 2003; Marttila *et al.*, 2005; Short *et al.*, 2006; Wu *et al.*, 2004). In recent years the ability to use CD46 has been disputed. Especially the results for Ad3p and Ad7p have been inconclusive. Evidence that Ad3p uses CD46 was presented by Sirena et al. (Sirena *et al.*, 2004), and also by Fleischli et al. (Fleischli *et al.*, 2007), who in addition claimed that Ad7p can use CD46. Marttila et al. (Marttila *et al.*, 2005) on the other hand argued that all species B types except Ad3p and Ad7p use CD46 and this conclusion is supported by the work of Tuve et al. (Tuve *et al.*, 2006). Gaggar

et al. concluded that Ad3p did not use CD46 as a high-affinity attachment receptor (Gaggar *et al.*, 2003).

Ad11p, Ad21, Ad34, Ad35, and Ad50 are established CD46-users. Three loops in the fiber knob of Ad11p have been shown to interact with CD46: the DG loop, the HI loop, and the IJ loop (Persson *et al.*, 2007). The Ad11p HI loop contains the arginine residues R279 and R280, which are critical for binding to the SCR1 and SCR2 domains of CD46 (Gustafsson *et al.*, 2006; Persson *et al.*, 2007).

Ad34 and Ad35 interact with CD46 as efficiently as Ad11p (Andersson *et al.*, 2010; Persson *et al.*, 2009). Comparing the fiber knobs of Ad11p to those of Ad34 and Ad35, the amino acid identity is only around 50%. Ad34 and Ad35 have serines in position 279 and there are also differences in the sequences of the three CD46-interacting loops.

The critical residues 279 and 280 and the structure of the HI loop are well conserved between Ad11p and Ad16 (Pache *et al.*, 2008). However, Ad16 has two additional residues in the FG loop (corresponding to the DG loop of Ad11p) and a deletion of two residues in the IJ loop as compared to Ad11p (Pache *et al.*, 2008). Pache et al. suggested that these differences, in particular the steric hindrance conferred by the longer FG loop, cause the affinity between the Ad16 fiber knob and CD46 to be about 70-fold lower than that between the Ad11p fiber knob and CD46. An insertion in the DG loop is also found in Ad3p. In addition, the latter has a lysine in position 279. This might result in a weaker interaction between the fiber knob and CD46. The fiber knobs of Ad3p and Ad7p, the two types representing the most non-conclusive CD46 binding patterns, are not particularly similar. Alignment of the fiber knob aa sequences reveal that there are distinct differences and the overall homology is only 46 % (Marttila *et al.*, 2005).

The fiber knob of Ad21 is highly homologous to that of Ad35, and like Ad35 there is a serine in the position corresponding to Arg279 in Ad11p. Ad21 has compared to Ad11p a more protruding DG loop and a shorter IJ loop, resulting in about 22-fold lower affinity for CD46 (Cupelli *et al.*, 2010).

Sialic acid

Four species D types causing epidemic keratoconjunctivitis, Ad8, Ad19, Ad37, and Ad54 use α2,3-linked sialic acid as a cellular receptor (Arnberg *et al.*, 2000; Burmeister *et al.*, 2004; Cashman *et al.*, 2004; Ishiko & Aoki, 2009). These adenovirus types seem to bind the negatively charged sialic acid in a charge dependent manner via a positively charged site on top of the fiber knob and in addition the *N*-acetyl group of sialic acid interacts with a hydrophobic pocket of the fiber knob (Arnberg *et al.*, 2002; Burmeister *et al.*, 2004).

HSPG

Heparan sulphate proteoglycans are abundantly expressed on most cells and are implicated as receptor molecules for several pathogens (Bernfield *et al.*, 1999; Olofsson & Bergström, 2005). It has been suggested that HSPGs interacts with Ad2 and Ad5 to mediate their binding to and infection of Chinese hamster ovary cells (Dechecchi *et al.*, 2001), but the full functionality of this interaction is not established.

Bridging factors

Recently it has been established that a number of soluble factors present in body fluids can act as bridges between adenoviruses and cell surface molecules and thereby facilitate infection (Baker *et al.*, 2007; Johansson *et al.*, 2007; Jonsson *et al.*, 2009; Shayakhmetov *et al.*, 2005).

Coagulation factors IX and X, as well as complement factor C4BP, enhance Ad5 infection of epithelial cells and hepatocytes via heparan sulphate proteoglycans (Jonsson *et al.*, 2009; Shayakhmetov *et al.*, 2005). Infection by Ad31 is also enhanced by coagulation factor IX (Jonsson *et al.*, 2009). Human lactoferrin has been shown to increase binding and infection of the species C adenoviruses specifically (Johansson *et al.*, 2007).

Internalization

Following binding to a primary receptor interaction between cell surface integrins and the RGD-motif located in the penton base of all adenovirus types except Ad40 and Ad41 occurs (Albinsson & Kidd, 1999; Wickham *et al.*, 1993). Ad40 and Ad41 undergo a delayed uptake in A549 cells (Albinsson & Kidd, 1999). Integrins known to function as co-receptors for adenoviruses include $\alpha_v\beta_3$, $\alpha_v\beta_5$ (Wickham *et al.*, 1993), $\alpha_v\beta_1$ (Li *et al.*, 2001), $\alpha_5\beta_1$ (Davison *et al.*, 1997), $\alpha_M\beta_2$, and $\alpha_L\beta_1$ (Huang *et al.*, 1996). Binding of integrins triggers a number of signalling events leading to endocytosis of the virus particle into clathrin coated vesicles (Greber, 2002; Varga *et al.*, 1991). Viral escape from the endosome occurs rapidly after endocytosis but is not very well understood. It is known that an acidic environment triggers the escape (Blumenthal *et al.*, 1986). Low pH in the early endosome releases pVI from the viral capsid and pIV aids the membrane disruption independently of pH (Wiethoff *et al.*, 2005). Penton base binding of integrin $\alpha_v\beta_5$ is also believed to promote permeabilization of the endosomal membrane (Wickham *et al.*, 1994). By means of dynein/dynactin/microtubule dependent mechanisms the partly dismantled virus capsid is transported to the nucleus where it docks to the nuclear pore complex (NPC) and the viral L3 23K protease degrades the capsid stabilizing pVI thereby finalizing the dismantling (Figure 5) (Greber *et al.*, 1997; Greber *et al.*, 1996; Greber *et al.*, 1993; Kelkar *et al.*, 2004; Suomalainen *et al.*, 1999). The viral capsid is believed to dock to the NPC by direct interactions between the hexon and the NPC protein CAN/Nup214 (Trotman *et al.*, 2001). The precursors of the core proteins TP and pVII are tightly associated with the adenoviral genome and contain nuclear localization signals (NLS) and are likely to play major roles in the import into the nucleus (Wodrich *et al.*, 2006; Zhao & Padmanabhan, 1988). Other factors identified as being involved in adenoviral DNA import are importins, histone H1, H1-import factors, and transportin (Hindley *et al.*, 2007; Saphire *et al.*, 2000; Trotman *et al.*, 2001).
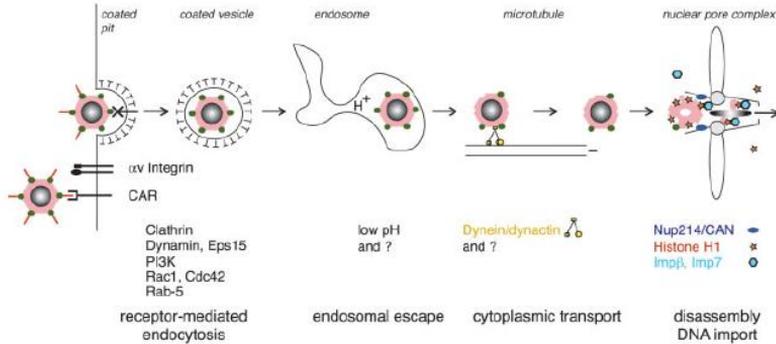
Figure 5. Infectious entry pathway for Ad2 and Ad5. From (Meier & Greber, 2004). Reproduced with permission from John Wiley & Sons Inc.

Genome organization and gene expression

The adenovirus genome consists of linear double stranded DNA approximately 34-36 kDa in size encoding about 40 proteins (Davison *et al.*, 2003). The organization of the genome is conserved between the genera and is characterized by a number of RNA polymerase II-dependent transcription units, the early E1A, E1B, E2, E3, and E4, the delayed early pIX, pIVa2 and E2 late, and the major late transcription unit (MLTU) (Figure 6) (Berk, 2007; Davison *et al.*, 2003). The MLTU transcript is further processed by polyadenylation and alternative splicing resulting in five or six mRNAs, L1-L5/L6 (Young, 2003). The adenovirus genome also encodes one or two small virus-associated RNAs (VA RNA I and II) transcribed by RNA polymerase III (Kidd *et al.*, 1995).
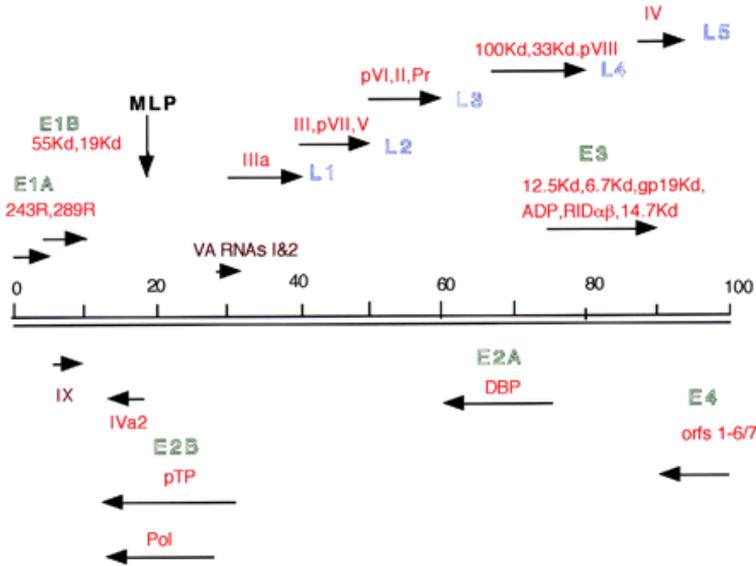
Figure 6. Transcription of the Ad2 and Ad5 genome. From (Russell, 2000). Reproduced with permission from the Society for General Microbiology.

Early transcription units

Activation of the early genes occurs when the viral DNA enters the nucleus. The products of the early genes adapt the cell for replication of viral DNA, expression of late genes and assembly of new virus particles.

E1A of Ad5 produces two major mRNAs, 12S and 13S, which encode the proteins E1A-243R and E1A-289R, respectively (Perricaudet *et al.*, 1979). The difference between these two proteins is an internal stretch of 46 amino acids unique to 13S. These proteins are essential for transcription of other early genes and promote expression of host cell proteins needed for DNA replication (Jones & Shenk, 1979). Two conserved regions, CR1-2, are common to 243R and 289R, whereas a third conserved region CR3 is unique to 289R. These conserved regions have distinct roles in the alteration of cellular morphology and transcriptional regulatory functions of E1A (Kimelman, 1986). Initial expression of E1A is induced by cellular

transcription factors like E2F, but E1A gene products also promote their own transcription (Kirch *et al.*, 1993; Kovesdi *et al.*, 1987). In addition to inducing transcription of other early viral genes the E1A proteins also induce a number of cellular genes and inhibit the transcription of others. The E1A proteins lack sequence specific DNA binding properties and exert their effect by interacting with cellular factors such as general or sequence specific transcription factors, co-activators and chromatin-modifying enzymes (Brockmann & Esche, 2003). E1A proteins bind to pRb-family proteins, release E2F and thereby promote entry of the cell into the S-phase (Hiebert *et al.*, 1991; Sidle *et al.*, 1996). The transforming effects of E1A gene products sensitize the cell to apoptosis.

E1B encodes the 19K and 55K proteins, both of which cooperate independently with E1A to induce transformation and counteract apoptosis. The small E1B protein is an inhibitor of p53 transcriptional repression as well as p53-dependent and p53-independent apoptosis (Debbas & White, 1993; Shen & Shenk, 1994). E1B-19K also counteracts apoptosis induced by TNF-α and anti-Fas antibody (Hashimoto *et al.*, 1991; White *et al.*, 1992). The 55K protein in complex with the E4ORF6 protein promotes degradation of p53 in proteasomes and thereby causes loss of the transcription stimulation activity of p53 (Harada *et al.*, 2002; Yew & Berk, 1992). This complex is also important for the transport of viral late mRNAs from the nucleus to the cytoplasm and for the translation of these mRNAs (Bridge & Ketner, 1990).

The **E2** region gives rise to three proteins directly involved in viral DNA replication, the DNA polymerase (AdPol), pTP, and the DNA binding protein (DBP). The highly conserved viral DNA polymerase is necessary for viral DNA replication with intrinsic 3´-5´ proofreading activity (Field *et al.*, 1984; Ikeda *et al.*, 1981). The pTP is required for DNA replication as it functions as a primer for initiation of replication by binding to the viral DNA polymerase (Brenkman *et al.*, 2002). DBP is a multifunctional protein essential for viral DNA replication. DBP has helix-destabilizing properties and it has been

suggested that it is required for unwinding the double-stranded template. DBP binds cooperatively to ssDNA without sequence specificity whereas binding to dsDNA is weak and non-cooperative (Monaghan *et al.*, 1994).

E3 gene products are involved in the evasion of the immune response. The 10.4K and 14.5K proteins as a complex are also known as RIDαβ. RIDαβ together with the 14.7K protein prevent cells from going into Fas ligand mediated or TNF-mediated apoptosis (Elsing & Burgert, 1998; Tollefson *et al.*, 1991; Tollefson *et al.*, 2001; Wold *et al.*, 1995). The E3 19K glycoprotein interferes with antigen presentation by sequestering major histocompatibility complex class I proteins in the endoplasmatic reticulum and thereby preventing cytotoxic T cell recognition (Burgert & Kvist, 1985; Burgert *et al.*, 1987). The 11.6K protein, also designated the adenovirus death protein (ADP), induces cell lysis and is necessary for viral release (Tollefson *et al.*, 1996).

The **E4** transcriptional unit contains at least six ORFs; ORF1, ORF2, ORF3, ORF4, ORF6, and ORF6/7 (Leppard, 1997). The roles of the encoded proteins are diverse, including transcriptional regulation, mRNA transport, translation activation, and apoptosis (Berk, 2007; Marcellus *et al.*, 1998).

Most human adenovirus genomes encode two species of virus-associated RNAs (**VA RNAs**), but some, including species B2 adenovirus types, have a single VA RNA gene. VA RNAs are transcribed by RNA polymerase III and inhibit interferon-induced PKR protein kinase activity which is activated by dsRNA (Kidd *et al.*, 1995; Ma & Mathews, 1996).

DNA replication

Replication of the adenoviral genome is initiated after the host cell has entered S-phase and the E2 gene products have been expressed. Replication of the viral DNA occurs in two stages. The inverted terminal repeats (ITRs) at each end of the DNA serve as origins of replication. In the first stage, synthesis starts at either terminus of the linear genome and proceeds

continuously to the other end. This way only one DNA strand serves as a template in the first stage. A complement to the other displaced parental strand is synthesized at the second stage of replication. The single stranded template circularizes through annealing of the self-complementary ends, resulting in a *panhandle* with the same structure as the termini of the double stranded genome, which allows it to be recognized by the same mechanism as the initiation of synthesis of the first strand (Berk, 2007). A complex of pTP and AdPol binds to a conserved region of the ITR and the pTP functions as a protein primer. The priming reaction is initiated by AdPol catalyzed pTP binding to deoxycytidine monophosphate (dCMP) at the 5´ end of the genome. The 3´-OH group of the pTP-dCMP complex then serves as a primer for 5´ to 3´ directed synthesis of the viral DNA by AdPol (Mysiak *et al.*, 2004). AdPol dissociates from pTP after synthesis of the first three or four nucleotides of the new DNA strand (King *et al.*, 1997). Elongation of the DNA chain also requires the viral protein DBP and the cellular topoisomerase I. DBP binds tightly and cooperatively to ssDNA and is believed to drive strand separation (Dekker *et al.*, 1997).

Delayed early transcription units

Immediately after the onset of DNA replication but before late gene expression has started three so-called delayed early viral proteins are produced, each from its own promoter; pIX, pIVa2, and E2 late. Protein IX is in addition to being a component of the capsid a transcription activator although not MLP specific (Rosa-Calatrava *et al.*, 2001); (Berk, 2007). Protein IVa2 is also a transcription activator (see Late transcription) and important for virus assembly (Lutz & Kedinger, 1996; Zhang *et al.*, 2001). The E2 late promoter is activated to increase the proteins needed for DNA replication, such as the DBP ((Berk, 2007).

Late transcription

The late genes in general code for structural proteins building up the viral capsid and are expressed after the onset of viral DNA replication. All late coding regions are organized into one large transcription unit that results in

an initial transcript of about 28 kb. This transcript is then processed into at least twenty distinct mRNAs grouped into five families, L1 to L5, depending on the use of common poly(A) addition sites (Berk, 2007). The major late promoter (MLP) controls expression of this large transcription unit. MLP is active at a low level even early in the infection cycle but its activity is strongly enhanced upon onset of viral DNA replication. Within the MLP unit there is an intermediate-phase promoter (L4P) embedded. This promoter triggers the progression into the late phase by expressing the L4-22K and L4-33K independently of the MLP. L4P is activated by proteins of E1A, E4 orf3 and IVa2, and by replication of the viral genome. IVa2 expression is itself dependent on viral replication and it acts as a transcription factor in activation of the MLP (Morris *et al.*, 2010; Toth *et al.*, 1992).

The **L1** region encodes the L1 52/55K protein and the precursor protein of pIII$_a$. L1 52/55K is required for encapsidation of viral DNA and promotes viral DNA replication and late gene expression.

L2 encodes four different proteins; pIII (penton base), precursor pVII, pV, and precursor pX.

L3 encodes precursor pVI, pII (hexon), and the 23K protease. An adenovirus particle contains around 10-30 copies of the cysteine protease that is required for uncoating of the viral particle during viral entry as well as for viral capsid assembly (Greber *et al.*, 1996; Weber, 1995)

L4 encodes the precursor protein of pVIII, the 100K, 33K, and 22K proteins. The 100K protein is required for correct folding of the hexon protein and the 33K protein is involved in viral assembly and shutting down host cell translation as well as in regulating late gene expression together with the 22K protein (Fessler & Young, 1999; Morris & Leppard, 2009; Oosterom-Dragon & Ginsberg, 1981; Ostapchuk *et al.*, 2006).

The only protein encoded within the **L5** region is the fiber protein (pIV).

Adenovirus assembly and release

It is generally believed that preformed hexon and penton capsomers build empty adenovirus capsids, into which the viral genome and core proteins subsequently are inserted. Monomeric hexon proteins are shortly after synthesis in the cytoplasm assembled into trimeric capsomers assisted by the L4 100K protein (Hong *et al.*, 2005). Nuclear import of hexon capsomers is facilitated by pVI (Wodrich *et al.*, 2003). Penton capsomers, built up by pentameric penton base and trimeric fiber, are assembled somewhat slower in the cytoplasm and are transported into the nucleus (Horwitz *et al.*, 1969). The viral genome is preferentially packed into the empty capsid with the left end of the genome first (Hammarskjöld & Winberg, 1980). Seven A-repeats located in regions in the left end of the genome, constituting the packaging domain, are required for Ad5 packaging (Ostapchuk & Hearing, 2005). Other proteins suggested to be involved in the packaging and assembly are L1 52/55K, pIVa2, and L4 22K (Gustin & Imperiale, 1998; Ostapchuk *et al.*, 2006; Zhang *et al.*, 2001). Several intermediate immature particles are produced during assembly, including young virions that precede the mature virions (Edvardsson *et al.*, 1976). Maturation of the particle into an infectious virion is achieved by cleavage of several structural precursor proteins by the viral 23K protease (Ostapchuk & Hearing, 2005). The mature viral particles are released from the cell upon lysis of the cellular membranes. At least three different systems appear to make the cell susceptible to lysis and viral escape. The viral 23K protease cleaves and rearranges cellular cytokeratins late in the infectious cycle, leading to weakened cytoskeletal stability of the cell (Chen *et al.*, 1993). High expression of the E3 encoded adenovirus death protein (ADP) in the late stages of infection results in cell death (Tollefson *et al.*, 1996; Tollefson *et al.*, 1992). The ADP is a membrane protein that localizes to the nuclear membrane, the endoplasmatic reticulum and the Golgi apparatus where it interacts with the MAD2B protein, a protein related to proteins that regulate mitosis. However, the exact mechanism following interaction between ADP and MAD2B and how this leads to cell death is not known (Ying & Wold, 2003). Excess amounts of soluble fiber proteins can be released from

infected cells and in the case of CAR interacting adenoviruses these may open the tight junctions and allow more efficient viral escape and access to uninfected cells (Levine & Ginsberg, 1967; Walters *et al.*, 2002).

The coxsackievirus- and adenovirus receptor, CAR, was originally designated the receptor for most adenoviruses (Bergelson *et al.*, 1998). Now it is known that not all, if any, adenovirus types use this protein as the primary receptor *in* vivo. Later it has also been discovered that CAR is expressed on the basolateral side of polarized cells and that it is a part of tight junctions between cells, but it is however not expressed on the apical side of polarized cells and would thus be inaccessible for incoming viruses, e.g. in the airways (Walters *et al.*, 1999). One recent hypothesis is that it is used by CAR-binding adenoviruses upon exit from the infected cell. Infected cells produce an excess of fiber proteins that are secreted basolaterally and can reach the tight junctions. Dissociation of CAR homodimers as a result of fiber-CAR interactions allows virions to move in-between cells and access surrounding cells (Walters *et al.*, 2002). This function of the interaction between the adenovirus fiber and CAR is plausible for the CAR-binding types but for the species B types that do not bind CAR this suggested mechanism is irrelevant.

Clinical and pathological aspects

Adenovirus infections are very common among humans as well as among other animals and are in general host species specific, although subclinical infections across species barriers have been reported (Wold & Horwitz, 2007). Adenoviruses gain access to the host by the mouth, the nasopharynx, or the ocular conjunctiva and are associated with a number of clinical manifestations in humans depending on the adenovirus type involved, such as upper respiratory illness, acute respiratory disease, gastroenteritis, hemorrhagic cystitis, and keratoconjunctivitis (Table 1) (Adrian *et al.*, 1986; De Jong *et al.*, 1999; Wadell, 1984; Wadell *et al.*, 1999). However, as many as 50% of all adenovirus infections are subclinical (Fox & Hall, 1980). The major initial sites of replication for entering adenoviruses are the tonsils,

adenoids and the surrounding epithelial cells (Wold & Horwitz, 2007). Adenovirus infections are in most cases lytic but persistence and latency can occur, the mechanism behind this is not fully understood (Fox *et al.*, 1969; Horvath *et al.*, 1986; Wold & Horwitz, 2007). Why different adenovirus types are capable of causing disease in some tissues and not in others is to a large extent unexplained.

Species C types are ubiquitous in young children and can be shed for months, especially in the stool. This is the reason for the endemic spread by the fecal-oral route to new susceptible children (Fox *et al.*, 1969).

Adenovirus infections are commonly self-limiting in otherwise healthy individuals, although an infection can result in severe disease. This is much more of a problem in immunocompromised individuals. This group is steadily growing as a result of increasing numbers of AIDS patients and patients undergoing immunosuppressive therapy for solid organ or hematopoietic stem cell transplantation, and also because of increased survival times of these patients. Immunocompromised individuals are at high risk of developing disseminated disease and multiple organ failure, and an adenovirus infection can become a serious life-threatening disease (Hierholzer, 1992; Janner *et al.*, 1990; Klinger *et al.*, 1998). Adenovirus is even described as a rare cause of encephalitis (Vincentelli *et al.*, 2010). Adenoviruses are an important cause of disease in immunocompromised children and case fatality rates of above 50% have been reported (Hierholzer, 1992). The incidence of adenovirus infections is significantly higher in pediatric bone marrow transplant (BMT) recipients than in adult BMT recipients (Baldwin *et al.*, 2000).

A number of different adenoviruses have been isolated from immunocompromised patients, most frequently from species A, B, or C (Hierholzer, 1992; Kojaoghlanian *et al.*, 2003; Michaels *et al.*, 1992). Species B types are predominantly associated with renal syndromes and species C types are usually associated with hepatitis in these patients. Infections with adenovirus type 31 (species A) have been increasingly reported and often occur in patients with infections involving multiple adenovirus types,

occasionally with lethal outcome (Hierholzer, 1992; Kroes *et al.*, 2007; Leruez-Ville *et al.*, 2006).

The immune response against adenoviruses is characterized by early cytokine and chemokine release. In murine models, alveolar macrophages and Kupffer cells play important roles in elimination of adenovirus vectors from liver and lung by uptake of the vector and release of inflammatory cytokines such as TNF-α, IL-6, and IL-8 (Nazir & Metcalf, 2005). High levels of these cytokines have also been detected in children with adenovirus infections (Mistchenko *et al.*, 1994).

Table 1. Tropism of human adenoviruses

Species	*Type*	*Tropism*
A	12, 18, 31	enteric, respiratory
B1	3, 7, 16, 21, 50	respiratory, ocular
B2	11, 14, 34, 35	renal, respiratory, ocular
C	1, 2, 5, 6	respiratory, ocular, lymphoid
D	8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51, (53, 54)	ocular, enteric
E	4	ocular, respiratory
F	40, 41	enteric
G	52	enteric

In addition to proinflammatory cytokines, induction of high levels of type I interferons (α and β) is an important part of the innate immune response against adenoviruses (Zhu *et al.*, 2007). Adenovirus antigens rapidly activate potent CD8^{+} and CD4^{+} T cell responses and viral capsid proteins activate B cells leading to production of neutralizing antibodies (Dai *et al.*, 1995). This is a strong immune response elicited by adenoviruses usually leading to a full recovery, but sometimes is part of the development of severe disease. It is also an issue in applications like gene therapy; in animal models as well as human clinical trials it has been shown that expression of transgenes from adenoviral vectors usually expires within 2 or 3 weeks even though vectors lacking all viral genes have been shown to sustain expression for as long as 84 days (Chen *et al.*, 1997; Huang & Yang, 2009).

Animal models for adenovirus infection

The need for functional mouse models for adenovirus infections is pronounced but today no such models exist. The cotton rat is semi-permissive for Ad infection and has been used as a model for pneumonia and oncolytic vectors (Prince *et al.*, 1993; Toth *et al.*, 2005). Quite recently the Syrian hamster was demonstrated to be a fully permissive Ad model and is used for studies on oncolytic vectors as well as antiviral drugs and vaccination trials (Diaconu *et al.*, 2010; Safronetz *et al.*, 2009; Thomas *et al.*, 2006). We have made an attempt to establish a CD46-transgenic mouse as a model for adenovirus infection. Ad5p (species C) is a well-documented CAR binding adenovirus type whereas Ad11p (species B) binds CD46. The murine CAR homolog and human CAR share over 90% amino acid identity and murine CAR serves as a receptor for human adenoviruses (Bergelson *et al.*, 1998; Tomko *et al.*, 1997). Expression of the CD46 homolog is limited to the testes of the mouse, and this protein has only 46% similarity to human CD46 (Tsujimura *et al.*, 1998). To mimic the human situation, where CD46 is expressed on all nucleated cells, we used transgenic mice that ubiquitously express human CD46 (Jonstone *et al.*, 1993; Kemper *et al.*, 2001; Mrkic *et*

al., 1998). Intravenous injection of Ad11p did not result in productive infection. Quantitative distribution analysis showed that the only significant difference between CD46-transgenic mice and wild-type mice was obtained in blood, where Ad11p remained in circulation of transgenic mice for a longer period of time than in wild-type mice. CD46 is clearly not a functional receptor for Ad11p in these mice. Ad5p was rapidly cleared from blood and sequestered to the liver in both transgenic and wild-type mice. The levels of detected Ad5 RNA and DNA increased in blood and in liver for at least 48 h indicating that replication might take place in a subset of permissive cells of the liver and it is likely that the infection is bridged by blood factors as have been shown for Ad5 in various systems (Baker *et al.*, 2007; Jonsson *et al.*, 2009; Shayakhmetov *et al.*, 2005).

Cell lines

Since most of the studies of anti-adenoviral compounds have been performed on Ad5 we found it necessary to include Ad5 in our evaluation of anti-adenoviral activity. The K562 cell line used for screening is a suspension cell line of erythroleukemic origin that is refractory to Ad5 infection but susceptible to Ad11p (Andersson *et al.*, 1979; Segerman *et al.*, 2000). These features made this cell line suitable for the screening process using the RCAd11pGFP vector (Sandberg *et al.*, 2009). A suspension cell line is more convenient to work with in a high throughput setting than an adherent cell line. But this cell line could not be used to study the effect on Ad5. A549 is an adherent continuous tumor cell line from a human lung carcinoma with epithelial cell properties that is susceptible to both Ad5 and Ad11p and more suitable for the verification of anti-adenoviral effect (Lieber *et al.*, 1976).

Antiviral drugs for DNA viruses

HSV and VZV inhibitors

Acyclovir (9-(2-hydroxyethoxymethyl)guanine) is a nucleoside analog that depends on the herpesvirus-encoded thymidine kinase for the first phosphorylation step and hence has a high specificity for HSV-1, HSV-2 and VZV. Subsequent phosphorylations to the triphosphate form that is required for termination of DNA chain elongation are carried out by cellular kinases (Figure 7). Acyclovir is administered orally, topically or intravenously (De Clercq, 2002; 2004).
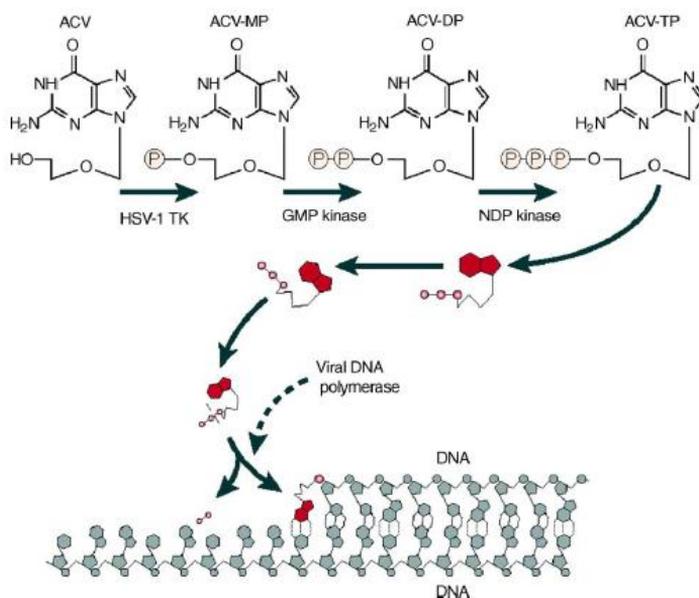
Figure 7. Mechanism of acyclovir inhibition of HSV-1. Adapted by permission from Macmillan Publishers Ltd: [Nat Rev Drug Discovery] ((De Clercq, 2002)), copyright (2002).

Valaciclovir (L-valine ester of acyclovir) is an oral prodrug of acyclovir and has the same mechanism (De Clercq, 2004).
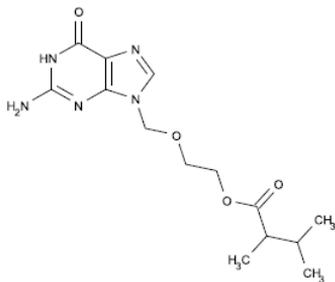
Penciclovir (9-(4-hydroxy-3-hydroxymethyl-but-1-yl)guanine) shares the mechanism of action with acyclovir but is only administered topically (De Clercq, 2004).
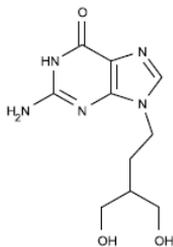
Famciclovir (diacetyl ester of 9-(4-hydroxy-3-hydroxymethyl-but-1-yl)-6-deoxyguanine) is an oral prodrug of penciclovir that following conversion acts as penciclovir (De Clercq, 2004).
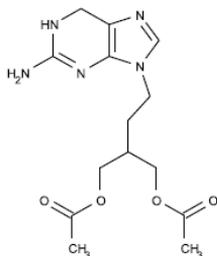
Idoxuridine (5-iodo-2´-deoxyuridine) is intracellulary phosphorylated to 5-iodo-2´-deoxyuridine-5´-trophosphate and incorporated into DNA (viral or cellular). Idoxuridine is topically administered (De Clercq, 2004).
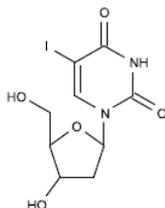
Trifluridine (5-trifluoromethyl-2´-deoxyuridine) inhibits the conversion of dUMP to dTMP by thymidylate synthase, following intracellular phosphorylation to the monophosphate (De Clercq, 2004).
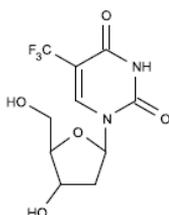
Brivudin ((*E*)-5(2-bromovinyl)-2´-deoxyuridine) is in the cell phosphorylated to the triphosphate form. The two first phosphorylation steps are dependent on the herpesvirus-encoded thymidine kinase. The triphosphate form of Brivudin can act as a competitive inhibitor or as alternate substrate and be incorporated into the viral DNA (De Clercq, 2004).
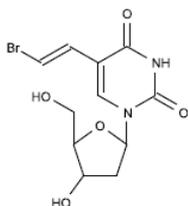
CMV inhibitors

Ganciclovir (9-(1,3-dihydroxy-2-propoxymethyl)guanine) following intracellular phosphorylation to the triphosphate and incorporation of the monophosphate at the 3´-end of the viral DNA chain acts as chain terminator. The first phosphorylation is dependent on the herpesvirus thymidine kinase or the cytomegalovirus (CMV) UL97 protein kinase. Ganciclovir is administered intravenously, orally or as intraocular implant (De Clercq, 2004).
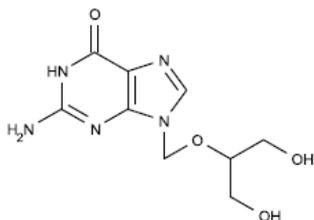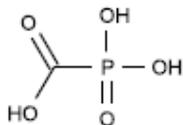

Valganciclovir (L-valine ester of ganciclovir) is an oral prodrug of ganciclovir (De Clercq, 2004).
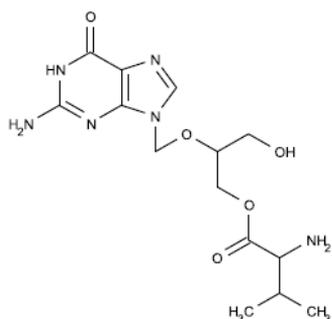
Foscarnet (trisodium phosphonoformate) is a pyrophosphate analog that interferes with binding of pyrophosphate to its binding site of the viral DNA polymerase (De Clercq, 2004).


Cidofovir ((*S*)-1-(3-hydroxy-2-phosphonyl-methoxypropyl)cytosine) targets the viral DNA polymerase. After intracellular phosphorylation to the diphosphate form it is incorporated at the 3´-end of the viral DNA chain and terminates elongation. Cidofovir is administered intravenously or topically (De Clercq, 2004).
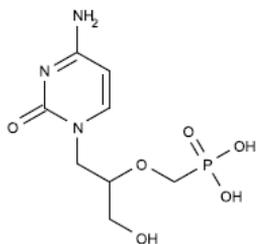

Fomivirsen (antisense oligodeoxynucleotide composed of 21 phosphorothioate-linked nucleosides) is complementary to the CMV immediate early 2 mRNA and hybridizes with the mRNA and blocks translation. The sequence is 5'-GCG TTT GCT CTT CTT CTT GCG-3' (De Clercq, 2004).

Ribavirin (1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide) in its monophosphate form targets IMP (inosine monophosphate) dehydrogenase, an enzyme that converts IMP to XMP (xanthine monophosphate), a vital step in de novo synthesis of GTP and dGTP. Ribavirin is administered orally or as aerosol (De Clercq, 2004).
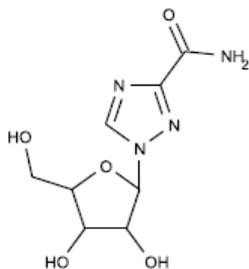

HBV inhibitors

Lamivudine ((-)-β-L-3′-thia-2′,3′-dideoxycytidine) acts as a chain terminator targeting the HBV (and HIV) reverse transcriptase, after intracellular phosphorylation to the triphosphate form and incorporation of the monophosphate into the viral DNA chain (De Clercq, 2004).
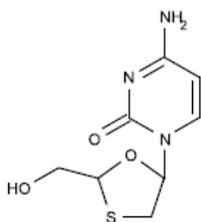
Adefovir dipivoxil (bis(pivaloyloxymethyl)ester of 9-(2-phosphonylmethoxyethyl)adenine) is an oral prodrug of adefovir that targets HBV(and HIV) reverse transcriptase and acts as a chain terminator in the diphosphate form (De Clercq, 2004).
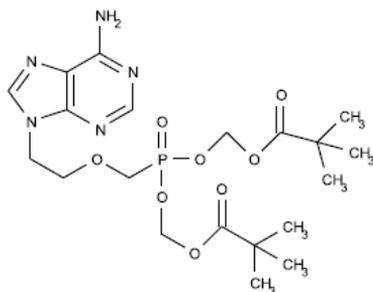
Emtricitabine ((-)-β-L-3´-thia-2´,3´-dideoxy-5-fluorocytidine) shares the mechanism of action with Lamivudine (De Clercq, 2004).
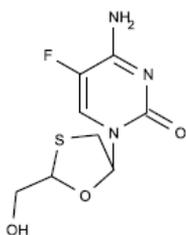
## Antiviral drugs for HIV treatment

It is not possible to cure Human Immunodeficiency Virus (HIV) infected individuals today but the viral load and progression of the infection to AIDS can be confined. Treatment is based on a combination of several drugs targeting different steps in the viral life cycle to avoid development of drug resistance, often referred to as highly active antiretroviral therapy (HAART). The anti-HIV drugs used today belong to seven classes with different mechanisms of action (De Clercq, 2010; Tsibris & Hirsch, 2010).

NRTI (nucleoside reverse transcriptase inhibitors) such as zalcitabine, emtriciatbin or stavudin.
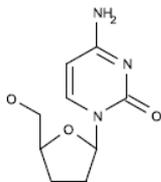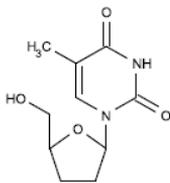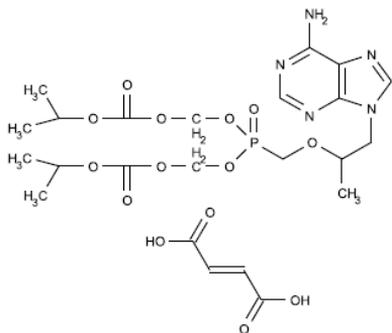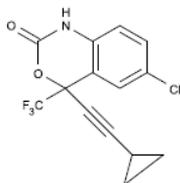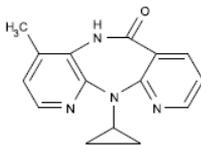

Zalcitabine   Stavudine

NtRTIs (nucleotide reverse transcriptase inhibitors) such as tenofovir disoproxil fumarate.

NNRTI (non-nucleoside reverse transcriptase inhibitors) such as efavirenz or nevirapine.

Efavirenz   Nevirapine

Protease inhibitors such as saquinavir, amprenavir or indinavir.

Amprenavir   Indinavir

Fusion inhibitors such as enfuvirtid which is a synthetic peptide of 36 amino accids. N-acetyl-L-tyrosyl-L-treonyl-L-seryl- L-leucyl-L-isoleucyl-L-histidyl-L-seryl-L-leucyl-L-isoleucyl-L-α-glutamyl-L-α-glutamyl-L-seryl-L-glutaminyl-L-asparaginyl-L-glutaminyl-L-glutaminyl-L-α- glutamyl-L-lysyl-L-asparaginyl-L-α-glutamyl-L-glutaminyl-L-alpha-glutamyl-L-leucyl-L-leucyl-L-α- glutamyl-L-leucyl-L-α-aspartyl-L-lysyl-L-tryptofyl-L- alanyl-L-seryl-L-leucyl-L-tryptofyl-L-asparaginyl-L- tryptofyl-L-fenylalaninamid.

Co-receptor inhibitors such as maravirok.
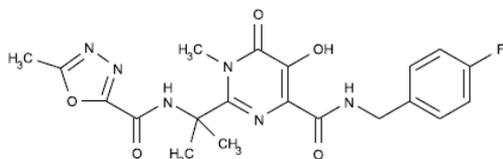
Integrase inhibitors such as raltegravir.

Antiviral drugs and adenoviruses

As of today, there is no formally approved anti-adenoviral agent available. Most compounds tested for *in vitro* activity against Ads are nucleoside or nucleotide analogs. The results from these tests are inconclusive. The use of different assays, different cell lines and different Ad types makes interpretation and comparison difficult. Cidofovir seems to be the most promising anti-adenoviral drug of the currently approved antiviral agents (De Clercq & Holy, 2005). Cidofovir is approved for treatment of CMV retinitis in AIDS patients and has also been shown to be effective against poxvirus and papillomavirus (Calista, 2000; De Clercq & Neyts, 2004). However, the effect of cidofovir on Ads *in vitro* appears to be serotype-dependent (Gordon *et al.*, 1991). The use of cidofovir has been associated with severe nephrotoxicity and hematological toxicity and it is not orally bioavailable (Plosker & Nobel, 1999). Creation of a number of lipid-ester derivatives of cidofovir that have increased oral bioavailability and diminished drug accumulation in the kidney have restored some of the faith in the area (Ciesla *et al.*, 2003). One such lipid-ester derivative called CMX001 has been very promising in animal experiments. Increase of bioavailability and cellular uptake are results of the lipid moiety, which is cleaved inside the cell to liberate cidofovir (Toth *et al.*, 2008). CMX001 is 5-fold more efficient *in vitro* than cidofovir against Ad31, 30-fold more against Ad8, 55-fold more against Ad5, 65-fold more against Ad7, and 200-fold more efficient against Ad3 (Hartline *et al.*, 2005).

A number of anti-HIV agents have been evaluated for their anti-adenoviral effect *in vitro* and zalcitabine and stavudine, both nucleoside reverse transcriptase inhibitors, were shown to be effective against adenoviruses while the non-nucleoside reverse transcriptase inhibitor nevirapine and the protease inhibitors indinavir and amprenavir were not (Uchio *et al.*, 2007).

The reason for the lack of efficient and specific antiviral agents against adenovirus is a complex issue. The closely intertwined mechanisms of cellular and viral DNA replication are evidently an obstacle when designing virus-specific drugs. Or maybe it is the mere fact that the diseases caused by adenoviruses have until recently been regarded as of minor importance.

Aims of the thesis

The first aim of this thesis was to investigate the role of CD46 as a receptor for the species B adenoviruses. Primary receptor usage is not always straight forward or easy to dissect. We designed a new bioassay to conclude which human adenoviruses can use CD46 as a functional receptor. Ad11p is an established CD46 user and it has recently been reported that another closely related species B type, Ad35, induces downregulation of CD46 on the cell surface upon binding. This could lead to increased sensitivity of the infected cell to complement mediated lysis. We set out to investigate if this is also true for Ad11p when down-regulation in Ad35 was not yet known.

Adenoviruses from most species, including species B, are a major and life-threatening problem for immunocompromised individuals. Antiviral agents specific against adenoviruses have not been developed thus far and therefore another aim of this thesis was to find and characterize compounds with inhibitory effect on adenoviruses.

Results and discussion

Paper I

Adenovirus interactions with CD46 on transgenic mouse erythrocytes

Andersson E.K., Mei Y.F., Wadell G.

Virology 2010, 402(1):20-5.

In this study we addressed the question of CD46 usage by all species B and two species D adenoviruses, some established CD46 users and others disputed, but all at some point reported to use CD46 as a cellular receptor.

The method we chose for this investigation was hemagglutination (HA) of CD46-transgenic mouse erythrocytes. The number of virus particles required for specific agglutination of these erythrocytes is a quantitative estimation of the strength of the interaction between the virus and CD46 on the surface of the erythrocytes. This is a unique system that allows studies of interactions at 37°C without the internalization problems encountered in other whole cell systems. The way to overcome this obstacle is usually by performing the experiment at 4°C and thereby risking cold denaturation of proteins.

We found that Ad11p together with Ad34 and Ad35 were by far the most efficient CD46 binders. Ad11p and Ad35 can with as few as 15 virus particles per erythrocyte establish agglutination. This remarkably low number indicates a very strong association with CD46. Ad21 and Ad50 did bind CD46 but less efficiently. Ad3p, Ad7p, Ad14, and Ad37 did not interact with CD46 specifically. The interaction between Ad16 and CD46 was unique as this adenovirus type was the only one that was eluted from the erythrocytes at +4 °C. In an early study of hemagglutination, Ad3, Ad7, and Ad14 but not Ad16 and Ad11 were found to elute from rhesus monkey erythrocytes; these cells have later been shown to express CD46 (Hsu *et al.*, 1997; Simon, 1962). These results would make sense if CD46 on rhesus monkey erythrocytes does in fact mediate agglutination by these adenoviruses. It is possible that there is a higher density of CD46 on rhesus erythrocytes than on transgenic mouse

erythrocytes (Hsu *et al.*, 1997; Kemper *et al.*, 2001); this could be the reason for the different results found in the present study. Pache et al. have shown that Ad16 binds to CD46 but with lower affinity than Ad11, which might explain how Ad16 can be eluted from CD46 mouse erythrocytes (Pache *et al.*, 2008). However, if the density of CD46 is much higher on rhesus erythrocytes, the increased avidity of Ad16 might prevent the elution phenomenon. In various studies, Ad3p, Ad7p, and Ad14 have been implicated as CD46 binders or non-binders. The HA results from 1962 suggest that these Ad types can bind to CD46 but with a very low affinity that allows agglutination of erythrocytes only if CD46 is abundantly expressed on the surface, and even then these types can be eluted. This is consistent with the findings of Persson et al. using Ad7p and Ad14 and also with the results of Gaggar et al. using Ad3p (Gaggar *et al.*, 2003; Persson *et al.*, 2009). The sequence differences between the fiber knobs of the species B types may account for some of the differences in CD46 binding capacity but do not explain them completely. The low amino acid identity of Ad34 and Ad35 compared to Ad11p is rather inconsistent with the interpretation that these three types are most capable of hemagglutination. And the similarity of Ad11p and the non-binders Ad11a and Ad14 adds to the inexplicability of the CD46 interactions of these fiber knobs. A recent study on Ad affinity for CD46 using surface plasmon resonance (SPR) found that the affinity of Ad21 for CD46 was around 20-fold lower than that of Ad11p and Ad35 (Cupelli *et al.*, 2010). This confirms our HA results for these three types, a corresponding difference in the minimal number of virus particles required for HA was observed for Ad11p, Ad21, and Ad35. SPR is an established procedure for measuring affinity, our bioassay is new and not established but offer qualitative aspects that are difficult to achieve in SPR procedures. Native CD46 is inserted in a cell membrane, although in a RBC, and the assay is performed at 37°C, which is difficult to perform using a nucleated permissive cell where binding will soon be followed by internalization and receptor dynamics as described in paper II.

Paper II

Adenovirus 11p downregulates CD46 early in infection

Gustafsson, D., Andersson, E.K., Hu, Y.L., Lindman, K., Marttila, M., Strand, M., Wang, L., Mei, Y.F.

For a number of pathogens including measles virus and Ad35, interaction with CD46 has been shown to downregulate its expression on the cell surface (Sakurai *et al.*, 2007; Schnorr *et al.*, 1995). This downregulation is likely to make the cells more susceptible to complement-mediated lysis, but not to the same extent as downregulation of CD55 and CD59 (Ajona *et al.*, 2007; Marie *et al.*, 2002). We have previously shown that Ad7 binds CD46 in a different way than Ad11p, if it binds at all (Gustafsson *et al.*, 2006). However, Fleischli et al. suggest that there may be a low-affinity binding of Ad7 to CD46 (Fleischli *et al.*, 2007). The purpose of the study described in this paper was to investigate the effect of binding of Ad11p or Ad7 on cell surface CD46, CD55, and CD59 levels. We discovered that Ad11p downregulates CD46 on the surface of K562 and A549 cells in a dose and time dependent manner. CD46 is downregulated already after 5 min and reaches its maximum 4 h after infection. Ad7 do not downregulate CD46 on K562 cells, but downregulation can to some extent be obseered on A549 cells after 8 h. However, there appears to be a general downregulation of CD46, CD55 and CD59 on A549 cells after 8 h, regardless of the infecting adenovirus type. This could possibly be explained by the fact that both Ad7 and Ad11p can replicate in these cells and that the lytic infection cycle leads to a general downregulation of receptors. In our previous studies of CD46 interactions we showed that the R279Q mutation in the fiber knob of Ad11p was sufficient to disrupt the high affinity binding of Ad11p to CD46 and create an interaction with affinity in the same range as Ad7 (Gustafsson *et al.*, 2006). In this study, recombinant Ad11p wild-type fiber knob downregulated CD46 efficiently and Ad11p R279Q fiber knob to a lesser extent, while Ad7 wild-type fiber knob did not significantly affect the CD46

level on K562 cells. The results obtained here indicate that if there in fact is a low-affinity interaction between Ad7 and CD46 the downstream implications of this interaction are different from those following Ad11p interaction and may be a part of the reason for the difference between the Ad7 and the Ad11p tropism and disease manifestations.

Paper III

Small molecule screening using a whole cell viral replication reporter gene assay identifies 2-[[2-(benzoylamino)benzoyl]amino]-benzoic acid as a novel anti-adenoviral compound

Andersson E.K, Strand M., Edlund K., Lindman K., Enquist P.A., Spjut S., Allard A., Elofsson M., Mei Y.F., Wadell G.
Antimicrobial Agents and Chemotherapy 2010, 54 (9), 3871-3877.

Adenoviruses are common pathogens and can cause serious disease and a number of symptoms but are very rarely life-threatening in immunocompetent individuals. That is however the case for immunocompromised individuals and as transplantation rates, cancer patients and AIDS patients are increasing, this is a rising problem. But still there are no approved antiviral drugs specific for adenoviruses available today. To address this we have screened 9800 small organic compounds for anti-adenoviral activity. Screening for putative antivirals depends on a robust method that allows a high throughput rate, usually including robotics which requires suspension cells such as hematopoietic cells. The assay used here was based on the replication-competent GFP expressing Ad11p vector, RCAd11pGFP, in a whole cell system, with the advantage of having no preconceived idea of the mechanism of action. The cell line used was K562, a hematopoietic suspension cell line expressing high amounts of CD46 but not CAR and hence it is susceptible to Ad11p but not Ad5 and suitable for screening with RCAd11pGFP. The screening campaign resulted in a number

of hits and based on high inhibitory effect in combination with low cytotoxicity twenty-four compounds were selected for further studies. These were confirmed to be inhibitory by a number of assays, each reflecting a different step in the adenoviral life cycle. Not many studies on anti-adenoviral effect have been based on Ad11p and we wanted to validate the hits on the more commonly studied Ad5. In addition the CD46-transgenic mouse, as well as the wild-type C57BL/6 mouse, appears to be semi-permissive to Ad5 but not to Ad11p and an animal model is crucial for further development and *in vivo* evaluation of potential drugs. Validation of the screening hit compounds was therefore performed in A549 cells that express both CD46 and CAR and are susceptible to both Ad5 and Ad11p.

The validation of the hits resulted in identification of 2-[[2-(benzoylamino)benzoyl]amino]-benzoic acid as a anti-adenoviral compound with inhibitory effect on adenovirus types representing all species. The mechanism of action is still not known, but it is obvious from binding experiments that attachment of the virus to the cellular receptor is not inhibited. The GFP-gene is located in the E1 region of the vector and expression of this is an early event in the adenoviral life cycle. The compounds were selected on basis of inhibition at this step and hence 2-[[2-(benzoylamino)benzoyl]amino]-benzoic acid could inhibit early gene expression. But 24 h after infection a full viral life cycle has taken place and inhibition of GFP could very well be inhibition of progeny virus. The inhibition is more prominent, although not complete, at the DNA replication step, perhaps partly as a result of the more sensitive QPCR method used. Expression of the structural hexon protein occurs late in the viral life cycle and inhibition of this step is less distinct than inhibition of the preceding DNA replication. This could be due to the fact that transcription and expression of hexon from a reduced number of templates can still occur.

The preparation of 2-[[2-(benzoylamino)benzoyl]amino]-benzoic acid that we purchased turned out to contain two additional compounds remaining after synthesis. This gave us an opportunity for an initial and very brief look into the structure-activity relationship of this compound. Neither the smaller compound A01 nor the bigger A03 (see fig. 2, paper III) was able to inhibit

adenoviral replication. It is possible to speculate that 2-[[2-(benzoylamino)benzoyl]amino]-benzoic acid exerts its effect by fitting into a pocket of the target protein, where A01 is too small to fill the required site and A03 is too bulky to fit. In conclusion 2-[[2-(benzoylamino)benzoyl]amino]-benzoic acid is a promising start and with the lead of the first analysis of the structure-activity relationship it has potential to be modified to an even more efficient drug.

Paper IV

Synthesis, biological evaluation, and structure-activity relationships of 2-[[2-benzoylamino)benzoyl]amino]-benzoic acid analogs as inhibitors of intracellular adenoviral replication.

Öberg, C.T., Andersson, E.K., Strand, M., Edlund, K., Tran, N.P.N, Mei, Y.F., Wadell, G., Elofsson, M.
Manuscript

2-[2-Benzoylamino)benzoylamino]-benzoic acid was identified as a potent adenoviral compound in a previous screening campaign. Here, initial attempts at optimizing this compound is addressed by screening a number of designed and synthesized compounds as well as 8 commercial, structurally similar compounds. We conclude that the *ortho*, *ortho* substituent pattern and the presence of a carboxylic acid in 2-[2-Benzoylamino)benzoylamino]-benzoic acid is favourable for this class of compounds and that the direction of the amide bonds as in 2-[2-Benzoylamino)benzoylamino]-benzoic acid is obligate. There appears to be room for some variability in the *N*-terminal moiety of the compound class, although the variability seems limited to substituted benzamides. One such benzamide, 32b, shows improved activity over 2-[[2-benzoylamino)benzoyl]amino]-benzoic acid (see Table 3, paper IV). Considering the anti-adenoviral effect of the anti-HIV drugs tested in another study it is difficult to draw any conclusions with respect to 2-[[2-benzoylamino)benzoyl]amino]-benzoic acid and its analogs since neither the

functional zalcitabine and stavudin nor the non-functional nevirapine, indinavir and amprenavir share much similarity with the molecules studied here (Uchio *et al.*, 2007).

The features required for anti-adenoviral effect have been further narrowed down and we are closing in on the optimization properties but additional analysis is needed to establish the full structure activity relationship for 2-[[2-(benzoylamino)benzoyl]amino]-benzoic acid and its analogs.

Concluding remarks

Among the seven species of adenoviruses, A-G, it is mainly types of species B that have been shown to use CD46 as a cellular receptor. It has been claimed that all species B types use CD46 but different studies by others have not been conclusive. As adenoviruses present a wide spectrum of avidity for CD46 the variable outcome of previous studies is therefore not unexpected and depends largely on the method used. We have addressed this issue using a new hemagglutination bioassay that mirrors the in vivo situation of the receptor expressed on a cell membrane interacting with the virus at 37°C. In disagreement with some other groups we conclude that not all species B adenoviruses use CD46 as a receptor. Comparing the amino acid sequence of the fiber knobs of viruses is not sufficient to determine the receptor usage, as we have shown for Ad11p and Ad11a. As it appears the CD46 binding types downregulate CD46 from the cell surface, possibly rendering the cell more susceptible to complement attack.

It is well known that human adenoviruses, including the species B types, can cause severe disease and at times even fatal infections in immunocompromised individuals. Nevertheless the mechanisms of infection are yet not fully understood and there is no specific treatment for adenovirus infections. Screening a library of small organic compounds for their ability to inhibit expression of an Ad11p-GFP vector replicating as efficiently as the wild type Ad11p resulted in the discovery of 2-[[2-benzoylamino)benzoyl]amino]-benzoic acid as an anti-adenoviral lead compound, later confirmed to inhibit human adenoviruses from species A-F.

The work of this thesis is a step to further understanding the mechanisms of adenovirus infections and a compound with potential to be developed into a functional adenovirus inhibiting drug was identified and characterized. The CD46-transgenic mouse semi-permissive to Ad5 could be a valuable and available tool for initial *in vivo* analysis of 2-[[2-benzoylamino)benzoyl]amino]-benzoic acid and its analogs.

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