# ONCOLYTIC ADENOVIRUSES FOR TREATMENT OF OVARIAN CANCER

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*to Vilma Angervo*

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# ABBREVIATIONS

| | |
|---|---|
| 5-FC | 5-fluorocytosine |
| 5-FU | 5-fluorouracil |
| ACE | angiotensin-converting enzyme |
| Ad3 | adenovirus serotype 3 |
| Ad5 | adenovirus serotype 5 |
| ADP | adenovirus death protein |
| AP-2 | clathrin adapter protein 2 |
| ATCC | American Type Culture Collection |
| bp | base pair |
| C4BP | complement C4-binding protein |
| CAR | coxsackie-adenovirus receptor |
| CD | cytosine deaminase |
| CE | carboxylesterase |
| CEA | carcinoembryonic antigen |
| cGMP | current good manufacturing practices |
| CMV | cytomegalovirus |
| cox-2 | cyclooxygenase-2 |
| CPE | cytopathic effect |
| CPT-11 | irinotecan |
| CR2 | constant region 2 |
| CXCR4 | CXC chemokine receptor 4 |
| dCTP | deoxycytidine triphosphate |
| ECM | extracellular matrix |
| EGF | epidermal growth factor |
| Ep-CAM | epithelial cellular adhesion molecule |
| Fab | antibody fragment |
| FCS | fetal calf serum |
| FGF2 | basic fibroblast growth factor |
| FIX | factor IX |
| GCV | ganciclovir |
| GFP | green fluorescent protein |
| Gy | gray |
| HRV5 | hypervariable region 5 |
| HSPG | heparan sulfate proteoglycan |
| hTERT | human telomerase reverse transcriptase |
| i.a. | intra-arterial |
| i.p. | intraperitoneal |
| i.t. | intratumoral |
| i.v. | intravenous |
| kb | kilobase |
| kD | kilodalton |
| KKTK motif | lysine-lysine-threonine-lysine motif |
| KOH | potassium hydroxide |
| LP-P | leukocyte plastin promoter |
| LRP | low-density lipoprotein receptor-related protein |
| luc | firefly luciferase |
| MK | midkine |

| | |
|---|---|
| MLP | major late promoter |
| MTS | 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium |
| NAb | neutralizing antibody |
| NMRI | Naval Medical Research Institute |
| OSP1 | ovarian-specific promoter-1 |
| PARP | poly(ADP-ribose) polymerase |
| PEG | polyethylene glycol |
| PET | positron emission tomography |
| pfu | plaque-forming unit |
| pRb | retinoblastoma protein |
| PSA | prostate-specific antigen |
| RGD motif | arginine-glycine-aspartic acid motif |
| s.c. | subcutaneous |
| sCAR | soluble CAR |
| SCCHN | squamous cell carcinoma of the head and neck |
| SCID | severe combined immunodeficiency |
| SLPI | secretory leukoprotease inhibitor |
| SPECT | single-photon emission tomography |
| TAG-72 | tumor-associated glycoprotein 72 |
| TK | thymidine kinase |
| TSP | tumor/tissue-specific promoter |
| VEGF | vascular endothelial growth factor |
| VP | viral particle |

# ABSTRACT

Virotherapy, the use of oncolytic properties of viruses for eradication of tumor cells, is an attractive strategy for treating cancers resistant to traditional modalities. Adenoviruses can be genetically modified to selectively replicate in and destroy tumor cells through exploitation of molecular differences between normal and cancer cells. The lytic life cycle of adenoviruses results in oncolysis of infected cells and spreading of virus progeny to surrounding cells for local amplification of input dose. Normal cells are spared due to lack of replication. Nevertheless, despite excellent preclinical data and proven safety in humans with these agents, several obstacles remain.

The potency of oncolytic adenoviruses might be limited due to poor transduction of target cells. Most adenoviral gene therapy strategies are based on serotype 5 (Ad5), which binds to the coxsackie-adenovirus receptor (CAR). However, expression of CAR is frequently low in many types of advanced cancers. Lack of CAR can be circumvented by substituting the knob domain of the Ad5 fiber with the serotype 3 (Ad3) knob. This allows binding and entry through the Ad3 receptor, which is expressed at high levels in most cancers.

Clinical trials with early-generation oncolytic viruses have indicated that complete elimination of solid tumor masses rarely occurs. A powerful approach for improving the efficacy of virotherapy is utilization of oncolytic adenoviruses in combination with conventional therapies such as chemotherapeutic agents. We evaluated the use of Ad5/3-Δ24, a serotype 3 receptor-targeted oncolytic adenovirus, in combination with gemcitabine or epirubicin against ovarian cancer. The combination of these agents showed synergistic cell killing *in vitro* compared with single treatments. Our results also indicate that gemcitabine reduces the initial rate of Ad5/3-Δ24 replication without affecting the total amount of virus produced. In an orthotopic murine model of peritoneally disseminated ovarian cancer, combining Ad5/3-Δ24 with either gemcitabine or epirubicin resulted in greater therapeutic benefit than either agent alone, and 60% of mice were cured. However, dose and sequencing of the agents were critical for efficacy *versus* toxicity, as some mice treated with Ad5/3-Δ24 and gemcitabine succumbed to treatment-related liver damage.

Another useful approach for increasing the efficacy of oncolytic agents is to arm viruses with therapeutic transgenes such as genes encoding prodrug-converting enzymes. We constructed Ad5/3-Δ24-TK-GFP, an infectivity-enhanced oncolytic adenovirus encoding the thymidine kinase (TK) – green fluorescent protein (GFP) fusion protein. This novel virus replicated efficiently on ovarian cancer cells, which correlated with increased GFP expression. Delivery of prodrug ganciclovir (GCV) immediately after infection abrogated viral replication, which might have utility as a safety switch mechanism. Oncolytic potency *in*

*vitro* was enhanced by GCV in one cell line, and the interaction was not dependent on scheduling of the treatments. However, in murine models of metastatic ovarian cancer, administration of GCV did not add therapeutic benefit to this highly potent oncolytic agent. Detection of tumor progression and virus replication with bioluminescence and fluorescence imaging provided insight into the *in vivo* kinetics of oncolysis.

For optimizing protocols for upcoming clinical trials, we utilized orthotopic murine models of ovarian cancer to analyze the effect of dose and scheduling of intraperitoneally delivered Ad5/3-Δ24. Weekly administration of Ad5/3-Δ24 did not significantly enhance antitumor efficacy over a single treatment. Our results also demonstrate that even a single intraperitoneal injection of only 100 viral particles significantly increased the survival of mice compared with untreated animals.

Improved knowledge of adenovirus biology has resulted in creation of more effective oncolytic agents for cancer gene therapy. However, with more potent therapy regimens an increase in unwanted side-effects is also possible. Therefore, inhibiting viral replication when necessary would be beneficial. We studied the antiviral activity of chlorpromazine and apigenin on adenovirus replication and associated toxicity *in vitro* in fresh human liver samples, normal cells, and ovarian cancer cells. Further, human xenografts in mice were utilized to evaluate antitumor efficacy, viral replication, and liver toxicity *in vivo*. Our data suggest that these agents can reduce replication of adenoviruses, which could provide a safety switch in case of replication-associated side-effects.

# LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

**I.** **Raki M**, Kanerva A, Ristimaki A, Desmond RA, Chen DT, Ranki T, Sarkioja M, Kangasniemi L, & Hemminki A: Combination of gemcitabine and Ad5/3-Δ24, a tropism modified conditionally replicating adenovirus, for the treatment of ovarian cancer. Gene Ther 2005; 12: 1198-1205.

**II.** **Raki M**, Särkioja M, Desmond RA, Chen D-T, Bützow R, Hemminki A, & Kanerva A: Oncolytic adenovirus Ad5/3-Δ24 and chemotherapy for treatment of orthotopic ovarian cancer. Gynecol Oncol 2008; 108; 166-172.

**III.** **Raki M**, Hakkarainen T, Bauerschmitz GJ, Särkioja M, Desmond RA, Kanerva A, & Hemminki A: Utility of TK/GCV in the context of highly effective oncolysis mediated by a serotype 3 receptor targeted oncolytic adenovirus. Gene Ther 2007; 14: 1380-1388.

**IV.** Kanerva A, **Raki M**, Ranki T, Särkioja M, Koponen J, Desmond RA, Helin A, Stenman U-H, Isoniemi H, Höckerstedt K, Ristimäki A, & Hemminki A: Chlorpromazine and apigenin reduce adenovirus replication and decrease replication associated toxicity. J Gene Med 2007; 9: 3-9.

# REVIEW OF THE LITERATURE

## 1. Introduction

Cancer is a major health problem, affecting the lives of millions of people globally. An estimated 11 million new cases are diagnosed each year, and almost 7 million cancer patients will eventually succumb to their disease (Parkin et al. 2005). Although knowledge of molecular background, diagnostic methods, and therapies for cancer has improved over the past decades, most cancer types continue to have a poor prognosis, and metastatic disease can be cured rarely. More efficient approaches and novel tools are therefore needed for the treatment of advanced cancer.

An increasing understanding of the molecular mechanisms that cause cancer has revealed the nature of cancer as a disease of the genes (Hanahan & Weinberg 2000). Human carcinogenesis is a multistep process. Most tumors arise from a series of accumulated, acquired genetic and epigenetic alterations, typically involving mutations in proto-oncogenes, and downregulation of tumor suppressor genes. A logical result of these findings is the idea to correct molecular defects to eliminate tumor cells. Alternatively, specific differences between malignant and normal cells could be utilized for targeting the antitumor effect to cancer cells.

Gene therapy aims at transfer of genes for correction of either genetic or somatic disease phenotypes, or for expression of molecules within or near target cells for a therapeutic effect (Brand 2009). In recent years, gene therapy has become a widely studied strategy for the treatment of diverse diseases. Although gene therapy was initially thought to be more suitable for the treatment of hereditary diseases, it has been increasingly exploited for the treatment of more complex diseases such as cancer. Cancer gene therapy involves a variety of heterogeneous approaches, the common factor of which is the transfer of genes encoding for proteins to deliver a therapeutic antitumor effect. Vehicles for gene transfer include both nonviral and viral vectors, such as adenovirus, retrovirus, adeno-associated virus, and herpes simplex virus (Pereboeva & Curiel 2004). Nonviral gene transfer is most commonly based on plasmid DNA, particle bombardment, or cationic liposomes. Viral gene delivery has already been optimized by evolution and is therefore generally more effective, while nonviral approaches are pharmacologically more attractive. Instead of delivering therapeutic transgenes, oncolytic potential of various viruses can be directly utilized for targeted destruction of tumor cells in an intriguing strategy called virotherapy.

## 2. Adenoviral cancer gene therapy

Since their first isolation from adenoid tissue in 1953 (Rowe et al. 1953), adenoviruses have become one of the most widely studied gene transfer tools in human gene therapy (McConnell & Imperiale 2004). At the moment, adenovirus-based systems are the most common vector type used in clinical studies worldwide (www.wiley.co.uk/genmed/clinical), representing 25% of all gene therapy trials.

Adenoviruses possess several features that render them attractive vectors for cancer gene therapy (Kanerva & Hemminki 2005). Adenoviruses are capable of efficient gene delivery to various cell types, including both dividing and quiescent cells. The molecular biology of adenovirus has been well characterized, and its safety has been demonstrated in a large number of clinical trials. Adenoviruses are ubiquitous viruses whose native pathogenesis typically involves mild upper respiratory tract, ocular, and gastrointestinal infections. Adenoviral vectors are stable, easy to manipulate, and production of high titers according to current good manufacturing practices (cGMP) is well established. Additionally, adenoviral DNA does not integrate into the host genome, resulting in a low risk of mutagenesis. Limited duration of viral gene expression may render adenoviruses less desirable for the treatment of hereditary diseases, where long-term expression is needed, but is adequate for cancer gene therapy, where the purpose is to kill target cells.

Adenoviruses are highly immunogenic, which can be advantageous if it leads to an antitumor immune response, but can be a disadvantage if the immune response blocks viral propagation or leads to toxicity. Variable expression of the adenovirus primary receptor on the surface of tumor cells presents another challenge for effective cancer gene therapy. Finally, adenoviruses do not inherently possess a mechanism for inactivation.

### *2.1 Adenovirus*

The adenovirus virion is a nonenveloped icosahedral particle with an outer protein shell surrounding an inner nucleoprotein core (Stewart et al. 1991, Stewart et al. 1993). The main structural components of the capsid include hexon, penton base, and fiber, along with a number of other minor proteins (figure 1). The triangular facets of the capsid are mainly composed of hexon trimers (Rux & Burnett 2004). Penton base units are located at each of the twelve vertices of the capsid anchoring homotrimeric protruding fibers responsible for the virus attachment to the cell surface. The linear, double-stranded 38-kb DNA genome is packaged within the capsid and is associated with histon-like core proteins.

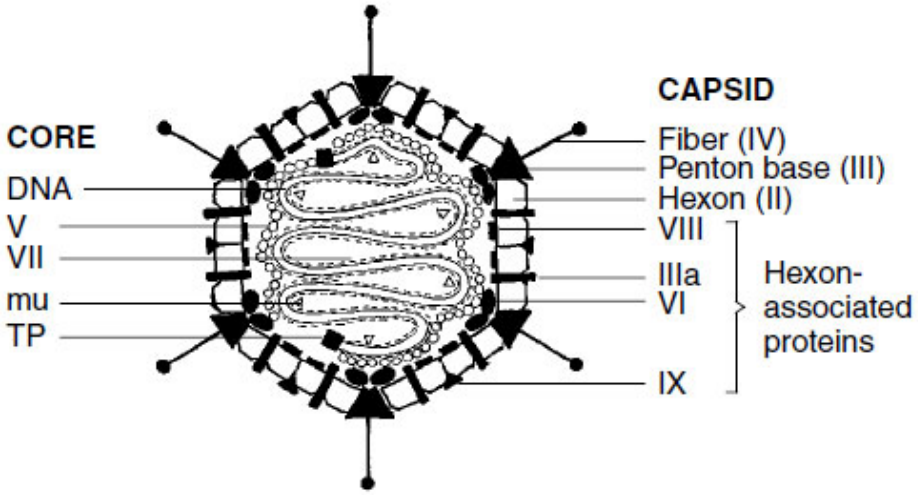


**Figure 1.** Structure of the adenovirus particle (Volpers & Kochanek 2004).

Over 50 different serotypes of human adenovirus have been classified into six subgroups (A-F) based on sequence homology and their ability to agglutinate red blood cells (McConnell & Imperiale 2004). The most commonly used vector for gene therapy, adenovirus serotype 5 (Ad5), belongs to subgroup C. Infection of Ad5 (figure 2) starts by high-affinity binding of the fiber globular knob domain to the cell surface coxsackie-adenovirus receptor (CAR), a type I transmembrane protein of the immunoglobulin superfamily (Bergelson et al. 1997). Members of all subgroups, except group B adenoviruses, utilize CAR as a primary receptor (Roelvink et al. 1998). Recently, members of subgroup B have been shown to bind either CD46, CD80/86, unidentified glycoprotein "receptor X", or combinations of these receptors (Gaggar et al. 2003, Short et al. 2004, Sirena et al. 2004, Marttila et al. 2005, Tuve et al. 2006). Sialic acid is also involved in the uptake of some subgroup D adenoviruses (Wu et al. 2003). In addition, heparan sulfate proteoglycans (HSPGs) have been suggested to promote adenovirus virion attachment to certain cell types, including liver cells, via the putative lysine-lysine-threonine-lysine (KKTK) motif on the fiber shaft (Dechecchi et al. 2001, Smith et al. 2003). However, recent studies argue against the role of the KKTK motif in determining infectivity towards hepatic cells *in vivo* (Shayakhmetov et al. 2005, Parker et al. 2006, Di Paolo et al. 2007). Further, mutation of the KKTK motif seems to affect more than just HSPG binding and might interfere with the correct folding of the fiber (Bayo-Puxan et al. 2006, Kritz et al. 2007).

Initial attachment is followed by internalization of the virus, mediated by secondary interaction of a penton base arginine-glycine-aspartic acid (RGD) motif and cellular $\alpha_v\beta$ integrins, which triggers endocytosis of the virion via clathrin-coated pits (Wickham et al. 1993, Stewart et al. 1997). In the endosome, the virus is disassembled followed by endosomal lysis (Greber et al. 1993). Thereafter, viral DNA is transported to the nucleus through a microtubule-mediated process, and viral genes are expressed (Leopold et al. 2000).

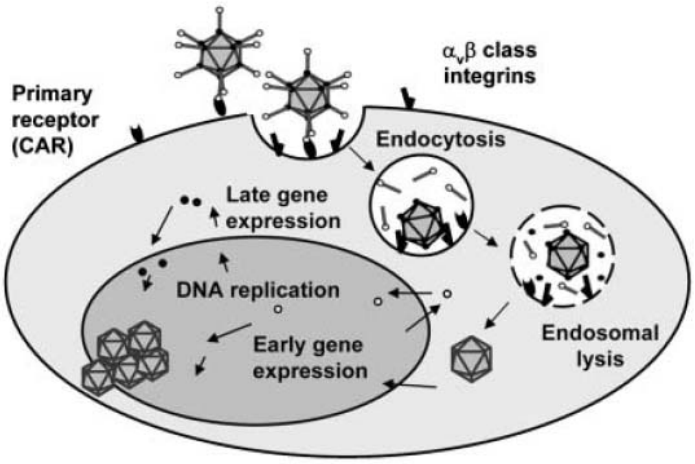


**Figure 2.** Schematic illustration of the Ad5 infection pathway. Initial attachment is mediated by coxsackie-adenovirus receptor (CAR), followed by an interaction with cellular integrins resulting in internalization of the virus via clathrin-mediated endocytosis. In the endosomes, viral genome is released and transported to nucleus for DNA replication (Kanerva & Hemminki 2005).

The adenoviral genome can be divided into immediate early (E1A), early (E1B, E2, E3, E4), intermediate (IX, IVa2), and late (L1-L5) genes (figure 3). Transcription of these genes can be defined as a two-phase event, early and late, occurring before and after virus DNA replication (Russell 2000). The first transcription unit to be expressed is E1A, whose products function to transactivate other early genes and to induce the cell to enter the S phase for replication of the viral genome (Berk 1986). The early gene region encodes mainly regulatory proteins necessary for viral replication, alteration of the host cell cycle, prevention of apoptosis, and interference with the host cell defense mechanisms (Russell 2000). After DNA replication, intermediate genes are produced, followed by expression of late genes driven by the major late promoter (MLP). These genes encode structural components of the virus as well as proteins involved in virion assembly.

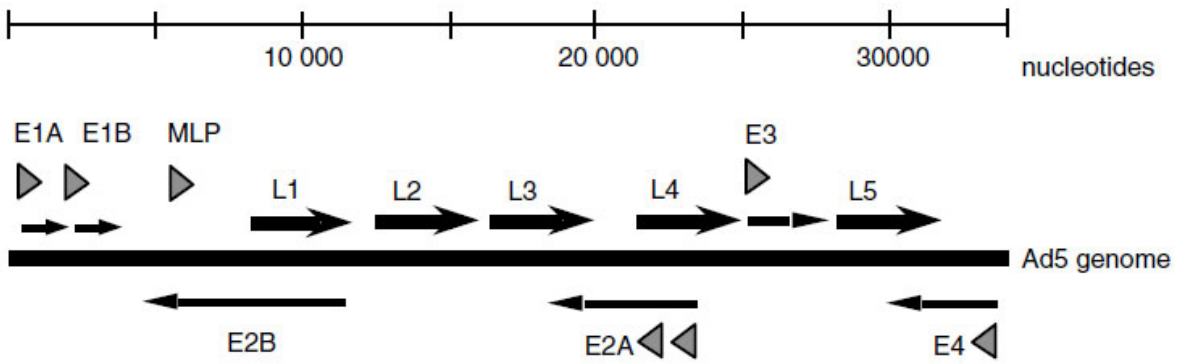


**Figure 3.** Map of the Ad5 genome and transcription units. Promoters are depicted by arrowheads; early (E) and late (L) genes are depicted by thin and heavy arrows, respectively. Arrows indicate the direction of transcription. MLP; major late promoter (Volpers & Kochanek 2004).

## *2.2 Targeting adenoviral vectors to cancer cells*

The key to successful cancer gene therapy lies in the efficient delivery of transgenes specifically to target cells. As viral tropism is mainly determined by the degree of receptor expression, cells producing low levels of CAR are refractory to Ad5 infection and gene transfer (Kim et al. 2002). Although CAR is ubiquitously expressed on a broad range of normal epithelial cells, production of CAR is frequently low in many tumor types, including ovarian cancer (Dmitriev et al. 1998, Miller et al. 1998, Cripe et al. 2001, Kanerva et al. 2002b, Shayakhmetov et al. 2002). Increased CAR expression appears to have a growth inhibitory effect on some cancer cell lines, while loss of CAR expression correlates with tumor progression and advanced disease (Okegawa et al. 2001). CAR is localized in tight junctions, which suggests a role on cell adhesion, and its expression may be cell cycle-dependent (Cohen et al. 2001, Seidman et al. 2001). Various strategies have been evaluated to modify adenovirus tropism in order to circumvent CAR deficiency, to enhance transduction of cancer cells, and to reduce infectivity of normal tissues (Saukkonen & Hemminki 2004, Glasgow et al. 2006, Campos & Barry 2007).

### 2.2.1 Transductional targeting via adapter molecules

Transductional targeting can be achieved by utilizing bispecific adapter molecules that block the interaction with CAR and redirect the virus to a novel receptor. Various molecules have been evaluated to physically bridge the vector to cell surface receptors: **1)** bispecific antibodies, **2)** chemical conjugates between antibody fragments (Fab) and cell-specific ligands, **3)** Fab-antibody conjugates using antibodies against target cell receptors, **4)** Fab-peptide ligand conjugates, and **5)** recombinant fusion proteins that incorporate Fabs and peptide ligands.

The first demonstration of adapter-based retargeting was carried out by utilizing bispecific conjugate consisting of an anti-fiber neutralizing Fab chemically linked to folate (Douglas et al. 1996). This approach resulted in CAR-independent uptake of virus via folate receptors highly expressed on the surface of a variety of malignant cells. Several other Fab-ligand conjugates targeted against, for instance, basic fibroblast growth factor (FGF2) receptor (Goldman et al. 1997, Rancourt et al. 1998, Gu et al. 1999), epidermal growth factor (EGF) receptor (Miller et al. 1998), epithelial cellular adhesion molecule (Ep-CAM) (Haisma et al. 1999, Heideman et al. 2001), tumor-associated glycoprotein 72 (TAG-72) (Kelly et al. 2000), or CD40 (Tillman et al. 1999, Hakkarainen et al. 2003) have been linked to an Ad5 fiber for enhanced transduction of cancer cells. Reynolds et al. (2000) succeeded in targeting

pulmonary endothelial cells *in vivo* after systemic administration of a virus modified with bispecific antibody against Ad5 fiber and angiotensin-converting enzyme (ACE).

An alternative approach to chemical conjugates utilized a single recombinant fusion molecule formed by a truncated, soluble form of CAR (sCAR) fused to either anti-CD40 antibody or EGF (Dmitriev et al. 2000, Hemminki et al. 2001b, Pereboev et al. 2002). To further increase the stability of the complex, a trimeric sCAR-fibritin-anti-erbB2 single-chain antibody molecule was created for targeting c-erbB2-positive cancer cells (Kashentseva et al. 2002). Recently, sCAR was fused to a single-chain antibody directed against carcinoembryonic antigen (CEA), resulting in increased gene expression in CEA-positive lung cells and reduced liver transduction after systemic delivery in mice (Everts et al. 2005, Li et al. 2007).

### 2.2.2 Transductional targeting via genetic manipulation

Another strategy for transductional targeting involves genetic manipulation of viral capsid proteins. Based on native adenovirus receptor recognition, the development of genetically targeted viruses has mainly concentrated on structural modifications of the fiber knob domain including: **1)** peptide incorporation into the fiber knob, **2)** pseudotyping of the fiber, and **3)** deknobbing of the fiber. In addition, other capsid locales have recently been evaluated for inclusion of targeting moieties.

Structural analysis of the major capsid proteins has facilitated the genetic incorporation of foreign peptides into exposed regions of the adenovirus capsid. Initial studies have demonstrated that short peptides can be inserted into the C terminus and HI loop of the fiber knob (Wickham et al. 1996a, Dmitriev et al. 1998, Krasnykh et al. 1998), the RGD-containing loop of the penton base (Wickham et al. 1996b), the hypervariable region 5 (HVR5) loop of the hexon (Crompton et al. 1994, Wu et al. 2005), and the C terminus of protein IX (Dmitriev et al. 2002). Adenoviruses with an integrin-binding RGD motif or heparan sulphate-binding polylysine residues (pK7) incorporated into the C terminus of the fiber knob have yielded positive results *in vitro* and *in vivo*, but insertion of larger peptides may result in inefficient packaging of the virion (Wickham et al. 1997, Wu et al. 2002). The HI loop can tolerate peptide insertions of up to 100 amino acids, with variable effects on virion integrity. Incorporation of RGD-4C peptide into the HI loop enhanced the transduction of Ad5 to a wide range of tumor cells, including ovarian cancer cells (Dmitriev et al. 1998, Vanderkwaak et al. 1999, Hemminki et al. 2001a, Hemminki et al. 2002b). Wu et al. (2002) created a double modified virus with a RGD motif in the HI loop and a pK7 motif in the C terminus. This virus resulted in enhanced infectivity towards ovarian cancer cells compared with viruses carrying a single targeting moiety (Wu et al. 2004). Other peptides have been incorporated into the HI

loop as well (Mizuguchi et al. 2001, Nicklin et al. 2001). In addition, the RGD motif has been successfully inserted into HRV5 of the hexon (Vigne et al. 1999) and into the C terminus of protein IX (Vellinga et al. 2004). Recently, the C terminus of protein IX has gained attention due to its ability to display large polypeptides and proteins on the surface of the virion. Instead of targeting, incorporation of large reporter enzymes allows noninvasive monitoring of viral functions (Le et al. 2004, Li et al. 2005, Matthews et al. 2006). However, most of the strategies utilizing direct ligand incorporation have resulted in vectors with expanded, rather than restricted, tropism. Incorporation of cell binding ligands isolated by phage display technique, or inserting mutations that ablate CAR binding could produce vectors with more specificity towards target cells (Nicklin et al. 2001, Nicklin et al. 2004, Work et al. 2004a).

Adenovirus fiber pseudotyping is a strategy that exploits the natural diversity of the adenovirus family. Genetic replacement of the entire fiber or the knob region of Ad5 with its structural counterpart from another serotype results in CAR-independent transduction of a variety of cell types (Havenga et al. 2002). Most of the studies have evaluated Ad5- or Ad2-based vectors with the fiber or knob derived from subgroup B or D adenoviruses. Adenovirus serotype 3 (Ad3) is a member of subgroup B and therefore recognizes a different receptor than Ad5 (Stevenson et al. 1995). Substitution of the knob domain of Ad5 with the corresponding domain of Ad3 allows binding and entry through the Ad3 receptor, which is expressed to a high degree on tumor cells (Krasnykh et al. 1996, Stevenson et al. 1997, Kanerva et al. 2002a). Ad5/3 chimeric virus displayed enhanced infectivity towards ovarian cancer cells and was able to partially avoid the effect of pre-existing neutralizing anti-Ad5 antibodies (Kanerva et al. 2002a, Kanerva et al. 2002b). Further, the biodistribution, liver toxicity, and blood clearance rates were comparable with wild-type Ad5 virus, suggesting excellent preclinical safety of this approach. Various pseudotyped adenoviruses, including fiber regions from serotypes Ad7 (Gall et al. 1996), Ad11 (Stecher et al. 2001), Ad16 (Goossens et al. 2001, Havenga et al. 2001), Ad17 (Chillon et al. 1999, Zabner et al. 1999), Ad35 (Shayakhmetov et al. 2000, Rea et al. 2001, Mizuguchi & Hayakawa 2002), and several others (Denby et al. 2004, Parker et al. 2007), have been constructed for improved transduction of a broad range of clinically relevant cell types. Moreover, this approach has exploited fiber elements from nonhuman viruses (Glasgow et al. 2004, Stoff-Khalili et al. 2005, Nakayama et al. 2006) and the fiber-like σ1 reovirus attachment protein (Mercier et al. 2004, Tsuruta et al. 2005, Tsuruta et al. 2007).

Finally, deletion of the entire knob region results in knobless fibers and total ablation of CAR binding. Addition of targeting ligand to such fibers allows more specific recognition of target cells. However, trimerization of the fiber protein, which is required for proper function of the fiber, is normally mediated by the knob domain. This problem has been solved by utilizing external trimerization signals, such as MoMuLV envelope glycoprotein trimerization

motif (van Beusechem et al. 2000), a neck region peptide of human lung surfactant protein D (Magnusson et al. 2001), or the foldon domain of bacteriophage T4 fibritin protein (Krasnykh et al. 2001).

### 2.2.3 Transcriptional targeting

Instead of changing tropism of adenoviruses, expression of viral genes or transgenes can be restricted to tumor cells by placing the desired gene under the control of tumor- or tissue-specific promoter (TSP). The gene is therefore expressed selectively in cells with high promoter activity. Additionally, promoters induced by the unique tumor environment, treatment, or certain chemicals can be used to regulate gene expression.

Several TSPs have been explored in order to limit the expression of transgenes specifically to ovarian cancer cells (Casado et al. 2001b, Saukkonen & Hemminki 2004). Leukocyte plastin (L-plastin) is expressed during tumorigenesis, especially in the context of gynecological cancers arising from estrogen-dependent tissues (Lin et al. 1993). Transcriptional control of adenovirus with the L-plastin promoter (LP-P) induced expression of a reporter gene in ovarian cancer cell lines and ascites samples, but little activity was seen in normal human tissues (Chung et al. 1999). In another study, suppression of ovarian tumor growth in mice was achieved when the suicide gene was placed under the control of LP-P (Peng et al. 2001).

Expression of cyclooxygenase-2 (cox-2) is normally low in most tissues, but can be highly induced in response to cell activation by hormones, proinflammatory cytokines, growth factors, and tumor promoters (Saukkonen et al. 2003). The cox-2 promoter has been investigated in the context of ovarian cancer, with promising results (Casado et al. 2001a).

The secretory leukoprotease inhibitor (SLPI) gene is expressed in several different carcinomas, while its expression in normal organs is low (Abe et al. 1997). High activity of the SLPI promoter was demonstrated in ovarian cancer cell lines, in primary ovarian tumor cells isolated from patient samples, and in an orthotopic murine model of ovarian cancer (Barker et al. 2003a). Intraperitoneal (i.p.) delivery of adenovirus armed with the SLPI-driven suicide gene resulted in increased survival of tumor-bearing mice after administration of a prodrug.

Various other TSPs have also been evaluated for the selectivity towards ovarian cancer cells, including CXC chemokine receptor 4 (CXCR4) (Zhu et al. 2004), DF3/MUC1 (Tai et al. 1999), mesothelin (Breidenbach et al. 2005), midkine (MK) (Casado et al. 2001a), and ovarian-specific promoter-1 (OSP1) (Bao et al. 2002).

Although TSPs have the potential to increase specificity and decrease toxicity of adenoviral gene therapy, vectors targeted with TSPs are still dependent on CAR for cell entry.

Therefore, viruses combining both transductional and transcriptional targeting moieties have been developed (Reynolds et al. 2001, Barker et al. 2003b, Work et al. 2004b).

### 2.2.4 Obstacles to systemic targeting

Despite advances in vector retargeting, several obstacles that inhibit the systemic delivery of adenoviruses remain. Although mechanisms of adenovirus attachment and uptake are relatively well understood *in vitro*, less is known about mechanisms governing *in vivo* infection. Studies have shown that CAR expression levels do not correlate with the virus biodistribution, and ablation of CAR binding does not have a significant impact on infectivity *in vivo* (Fechner et al. 1999, Alemany & Curiel 2001).

The majority of intravascularly administered adenovirus is rapidly cleared from the blood circulation, accumulating in liver tissues (Alemany et al. 2000). Uptake of vector particles by Kupffer cells, resident macrophages in the liver, decreases the amount of virus available for therapeutic purposes. Therefore, high viral doses are required for effective transduction of target tissues, which can result in acute toxicity and compromise the safety of the patient. Recently, several blood factors were identified that promote CAR-independent infection of murine hepatocytes (Shayakhmetov et al. 2005, Parker et al. 2006, Baker et al. 2007). Intravascularly delivered adenovirus interacts directly with vitamin K-dependent coagulation zymogens (FVII, FIX, FX, protein C) and complement C4-binding protein (C4BP), which form a bridge between virus particles and hepatic HSPGs or low-density lipoprotein receptor-related protein (LRP). Recent studies suggest that adenovirus binding to FIX occurs via Ad5 hexon protein instead of the fiber knob (Kalyuzhniy et al. 2008, Waddington et al. 2008). Besides plasma proteins, adenovirus can also have direct interactions with host blood cells, preventing access of the vector to target cells (Cotter et al. 2005, Lyons et al. 2006, Stone et al. 2007).

In addition to sequestration by nontarget tissues, systemic delivery of adenovirus triggers innate immunity, including induction of cytokines, inflammation, transient liver toxicity, and thrombocytopenia (Muruve 2004). These responses represent a major barrier for clinical gene therapy and result in part from the uptake of vector particles by Kupffer cells. Initial responses are triggered by the actual viral capsids, which are independent of the transcription of viral genes. Innate host responses are followed by activation of the adaptive immune system and production of neutralizing antibodies (NAbs) (Bessis et al. 2004). Pre-existing NAbs derived from previous exposure to adenovirus through either natural infection or administration of the vector can significantly reduce the effect of therapy.

Furthermore, viral spread in the solid tumor mass can be limited by physical barriers, including extracellular matrix (ECM) and connective tissue (Harrison et al. 2001, Cheng et al.

2007a). In addition to physical limitations, solid tumors contain physiologically different sub-compartments, such as necrotic, hypoxic, or hyperbaric regions, which can restrict the dissemination of the virus.

## 3. Oncolytic virotherapy

Due to safety concerns, gene therapy approaches have traditionally been based on viruses that are unable to replicate in infected cells. Although replication-deficient viruses expressing therapeutic transgenes have provided high preclinical efficacy and good clinical safety data, trials have demonstrated that the utility of these agents may be limited when faced with advanced and wide-spread disease. Viruses that replicate and spread specifically inside the tumor have been suggested as a way of improving penetration of and dissemination within solid tumor masses (Liu & Kirn 2008). Oncolytic viruses used in various cancer gene therapy approaches take advantage of tumor-specific changes that allow preferential replication of the virus in target cells (figure 4). The viral replication cycle causes oncolysis of the cell, resulting in the release of newly generated virions and subsequent infection of neighboring cells. Normal tissue is spared due to lack of replication. Thus, the antitumor effect is delivered by the actual replication of the virus. Therefore, a transgene is not necessarily required, but can be used for additional efficacy. The viral replication cycle allows dramatic local amplification of the input dose, and in theory, the oncolytic process continues as long as target cells persist.

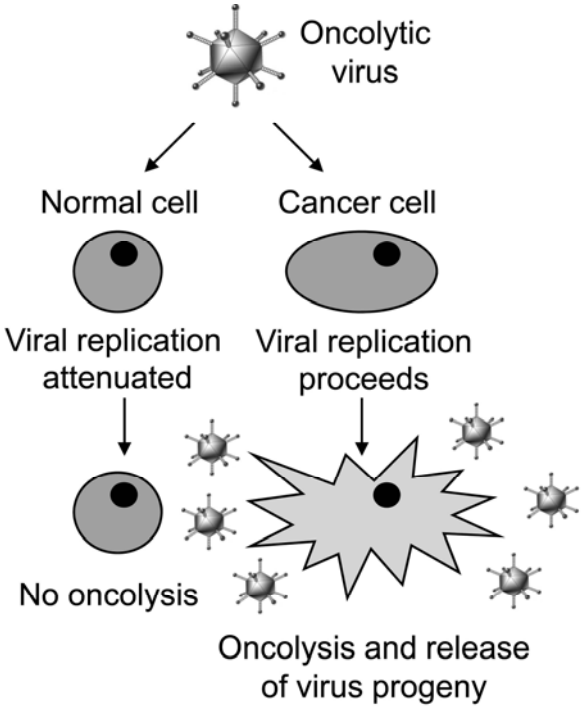


**Figure 4.** Principle of oncolytic virotherapy. Viral infection of cancer cells results in replication, oncolysis and release of virions to surrounding cells. Normal cells are spared due to lack of replication.

Various approaches exist for achieving selective viral replication in tumor cells. As the cellular changes induced by viral infection are often similar to changes acquired during tumor development, it is not surprising that some viruses, such as measles and vesicular stomatitis virus, have inherent selectivity for cancer cells. However, many popular oncolytic viruses, including adenovirus, are genetically modified to replicate specifically in tumor cells (Kanerva & Hemminki 2005). One way to restrict replication of adenoviruses to malignant tissues is to limit the expression of the viral E1A gene product to cancer cells with TSPs. Another strategy involves engineering deletions in viral genes critical for efficient replication in normal cells but not in cancer cells.

## *3.1 Type I oncolytic adenoviruses*

With type I oncolytic adenoviruses, tumor-specific replication is achieved by introduction of loss-of-function mutations to the virus genome that require specific cellular factors to compensate for the effects of these mutations. Most approaches are based on deletions in E1A or E1B adenoviral genes, resulting in mutant proteins unable to bind the cellular proteins necessary for viral replication in normal cells, but not in cancer cells (Kanerva & Hemminki 2005).

The first and most widely studied oncolytic adenovirus, ONYX-015 (*dl*1520), carries two deletions in the gene coding for the E1B-55 kD protein (Bischoff et al. 1996). One purpose of this protein is binding and inactivation of tumor suppressor protein p53 in infected cells, for induction of the S phase, which is required for effective virus replication (figure 5). Thus, this virus may have selectivity for cells with an aberrant p53-$p14^{ARF}$ pathway, a common feature of human tumors (Hollstein et al. 1991). Initial studies suggested that this agent replicates selectively in cancer cells lacking functional p53 (Bischoff et al. 1996, Heise et al. 1997, Heise et al. 1999, Rogulski et al. 2000a). However, contradicting studies have suggested that cells with functional p53 also support ONYX-015 replication (Goodrum & Ornelles 1998, Rothmann et al. 1998). Clearly, factors independent of p53 play critical roles in determining the sensitivity of cells to ONYX-015. Loss of function of $p14^{ARF}$, which normally stabilizes p53, can result in inactivation of p53 (Ries et al. 2000). Adenoviral proteins other than E1B-55 kD, including E4orf6, E1B-19 kD, and E1A, have effects on p53 function (Dobner et al. 1996). Further, the functions of E1B-55 kD are not limited to p53 binding, but include also mRNA transport (Yew et al. 1994), and therefore, the virus replicates inefficiently compared with the wild-type adenovirus. Nevertheless, taken together, ONYX-015 data seem to suggest more effective replication in tumor cells than in normal cells (Kirn 2001).

Ad5-Δ24 and *dl*922-947 are closely related viruses that carry a 24-bp deletion in constant region 2 (CR2) of the E1A gene (Fueyo et al. 2000, Heise et al. 2000a). This domain of the

E1A protein is responsible for binding of the retinoblastoma protein (pRb) for induction of the S phase and DNA replication (Whyte et al. 1988, Whyte et al. 1989). In normal cells, pRb acts as a tumor suppressor by inhibiting cell cycle progression via binding to E2F, a transcriptional activator that promotes expression of genes necessary for driving the cell into S phase (Nevins 1992). Adenoviral E1A binds to pRb and releases repression of E2F, allowing it to activate its target genes. However, viruses with CR2 deletion in E1A are unable to bind pRb and have reduced ability to overcome the G1/S checkpoint. Therefore, they replicate selectively in cells deficient in the pRb-p16 pathway, including most cancer cells (Sherr 1996, D'Andrilli et al. 2004). These viral agents are promising anticancer agents, as they are not attenuated in comparison with wild-type viruses, and some reports suggest that they may be even more oncolytic than wild-type viruses in tumor cells (Heise et al. 2000a). However, safety of this approach has not yet been demonstrated in currently ongoing human trials (www.wiley.co.uk/genmed/clinical).

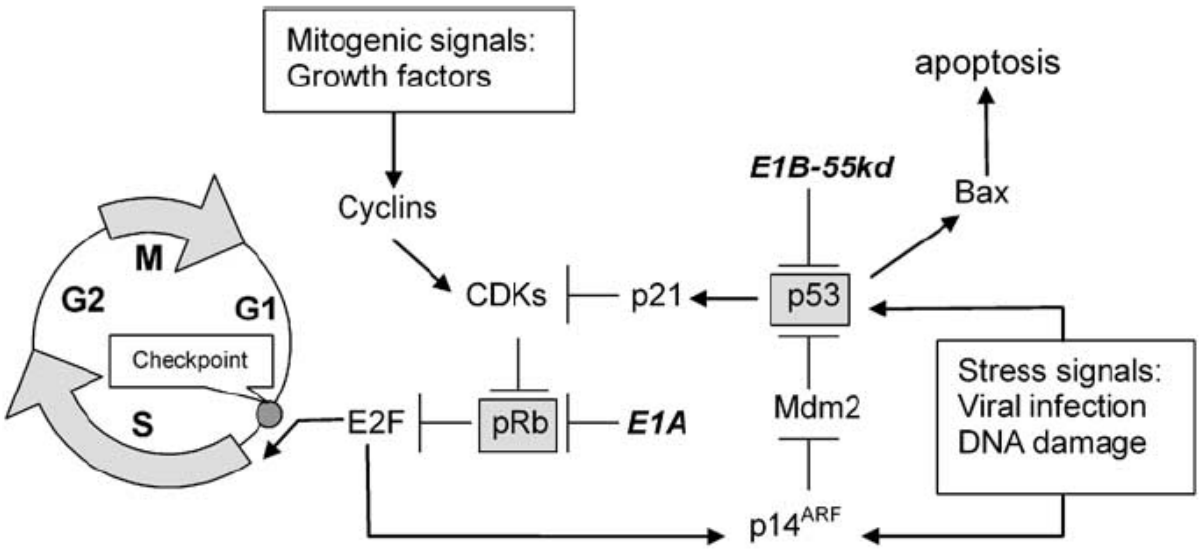


**Figure 5.** Intervention by viral proteins leads to loss of cell cycle control. Tumor suppressor protein p53 is upregulated and activated upon stress signals such as DNA damage or viral infection. p53 can act as a transcription factor to activate expression of genes that induce apoptosis or cell cycle arrest. Adenoviral E1B-55 kD binds to and inactivates p53 leading to progression into the S phase. Retinoblastoma protein (pRb) regulates the G1/S cell cycle checkpoint. pRb exerts its effects by binding to and inhibiting transcription factor E2F, which induces expression of genes needed for DNA synthesis. Adenoviral E1A binds to pRb releasing repression of E2F, allowing it to activate its target genes and leading to progression into the S phase. However, targeted deletions in E1B-55 kD or E1A result in mutant proteins unable to bind p53 or pRb, respectively. Therefore, modified viruses replicate only in cells deficient in these pathways, including most cancer cells (Everts & van der Poel 2005).

## 3.2 Type II oncolytic adenoviruses

With type II oncolytic adenoviruses, TSPs replace endogenous viral promoters, restricting viral replication to target tissues expressing the TSP. Usually, the promoter is placed to control E1A, but alternatively, or in addition, other early genes can also be regulated. Various TSPs have been used to limit viral replication to desired tissues, including alpha-fetoprotein (Hallenbeck et al. 1999, Li et al. 2001), cox-2 (Yamamoto et al. 2003, Kanerva et al. 2004), CXCR4 (Rocconi et al. 2007), DF3/MUC1 (Kurihara et al. 2000), E2F (Tsukuda et al. 2002, Jakubczak et al. 2003, Ryan et al. 2004), human telomerase reverse transcriptase (hTERT) (Uchino et al. 2005), IAI.3B (Hamada et al. 2003), mesothelin (Tsuruta et al. 2008), MK (Ono et al. 2005), prostate-specific antigen (PSA) (Rodriguez et al. 1997, Yu et al. 1999), SLPI (Maemondo et al. 2004, Rein et al. 2005), survivin (Zhu et al. 2005), and tyrosinase (Nettelbeck et al. 2002, Banerjee et al. 2004, Ulasov et al. 2007).

Oncolytic adenoviruses combining both type I and type II strategies have been constructed (Doloff et al. 2008). Such double-control of viral replication is advantageous over a single-control approach because specificity may be gained without loss of efficacy.

## 3.3 Clinical trials with oncolytic adenoviruses

The first cancer trials with replicating adenoviruses were done already in the 1950s (Smith et al. 1956). Ten different serotypes of wild-type adenoviruses were applied intratumorally (i.t.), intra-arterially (i.a.), by both routes, or intravenously (i.v.) into patients with cervical carcinoma. No significant toxicity was reported. A “marked to moderate local tumor response” was described in over half of the patients. However, systemic responses were not detected, and all patients eventually had tumor progression. Although these clinical studies were not performed according to current clinical research standards, they suggest that oncolytic adenoviruses can be safely administered to humans, and that viruses can replicate in tumors for therapeutic effect.

The safety and antitumor efficacy of ONYX-015 has been tested in numerous clinical trials with various tumor types and several routes of administration, with and without concomitant conventional treatments (table 1). The goal has been to sequentially increase systemic exposure to the virus after safety with localized delivery has been shown. Following demonstration of safety and biological activity by the i.t. route, trials were initiated to study intracavitary, i.a., and eventually i.v. administration of ONYX-015 (Kirn 2001). In summary, ONYX-015 has been well tolerated even at the highest doses administered by any route of administration. The lack of clinically significant toxicity has been remarkable. Flu-like symptoms have been the most common toxicities, and have been more frequent in patients receiving intravascular treatment. Unfortunately, single-agent antitumoral activity has mostly

been limited to head and neck cancers (Nemunaitis et al. 2000, Nemunaitis et al. 2001b). However, favorable and potentially synergistic interaction with chemotherapy has been discovered in multiple tumor types, and by multiple routes of administration (Khuri et al. 2000, Lamont et al. 2000, Reid et al. 2001, Reid et al. 2002, Hecht et al. 2003, Galanis et al. 2005).

Recently, i.t. injection of H101, an oncolytic adenovirus closely related to ONYX-015, was evaluated in a phase III trial against squamous cell carcinoma of the head and neck (SCCHN) or esophagus in China (Xia et al. 2004). Combination treatment with 5-fluorouracil (5-FU) and cisplatin resulted in a doubling of the response rate compared with chemotherapy alone (78.8% *versus* 39.6%). This is the first randomized demonstration of safety and efficacy of oncolytic viruses in humans. The virus dose, chemotherapy regimen, injection procedure, and results were very similar to an earlier independent phase II trial performed in USA (Khuri et al. 2000). As a result, H101 has been approved by the Chinese government for use in combination with chemotherapy for the treatment of head and neck cancers.

**Table 1.** Clinical trials with ONYX-015 and H101.

| Phase | Cancer | Pts | Route | Combination | Results | Ref. |
|---|---|---|---|---|---|---|
| I | SCCHN | 22 | i.t. | - | $1x10^{11}$ pfu<br>3 PR, 2 MR | Ganly et al. 2000 |
| I | pancreatic | 23 | i.t. | - | $1x10^{11}$ pfu<br>no responses | Mulvihill et al. 2001 |
| I | metastatic solid tumor | 10 | i.v. | - | $2x10^{13}$ VP<br>no responses | Nemunaitis et al. 2001a |
| I | CLM | 11 | i.ha. | 5-FU and leukovorin | $2x10^{12}$ VP<br>1 PR with combination | Reid et al. 2001 |
| I | ovarian | 16 | i.p. | - | $1x10^{11}$ pfu for 5d<br>no responses | Vasey et al. 2002 |
| I | colon | 5 | i.v. | 5-FU and CPT-11 | $2x10^{12}$ VP<br>no responses | Nemunaitis et al. 2003 |
| I | metastatic solid tumor | 5 | i.v. | IL-2 | $2x10^{11}$ VP<br>no responses | Nemunaitis et al. 2003 |
| I | malignant glioma | 24 | intra-cerebral | - | $1x10^{10}$ pfu<br>no responses | Chiocca et al. 2004 |
| I | metastatic solid tumor | 9 | i.v. | enbrel | $1x10^{12}$ VP<br>weekly for 4w<br>no responses | Nemunaitis et al. 2007 |
| I-II | HCC and CLM | 16 | i.t<br>i.ha.<br>i.v. | 5-FU in phase II | $3x10^{11}$ pfu<br>no responses | Habib et al. 2001 |

| | | | | | | |
|---|---|---|---|---|---|---|
| I-II | pancreatic | 21 | i.t. | gemcitabine | $2x10^{11}$ VP<br>8 injections over 8w<br>2 PR | Hecht et al. 2003 |
| I-II | premalignant oral dysplasia | 22 | m.w. | - | $1x10^{11}$ pfu for 5d followed by 1 dose/w for 5w<br>2 CR, 1 PR | Rudin et al. 2003 |
| I-II | advanced sarcoma | 6 | i.t. | MAP | $5x10^{10}$ pfu<br>1 PR | Galanis et al. 2005 |
| I-II | metastatic colorectal | 24 | i.ha | 5-FU and leukovorin | 2 PR | Reid et al. 2005 |
| II | SCCHN | 37 | i.t. | 5-FU and cisplatin | $1x10^{10}$ pfu for 5d<br>8 CR, 11 PR | Khuri et al. 2000 |
| II | SCCHN | 14 | i.t. | 5-FU and cisplatin | $1x10^{10}$ pfu for 5 d<br>3 CR, 3 PR | Lamont et al. 2000 |
| II | SCCHN | 37 | i.t. | - | 1x $10^{10}$ pfu twice/d for 10d<br>2 CR, 3 PR | Nemunaitis et al. 2000 |
| II | SCCHN | 40 | i.t. | - | $2x10^{11}$ VP twice/d for 10d<br>3CR, 2 PR | Nemunaitis et al. 2001b |
| II | HCC | 10 | i.t.<br>i.v. | - | $3x10^{11}$ pfu<br>1 PR | Habib et al. 2002 |
| II | gastrointestinal cancer metastatic to liver | 27 | i.ha. | 5-FU and leukovorin | $2x10^{12}$ VP<br>3 PR | Reid et al. 2002 |
| II | hepatobiliary carcinoma | 20 | i.t.<br>i.p. | - | $3x10^{10}$ pfu<br>1 PR | Makower et al. 2003 |
| II | metastatic colorectal | 18 | i.v. | - | $2x10^{12}$ VP every 2w<br>no responses | Hamid et al. 2003 |
| II | SCCHN | 15 | i.t. | - | $1x10^{10}$ pfu<br>virus detected in 10/15 tumors | Morley et al. 2004 |
| II (H101) | late stage cancer | 50 | i.t. | 5-FU and cisplatin | $5x10^{11}$ VP daily for 5d<br>3 CR and 11 PR | Lu et al. 2004 |
| III (H101) | SCCHN | 123 | i.t. | 5-FU and cisplatin or adriamycin | $1.5x10^{12}$ VP daily for 5d<br>79% response in combination treatment | Xia et al. 2004 |

Abbreviations: Pts, patients; SCCHN, squamous cell carcinoma of the head and neck; CLM, colorectal liver metastasis; HCC, hepatocellular carcinoma; i.t., intratumoral; i.p., intraperitoneal; i.v., intravenous; i.ha., intrahepatic artery; m.w., mouth wash; 5-FU, 5-fluorouracil; CPT-11, irinotecan; IL-2, interleukin 2; MAP, mitomycin-C-doxorubicin-cisplatin; pfu, plaque forming units; VP, viral particle; CR, complete response; PR, partial response; d, days; w, weeks.

In general, clinical data with E1B-55 kD-deleted viruses point to the need for more effective and selective viruses. Two phase I trials have been completed with a derivative of ONYX-015 that expresses bacterial cytosine deaminase (CD) and herpes simplex virus thymidine kinase (TK) suicide genes (Freytag et al. 2002a, Freytag et al. 2003) (table 2). Intraprostatic Ad5-CD/TKrep combined with prodrug treatment resulted in a transient drop in PSA levels, but no long-term responses were observed. Recently, Freytag et al. (2007b) carried out a phase I trial with their second-generation oncolytic adenovirus armed with improved suicide genes and adenovirus death protein (ADP) in combination with radiotherapy. No dose-limiting toxicities were observed after the treatment.

PSA and rat probasin promoters have been utilized for prostate cancer-specific replication. CG7060 has a PSA promoter and enhancer controlling E1A expression (Rodriguez et al. 1997). In a phase I trial of locally recurrent prostate cancer, CG7060 was well tolerated following intraprostatic injection and resulted in dose-dependent reductions in PSA in some patients (DeWeese et al. 2001). Similar results were obtained in a phase I/II dose escalation trial with intraprostatic CG7870 (DeWeese et al. 2003), which has a rat probasin promoter to control E1A and a PSA promoter and enhancer to drive E1B (Yu et al. 1999). In contrast to CG7060, this virus has an intact E3 region. CG7870 was recently delivered i.v. to patients with hormone-refractory metastatic prostate cancer (Small et al. 2006). Although PSA levels decreased in some patients, neither partial nor complete formal PSA responses were observed.

**Table 2.** Clinical trials with other oncolytic adenoviruses.

| Virus | Phase | Cancer | Pts | Route | Combination | Results | Ref. |
|---|---|---|---|---|---|---|---|
| Ad5-CD/TK*rep* | I | prostate | 16 | i.t. | GCV and 5-FC | $1x10^{12}$ VP<br>≥50% PSA decrease in 3/16 patients | Freytag et al. 2002a, Freytag et al. 2007c |
| Ad5-CD/TK*rep* | I | prostate | 15 | i.t. | GCV, 5-FC and radiation | $1x10^{12}$ VP<br>significant decline in PSA level in all patients | Freytag et al. 2003 |
| Ad5-yCD/ *mut*$TK_{SR39}$ *rep*-ADP | I | prostate | 9 | i.t. | GCV, 5-FC and radiation | $1x10^{12}$ VP x 2<br>significant decline in PSA level in all patients | Freytag et al. 2007b |
| CG7060 (CV706) | I | prostate | 20 | i.t. | - | $1x10^{13}$ VP<br>50% PSA decrease in two highest dose levels | DeWeese et al. 2001 |
| CG7870 (CV787) | I | prostate | 23 | i.v. | - | $6x10^{12}$ VP<br>no responses | Small et al. 2006 |

Abbreviations: Pts, patients; i.t., intratumoral; i.v., intravenous; GCV, ganciclovir; 5-FC, 5-fluorocytosine; PSA, prostate specific antigen; VP, viral particle.

# 4. Improving safety and efficacy of oncolytic virotherapy

Oncolytic adenoviruses have demonstrated tremendous efficacy as single agents in preclinical model systems featuring human xenograft tumors in immune-deficient mice. Unfortunately, while the safety of this approach has been excellent in human clinical trials, complete eradication of solid tumors rarely occurs (Liu & Kirn 2008). Therefore, efforts are underway to improve the potency of these agents. Several strategies are currently being explored to enhance the transduction of target cells and effective penetration of tumor masses. Furthermore, maximizing the clinical benefits to patients might require combination therapies with conventional approaches. Finally, an important aspect for the future is to improve safety of oncolytic agents, as treatment with more potent viruses and combination regimens may increase the risk for undesired side-effects.

## *4.1 Targeting oncolytic adenoviruses to cancer cells*

Various previously described targeting moieties have been introduced into oncolytic adenoviruses to increase the transduction of tumor cells. Ad5-Δ24-RGD features a 24-bp deletion in E1A and a RGD motif in the fiber (Suzuki et al. 2001). This virus has displayed enhanced oncolytic potency in many tumor types, especially against glioma (Lamfers et al. 2002, Fueyo et al. 2003, Lamfers et al. 2007, Alonso et al. 2008) and gynecological cancers (Bauerschmitz et al. 2002, Lam et al. 2003, Bauerschmitz et al. 2004). Ad5-Δ24-RGD was able to replicate efficiently in ovarian cancer primary cell spheroids and resulted in improved survival in an orthotopic model of ovarian cancer. Recently, the safety profile of i.p.-delivered Ad5-Δ24-RGD was evaluated (Page et al. 2007), and the virus is currently in clinical trials for the treatment of recurrent malignant glioma and ovarian cancer (www.wiley.co.uk/genmed/clinical).

Ad5/3-Δ24 is another pRb-p16 pathway selective adenovirus, whose knob region has been pseudotyped from Ad5 to Ad3. This Ad3 receptor-targeted oncolytic agent has previously been demonstrated to deliver a powerful antitumor effect to ovarian cancer cells *in vitro*, to clinical ovarian cancer specimens, and in orthotopic models of ovarian cancer (Kanerva et al. 2003, Lam et al. 2004). In addition, Ad5/3-Δ24 has shown increased therapeutic efficacy in other tumor types as well (Kangasniemi et al. 2006, Guse et al. 2007b). Ad5/3 fiber chimeric oncolytic adenoviruses based on SLPI (Rein et al. 2005), CXCR4 (Rocconi et al. 2007), survivin (Zhu et al. 2008), and mesothelin (Tsuruta et al. 2008) promoters have been recently studied as promising candidates for treatment of metastatic ovarian cancer. Further, even triple-targeted adenoviruses have been developed in order to enhance infectivity and specificity towards cancer cells. Bauerschmitz et al. (2006)

engineered an Ad3 receptor-targeted oncolytic adenovirus featuring cox-2 promoter-driven variants of the E1A gene. Ad5/3-cox2LΔ24 demonstrated increased selectivity in ovarian cancer cells compared with normal cells and a therapeutic benefit in ovarian cancer xenografts (Bauerschmitz et al. 2006, Guse et al. 2007a).

## *4.2 Combination with conventional therapies*

A powerful approach for increasing the efficacy of virotherapy is utilization of oncolytic adenoviruses in combination with traditional anticancer therapies in a multimodal antitumor approach (Post et al. 2003). Oncolytic tumor killing differs mechanistically from conventional therapies, and therefore, additive or even synergistic effects are possible. The toxicity profiles of the treatments may be different and could result in enhanced therapeutic efficacy without increased side-effects. Cross-resistance, common for chemotherapeutics, is unlikely since agents have different mechanisms of cell killing. Finally, it may be possible to use lower treatment doses and still achieve efficacy.

Several preclinical studies with various tumor models have demonstrated improved therapeutic efficacy when ONYX-015 has been combined with standard chemotherapeutic drugs such as 5-FU, cisplatin, paclitaxel, or doxorubicin (Heise et al. 1997, Heise et al. 2000b, You et al. 2000, Portella et al. 2002). Heise et al. (2000b) suggested that the synergistic effects were highly dependent on sequencing of the agents. Treatment of SCCHN and ovarian cancer xenografts with ONYX-015 prior to or simultaneously with chemotherapy was superior to chemotherapy followed by virus treatment. ONYX-015 has also been used in conjunction with chemotherapy in several human clinical trials, providing evidence of synergistic interactions and safety of the approach (table 1). The most encouraging results were seen in a phase II trial of i.t.-delivered ONYX-015 in combination with 5-FU and cisplatin against SCCHN (Khuri et al. 2000). The treatment resulted in 27% complete and 36% partial responses. The combined treatment was well tolerated, with no apparent increase in toxicity compared with a single treatment. Similar results were obtained with the related virus H101 (Lu et al. 2004, Xia et al. 2004). Ad5-Δ24 has been successfully combined with CPT-11 for the treatment of glioma cells *in vitro* and *in vivo* (Gomez-Manzano et al. 2006). Administration of Ad5-Δ24 potentiated the chemotherapy-mediated antitumor effect without modifying the replicative phenotype of the virus. The use of Ad5-Δ24-RGD and everolimus has also been evaluated (Alonso et al. 2008). Combination treatment resulted in induction of autophagy *in vitro* and an enhanced antiglioma effect in tumor-bearing animals. Icovir-5 is an Ad5-Δ24-RGD-based oncolytic adenovirus containing an insulated form of the E2F-1 promoter and the Kozak sequence upstream of viral E1A (Alonso et al. 2007a, Cascallo et al. 2007). Recently, Icovir-5 was given in conjunction with everolimus and temozolomide

against glioma, with promising results (Alonso et al. 2007b). Numerous other preclinical studies have suggested enhanced cell killing and synergistic activity when oncolytic adenoviruses and chemotherapeutic agents are combined (Li et al. 2001, Yu et al. 2001, Fujiwara et al. 2006, Hoffmann et al. 2006, Mantwill et al. 2006, Pan et al. 2007a, Pan et al. 2007b, Ganesh et al. 2008a, Lei et al. 2008).

The combination of ONYX-015 and radiotherapy has exhibited an additive antitumor effect in colon carcinoma (Rogulski et al. 2000a) and glioma (Geoerger et al. 2002) animal models, as well as in thyroid cancer cells *in vitro* (Portella et al. 2003) Importantly, the level of viral replication *in vitro* after radiation exposure was not significantly reduced even with 20 Gy. Other oncolytic adenoviruses that have been studied with radiotherapy include Ad5-CD/TKrep and its second-generation variant (Freytag et al. 1998, Rogulski et al. 2000b, Freytag et al. 2002b, Freytag et al. 2003, Freytag et al. 2007a, Freytag et al. 2007b), CG7060 (Chen et al. 2001), CG787 (Dilley et al. 2005), Ad5-Δ24 (Idema et al. 2007), and Ad5-Δ24-RGD (Lamfers et al. 2002, Lamfers et al. 2007).

## *4.3 Armed oncolytic adenoviruses*

A useful approach for further enhancing the oncolytic potency of replicating agents is to arm viruses with therapeutic transgenes such as genes encoding prodrug-converting enzymes (figure 6), a.k.a. suicide genes (Hermiston & Kuhn 2002). The TK/GCV system is one of the most studied prodrug strategies in gene therapy. The approach is based on delivery of a gene encoding herpes simplex virus TK, which can convert systemically administered nonharmful prodrug ganciclovir (GCV) into a cytotoxic metabolite. TK phosphorylates GCV to its corresponding monophosphate, and cellular kinases further phosphorylate it into a toxic triphosphate form (Miller & Miller 1980). This can be incorporated into DNA, resulting in chain termination, DNA damage, and ultimately cell death (Ilsley et al. 1995, Haynes et al. 1996, Thust et al. 1996). This system is associated with killing of uninfected neighboring cells (bystander effect) (van Dillen et al. 2002). Reports combining the TK/GCV system with replicating adenoviruses have been rather controversial. Some studies demonstrate that the antitumor efficacy of TK-expressing oncolytic adenoviruses is enhanced by treatment with GCV (Wildner et al. 1999a, Wildner et al. 1999b, Nanda et al. 2001). Other reports suggest that GCV does not further improve the oncolytic potential of replicating adenoviruses, at least not *in vivo* (Morris & Wildner 2000, Rogulski et al. 2000b, Wildner & Morris 2000, Lambright et al. 2001, Hakkarainen et al. 2006). Interestingly, most studies showing enhanced efficacy with the TK/GCV system have been performed with viruses with low oncolytic potential, but its utility in combination with highly effective infectivity-enhanced viruses is unknown.

Other common prodrug-converting enzymes utilized in the context of replicating adenoviruses include bacterial or yeast CD (Bernt et al. 2002, Fuerer & Iggo 2004, Conrad et al. 2005, Liu & Deisseroth 2006) and rabbit carboxylesterase (CE) (Stubdal et al. 2003, Oosterhoff et al. 2005), which are combined with 5-fluorocytosine (5-FC) and irinotecan (CPT-11), respectively. Double suicide gene therapy with the TK and CD fusion gene has proven useful both preclinically (Freytag et al. 1998, Rogulski et al. 2000b, Freytag et al. 2002b, Barton et al. 2006, Freytag et al. 2007a), and in clinical trials (Freytag et al. 2002a, Freytag et al. 2003, Freytag et al. 2007b).

Finally, transgenes encoding tumor suppressor proteins (p53), immunostimulatory factors (cytokines), or proteins that target the tumor microenvironment (relaxin, matrix metalloproteinases) have been successfully coupled with the lytic capability of the replicating adenovirus (Sauthoff et al. 2002, van Beusechem et al. 2002, Geoerger et al. 2004, Kim et al. 2006, Ramesh et al. 2006, Su et al. 2006, Zhao et al. 2006, Cheng et al. 2007b, Ganesh et al. 2007, Lei et al. 2008, Luo et al. 2008) .

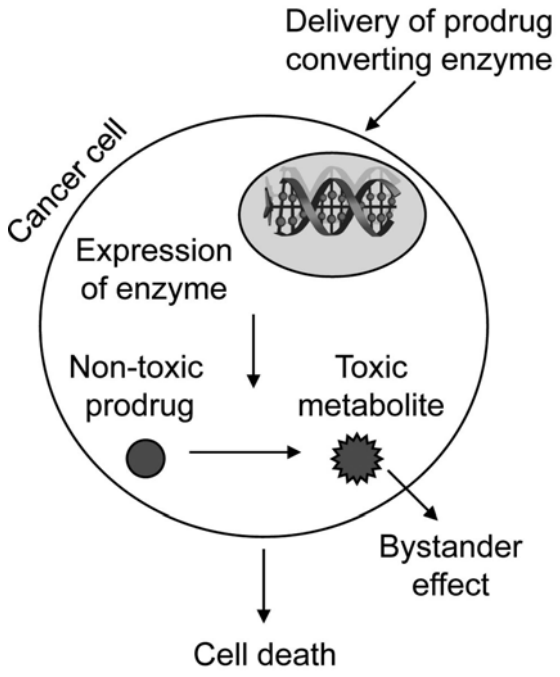


**Figure 6.** Principle of gene-directed enzyme prodrug therapy. Delivery and expression of a suicide gene results in conversion of a non-toxic prodrug into a cytotoxic metabolite. This leads to cell death and eradication of surrounding cells (bystander effect).

## *4.4 Safety switch strategies*

Improved understanding of adenovirus biology and its interactions with cellular proteins and the immune system has allowed creation of more potent viruses for cancer gene therapy. However, with more effective therapy regimens, an increase in undesired side-effects may occur. The potential for adenovirus-associated toxicity is corroborated by reports of severe toxicity in immunocompromised patients (Kojaoghlanian et al. 2003), and a fatality in an adenoviral gene therapy trial (Raper et al. 2003). Therefore, termination of viral replication when necessary would be useful (safety switch).

Adenoviruses do not inherently possess a mechanism for inactivation. Further, no formally approved antiviral therapy exists for adenovirus infections, although some promising agents have emerged. Most compounds reported to have anti-adenovirus activity, such as cidofovir and ribavirin, are nucleoside or nucleotide analogs that target the adenovirus DNA polymerase and subsequently inhibit DNA replication (Naesens et al. 2005, Lenaerts & Naesens 2006). Cidofovir has been successfully used in clinical studies for adenovirus infections (Ljungman et al. 2003), and its antiviral activity against oncolytic adenovirus Telomelysin has been evaluated *in vitro* (Ouchi et al. 2008). Administration of cidofovir suppressed the cytopathic effect and decreased the E1A copy number in lung cancer cells. Other pharmacological agents with antiviral activities include the antipsychotic agent chlorpromazine and the natural bioflavonoid apigenin. Chlorpromazine prevents endosomal uptake of viruses by inhibiting the assembly of clathrin adapter protein AP-2 on clathrin coated pits, which causes the pits to disappear from the cell surface (Wang et al. 1993). Apigenin is a potent cytostatic agent (Patel et al. 2007) that exerts its effects on the cell cycle by activating a reversible G2/M and G0/G1 arrest, inhibiting the induction of S phase and DNA synthesis (Sato et al. 1994, Lepley & Pelling 1997, Wang et al. 2000, Yin et al. 2001).

Another mechanism for increasing safety is to use regulatable gene expression systems (Goverdhana et al. 2005). Promoters that are responsive to a variety of environmental or physiological changes can be placed to drive expression of a transgene or viral genes. Promoters activated by radiation or hypoxia have been commonly used in gene therapy (Hallahan et al. 1995, Ido et al. 2001, Binley et al. 2002, Greco et al. 2006, Mezhir et al. 2006). Regulation can also be achieved by inducing promoters with certain chemical agents such as tetracycline (Hillen & Berens 1994, Fechner et al. 2003). Recently, Kanerva et al. (2008) evaluated the effect of anti-inflammatory agents on the activity of cox-2 and vascular endothelial growth factor (VEGF) promoters.

Finally, as many common prodrugs act by inhibiting DNA synthesis, suicide genes may also be utilized as a fail-safe mechanism in the case of uncontrolled viral replication (Wildner et al. 2003).

### *4.5 Other approaches*

Adenoviruses are highly immunogenic in nature, and immune responses towards the vector are a major hurdle for long-term gene expression and oncolytic potency. Successful gene transfer applications usually require readministration of virus, the efficacy of which may be inhibited by induction of NAbs. A serotype switch is a straightforward strategy for facilitating retreatment with adenoviruses. Treatment is based on alternating related viruses with capsids from different serotypes or even different species with the rationale that second administration would not encounter a neutralizing response (Mastrangeli et al. 1996, Moffatt et al. 2000, Sarkioja et al. 2008). Adenoviruses can be masked with synthetic molecules to circumvent recognition by the immune system. Vectors coated with polyethylene glycol polymers (PEGs) (O'Riordan et al. 1999, Croyle et al. 2001, Fisher et al. 2001) or encapsulated into silica gel implants (Kangasniemi et al. 2009) have been utilized to evade the neutralizing effect. An intriguing recent approach to improve the bioavailability of vectors while remaining hidden from the immune system takes advantage of tumor-homing cells as carriers of oncolytic adenoviruses (Komarova et al. 2006, Hakkarainen et al. 2007). Further, pre/co-administration of vector with immunosuppressive agents, such as cyclophosphamide, results in transient block of NAbs and can increase the duration of transgene expression (Smith et al. 1996, Lamfers et al. 2006). Other methods that have been explored to avoid immune attack towards adenovirus involve depletion of Kupffer cells (Schiedner et al. 2003, Ranki et al. 2007) and physical removal of NAbs by using immunopheresis, or an adenovirus capsid protein column (Rahman et al. 2001).

An alternative approach to ECM-modifying transgenes is pre/co-treatment with proteolytic enzymes or vaso-active compounds that alter the tumor microenvironment (Kuriyama et al. 2001, Cairns et al. 2006, Ganesh et al. 2008b). Furthermore, increasing oxygenation of tumors to relieve hypoxia and to lower the interstitial pressure has been proposed to facilitate drug delivery and dissemination by normalizing the vasculature (Minchinton & Tannock 2006).

## 5. Ovarian cancer

Ovarian cancer is the leading cause of mortality from gynecological malignancies in developed countries (Parkin et al. 2005). In Finland, it is the ninth most common cancer among women, with 457 new cases in 2006, and the fifth cancer-related cause of death (www.cancerregistry.fi). The median age at diagnosis is 62 years, and the risk for disease increases with age.

Ovarian carcinomas, tumors originating from the surface epithelium, account for approximately 85% of all malignant ovarian tumors and exhibit several histological subtypes with different pathogenesis and outcome (Heintz et al. 2001). The most common histological types of epithelial ovarian carcinoma are serous, endometroid, and mucinous carcinoma, representing 50%, 15%, and 15% of all cases, respectively. Due to mild early symptoms and lack of available screening methods, most cases of ovarian cancer are diagnosed at an advanced stage resulting in poor prognosis. Approximately 75% of patients are at International Federation of Gynecology and Obstetrics (FIGO) stages II-IV at the time of diagnosis. Long-term survival of patients with metastatic disease rarely exceeds 15-30%.

The most common approach for the treatment of ovarian cancer is cytoreductive surgery, followed by systemic chemotherapy (McGuire & Markman 2003). Today, a combination therapy with carboplatin and paclitaxel is utilized as a first-line chemotherapy regimen in most cases. Although these therapies often provide initial responses, drug resistance eventually occurs and disseminated disease cannot be cured. Therefore, the management of recurrent ovarian cancer remains difficult, and novel treatment approaches are needed. Adenoviral cancer gene therapy is an attractive modality for ovarian cancer, as tumor tissues preferentially disseminate throughout the peritoneal cavity, allowing a degree of compartmentalization and creating a rationale for direct locoregional delivery. Some of the harmful side-effects of systemic treatment may thus be circumvented. Growing evidence suggests that patients with microscopic residual disease might benefit from i.p. treatment (Armstrong et al. 2006).

## *5.1 Adenoviral gene therapy trials for ovarian cancer*

Most adenoviral gene therapy trials for ovarian cancer have been carried out with replication-deficient vectors encoding a therapeutic transgene. Mutation of the p53 tumor suppressor gene is one of the most frequent genetic changes in cancer. Alterations of the p53 gene have been found in nearly 60% of advanced ovarian cancers (Shahin et al. 2000). Preclinical studies have demonstrated that adenovirus-mediated delivery of wild-type p53 inhibits growth of ovarian cancer cells both *in vitro* and *in vivo* (Santoso et al. 1995, Mujoo et al. 1996). Adp53, a recombinant adenovirus encoding human wild-type p53, was evaluated in a phase I/II trial, and i.p. virus treatment was well tolerated (Buller et al. 2002a, Buller et al. 2002b). In phase I trial, patients received a single injection of the virus, while in phase I/II they were treated with multiple doses of up to $7.5 \times 10^{13}$ viral particles (VP) for five consecutive days. Gene transfer and biological acitivity were also demonstrated (Wen et al. 2003). Promising data led to a randomized phase II/III trial, where Adp53 was given i.p. in combination with chemotherapy (Zeimet & Marth 2003). However, the first interim analysis suggested a lack of

therapeutic effect and increased toxicity, and the study was closed. Recently, Wolf et al. (2004) completed a phase I trial of multiple-dose Adp53 given i.p. to patients with chemo-resistant ovarian cancer. Again, the treatment was well tolerated, but no response was detected.

Growth factor receptors can be utilized as a target for replacement or inactivation. Deshane et al. (1994) constructed a gene that encodes an intracellular single-chain antibody (intrabody) against the growth factor receptor erbB2 (HER-2/neu). The receptor is highly expressed in 10-15% of ovarian cancers and has a correlation with poor prognosis (Slamon et al. 1989). Adenovirus (Ad21)-mediated transfer of an anti-erbB2 intrabody to ovarian tumors resulted in induction of apoptosis and cytotoxicity *in vitro*, and enhanced efficacy and survival in animal models of ovarian cancer (Deshane et al. 1995a, Deshane et al. 1995b, Deshane et al. 1996). The intrabody strategy was also evaluated in a phase I trial (Alvarez et al. 2000a). Treatment with Ad21 i.p. up to a dose of $1 \times 10^{11}$ plaque-forming units (pfu) was well tolerated without dose-limiting toxicity. Gene transfer and expression of transgene were demonstrated, but no response was detected.

Based on promising preclinical results (Rosenfeld et al. 1995, Tong et al. 1996), Alvarez et al. (2000b) utilized i.p. delivery of a replication-deficient adenovirus (AdHSV-TK) combined with i.v. GCV. A single viral dose from $1 \times 10^{9}$ to $1 \times 10^{11}$ pfu was followed by 14 days of GCV. No dose-limiting virus-related side-effects were seen, and 38% of patients had stable disease. However, no objective responses were detected. The presence of transgene could be detected from ascites samples of patients. Another phase I study combined i.p. delivery of AdHSV-TK with i.v. administration of acyclovir and topotecan (Hasenburg et al. 2000). With doses ranging from $2 \times 10^{10}$ to $2 \times 10^{13}$ VP, no dose-limiting adverse effects were seen, and the most common side-effect was myelosuppression, with grade 3-4 thrombocytopenia and neutropenia most likely related to chemotherapy. The median survival of these patients was 18.5 months (Hasenburg et al. 2001).

The only completed ovarian cancer trial with oncolytic adenovirus was carried out with ONYX-015 (Vasey et al. 2002). In the phase I trial, 16 patients received 1-4 cycles of ONYX-015 i.p. on five consecutive days at doses from $1 \times 10^{9}$ to $1 \times 10^{11}$ pfu. Treatment resulted in grade 3 abdominal pain and diarrhea in one patient, but the maximum-tolerated dose was not reached. The presence of viral DNA was detected for up to 10 days after the final infusion, suggesting viral replication. Disappointingly, no clinical or radiological responses were observed in any of the patients.

Importantly, none of the above-described trials were randomized. Recent randomized, controlled trials with i.t.-delivered AdHSV-TK + GCV (Immonen et al. 2004) and Adp53 + radiation (Pan et al. 2008) against glioma and nasopharyngeal carcinoma, respectively,

demonstrate that even treatment with replication-deficient first-generation vectors can result in enhanced antitumor efficacy and increased survival.

# AIMS OF THE STUDY

**1.** To evaluate the antitumor efficacy of Ad5/3-Δ24 in combination with conventional chemotherapeutic agents against ovarian cancer *in vitro* and *in vivo* (**I-II**).

**2.** To analyze the effect of dose and scheduling of i.p.-delivered Ad5/3-Δ24 *in vivo*. We investigated whether multiple i.p. injections are superior to a single dose, and determined the lowest effective dose of the virus (**II**).

**3.** To construct Ad5/3-Δ24-TK-GFP, a serotype 3 receptor-targeted pRb-p16 pathway-selective oncolytic adenovirus containing the herpes simplex virus thymidine kinase-green fluorescent protein (TK-GFP) fusion gene in the partly deleted E3 region. Functionality of the virus was examined *in vitro* as well as *in vivo* (**III)**.

**4.** To evaluate the effect of chlorpromazine, an inhibitor of clathrin-dependent endocytosis, and apigenin, a cell cycle regulator, on adenovirus replication and associated toxicity *in vitro* and *in vivo* (**IV**).

# MATERIALS AND METHODS

## 1. Cell lines and fresh human liver tissue

All cell lines (table 3) were cultured in the recommended growth medium supplemented with 10% FCS, 2 mM L-glutamine, and penicillin-streptomycin solution. Cells were maintained in a humified atmosphere at 37°C and 5% $CO_2$.

**Table 3.** Human cell lines used in this study.

| Cell line | Description | Source | Study |
|---|---|---|---|
| 293 | Transformed embryonic kidney cells | ATCC (Manassas, VA, USA) | I-IV |
| A549 | Lung adenocarcinoma cells | ATCC (Manassas, VA, USA) | I-IV |
| MDAH 2774 | Ovarian adenocarcinoma cells | ATCC (Manassas, VA, USA) | III |
| SKOV3.ip1 | Ovarian adenocarcinoma cells | Dr. J Price (M.D. Anderson Cancer Center, Houston, TX, USA) | I-IV |
| SKOV3-luc | Ovarian adenocarcinoma cells expressing firefly luciferase | Dr. R Negrin (Stanford Medical School, Stanford, CA, USA) | III |
| Hey | Ovarian adenocarcinoma | Dr. J Wolf (M.D. Anderson Cancer Center, Houston, TX, USA) | I-IV |
| OV-4 | Ovarian adenocarcinoma | Dr. TJ Eberlein (Harvard Medical School, Boston, MA, USA) | III-IV |

Permission for studies on human tissue was obtained from the ethics committee prior to experiments. Fresh liver samples (**IV**) were received from healthy donor livers that were to be transplanted into recipients (Department of Surgery, Helsinki University Central Hospital). Livers were kept on ice with ViaSpan solution (Bristol-Myers Squibb Ab, Bromma, Sweden) until slicing. Precision-cut slices (250 μm) were cut with a Vibratome 1000 Plus sectioning system (Vibratome, St. Louis, MO, USA) and cultured on a rocker at 37°C and 5% $CO_2$ in oxygenated William's Medium E supplemented with 25 mM D-glucose and 50 μg/ml gentamycin.

## 2. Recombinant adenoviruses

To create oncolytic Ad5/3-Δ24-TK-GFP, we utilized a plasmid pTHSN-TGL. Briefly, the plasmid was constructed by digesting pTHSN (Kanerva et al. 2005), a plasmid containing the E3 region of the adenoviral genome, with SunI/MunI, and inserting TK-GFP (Loimas et al. 1998) into the resulting 965-bp 6.7K/gp19K deletion of E3A (Hawkins et al. 2001). pAdEasy-1.5/3-Δ24-TGL was generated by homologous recombination in *Escherichia coli* BJ5183

cells (Qbiogene Inc., Irvine, CA, USA) between FspI-linearized pTHSN-TGL and SrfI-linearized pAdEasy-1.5/3-Δ24, a rescue plasmid containing the Ad3 knob and a 24-bp deletion in E1A (Kanerva et al. 2005). The genome of Ad5/3-Δ24-TK-GFP was released by PacI digestion and subsequent transfection of A549 cells.

To construct the E1-deleted control virus Ad5/3-TK-GFP, we used PCR amplification to engineer BglII/XhoI restriction sites around the TK-GFP gene: 5'-ACAGATCTCTAGAGGATCTTGGTGGCGTGAA-3' and 5'-TACTCGAGCTAGAGGATCCCCGGCCG-3'. pShuttle-CMV (He et al. 1998) was digested with BglII/XhoI, and the TK-GFP fragment was ligated into the multiple cloning site under the control of the CMV immediate early promoter to generate pShuttle-CMV-TGL. Homologous recombination was performed between pAdEasy-1.5/3 and PmeI-linearized pShuttle-CMV-TGL to construct pAdEasy-1.5/3-TGL. The genome of Ad5/3-TK-GFP was released by PacI and transfected into 293 cells.

Ad5/3-Δ24-Δgp19K, an oncolytic virus containing a 965-bp deletion in E3A, was constructed as above, but without ligating TK-GFP into pTHSN. Characterization and production of Ad5/3luc1 and Ad5/3-Δ24 have been previously described (Kanerva et al. 2002a, Kanerva et al. 2003).

All recombinant adenoviruses are listed in table 4. Oncolytic and replication-deficient viruses were amplified on A549 and 293 cells, respectively, and purified on double cesium chloride gradients. The VP concentration was measured at 260 nm, and infectious particles were determined by standard plaque assay or $TCID_{50}$ on 293 cells. Modified viral regions were confirmed by polymerase chain reaction (PCR) and sequencing.

**Table 4.** Recombinant adenoviruses used in this study.

| Virus | Description | Source | Study |
|---|---|---|---|
| Ad5/3luc1 | Ad5 virus with deleted E1/E3<br>luciferase gene under CMV promoter replacing E1<br>chimeric fiber with receptor-binding knob domain of Ad3 | (Kanerva et al. 2002a) | I-II |
| Ad5/3-TK-GFP | Ad5 virus with deleted E1/E3<br>TK-GFP gene under CMV promoter replacing E1<br>chimeric fiber with receptor-binding knob domain of Ad3 | This study | III |
| Ad5/3-Δ24 | Ad5 virus with 24-bp deletion in CR2 of E1A<br>chimeric fiber with receptor-binding knob domain of Ad3 | (Kanerva et al. 2003) | I-IV |
| Ad5/3-Δ24-TK-GFP | Ad5 virus with 24-bp deletion in CR2 of E1A<br>TK-GFP gene under E3 promoter in partly deleted E3<br>chimeric fiber with receptor-binding knob domain of Ad3 | This study | III |
| Ad5/3-Δ24-Δgp19K | Ad5 virus with 24-bp deletion in CR2 of E1A<br>partly deleted E3<br>chimeric fiber with receptor-binding knob domain of Ad3 | This study | III |

## 3. Agents

Gemcitabine (Gemzar®) was purchased from Eli Lilly and Company (Indianapolis, IN, USA) (**I-II**). The stock solution was prepared in 0.9% NaCl. Epirubicin (Farmorubicin®) 2 mg/ml solution was obtained from Pharmacia (Vantaa, Finland) (**II**). GCV was purchased from Roche (Espoo, Finland) (**III**). Stock solution 50 mg/ml was prepared in sterilized water. Apigenin and chlorpromazine were purchased from Sigma-Aldrich Finland (Helsinki, Finland) (**IV**). Apigenin stock solution 50 mg/ml was prepared in 1M KOH for *in vitro* studies. For *in vivo* experiments, 25 mg/ml stocks of chlorpromazine and apigenin were prepared in 1% Tween 80 and 0% growth medium, respectively. All agents were further diluted in growth medium immediately before use.

## 4. *In vitro* experiments

### *4.1 Cytotoxicity assay* (I-IV)

Cells were seeded at a density of 1 x $10^4$ cells/well on 96-well plates and cultured overnight. Briefly, cells were either infected with variable concentrations of viruses or treated with agents, or treated with a combination of both. Detailed description of doses and schedules utilized in different studies can be found in the original publications. Cells were incubated at 37°C until almost complete cell killing was visually evident. Thereafter, cell viability was measured with the CellTiter 96 $AQ_{ueous}$ One Solution Cell Proliferation Assay (MTS assay) (Promega, Madison, WI, USA).

### *4.2 Quantitation of viral replication* (I, III-IV)

Ovarian adenocarcinoma cells were seeded on 6-well plates. The next day, cell monolayers were infected with viruses at 10 VP/cell in 1 ml of growth medium with 2% FCS. Detailed descriptions of administration of gemcitabine **(I)**, GCV **(III)**, chlorpromazine or apigenin **(IV)** can be found in the original publications. Cells and medium were harvested 24-96 h post-infection, lysed by three freeze-thaw cycles, and centrifuged at 4000 rpm for 10 min. The amount of infectious particles present in the resulting supernatants was determined by plaque assay or $TCID_{50}$ on 293 cells.

Liver slices were preincubated with chlorpromazine or apigenin for 1h before administration of $10^7$ VP of viruses **(IV)**. Slices and supernatant were frozen separately at the indicated time-points. After gradual thaw on ice, slices were minced, combined with

supernatant, and freeze-thawed three times. After centrifugation, supernatant was titered on 293 cells.

### *4.3 Correlation of viral replication and GFP expression* (III)

Ovarian adenocarcinoma cells were plated at 1 x $10^4$ cells/well on 96-well plates and cultured overnight. Cells in 10 replicates were infected with viruses at 0.0001-1000 VP/cell in 100 µl of growth medium with 2% FCS. Cells were followed daily for 10 days for CPE, and the amount of infectious particles was calculated based on CPE data by the modified $TCID_{50}$ method. Cells were imaged daily with a Typhoon 9400 variable mode imager (GE Healthcare Life Sciences, Helsinki, Finland) for expression of GFP and images were analyzed with ImageQuant 5.20 software (GE Healthcare Life Sciences, Helsinki, Finland).

## 5. Murine models of ovarian cancer

### *5.1 Mice* (I-IV)

Three to four-week-old female mice were obtained from Taconic (Ejby, Denmark) and quarantined for 2 weeks. All animal experiments were approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland.

Tumors were inoculated i.p. in C.B-17 SCID (severe combined immunodeficiency) mice and followed for survival. The health of the mice was monitored daily, and mice were killed when there was evidence of pain or distress. Subcutaneous (s.c.) tumors were established in flanks of NMRI (Naval Medical Research Institute) nude mice and followed for tumor growth. Tumors were measured with a caliper, and mice were killed when tumors reached a diameter of 10 mm. Tumor size was calculated by using the formula 0.5 x length x $width^2$. All agents were diluted in 500 µl of growth medium for i.p. and 50 µl for i.t. injections. Untreated control mice received only growth medium.

### *5.2 Comparison of single and weekly delivery* (II)

An orthotopic model of ovarian cancer was developed by injecting 3 x $10^6$ Hey cells i.p. On day 3 after cell injection, mice were treated i.p. with 1 x $10^8$ VP, or the same dose was given weekly on days 3, 10, 17, and 24. In another experiment, the tumors were established by injecting 1 x $10^7$ SKOV3.ip1 cells i.p. and treated with a single i.p. injection of 1 x $10^8$ VP on day 10, or the same dose was administered weekly on days 10, 17, 24, and 31.

### *5.3 Determination of lowest effective dose* (II)

Mice were injected i.p. with 1 x $10^7$ SKOV3.ip1 cells, and carcinomatosis was allowed to develop. On day 10, the control group received growth medium i.p., and treatment groups were injected i.p. with $10^2$-$10^6$ VP.

### *5.4 Combination with chemotherapy* (I-II)

Mice were injected i.p. with 1 x $10^7$ SKOV3.ip1 cells and randomized into six treatment groups: **1)** growth medium i.p. on day 10, **2)** 3 x $10^7$ VP of virus i.p. on day 10, **3)** 80 mg/kg gemcitabine i.p. on days 10, 13 and 16. **4)** virus and gemcitabine simultaneously at the same doses and schedules as above, **5)** virus on day 10 and gemcitabine on days 11, 14, and 17, and **6)** gemcitabine on days 10, 13, and 16 and virus on day 11 **(I)**.

In another study, mice received 3 x $10^6$ Hey cells i.p. On days 3, 10, 17, and 24, 40 mg/kg gemcitabine and 1 x $10^8$ VP were administered i.p. either alone or together. Alternatively, 1 mg/kg epirubicin and 1 x $10^8$ VP were given i.p. alone or in combination on days 3, 10, and 17 **(II)**.

### *5.5 Combination with ganciclovir* (III)

S.c. xenografts were established by inoculating 5 x $10^6$ SKOV3-luc cells in both flanks of nude mice. When tumors reached a diameter of approximately 4-5 mm, mice were randomized into six treatment groups. 1 x $10^8$ VP were delivered i.t. on days 7, 8, and 9. A second round of viruses was given on days 19, 20, and 21. 50 mg/kg GCV was administered i.p. daily, starting 48h after the last virus injection, over one-week periods on days 11-17 and 23-29.

An i.p. model was developed by injecting 5 x $10^6$ SKOV3-luc cells i.p. into SCID mice. On day 5, mice were treated i.p. with 1 x $10^8$ VP. 50 mg/kg GCV was given daily for 14 days, starting 48h after virus delivery.

### *5.6 Noninvasive imaging* (III)

Mice were imaged using an IVIS 100 imaging system (Xenogen, Alameda, CA, USA) to detect expression of luciferase or GFP. For bioluminescence imaging, 150 mg/kg D-luciferin (Promega, Madison, WI, USA) was injected i.p., and images were captured 10 min after injection. Photographic and bioluminescence/fluorescence images were overlaid by using Living Image software 2.50 (Xenogen, Alameda, CA, USA). Total flux (photons/s) was measured by drawing regions of interest (ROIs) around tumor areas, enclosing all emitted

signals. Background was subtracted by measuring same-sized ROIs in areas without light emission.

### *5.7 Inhibition of viral replication* (IV)

5 x $10^6$ Hey cells were injected in both flanks of nude mice. Mice were treated i.t. with 3 x $10^8$ VP on days 0, 2, and 4. Mice received 200 μg chlorpromazine or 250 μg apigenin i.p. daily.

In replication assay, Hey tumors were established as above. Mice were treated i.t. with 3 x $10^8$ VP on day 0. Chlorpromazine or apigenin was given as above. Tumors were harvested and homogenized, and virions were released by three freeze-thaw cycles. Infectious particles were determined by $TCID_{50}$.

For development of a toxicity model, 1 x $10^7$ SKOV3.ip1 cells were injected i.p., after which mice received 3 x $10^7$ VP i.p. on day 10. Chlorpromazine or apigenin was thereafter administered daily. Further, all mice received 80 mg/kg gemcitabine i.p. on days 11, 14, 21, and 24.

### *5.8 Histopathology* (I-II, IV)

Livers and residual tumors were collected and fixed in buffered formalin (10%), embedded in paraffin, and cut into 5 μm sections. Deparaffinized specimens were stained with hematoxylin and eosin. Histopathology was scored blinded by an independent pathologist.

## 6. Statistical analysis (I-IV)

In combination studies *in vitro* (**I-II**), Chou and Talalay's (1983) median-effect method was used to calculate combination index (CI) values under the assumption of mutually nonexclusive drug interactions using S-PLUS 6.0 (Insightful Corporation, Seattle, WA, USA). CI <1 indicates synergism, CI=1 additivity, and CI>1 antagonism. One sample t-test was performed to determine whether the mean CI values from separate experiments at multiple-effects levels were significantly different from a value of 1.0.

One-way analysis of variance with Bonferroni's post-hoc test for multiple comparisons was used for statistical analysis of the effect of GCV on cell viability and tumor GFP expression data (**III**) with SPSS 14.0 (SPSS Inc., Chicago, IL, USA).

The effects of chlorpromazine or apigenin on replication and cell viability (**IV**) were analyzed by using bootstrap multiple comparisons of means in analysis of variance (PROC

MULTITEST SAS 9.1 (SAS Institute Inc., Cary, NC, USA). The levels of viral replication were log-transformed for normality.

Survival data (**I-IV**) were plotted in a Kaplan-Meier curve, and groups were compared pair-wise with log-rank test using SPSS 14.0. A analyse of tumor volume (**III-IV**) and photon emission (**III**) were performed using a repeated measures model with PROC MIXED using SAS 9.1. Measurements were log-transformed for normality. The effects of treatment group, time in days, and the interaction of treatment group and time were evaluated by F-tests. Curvature in the models was tested by a quadratic term for time. The *a priori* planned comparisons of specific differences in predicted treatment means averaged over time and at the last time-point were computed by t-statistics. For all analyses, $P<0.05$ was deemed statistically significant.

# RESULTS AND DISCUSSION

## 1. Tropism modified oncolytic adenovirus Ad5/3-Δ24 in combination with chemotherapy (I-II)

Ad5/3-Δ24 is a serotype 3 receptor-targeted pRb-p16 pathway-selective oncolytic adenovirus, which has previously been shown to have enhanced antitumor effect in the context of ovarian cancer (Kanerva et al. 2003, Lam et al. 2004). Nevertheless, when tested in a highly sensitive *in vivo* imaging assay, evidence emerged that tumors may be able to gain resistance even to this highly potent treatment (Kanerva et al. 2005). Therefore, we hypothesized that the therapeutic efficacy of Ad5/3-Δ24 might be improved when used in combination with chemotherapeutic agents.

Most patients with advanced epithelial ovarian cancer will relapse after first-line chemotherapy with platinum and taxane compounds. Thus, we chose gemcitabine and epirubicin in our experiments as examples of commonly used second-line treatment options for platinum/taxane-resistant ovarian cancer (Harries & Gore 2002).

### *1.1 Combination of Ad5/3-Δ24 and gemcitabine in vitro and in vivo* (I-II)

The deoxycytidine analog gemcitabine has been used to treat solid tumors, and the drug has demonstrated a therapeutic response in a variety of malignancies, including ovarian cancer (Lund et al. 1995, Shapiro et al. 1996, Fowler & Van Le 2003, Markman et al. 2003). The drug acts through several different mechanisms that result in self-potentiation of its activity (Plunkett et al. 1995). Specifically, gemcitabine is phosphorylated by cellular deoxycytidine kinase to mono-, di- and triphosphate forms, of which the latter is the major cellular metabolite. It competes with endogenous deoxycytidine triphosphate (dCTP) for incorporation into DNA, resulting in chain termination and subsequent inhibition of DNA replication and repair mechanisms (Huang et al. 1991). In addition, the diphosphate form of the drug decreases the pool of cellular DNA precursors, including dCTP, available for DNA synthesis. Reduction in dCTP increases incorporation of the drug into DNA and potentiates its cytotoxicity. Reduction of dCTP may also release more deoxycytidine kinase from feedback inhibition and increase phosphorylation of gemcitabine (Heinemann et al. 1992).

To determine the potential interaction between Ad5/3-Δ24 and gemcitabine *in vitro*, the cell killing effect of the combination treatment was compared with single treatments on established ovarian adenocarcinoma cell lines, and synergism was quantitated with Chou and Talalay's (1983) median-effect method. On SKOV3.ip1 cells, analysis revealed significant

synergy ($P$=0.003) when gemcitabine was given concomitantly with Ad5/3-Δ24 (figure 1c in **I**). Infection of cells at 10 VP/cell resulted in 95% survival, whereas 0.2 μg/ml gemcitabine showed 62% survival. However, when Ad5/3-Δ24 and gemcitabine were administered simultaneously at these concentrations, cell survival decreased to 32%. We also evaluated whether sequencing of the agents would have an effect on cytotoxicity. Synergistic activity was not seen when the virus was given 24h before or after gemcitabine ($P$>0.2) (figure 1d and 1e in **I**). With regard to Hey cells, viral infection 24h after gemcitabine yielded synergy at a dose of 0.2 μg/ml (Figure 2e in **I**), but when other doses were taken into the analysis, significant overall synergy was not evident ($P$>0.1). This was also the case when cells were infected simultaneously or before gemcitabine treatment ($P$>0.2). Ad5/3luc1 was used as a replication-deficient control, and it did not cause oncolysis or potentiation of gemcitabine.

As human adenoviruses do not replicate productively in murine tissues (Blair et al. 1989), human xenografts in mice were utilized for *in vivo* studies. The antitumor efficacy of combination treatment was evaluated in orthotopic murine models of ovarian cancer. Mice were followed for survival and livers were collected and processed for histopathological analysis. In the SKOV3.ip1 model (figure 4 in **I**), the median survival of untreated mice was 30 days, and gemcitabine increased the median survival to 39 days ($P$<0.0001). Mice in these groups died due to disease progression. In groups receiving the combination regimen, a number of early deaths were seen. These were probably due to liver toxicity, manifested as either centrilobular or total necrosis of the liver (figure 5 in **I**). In one mouse treated with Ad5/3-Δ24 24h prior to gemcitabine administration, we also saw evidence of hepatic extramedullary hematopoiesis. One possible reason for this finding is bone marrow damage (Harada et al. 1999). Nevertheless, simultaneous administration of Ad5/3-Δ24 with gemcitabine improved the survival of mice in comparison with no treatment ($P$<0.0001) or gemcitabine alone ($P$=0.0084). While the median survival of mice treated with Ad5/3-Δ24 was 89 days ($P$<0.0001 for both mock and gemcitabine), the median survival was not reached for Ad5/3-Δ24 given 24h after gemcitabine, as 60% of the mice were alive at the end of the experiment on day 130 ($P$=0.0003 and $P$=0.0002 for mock and gemcitabine, respectively). None of the mice treated with virus alone or in combination with gemcitabine died due to i.p. relapse. Instead, mice died due to toxicity of the treatment or were killed because of s.c. tumors at the injection site.

Combination treatment was also tested in a more aggressive Hey ovarian carcinoma model (figure 5a in **II**). All treatment schedules resulted in significantly enhanced survival ($P$<0.001) compared with untreated mice, whose median survival was 23 days. Animals receiving Ad5/3-Δ24 and gemcitabine as a single treatment had an improved median survival of 31 and 38 days, respectively, and these treatments did not differ significantly ($P$=0.096). The combination resulted in superior antitumor efficacy compared with Ad5/3-Δ24 ($P$<0.001)

or gemcitabine ($P$=0.012) alone, and median survival increased to 54 days. In histopathological studies (figure 5b and 5c in **II**), such changes as apoptosis, diffuse necrosis, and fibrotic strias were more prominent in the treated tumors, while the group injected with growth medium only showed reticular necrosis and necrotic foci (tumor necrosis). However, treatment-related deaths were circumvented by utilizing lower doses of gemcitabine. In the immune-deficient mice used here, antiviral antibodies are not formed, and liver damage is possible. Virus replication might be more abrogated in immunocompetent systems, resulting in less hepatic toxicity, while antitumor efficacy might remain due to relative protection from the immune system provided by the tumor environment.

### *1.2 Effect of gemcitabine on Ad5/3-Δ24 replication* (I)

One possible mechanism for synergy between Ad5/3-Δ24 and gemcitabine might be that chemotherapeutic agents increase the level of viral replication (Yu et al. 2001). Therefore, we evaluated the effect of gemcitabine on Ad5/3-Δ24 replication on ovarian cancer cells. Cells were infected with the virus alone or in combination with gemcitabine, and the amount of the virus at different time-points was determined. The number of infectious particles increased more rapidly after infection with Ad5/3-Δ24 alone than with combination treatment (figure 3 in **I**). The difference was more than 1000-fold 24h after infection and approximately 100-fold after 48h and 72h. However, a plateau was reached for Ad5/3-Δ24 already after 48h, whereas viral replication continued to increase in cells treated with the combination regimen. The total amount of virus production was similar in both cases approximately 96 h post-infection. Viral infection 24h after administration of gemcitabine was also performed, and the number of infectious particles measured as above. Again, Ad5/3-Δ24 alone resulted in more than 1000-fold and 100-fold virus production at 24h and 48h after infection, respectively. Instead of increasing the level of viral replication, our results suggest that gemcitabine reduces the rate of Ad5/3-Δ24 replication early after infection.

### *1.3 Combination of Ad5/3-Δ24 and epirubicin in vitro and in vivo* (II)

Epirubicin is a derivative of a commonly used anthracycline, doxorubicin, but causes less cardiotoxicity (Minotti et al. 2004). The drug has been utilized to treat various cancer types, including platinum-resistant ovarian cancer (Pelaez et al. 1996, Vermorken et al. 1999, Vermorken et al. 2000). The precise mechanism of action of epirubicin, like other anthracyclines, is a matter of controversy. Briefly, the drug intercalates into DNA strands, resulting in a complex formation that prevents DNA synthesis (Minotti et al. 2004). It also inhibits topoisomerase II activity by stabilizing the DNA-topoisomerase II complex (Tewey et

al. 1984). Prevention of religation of cleaved DNA leads to double-strand breaks and cell death.

The cytotoxicity of Ad5/3-Δ24 and epirubicin *in vitro* was analyzed on established ovarian adenocarcinoma cells. Ad5/3-Δ24 infection of cells with 0.1 VP/cell resulted in 88% cell survival, whereas 0.25 μg/ml epirubicin resulted in 63% viability (figure 3b in **II**). However, when both agents were administered together at these doses, the amount of living cells decreased to 37%. Thus, the combination resulted in significantly improved cancer cell killing compared with single treatments ($P$=0.002). Again, Ad5/3luc1 was used as a replication-deficient control and it did not cause oncolysis (figure 3a in **II**).

To examine whether the combination works synergistically also *in vivo*, mice with advanced ovarian cancer were treated either with single agents or the combination regimen (figure 4 in **II**). All treatment schedules resulted in significantly improved survival ($P$<0.001) compared with untreated mice, which had a median survival of 25 days. Treatment with Ad5/3-Δ24 or epirubicin alone enhanced the survival to 32 or 37 days, respectively. No significant difference existed between single therapies ($P$=0.565). However, the combination resulted in greater efficacy than the virus ($P$=0.030) or epirubicin ($P$=0.022) alone. Median survival increased to 42 days and two mice were alive at the end of the experiment on day 50.

### *1.4 Possible mechanisms for synergistic interactions* (I-II)

The mechanism for synergistic activity between oncolytic adenoviruses and chemotherapeutic agents is currently unknown, but several hypotheses can be put forth. First, chemotherapeutic agents may affect the level of viral replication. Increased viral production was reported when oncolytic adenovirus CV787 was combined with taxane compounds (Yu et al. 2001). Low concentration of paclitaxel has also been shown to improve transduction of recombinant adenovirus in a dose-dependent manner, resulting in more efficient viral replication (Nielsen et al. 1998). On the other hand, several other studies have demonstrated that cytotoxic drugs, such as cisplatin or CPT-11, have no effect on the replication kinetics of oncolytic viruses (Heise et al. 2000b, Khuri et al. 2000, Gomez-Manzano et al. 2006, Ganesh et al. 2008a). These results are logical since different agents work by different mechanisms of action. Our findings suggest that gemcitabine reduces the rate of Ad5/3-Δ24 replication early after infection, but does not affect the total virus yield. It is not clear how slower replication increases antitumor efficacy, but perhaps enhanced tumor penetration and dissemination before oncolysis, and formation of subsequent necrotic areas that are unpenetrable to the virus are involved. Further, slower replication could be advantageous with regard to toxicity, resulting in less liver damage. However, slow replication could be disadvantageous when faced with a mounting immune response.

Second, oncolytic virus may augment the antitumor activity of chemotherapeutics. Adenoviral E1A protein expression has been shown to increase cellular sensitivity to chemotherapeutic agents in both a p53-dependent and a p53-independent manner (Lowe & Ruley 1993, Sanchez-Prieto et al. 1996). The initial molecular mechanism of E1A-mediated tumor suppression was downregulation of HER-2/neu proto-oncogene (Yu et al. 1991). Ueno et al. (2000) demonstrated that taxane-resistant HER-2/neu-overexpressing SKOV3.ip1 cells became sensitive to paclitaxel treatment after downregulation of HER-2/neu by E1A. Suppression of NF-κB activation is another mechanism by which E1A promotes apoptosis in cells exposed to apoptotic stimuli (Shao et al. 1997). NF-κB is inactive in association with unphosphorylated IκB. In response to harmful stimuli, such as chemotherapy, IκB is phosphorylated and degraded, leading to activation of NF-κB, which functions as a transcriptional factor for the expression of survival genes. E1A inhibits NF-κB activation by stabilizing the unphosphorylated form of IκB (Shao et al. 1999). E1A also suppresses the expression of poly(ADP-ribose) polymerase (PARP), which plays an important role in DNA repair. Recently, E1A was shown to sensitize hepatocellular carcinoma cells to gemcitabine (Lee et al. 2003). Interestingly, as a mechanism for gaining resistance, NF-κB and PARP are induced in cancer cells treated with gemcitabine, while E1A inhibits NF-κB and PARP, consecutively resulting in sensitization of cells to gemcitabine (Lee et al. 2003). These aspects may also partially account for the hepatic toxicity seen here. Although human adenoviruses do not productively replicate in murine cells, E1A expression and protein production do ensue (Hallden et al. 2003). Theoretically, this could sensitize normal cells to gemcitabine-mediated damage.

Expression of CAR has been reported to be induced with certain chemotherapeutic agents such as gemcitabine and etoposide (Hemminki et al. 2003). Although increased receptor expression has not been shown for the Ad3 receptor, it could potentially play a role. CD46 has been suggested as a possible Ad3 receptor (Sirena et al. 2004, Fleischli et al. 2007), but other reports disagree (Gaggar et al. 2003, Short et al. 2004, Marttila et al. 2005). Once the Ad3 receptor is identified, possible upregulation mediated by chemotherapeutic agents can be investigated.

Finally, and more unlikely, each agent could work independently on different cell populations. Chemotherapeutics may also be able to kill tumor-surrounding stromal cells of murine origin, particularly when sensitized with E1A.

## 2. Evaluation of dose and scheduling of intraperitoneally delivered Ad5/3-Δ24 in ovarian cancer xenografts (II)

Ad5/3-Δ24 is currently under development for human clinical testing. To optimize protocols for upcoming clinical trials, determination of wheter multiple rounds of treatment are superior to a single dose is warranted. Each ovarian cancer cell infected *in vitro* rapidly produces thousands of new virions. Therefore, we performed *in vivo* dose de-escalation to test whether virus amplification would translate into efficacy at very small doses, which would be promising with regard to clinical trials for patients suffering from advanced ovarian cancer. To mimic the clinical situation, we utilized orthotopic murine models of peritoneally disseminated ovarian cancer to compare survival of mice treated i.p. with either a single viral dose or weekly delivery. Further, the lowest effective dose of i.p.-delivered Ad5/3-Δ24 was determined.

### *2.1 Comparison of single and weekly delivery of Ad5/3-Δ24* (II)

Adenoviruses are common pathogens and pre-existing immunity is thought to rapidly clear the viruses, and thus, multiple dosing is probably required for successful virotherapy (Bessis et al. 2004). Gene therapy trials for ovarian cancer have usually relied on i.p. delivery of the agents to patients with peritoneally disseminated disease. The peritoneal cavity of most ovarian cancer patients contains malignant ascites fluid with NAbs against adenovirus (Hemminki et al. 2002a). These antibodies might inhibit the initial viral infection and further spreading of virions. However, previous studies have suggested partial escape of Ad5/3 pseudotyped viruses from pre-existing Nabs, perhaps due to the chimeric nature of the fiber (Kanerva et al. 2002b). Further, repeated delivery of highly potent, infectivity-enhanced viral agents might lead to toxicity. Therefore, we evaluated *in vivo* whether the therapeutic efficacy of a single Ad5/3-Δ24 injection differs from that of the weekly delivery schedule.

Treatment with a single Ad5/3-Δ24 injection was compared with multiple injections in orthotopic murine models of ovarian cancer. Peritoneally disseminated carcinomatosis was allowed to develop, and Ad5/3-Δ24 was given i.p. either as a single or a weekly dose of 1 x $10^8$ VP (figure 1 in **II**). Both treatment schedules resulted in significantly improved survival compared with untreated mice ($P$<0.001) in both Hey and SKOV3.ip1 *in vivo* models. However, administration of a single viral dose did not differ significantly from several doses in either model ($P$=0.900 and $P$=0.654, respectively), suggesting effective *in vivo* replication. In aggressively growing Hey xenografts, the median survival of untreated mice was 23 days, while single and weekly virus treatments increased the median survival to 35 and 31 days,

respectively. All mice died due to tumor progression in the peritoneal cavity. In the SKOV3.ip1 model, untreated mice achieved a median survival of 37 days, while single and weekly injection schedules enhanced the median survival to 58 and 75 days, respectively. Most of the treated mice died due to disease progression, but in a few cases there was evidence of hepatic toxicity in both treated groups, a known side-effect of persistent virus replication. Our results are consistent with a previous report where single treatment with Ad5/3-Δ24 was suggested to be as effective as delivery on three consecutive days (Kanerva et al. 2003). However, these survival studies were not carried out in the same experiment, which makes direct comparison difficult.

## *2.2 Determination of lowest effective dose of Ad5/3-Δ24* (II)

The lytic life cycle of adenoviruses results in oncolysis of infected cells and spreading of virus progeny to surrounding cells and, at least in theory, this process would be expected to continue as long as target cells persist. Further, the intensity of immune responses induced by adenoviruses is highly dependent on the vector dose (Bessis et al. 2004). Therefore, we examined whether Ad5/3-Δ24 infection with very small doses could lead to therapeutic efficacy.

The lowest effective i.p. dose of Ad5/3-Δ24 was evaluated in mice with orthotopic SKOV3.ip1 tumors. Because $10^8$ VP had previously been found to be effective, dose de-escalation started from $10^6$ and was continued to $10^2$ VP. Although not significant, there seemed to be a trend towards dose-dependency since treatment with the highest dose of $10^6$ VP resulted in the highest survival rates and a median survival of 83 days (figure 2 in **II**). However, even the lowest viral dose of $10^2$ VP with a median survival of 54 days was sufficient to increase the survival of mice significantly compared with untreated animals ($P$=0.001). Slight variations in median survival did not result in different long-term survival, as treatment with all doses enhanced the survival significantly compared with untreated animals.

These findings are consistent with potent amplification of the agent. However, the survival curves appear to somewhat favor the higher dose. Although not demonstrated before *in vivo*, even a small amount of virus can amplify exponentially and kill large numbers of tumor cells. Nevertheless, given the different tumor characteristics, these findings might have been different for other cancer cell lines. Further, results obtained from immune-deficient mice do not correlate with the actual situation in clinics, where low doses might be rapidly cleared by the immune system.

## 3. Tropism modified oncolytic adenovirus Ad5/3-Δ24-TK-GFP and utility of TK/GCV suicide gene system (III)

Arming oncolytic adenoviruses with therapeutic transgenes and enhancing transduction of tumor cells are useful strategies for eradication of advanced tumor masses (Hermiston & Kuhn 2002, Kanerva & Hemminki 2005). The TK/GCV prodrug system is a classic strategy based on conversion of systemically administered prodrug GCV to cytotoxic metabolite by cellular TK. This approach has been promising when coupled with viruses featuring considerably low oncolytic potential (Wildner et al. 1999a, Wildner et al. 1999b), but its utility in the context of highly effective infectivity-enhanced viruses is unknown. Therefore, we constructed a serotype 3 receptor-targeted, pRb-p16 pathway-selective oncolytic adenovirus containing a fusion gene encoding TK and GFP in the partly deleted E3 region under the control of endogenous adenoviral gene expression machinery. The aim was to examine whether the TK/GCV system increases the oncolytic potency of our newly generated virus. Further, we hypothesized that we can follow tumor progression and virus spreading by noninvasive imaging technology.

### *3.1 Verification of virus replication and transgene production* (III)

An approximately 2-kb TK-GFP fusion gene (Loimas et al. 1998) was inserted into the 6.7K/gp19K-deleted E3A region of a serotype 5-based oncolytic adenovirus featuring the serotype 3 fiber knob, resulting in a novel virus Ad5/3-Δ24-TK-GFP (figure 1a in **III**). This insertion site has previously been demonstrated to be ideal for expression of transgenes because of tight linkage to virus replication (Hawkins et al. 2001, Kanerva et al. 2005). Transgene production begins approximately 8h post-infection, and a heterologous promoter or poly-A is not required. Both of these features are useful for retaining the highest possible virus replication, and space is saved for maximal transgene size. Ad5/3-TK-GFP, a virus with a CMV promoter-driven transgene in the deleted E1 region, was constructed as a replication-deficient control.

To determine whether insertion of the fusion gene had an effect on virus replication, Ad5/3-Δ24-TK-GFP was compared with Ad5/3-Δ24-Δgp19K and Ad5/3-Δ24 (figure 1b in **III**). The former is an isogenic virus featuring the corresponding deletion without a transgene, and the latter contains an intact E3 region. Ad5/3-Δ24-TK-GFP replicated efficiently and resulted in complete killing of all analyzed ovarian cancer lines. However, the replication rate was slightly slower, and total production of functional virions remained 1-2 log lower levels when compared with the other oncolytic viruses. This may have been due to transgene production competing for the cellular resources needed for amplification of the viral genome.

Alternatively, because our transgene is 1 kb longer than the deleted E3 fragment, the larger genome size may have slowed replication to some extent. Slower replication was not caused by deletion of the 6.7K/gp19K gene. In contrast, infection with the isogenic Ad5/3-Δ24-Δgp19K harboring the same deletion but lacking a transgene resulted in higher replication levels and more efficient oncolysis than Ad5/3-Δ24, whose genome is closer to a wild-type adenovirus. These results highlight the impact of genome size on speed of adenovirus replication.

To confirm expression of the TK-GFP fusion product, Ad5/3-Δ24-TK-GFP- and Ad5/3-TK-GFP-infected ovarian cancer cells were imaged daily for GFP expression (supplementary figure 1 in **III**). Ad5/3-Δ24-TK-GFP-infected cells expressed more GFP than cells infected with the replication-deficient virus, and the proportion of GFP-positive cells increased over time. By day 10, expression of GFP was seen in cells infected with only 0.001 VP/cell. These data suggest that higher amounts of fusion protein are produced from replicating viruses, and that coupling of transgene expression to replication allowed detection of replication by analysis of GFP.

## *3.2 Effect of GCV on virus replication* (III)

It has been proposed that GCV might block viral proliferation and subsequent spreading of oncolytic viruses (Morris & Wildner 2000, Rogulski et al. 2000b, Wildner & Morris 2000, Nanda et al. 2001). These proposals are not surprising since the toxic form of the drug acts by incorporating into DNA, thus competing with endogenous DNA precursors. Thereafter, elongation of DNA strands is prevented and further DNA synthesis inhibited (Ilsley et al. 1995, Haynes et al. 1996, Thust et al. 1996).

We evaluated the kinetics of Ad5/3-Δ24-TK-GFP in the presence of GCV. Ovarian cancer cells were infected with Ad5/3-Δ24-TK-GFP and GCV was added according to different schedules (figure 2 in **III**). When GCV was given 1h post-infection, practically no infectious particles were formed, indicating that GCV has a dramatic inhibitory effect on viral replication when given immediately after the virus. Such a safety switch mechanism might be useful if it becomes necessary to abrogate virus replication (Wildner 2003) Administration of GCV 24h post-infection resulted in a 10-fold reduction in the amount of viable virus at 48h and 72h after infection. A plateau was reached for Ad5/3-Δ24-TK-GFP after 48h and therefore, it was logical that GCV added at this time-point did not decrease the number of infectious particles. These results are in parallel with previous studies suggesting GCV-induced inhibition of viral replication (Morris & Wildner 2000, Rogulski et al. 2000b, Wildner & Morris 2000, Nanda et al. 2001). Further, our results indicate the importance of GCV scheduling in order to achieve a balance between a GCV-mediated decrease in

cytotoxicity by inhibition of viral replication and a GCV-mediated increase in cytotoxicity by bystander effect.

### *3.3 Oncolytic potency in the presence of ganciclovir in vitro* (III)

To evaluate whether the TK/GCV system can improve the efficacy of oncolytic virotherapy *in vitro*, ovarian cancer cells were infected with Ad5/3-Δ24-TK-GFP or Ad5/3-TK-GFP and exposed to GCV at 1h, 24h, 48h, 72h or 96h post-infection. MTS assay was used as an indicator of cell viability. On SKOV3.ip1 cells, Ad5/3-Δ24-TK-GFP infection in combination with GCV resulted in significantly enhanced cell killing compared with virus alone in all analyzed treatment schedules (supplementary figure 2 in **III**). When GCV was given 48h after infection with 1 VP/cell of Ad5/3-Δ24-TK-GFP, virus alone resulted in 87% cell viability, whereas combination treatment decreased the proportion of living cells to 10% ($P$<0.001) (figure 3a in **III**). SKOV3.ip1 cells are known to be sensitive to the bystander effect (Hakkarainen et al. 2005), which may explain why GCV increased cell killing despite abrogation of virus replication.

With Hey and OV-4 cells (figure 3b and 3c in **III**), replication of Ad5/3-Δ24-TK-GFP was rapid and administration of GCV 48h post-infection did not significantly increase cytotoxicity. Other time-points yielded similar data. In a recent study, infection with another oncolytic adenovirus, Ad5-Δ24TK-GFP, resulted in enhanced cell killing of both Hey and OV-4 cells when combined with GCV (Hakkarainen et al. 2006). Entry of Ad5-Δ24TK-GFP into target cells is dependent on CAR expression, which has been demonstrated to be low in Hey and OV-4 cells (Kanerva et al. 2002a). Therefore, less cells were infected by the CAR-binding Ad5-Δ24TK-GFP, and the need for the GCV-induced bystander effect was more urgent. Further, expression of TK-GFP was driven by the CMV promoter placed in the deleted E3 region, which might not be optimal for effective replication because both deletion of the entire E3 and CMV-directed expression are known to reduce production of new virions (Suzuki et al. 2002).

GCV was useful in combination with replication-deficient Ad5/3-TK-GFP with all cell lines and schedules compared with virus alone ($P$<0.001 at 100 VP/cell). However, oncolytic virus alone led to more efficient cell killing than E1-deleted virus combined with GCV. Our *in vitro* data suggest that the utility of TK/GCV-mediated cell killing is most evident in the context of replication-deficient viruses. In contrast to the effect on virus replication, GCV timing did not influence cell killing dramatically. However, allowing the virus to replicate prior to GCV treatment is useful because of the inhibitory effect on replication and the subsequently reduced transgene production.

### *3.4 Antitumor efficacy in subcutaneous and intraperitoneal murine models of ovarian cancer* (III)

The antitumor efficacy of Ad5/3-Δ24-TK-GFP and feasibility of TK/GCV system was analyzed in human xenografts of ovarian cancer. To study whether flank tumor growth in nude mice might be inhibited, s.c. SKOV3-luc tumor-bearing mice were treated i.t. with two cycles of Ad5/3-Δ24-TK-GFP or replication-deficient Ad5/3-TK-GFP, and GCV was administered i.p. for one week starting 48h after the last viral injections (figure 4a in **III**). Both viruses alone and together with GCV reduced tumor growth significantly compared with untreated animals (all $P<0.006$). Further, both Ad5/3-Δ24-TK-GFP alone and coupled with GCV were more efficient in inhibiting tumor growth than E1-deleted Ad5/3-TK-GFP with GCV ($P<0.001$). Interestingly, GCV did not provide an additional therapeutic benefit when combined with Ad5/3-Δ24-TK-GFP ($P=0.495$).

Therapeutic efficacy was also evaluated in an orthotopic murine model of ovarian cancer. SCID mice with peritoneal SKOV3-luc carcinomatosis were injected i.p. with Ad5/3-Δ24-TK-GFP or Ad5/3-TK-GFP followed by daily GCV for two weeks starting 48h after virus injection (figure 6a in **III**). The median survival of untreated mice was 32 days, and treatment with replication-deficient Ad5/3-TK-GFP together with GCV increased it to 36 days ($P=0.001$). Oncolytic Ad5/3-Δ24-TK-GFP alone or in combination with GCV resulted in median survival of 46 and 47 days, respectively, both of which were significantly improved compared with untreated animals ($P<0.001$). However, GCV did not add a significant survival benefit relative to Ad5/3-Δ24-TK-GFP alone ($P=0.481$). Again, treatment with Ad5/3-Δ24-TK-GFP alone led to significantly enhanced survival compared with E1-deleted Ad5/3-TK-GFP combined with GCV ($P=0.023$).

In summary, our *in vivo* data demonstrate that TK/GCV does not augment antitumor efficacy, as delivery of GCV failed to reduce tumor growth or increase survival compared with oncolytic virus alone. This data is in accordance with other studies combining oncolytic adenoviruses with the TK/GCV system (Morris & Wildner 2000, Rogulski et al. 2000b, Wildner & Morris 2000, Lambright et al. 2001, Hakkarainen et al. 2006). High oncolytic potency might reduce the utility of prodrug conversion because of the strong lytic effect caused by virus dissemination. Prodrug conversion might also affect virus replication, which could work against oncolysis. Other arming approaches could therefore be more useful in the context of highly potent oncolytic adenoviruses (Boland et al. 2000, Erbs et al. 2000, Lukashev et al. 2005, Oosterhoff et al. 2005, Ramesh et al. 2006).

### *3.5 Noninvasive imaging of tumor growth and virus replication* (III)

Noninvasive imaging techniques provide a useful tool for preclinical and clinical gene therapy research as well as for diagnostic use (Min & Gambhir 2004). In this approach, reporter gene expression is monitored in living organisms, which allows more efficient utilization of orthotopic model systems and offers the possibility of performing repeated measurements. We utilized noninvasive bioluminescence and fluorescence imaging to follow tumor development and virus dissemination in mice.

Luciferase-expressing SKOV3-luc ovarian cancer cells were detected with bioluminescence imaging after administration of D-luciferin. The advantage of bioluminescence is minimal background noise, as luciferase is not a natural constituent of mammalian organisms (Min & Gambhir 2004). The total flux of emitted light was measured (figures 4b and 6b in **III**), and the extent of disease was visualized with pseudocolor images of luciferase expression (figures 7 and 9, figures 4c and 6c in **III**). In s.c. model, good correlation was seen between tumor size and photon emission data. Administration of GCV did not significantly affect photon flux compared with viruses alone ($P$=0.899 for Ad5/3-Δ24-TK-GFP and $P$=0.061 for Ad5/3-TK-GFP). Results with i.p. xenografts were in accordance with survival data, as photon flux from mice treated with Ad5/3-Δ24-TK-GFP alone or in combination with GCV did not differ significantly ($P$=0.707). However, both treatments resulted in a smaller tumor load than in untreated mice ($P$<0.002).

Replication and spreading of TK-GFP expressing viruses in flank tumors were followed with fluorescence imaging of GFP expression (figure 8, figure 5 in **III**). Signals emitted from Ad5/3-Δ24-TK-GFP-treated tumors seemed higher than in tumors injected with Ad5/3-TK-GFP, indicating effective replication of the virus, although differences were not statistically significant. Utilization of fluorescence imaging to monitor transgene expression might be problematic. Since mammalian tissues absorb light that is used to excite GFP, the tissues also fluoresce when excited at these wavelengths known to have limited penetration in mammalian tissues. The combination of absorption of specific signal and autofluorescence can result in a poor and noisy signal (Bogdanov & Weissleder 2002). This phenomenon was also evident in our studies, as we were unable to detect GFP at all time-points due to high background fluorescence levels. In addition to its therapeutic value, TK could also be used as a reporter gene. Radionuclide imaging with positron emission tomography (PET) or single photon emission tomography (SPECT) allows noninvasive detection of TK gene transfer and expression (Tjuvajev et al. 1996, Tjuvajev et al. 1998).

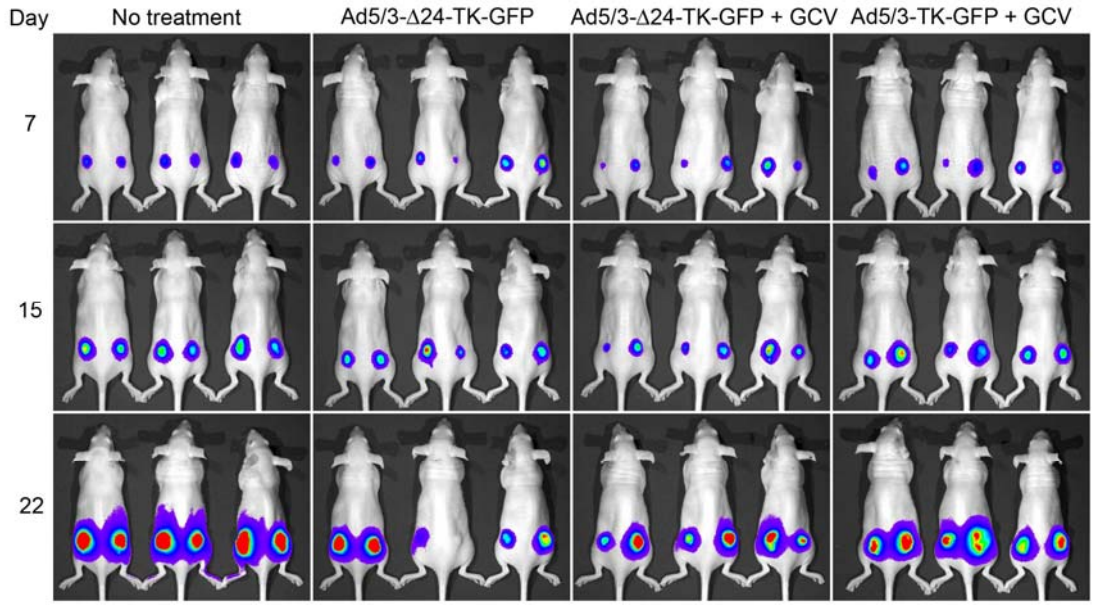


**Figure 7.** Bioluminescence imaging of luciferase-expressing ovarian cancer cells in subcutaneous model of ovarian cancer. Pseudocolor images of s.c. tumors in flanks of nude mice treated with two rounds of 1 x $10^8$ VP Ad5/3-Δ24-TK-GFP or Ad5/3-TK-GFP i.t. on days 7-9 and 19-21. 50 mg/kg GCV was injected i.p. 48h after viral injections (days 11-17 and 23-29). Color scale minimum and maximum have been adjusted so that they are identical in each image.

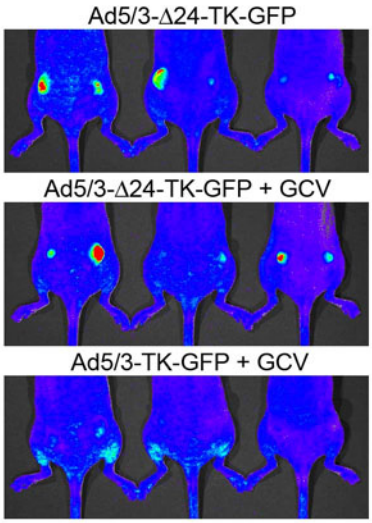


**Figure 8.** Fluorescence emitted from Ad5/3-Δ24-TK-GFP- or Ad5/3-TK-GFP-injected tumors on day 22. Color scale minimum and maximum have been adjusted so that they are identical in each image.

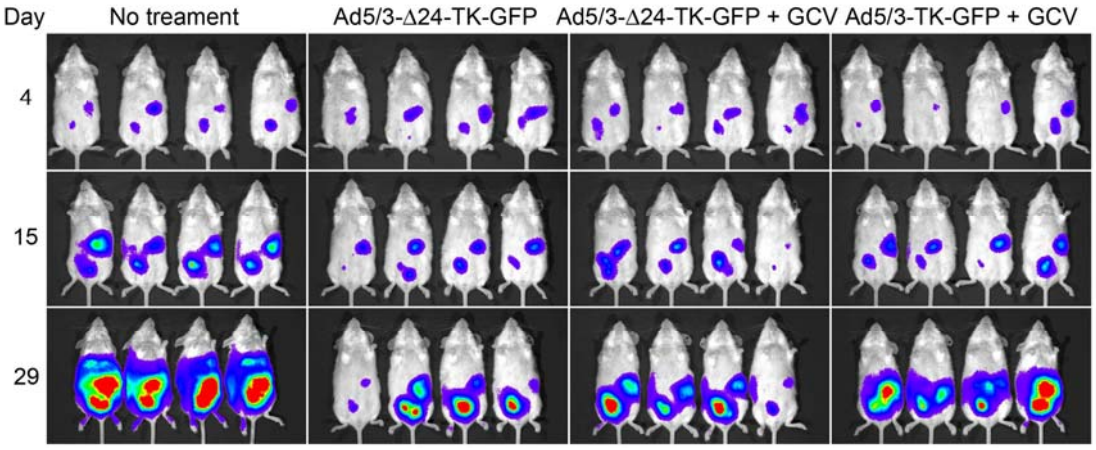


**Figure 9.** Bioluminescence imaging of luciferase-expressing ovarian cancer cells in a murine model of peritoneally disseminated ovarian cancer. Pseudocolor images of i.p. tumor burden in SCID mice treated with 1 x $10^8$ VP Ad5/3-Δ24-TK-GFP or Ad5/3-TK-GFP i.p. on day 5 followed by 50 mg/kg GCV i.p. daily for two weeks starting on day 7. Color scale minimum and maximum have been adjusted so that they are identical in each image.

## 4. Inhibition of virus replication and associated toxicity with pharmacological agents (IV)

Oncolytic adenoviruses have been well tolerated in cancer trials at doses of up to 2 x $10^{13}$ VP (Nemunaitis et al. 2001a). However, most studies have been carried out with E1B-55 kD-deleted ONYX-015, which is somewhat attenuated even in tumor cells compared with the Ad5 wild-type (Kirn 2001). Newer generation agents replicate similarly or even more rapidly than the wild-type adenovirus (Fueyo et al. 2000, Heise et al. 2000a, Kanerva et al. 2003). More potent oncolytic adenoviruses could mean more unwanted side-effects, especially in immunosuppressed patients. We hypothesized that we may be able to increase the safety of oncolytic adenoviruses by developing means for pharmacological intervention for reduction of replication, which might be useful in the event of severe side-effects in trials.

We evaluated the antiviral activity of the antipsychotic agent chlorpromazine and the natural bioflavonoid apigenin. Chlorpromazine has been used for decades in millions of humans and its safety and side-effect profile are well known (Lehman et al. 2004). Apigenin has also been studied in clinical trials, with good safety data (Nielsen et al. 1999). These agents possess different mechanisms of action since chlorpromazine works at the transductional level by inhibiting clathrin-mediated endocytosis (Wang et al. 1993), and apigenin blocks DNA synthesis (Sato et al. 1994, Lepley & Pelling 1997, Wang et al. 2000, Yin et al. 2001).

### *4.1 Reduction of replication in normal tissues in vitro* (IV)

The *in vitro* replication of Ad5 wild-type adenovirus and oncolytic adenovirus Ad5/3-Δ24 in combination with drugs was analyzed in fresh human liver explants. Wild-type virus alone displayed replication over 48h, and chlorpromazine reduced the titer 8-fold ($P$=0.0271) at the last time-point (figure 1a in **IV)**. Further, lower liver aspartate transaminase (AST) levels measured from chlorpromazine-treated livers suggested reduced hepatocyte damage (figure 1e in **IV**). Importantly, as Ad5/3-Δ24 is in development for human trials, it did not replicate productively in the human liver explants (figure 1b in **IV**). Nevertheless, even marginal replication was further attenuated by chlorpromazine, as evidenced by decreasing titer at later time-points. In nonmalignant E1-transformed 293 cells, both viruses replicated efficiently, and administration of chlorpromazine reduced replication up to 1960-fold ($P$=0.0056 for wild-type, and $P$=0.0040 for Ad5/3-Δ24) at 48h post-infection (figures 1c and 1d in **IV**).

## *4.2 Reduction of replication in cancer cells in vitro* (IV)

Next, viral replication coupled with pharmacological agents was examined in tumor cells. In ovarian adenocarcinoma Hey cells, apigenin reduced replication of both wild-type and Ad5/3-Δ24, with a significant difference ($P$=0.0156 and $P$=0.0478, respectively) at 72h post-infection (figures 2a and 2b in **IV**). However, the effect was less pronounced in another ovarian cancer cell line, OV-4 (figures 2c and 2d in **IV**). Presumably, genetic heterogeneity between OV-4 and Hey resulted in variation in replication attenuation with the drugs. The close association of replication with cell killing was corroborated in a longitudinal assay, where apigenin reduced the activity of Ad5/3-Δ24, resulting in increased cell viability (figure 2e in **IV**). Carette et al. (2005) reported a reduction of 3 to 4 orders of magnitude in oncolytic adenovirus DNA copy number with apigenin treatment in a quantitative PCR assay, and also the expression of transgene was reduced up to 4 logs in various cancer cell lines. Of note, in those experiments, the apigenin concentration was 8 times higher than the concentration that we utilized.

Interestingly, although chlorpromazine inhibited viral replication in normal tissues, an inhibitory effect was not evident in cancer cells. Administration of apigenin, by contrast, had the opposite effect. This could be explained by molecular differences between normal and malignant cells. Hey cells are known to proliferate very rapidly and efficiently *in vitro*, which might partly explain the stronger inhibitory effect by apigenin since it arrests the cell cycle, thus preventing DNA synthesis.

## *4.3 Reduction of replication and toxicity in vivo* (IV)

In a s.c. human xenograft of ovarian cancer, both apigenin and chlorpromazine were found to decrease the antitumor efficacy of Ad5/3-Δ24 (both $P$<0.0001) (figure 3a in **IV**). This was due to reduced replication of the virus (figure 3b in **IV**). Production of new viruses was reduced 36-fold with chlorpromazine. An 11-fold decrease was seen also with apigenin. Neither apigenin nor chlorpromazine resulted in therapeutic benefit by itself, although apigenin has been proposed to possess anticancer activity in several cancer types (Patel et al. 2007). We do not fully understand the reason for the discrepancy in the effect of chlorpromazine on Hey cells with Ad5/3-Δ24 *in vitro* and *in vivo*. However, because this virus replicates very rapidly *in vitro*, the speed of replication may actually become counterproductive to packaging of functional virions. Theoretically, cells might be lysed before producing the maximum number of virions. Thus, slowing of the speed of replication may not necessarily be seen as reduction of *in vitro* virion production. Nevertheless, tumor penetration and i.t. dissemination might be improved by rapid replication and release, and these aspects cannot be assessed *in vitro*.

Therefore, we feel that the *in vivo* data gives a more complete and relevant picture of the actions of the drugs.

To assess adenovirus-associated toxicity, we utilized a murine model where peritoneally disseminated human ovarian cancer is first inoculated and then cured by injecting Ad5/3-Δ24 and gemcitabine, a combination that has previously resulted in toxicity and liver damage. In this study, mice succumbed to liver necrosis, foamy degeneration, and steatosis. Further, the few surviving hepatocytes displayed large nuclei (figure 4b in **IV**). Of note, apigenin- or chlorpromazine-treated mice displayed less liver damage (figure 4c and 4d in **IV**). When all evaluable livers were analyzed in a blinded manner, liver toxicity was present more often in the control group than in mice receiving apigenin or chlorpromazine ($P$=0.0213).

These data suggest that apigenin and chlorpromazine can reduce the replication of adenoviruses *in vitro* and *in vivo*, which could provide a safety switch in case of unwanted side-effects. Compounding the need for an antidote, virus replication may persist for weeks, as opposed to most conventional medicines that have short half-lives.

# SUMMARY AND CONCLUSIONS

In this study, we evaluated different approaches for improving safety and efficacy of oncolytic virotherapy against human ovarian adenocarcinoma.

We examined the antitumor efficacy of Ad5/3-Δ24, a serotype 3 receptor-targeted pRb-p16 pathway-selective oncolytic adenovirus, in combination with conventional chemotherapeutic agents. We observed synergistic activity in ovarian cancer cells *in vitro* when Ad5/3-Δ24 was given with either gemcitabine or epirubicin, common second-line treatment options for ovarian cancer. Our results also suggest that gemcitabine decreases the level of Ad5/3-Δ24 replication early after infection. In murine xenografs of peritoneally disseminated ovarian cancer, delivery of Ad5/3-Δ24 with either gemcitabine or epirubicin resulted in a greater therapeutic efficacy than single agents. However, dosage and timing of gemcitabine treatment played a major role in defining the treatment outcome, as some mice with certain schedules died due to treatment-related liver damage. This was especially the case when Ad5/3-Δ24 was given 24h prior to gemcitabine treatment, indicating that toxicity might result due to chemosensitizing activity of adenoviral E1A. Importantly, administration of Ad5/3-Δ24 24h after gemcitabine resulted in dramatically enhanced survival, and 60% of mice were tumor-free at the end of the experiment. Considering the synergistic effect that many gene therapy approaches have with existing treatments, it is likely that the first routine clinical applications will be combination treatments with traditional modalities.

We analyzed the feasibility of the TK/GCV system in the context of infectivity-enhanced oncolytic adenoviruses. Infection with Ad5/3-Δ24-TK-GFP, a novel virus harboring the TK-GFP fusion gene, resulted in complete killing of ovarian cancer cells. However, Ad5/3-Δ24-TK-GFP replicated at slightly lower levels than control viruses, possibly due to transgene expression. Alternatively, the larger genome size may have slowed replication. Replication was associated with increased GFP expression, indicating effective production of the transgene. Administration of GCV immediately after Ad5/3-Δ24-TK-GFP infection caused almost total inhibition of DNA replication. However, oncolytic potency *in vitro* was enhanced after exposure to the GCV cell line-dependently and regardless of timing. Unfortunately, administration of GCV did not further improve therapeutic efficacy of this highly potent oncolytic agent in murine models of metastatic ovarian cancer. Finally, we utilized noninvasive bioluminescence and fluorescence imaging to monitor tumor development and virus replication in living mice.

As many common chemotherapeutic agents and prodrugs act through inhibition of DNA synthesis, optimizing treatment protocols becomes an important issue. Our results demonstrate that correct scheduling of the agents is required to achieve the maximum benefit

from both agents. The key factor appears to be a balance between a drug-induced increase in cell killing due to its cytotoxic mechanisms and a drug-induced reduction in cell killing due to inhibition of viral replication.

Orthotopic murine models of ovarian cancer were utilized to study the effect of dose and scheduling of intraperitoneally delivered Ad5/3-Δ24. Treatment outcome after administration of a single dose did not differ significantly from delivery of multiple rounds of Ad5/3-Δ24. Dose de-escalation suggested a trend towards dose-dependency since treatment with the highest dose resulted in the highest survival rates. However, even the lowest viral dose of 100 VP was sufficient to increase the survival of mice significantly compared with untreated animals. These results are in accordance with the potent amplification of the agent.

Finally, we evaluated whether safety of Ad5/3-Δ24 could be improved by developing means for pharmacological intervention for reduction of replication, which might be useful in the event of severe side-effects in trials. The antiviral effect of chlorpromazine and apigenin was analyzed in fresh human liver samples as well as in established normal cells and cancer cells *in vitro*. Further, we utilized murine models of ovarian cancer to evaluate antitumor efficacy, viral replication, and liver toxicity in the presesence of drugs *in vivo*. Our data show that these agents can decrease the rate of adenoviral replication, which could provide a safety switch in case of treatment-associated side-effects.

In conclusion, we demonstrate that Ad5/3-Δ24 is a useful oncolytic agent for treatment of ovarian cancer either alone or in combination with conventional chemotherapeutic drugs. Insertion of genes encoding prodrug-converting enzymes into the genome of Ad5/3-Δ24 might not lead to enhanced antitumor efficacy with this highly potent oncolytic virus. As a safety feature, viral activity can be inhibited with pharmacological substances. Clinical trials are however needed to confirm if these preclinical results can be translated into efficacy in humans. Promising safety data seen here, and in previous publications suggest that clinical evaluation of the agent is feasible. Importantly, Ad5/3-Δ24 is currently heading towards clinical trial against recurrent ovarian cancer.

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