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Fakulteten för Veterinärmedicin och husdjursvetenskap
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Novel anticancer combination therapies using replication-selective oncolytic adenoviruses and chemotherapeutics: investigating cell death mechanisms

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SAMMANFATTNING

Cancer i bukspottkörteln är en förödande sjukdom med dålig prognos och hög mortalitet. Majoriteten av cancertyperna är resistenta mot cytostatikapreparatet gemcitabin, som ändå används för dess palliativa effekt. För närvarande är sjukdomen obotlig och det är av högsta vikt att potenta behandlingsalternativ utvecklas. Onkolytiska replikationsselektiva adenovirus i kombination med cytostatika är en ny och lovande metod. Tidigare studier har visat att två adenovirala mutanter, Ad5Δ19K och Ad5ΔΔ, ökade celldöden av cancerceller i bukspottkörteln i kombination med gemicitabin.

Syftet med denna studie var att, baserat på dessa fynd, ytterligare utreda Ad5ΔΔ i kombination med chemoterapeutiska läkemedel samt bestämma mekanismerna bakom ökad celldöd. Ad5ΔΔ är blockerad i den virala E1ACR2-regionen. E1A är essentiell för S-fasinduktion och blockeringen gör att viruset inte kan replikera i normala celler. Parallellt med studierna av Ad5ΔΔ analyserades vildtypsformen av Ad5 för att bestämma skillnader i celldödseffektivitet. Det visades att Ad5ΔΔ lyserade bukspottkörtelcancerceller *in vitro*, ökade celldöden i kombination med gemcitabin och irinotekan samt inducerade förändringar i cellcykeln hos infekterade celler. Celler infekterade med Ad5ΔΔ uppvisade en ökad polyploid cellpopulation vilket inte kunde observeras hos celler infekterade med vildtyp. Dessutom påverkades uttrycket av virala gener av de chemoterapeutiska läkemedlen samt ökade uttrycket av proteiner involverade i mitos.

Ytterligare kunskap om mekanismerna bakom ökad celldöd till följd av Ad5ΔΔ i kombination med cytostatika kan bidra till att vi besegrar läkemedelsresistent bukspottkörelcancer i framtiden.

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ABSTRACT

Pancreatic cancer is a devastating disease with poor prognosis and high mortality. The majority of cancers show resistance to the chemotherapeutic drug gemcitabine, which is used mainly for palliative effects. Currently, there is no cure for pancreatic cancer and development of more potent treatments are crucial. Use of oncolytic replication-selective adenoviruses is a novel and promising approach. Previous studies have demonstrated that two adenoviral mutants, Ad5Δ19K and Ad5ΔΔ, enhanced cell-killing in pancreatic cancer cells in combination with gemcitabine.

Based on these findings, this study was aimed at further investigations of Ad5ΔΔ in combination with chemotherapeutic drugs and to determine the mechanisms causing potential enhancement of cell-killing. Ad5ΔΔ has deletions in the viral E1ACR2 region. E1A is essential for induction of S-phase and the deletion makes the virus replication-defective in normal cells. The wild type Ad5 (Ad5tg) virus was analysed in parallel in all experiments to determine differences in cell-killing efficacy. It was demonstrated that Ad5ΔΔ lysed pancreatic cancer cells *in vitro*, enhanced cell-killing in combination with gemcitabine and irinotecan and induced aberrations in cell cycle progression of infected cells. Cells infected with Ad5ΔΔ showed an increase in polyploid cells, which was not observed with Ad5tg. In addition, the presence of chemotherapeutic drugs affected viral gene expression and increased expression of proteins involved in mitosis.

Further understanding of the mechanisms involved in the enhancement of cell-killing observed for Ad5ΔΔ and chemotherapeutic drugs might aid in overcoming drug resistance in pancreatic cancers in the future.

ABBREVIATIONS

Ad5tg	–	Wild type adenovirus type 5
Ad5ΔΔ	–	E1ACR2- and E1B19K deleted adenovirus type 5
Ad5Δ19K	–	E1B19K deleted adenovirus type 5
CAR	–	Coxsackievirus and adenovirus receptor
CPE	–	Cytopathic effect
CR	–	Conserved Region
CT	–	Cycle threshold
Bcl2	–	B-cell lymphoma 2
MHC I	–	Major Histocompatibility Complex Class I
DMEM	–	Dulbecco's Modified Eagle Medium
EC_{50}	–	Half maximum effective concentration
E1	–	Early Region 1
FACS	–	Flow cytometry assay
FCS	–	Foetal calf serum
GAPDH	–	Glyceraldehyde 3-Phosphate Dehydrogenase
Gem	–	Gemcitabine
HUVEC	–	Human umbilical-vein endothelial cells
Iri	–	Irinotecan
kRas	–	kRatSarcoma protein
MAD 2	–	Mitotic arrest deficient protein 2
MOI	–	Multiplicity of infection
MTS	–	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphel)-2-(4-sulfophenyl)-2H-tetrazolium
NHBC	–	Normal human bronchial epithelial cells
nM	–	Nano molar
uM	–	Micro molar
mM	–	Milli molar
PBS	–	Phosphate buffered saline
pRB	–	Phosphorylated retinoblastoma
PFU	–	Plaque forming unit
PMS	–	Phenazine methosulfate
PI	–	Propidium iodide
ppc	–	Particles per cell
qPCR	–	Quantitative polymerase chain reaction
$TCID_{50}$	–	Tissue culture infective dose at 50%
5-FU	–	5-fluorouracil

PLEASE NOTE: In some graphs Ad5ΔΔ is named Ad5DD.

INTRODUCTION

Pancreatic Cancer

Pancreatic cancer is the 11th most common form of cancer in the UK (Cancer Research UK http://info.cancerresearchuk.org) and the 4th most common cause of cancer death worldwide (Mariharan, D. *et al*. 2008). Among the 21 most common forms of cancer in England, pancreatic cancer shows the lowest relative survival rate, of less than 3%, after 5 years (Office for National Statistics 2010). In comparison to other forms of cancer, survival and development of efficient therapies targeting pancreatic cancer have not significantly improved over the last 30 years (Cancer Research UK). Since the symptoms are vague and diffuse, clinical presentation and diagnosis often occur first when the cancer is at an advanced stage. It is common that metastases are already present when the patient is diagnosed. Currently, the only curative treatment to date is radical surgery, but due to the often late diagnosis, inconvenient anatomical location and development of metastases, it is almost impossible to resect all tumors. In addition, pancreatic cancers are highly resistant to the currently available chemotherapeutic drugs such as gemcitabine and 5-fluorouracil (5-FU). Therefore, it is imperative to develop new therapies.

The majority of pancreatic cancers are ductal adenocarcinomas. Of these, 50-75% are mutated in the p53 gene, allowing cells to bypass DNA damage checkpoints and the induction of apoptosis. Approximately 90% of all pancreatic cancers have an activating mutation of the oncogene K-ras and an inactivating mutation of the tumour-suppressor gene CDKN2A. The activation of K-ras results in an aberrant activation of signalling pathways promoting cell proliferation and survival. Inactivation of CDKN2A leads to loss of the tumour suppressor gene p16, an important regulator of the progression from G_1 to S-phase in the cell cycle (Hidalgo, M. 2010). Together,

these and other common cancer-related mutations lead to failure in growth arrest, proliferation and promotion of metastasis (Morton, P J. *et al.* 2010).

Chemotherapeutic drugs act palliatively, but do not cure pancreatic cancer. 5-FU) was the only drug used in pancreatic cancer for many decades. In 1997, another chemotherapeutic drug with significantly better clinical responses emerged: gemcitabine (Li, J & Wasif, S M. 2009). Gemcitabine (2', 2'-difluorodeoxycitidine) is a pyrimidine nucleoside analogue, which incorporates in DNA and RNA stopping replication and translation respectively, ultimately leading to apoptosis. It mainly targets cells in S-phase but also inhibits cell progression from G_1 to S-phase. Since 1997, gemcitabine has been the drug of choice for advanced pancreatic cancer. However, despite the benefits in comparison to 5-FU, the overall objective responses are modest. Only 25% of patients actually benefit from gemcitabine therapy, since many types of pancreatic cancer show resistance to the drug. The mechanisms for sensitivity and resistance to gemcitabine have been widely investigated in order to identify molecular markers for treatment responses (Akada, M. *et al.* 2005). Mutant p53 **(**Galmarini, CM. *et al.* 2002) (presents in the majority of pancreatic cancers) and Bcl-XL **((**Shi, X. *et al.* 2002) & (Schniewind, *et al.* 2004)**),** both involved in apoptosis, have been identified as possible molecular markers of chemoresistance to gemcitabine. It has also been demonstrated that Bcl2/adenovirus E1B 19kDa protein interacting protein (BNIP3) is expressed at lower levels in pancreatic cell lines resistant to gemcitabine. BNIP3 is a proapoptotic protein in the Bcl-2 family (Akada, M. *et al.* 2005).

Various gemcitabine based combination chemotherapies have been designed trying to improve efficacy, but none has been demonstrated to be superior to gemcitabine mono therapy. One of the chemotherapeutic drugs tested in combination with gemcitabine is irinotecan (camptothecin), normally used for the treatment of colorectal cancer (Li, J & Wasif, S M. 2009). Irinotecan inhibits DNA Topoisomerase I, an enzyme essential for religation of single strand breaks during DNA-replication, the cytotoxic effect targets mainly cells in S-phase with active DNA synthesis, but also cells in G_2-phase (Pommier, Y. 2006).

Oncolytic adenovirus as therapeutics

Viral gene therapy is a promising developing field in the battle against cancer. Adenoviruses have been developed as efficient tools for cancer treatments during the last decade, because of efficient transgene delivery, transduction of both dividing and non-dividing cells, ease of production to high titres and extensive understanding of the viral life cycle ((Wu, Q. *et al.* 2001) & (Douglas, JT. 2007)). Through manipulating the viral genome, for example via the deletions or insertions of genes, the virus can be tailored to match specific gene mutations in the cancer cell ((Young, S L. *et al* 2006) & (Anurag, S. *et al.* 2009)). In addition, the virus does not integrate in the host genome and has been proven safe in humans with promising efficacy in many clinical trials (Douglas, JT. 2007). Adenoviruses can accommodate up to 2kB (5%) of foreign DNA without significant effects on stability and infectivity (Wu, Q. *et al.* 2001). Adenovirus serotypes 5 and 2, have been most frequently used in cancer gene therapy to date **(**Anurag, S. *et al.* 2009).

Human adenovirus is specific to humans and does not replicate in murine cells. Adenoviral *in vivo* models on cancer must therefore be performed using immunodeficient mice with human tumour xenografts. Other limitations for the therapeutic uses of Ad5 are the difficulties with systemic delivery in humans. Human erythrocytes bind and inactivate Ad5 to protect against systemic infection. In contrast to mouse erythrocytes, it was demonstrated that human erythrocytes express coxsackievirus and adenovirus receptor (CAR). This is the same receptor as the virus uses for cell entry during infection, resulting in Ad5 sequesteration by erythrocytes and final elimination by the liver (Carlisle, C. R. *et al.* 2009) Until this complex formation has been overcome, for example by detargeting of viruses, anti-cancer therapy using Ad5 must be delivered non-systemically, for example *via* intratumoral or intracavitary injections. This limits the possibilities to reach all tumours.

Adenovirus

The name *adeno* is derived from where this virus was first isolated, the adenoid tissue, in 1956 (Shenk, T E. 2001). Infection with adenovirus in immuno-competent individuals is mild. Dependant on the serotype the virus can cause upper respiratory tract infections, gastroenteritis or keratoconjunctivitis, and symptoms similar to a mild flu (Wu, Q. *et al.* 2001). The human adenovirus has been divided into 51 serotypes, based on their resistance to neutralisation by antisera. The serotypes are then classified into subgroups based on their ability to agglutinate erythrocytes. Adenovirus type 2 and 5 are the most studied serotypes, and therefore are the most important in adenoviral gene therapy (Shenk, T E. 2001). The Ad5 genome is a linear, double-stranded DNA of approximately 36kb. The genome is surrounded by an icosahedral capsid. The capsid can be described as a protein shell composed of predominantly three proteins: hexon, penton base and fiber (Wu, Q. *et al.* 2001).

Adenovirus can infect all epithelial cells. When entering the cell, the fiber knob attaches to CAR leading to receptor-mediated endocytosis. MHC I can probably also serve as a receptor for Ad5 (Shenk, T E. 2001). The viral genome is released from the viral capsid in the endosome and transported to the nucleus via microtubules (Fig. 1). Once the genome is in the nucleus the early genes (E1-4) are expressed (Hakkarainen, T & Hemminki, A. 2001). Viral replication leads to cell lysis and release of new adenoviruses.

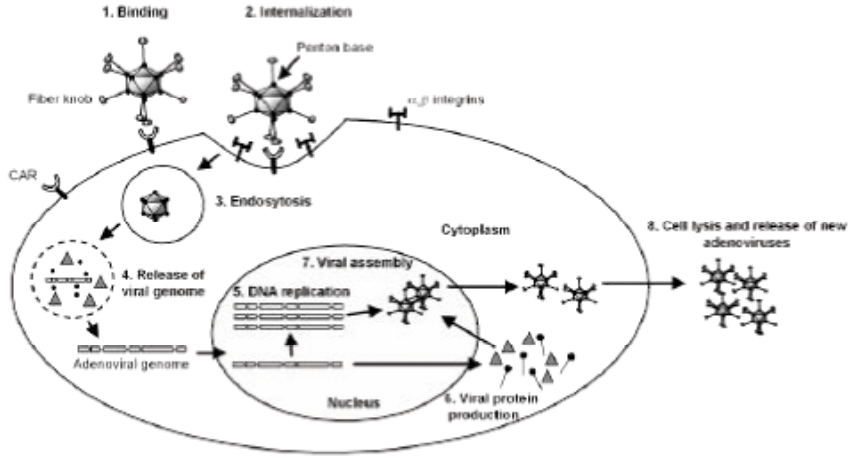


Fig. 1 The adenoviral life cycle. The fiber knob attaches to CAR leading to receptor-mediated endocytosis. The viral genome is released from the viral capsid in the endosome and transported to the nucleus via microtubules. Viral gene expression starts once the genome has reached the nucleus. Replication leads to lysis of the cell and release of new viruses (Hakkarainen, T & Hemminki, A. 2001).

Viruses manipulate their host cells by expressing viral proteins that can either induce or inhibit cellular functions to enable the viral life cycle to proceed. Through this viral mimicry, the virus can prevent apoptosis and evade the host's immune-response (Young, S. L. *et al.* 2006). The adenoviral genome (Fig. 2) has been described in terms of early (E) and late (L) genes/proteins depending on when they are expressed in the infection cycle. This does not always hold true, since some genes are expressed at low level throughout the entire replicative cycle, but it can be used to simplify the understanding of the genome (Shenk, T E. 2001).

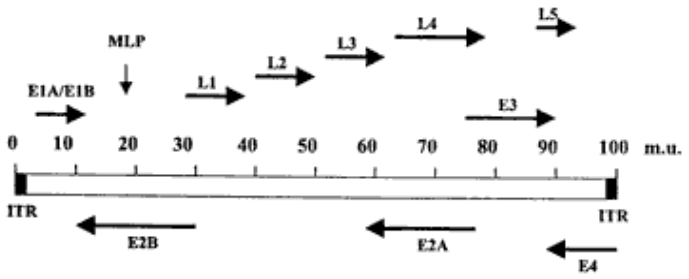


Fig. 2 Schematic diagram of the adenoviral genome (Ad5). The length of genome is divided into 100 measurement units (m.u), two inverted terminal repeats (ITR) a major late promotor (MLP) and the early (E1-E4) and late (L1-L5) structural genes. Arrows indicate the direction of transcription (Wu, Q. *et al* 2001).

The adenoviral genome carries four early transcription units: E1 (A and B), E2, E3 and E4.

The E1A gene is the first gene to be expressed when the viral genome has reached the nucleus. It induces expression of other early genes and forces the cell to enter S phase. Retinoblastoma protein (Rb) is a negative controller of cell cycle progression. It has a pocket domain through which it binds and inactivates E2F, a transcription factor inducing S phase. When Rb binds to E2F, the progression from G_1 to S phase is inhibited (Fig 3). The adenoviral E1A proteins, 12- and 13S, both contain two independent domains, which can bind Rb: E1ACR1 and -2. The interaction is not yet fully understood, but it seems as if E1ACR1 binds to the same site as E2F whilst E1ACR2 binds to another site on Rb. It has been shown that E1ACR2 is the essential domain for the formation of the Rb complex (Fattaey, R A. *et al.* 1993). When E1ACR2 binds Rb, E2F is released and the cell pushed into S-phase.

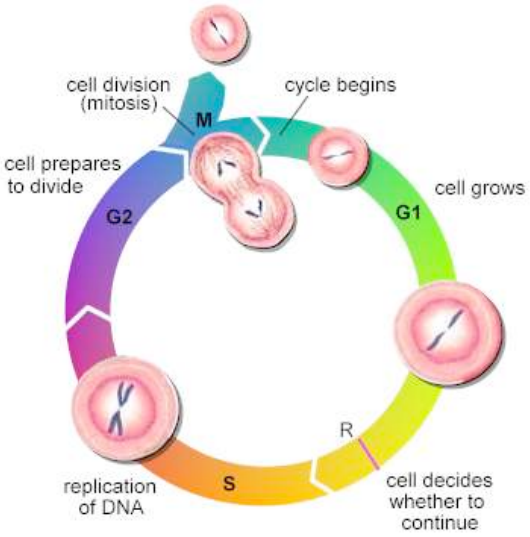


Fig. 3 Normal cell cycle. Checkpoint R regulates progression from G_1-S. pRB is a negative controller of this checkpoint.
(http://kirschner.med.harvard.edu/files/html/research.shtml)

The E1B gene encodes two proteins, E1B-55K and E1B-19K, which block apoptosis that is induced by E1A. The E2 proteins function directly in viral DNA replication. The E3 region encodes several proteins that modulate the host immune-response to protect the infected cell from cytokine-induced cell-killing and the E4 proteins mediate transcriptional regulation, modulate DNA replication and prevent activation of the DNA-damage response. (Shenk, T E. 2001).

Oncolytic replication-selective adenoviral mutants

The rationale for the use of replication-selective oncolytic viruses is that the mutants can lyse cancer cells but spares normal cells. There are different ways to achieve this. One way is to construct a virus that is replication-attenuated in the intracellular environment of a normal cell but has its full efficacy in the cancer cell, an oncolytic replication-selective virus. The viral mutant can thus replicate and lyse cancer cells and spread within the tumour with limited amplification and cytotoxicity in normal tissue (Fig. 4). Infected normal cells subsequently undergo apoptosis and viral spread in non-cancer cells is limited. Cycling normal cells provide a similar environment as cancer cells,

and the virus could potentially replicate in these cells but not in quiescent cells (Young, S. L. *et al.* 2006).

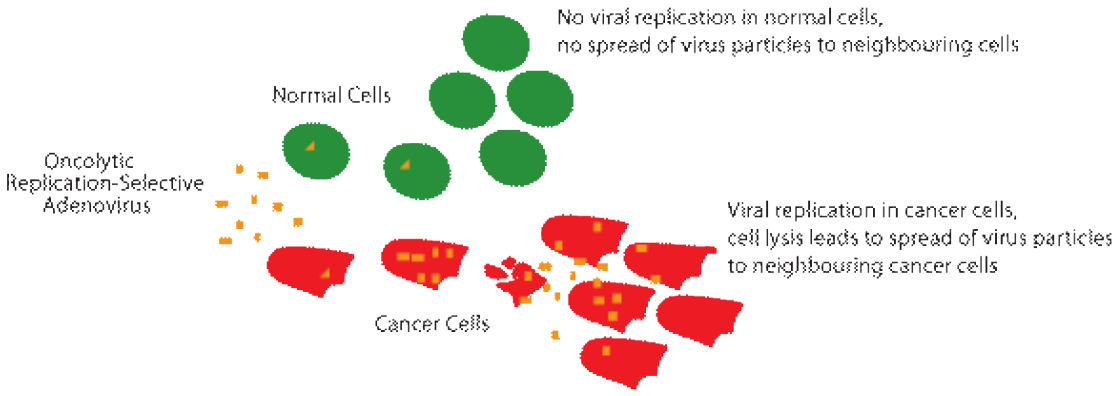


Fig. 4 The theoretical principle of an oncolytic replication-selective adenovirus in action.

For the host, the apoptotic pathway is an important defence-mechanism in virus-infected cells. Apoptosis is induced in response to the presence of viral DNA and E1A. To inhibit induction of apoptosis viral proteins are expressed (E1B, E4). In cancer cells, these apoptotic pathways are commonly deregulated. The adenoviral gene E1B encodes two proteins, E1B19K and E1B55K, which block apoptosis. The first oncolytic replication-selective adenoviral mutant to be evaluated clinically, ONYX-015 (*dl*1520), was deleted in the E1B55K gene (Aghi, M & Martuza, RL. 2005). ONYX-015 was engineered to selectively replicate in and lyse p53-deficient cancer cells, not being able to inhibit the functional p53 in normal cells (Khuri, R F. *et al.* 2000). The E1B55K protein binds p53 and antagonizes the ability of p53 to block cell cycle progression and induce apoptosis by inhibiting its activation function. E1B55K also collaborates with E1A to activate quiescent cells to progress in cell cycle (Shenk, T E. 2001). However, it was later discovered that E1B55K was essential for viral mRNA export making ONYX-015 too attenuated resulting in poor efficasy (O'Shea, C C. 2005).

The second E1B protein, E1B19K, is a viral Bcl-2 homolog that inactivates the pro-apoptotic Bcl-2 family members, BAX and BAK, so that apoptosis is inhibited (Berk, J A. 2005). By deleting this gene, the virus cannot prevent

apoptosis in normal cells. Mutants lacking E1B19K (ΔE1B19K) have been shown to improve efficacy of DNA-damaging drugs such as gemcitabine through increasing apoptosis-like cell death (Leitner, S. *et al.* 2009)

Recently, an oncolytic mutant was engineered (Fig. 5), a double deleted Ad5 virus, Ad5ΔΔ, with deletions in the ΔE1ACR2 region for tumour selectivity and deletions in the ΔE1B19K gene for attenuated replication in normal cells (Oberg, D. *et al.* 2010). Ad5ΔΔ has been shown to have high cell-killing activity in pancreatic carcinomas (Oberg, D. *et al.* 2010) and has been demonstrated to enhance cell-killing in combination with the chemotherapeutic drugs gemcitabine and irinotecan *in vitro* (Dr. Cherubini & Kallin, unpublished work). This synergistic effect was previous demonstrated in the single deleted Ad5Δ19K (Leitner, S *et al*. 2009).

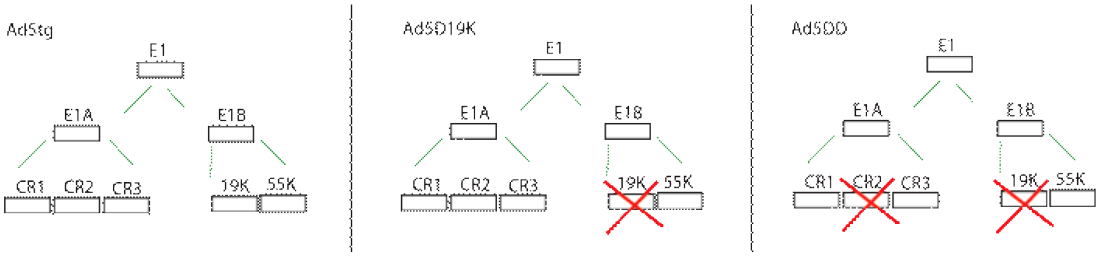


Fig. 5 Schematic drawing of localisation of deletions in Ad5Δ19K and Ad5ΔΔ compared to Ad5tg (adapted from: Wu, Q. *et al* 2001 & Oberg, D. *et al* 2010).

Tumour selectively by E1ACR2-deletion. As described above, the E1ACR2-domain competes with E2F for access to the Rb pocket domain, blocks the binding site for E2F and forms a complex with Rb. Free E2F activates the transcription of a series of genes important for S phase and cell growth (Fig. 6). When E1ACR2 is deleted, it cannot bind to Rb and is unable to stimulate cellular DNA synthesis (Shenk, T E. 2001). In most cancers, Rb is phosphorylated (pRb) and thereby inactivated, free E2F can activate transcription and cell cycle progression.

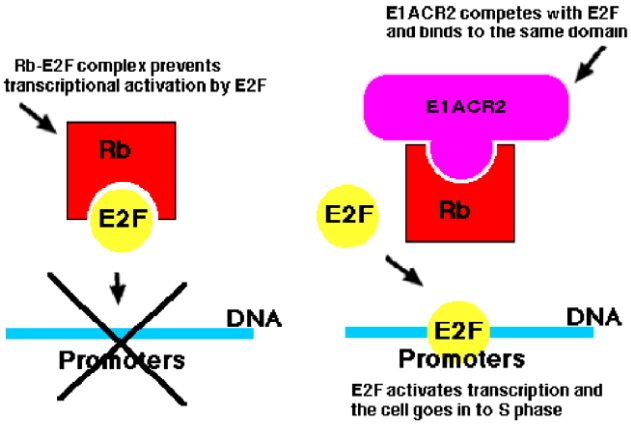


Fig. 6 The adenoviral E1ACR2 competes with E2F for access to the Retinoblastoma protein pocket domain. Free E2F activates transcription and induces S-phase. (adapted from: http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/T/TumorSuppressorGenes.html)

To date, most oncolytic viruses evaluated in the clinic have E3-deletions, to shorten the viral survival. E3 is an immunoregulatory gene, which prolongs the virus $T_{1/2}$ in vivo. Thus, it has been shown that oncolytic mutants with intact E3-region replicate more efficiently and show enhanced anti-tumour effect ((Wang, Y. *et al.* 2003) & (Oberg, D. *et al.* 2010)). Both Ad5Δ19K and Ad5ΔΔ have an intact E3 gene.

Project aims

The adenoviral mutant Ad5ΔΔ (E1ACR2- and E1B19K- deleted) in combination with the chemotherapeutic drugs gemcitabine and irinotecan has been shown to enhance cell-killing in pancreatic cancer cells. This project was aimed at establishing whether this effect also was observed in additional pancreatic cancer cell lines and if so, investigating mechanisms causing the more than additive increase in cell-death.

- Does Ad5ΔΔ lyse pancreatic cancer cells efficiently?
- Does Ad5ΔΔ sensitize cancer cells to the chemotherapeutic drugs gemcitabine and irinotecan?
- How does Ad5ΔΔ in combination with drugs affect cell cycle progression and cellular protein expression?
- Is Ad5ΔΔ replication-attenuated in normal human umbilical-vein endothelial cells?
- How do the combination treatments kill the cancer-cells?

Finding a new treatment for pancreatic cancer is crucial. The results of the experiments in this project will, hopefully, bring the Ad5ΔΔ virus a step closer to clinical trials as a new treatment for pancreatic cancer. Further understanding of the mechanisms behind the greatly improved efficacy observed for Ad5ΔΔ in combination with chemotherapeutic drugs might aid in the understanding of drug resistance.

MATERIALS AND METHODS

Cell lines and normal cells. Human pancreatic adenocarcinoma cell lines used for this project were MIA-PaCa-2 and AsPC-1 (Table 1) provided from Cell Services, Clare Hall, Cancer Research UK. MIA-PaCa-2 and AsPC-1 are classified as intermediate sensitive to gemcitabine (Akada, M. *et al.* 2005).

Cell line	*Differentiation*	*Source*	*p53*	*K-ras*	*CDKN2A*	*SMAD4*
MIA-PaCa-2	Poor	Primary tumor	Mutation (-) *Point mutation*	Mutation (+) *Point mutation*	Mutation (-) *Homozygous deletion*	None *Wild type*
AsPC-1	Moderate	Ascites	Mutation (-) *Frameshift*	Mutation (+) *Point mutation*	Mutation (-) *Frameshift*	Mutation (-) *Point mutation*

Table 1 Genetic aberrations in MIA-PaCa-2 and AsPC-1. Activating (+) or inactivating (-) mutations (Moore *et al*. 2000 and Sipos *et al*. 2002).

JH293 cells, a subclone of the human embryonic kidney cell line HEK293 (Wang, Y. *et al.* 2005) was used for half maximum tissue culture infective dose ($TCID_{50}$), (Cell Services, Cancer Research UK) were cultured according to the manufacturer's instructions.

Normal human umbilical vein endothelial cells, HUVEC, were cultured in endothelial cell basal medium-2 (EBM-2) according to the manufacturer's instructions (Lonza Clonetic®).

Cell culture. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose (4.5 g/L) (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% foetal calf serum (FCS) (PAA Laboratories GmbH,

Pasching, Austria) and 100 units/mL penicillin, 100mg/L streptomycin (PAA Laboratories GmbH, Pasching, Austria). The cells were incubated in 37˚C with CO_2 levels kept constant at 5% for MIA-PaCa-2, and 10% for AsPC-1. The cells were propagated twice a week and transferred into new flasks before reaching confluency. The media was aspired and the cells washed with phosphate buffered saline (PBS), before adding the enzyme trypsin to deattach the monolayer. Once the cells were deattached the enzymatic reaction was stopped through addition of 10% FCS DMEM. Cells needed for experiments were plated at the time of propagation.

Adenoviruses. The following mutants of adenovirus type 5 were used: Ad5tg (Ad5 wild-type), Ad5ΔΔ (Ad5tg with E1ACR2- and E1B19K-deletions) and Ad5Δ19K (Ad5tg with E1B19K-deletion). The Ad5tg genome was derived from the pTG3602 plasmid (Dr. M. Methali, Transgene) and all mutants were generated by homologous recombination with shuttle plasmids containing the respective E1ACR2 and E1B19K deletions as described (Oberg, D. *et al.* 2010). All the mutants are replication-competent.

Chemotherapeutic drugs. Gemcitabine (2',2'-difluorodeoxycitidine, Gemzar®, Eli-Lilly, Indianapolis, IN) and irinotecan (camptothecin, Irinotecan HCL, Hospira UK Limited) were used in this project. The stock concentration for gemcitabine and irinotecan was 100mM and 34mM respectively. Gemcitabine was stored at -20˚ and irinotecan at room temperature.

Cell-killing assays. The MTS assay (3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphel)-2-(4-sulfophenyl)-2H-tetrazolium) is a colorimetric assay that measures the changes in colour caused by mitochondrial enzymes active only in live cells. In the presence of PMS (phenazine methosulfate), mitochondrial enzymes convert MTS to formazan with an absorbance at 490nm. The absorbance value is directly proportional to the number of live cells enabling quantification of cell death.

The MTS assay was used to determine cell-killing 72 hours after viral infection and/or treatment with chemotherapeutic drugs. Dose response curves were generated to both virus and drugs to determine 50% and 20% cell death for EC_{50}- and EC_{20}- values respectively. The chemotherapeutic drug/viral concentration that resulted in 20% or less cell death alone, was used as the constant concentration in the combination treatment. Each data point was generated from triplicate samples and the experiment repeated up to 4 times for each condition.

Infection for cell-killing assay

Cell lines analysed: MIA-PaCa-2 and AsPC-1

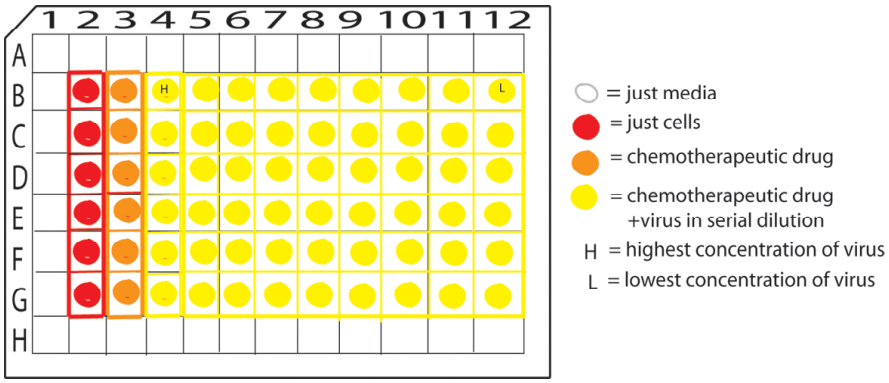
10000 cells per well were seeded on sterile 96-wells plates in 10% FCS DMEM (100μl), 24 hours before infection/treatment. The outer rows were filled with 100μl media alone, to help maintain humidity. Each condition was tested in triplicates: three wells with the same conditions. On the day of infection/treatment, virus and chemotherapeutic drugs were prepared in DMEM supplemented with 2% FCS. The MTS assay was first set up for virus and chemotherapeutic drug respectively and then for combination treatments. The concentrations used in the combination treatments were based on virus and chemotherapeutic drug dose-response curves and were selected at a dose that resulted in <20% cell death on its own.

The serial dilution for virus started at $1x10^5$ particles per cell (ppc) with ten 5-fold dilutions. The chemotherapeutic drugs were diluted 3-fold 10 times, starting at concentrations 100μM, 40μM, 10μM and 1μM for gemcitabine, and 3.4mM and 1mM for irinotecan.

Combination treatment with constant concentration of chemotherapeutic drug and serial dilution of virus. The concentrations of chemotherapeutic drug were prepared in 2% FCS DMEM. Two different concentrations of each chemotherapeutic drug were used in each combination experiment, causing <20% cell death alone. 90μl of the chemotherapeutic drug solution was added to each well. 10μl from each viral dilution was added to the wells, all in

triplicates. Three rows were left without any virus: one with just the chemotherapeutic drug solution as control for drug-induced cell death, one with just cells as control for maximum cell viability in cells alone and one with just media background control (Fig. 7 A and B).

A



B

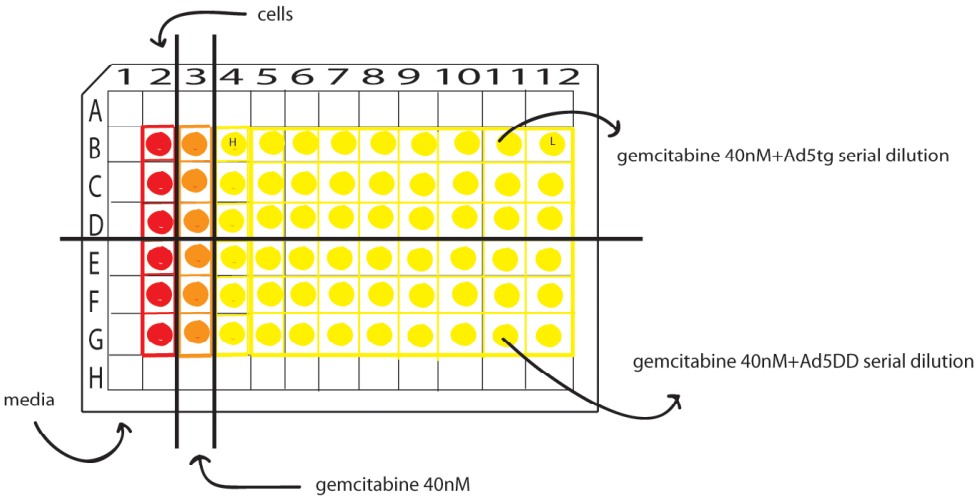
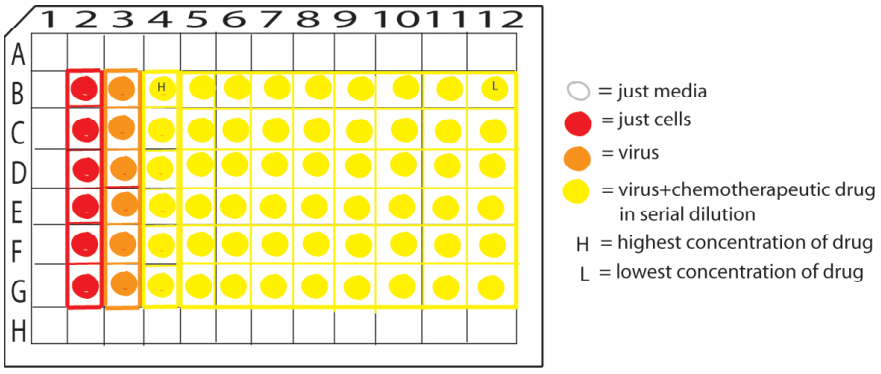


Fig. 7 A. Organisation of combination treatment with constant chemodrug and serial dilution of virus on the 96-well plate. **B**. Example of 40nM gemcitabine and serial dilutions of Ad5tg and Ad5ΔΔ respectively, in triplicates.

Combination treatment with constant concentration of virus and serial dilution of chemotherapeutic drug. The virus dilutions were prepared in 2% FCS DMEM. Two different concentrations of each virus were used, based on EC_{20}-values in previous MTS-assays. The concentration of virus on its own resulted in <20% cell death. The experiment was performed as described above with the exception that one row contained just virus instead of just chemotherapeutic drug (Fig. 8 A and B).

A



B

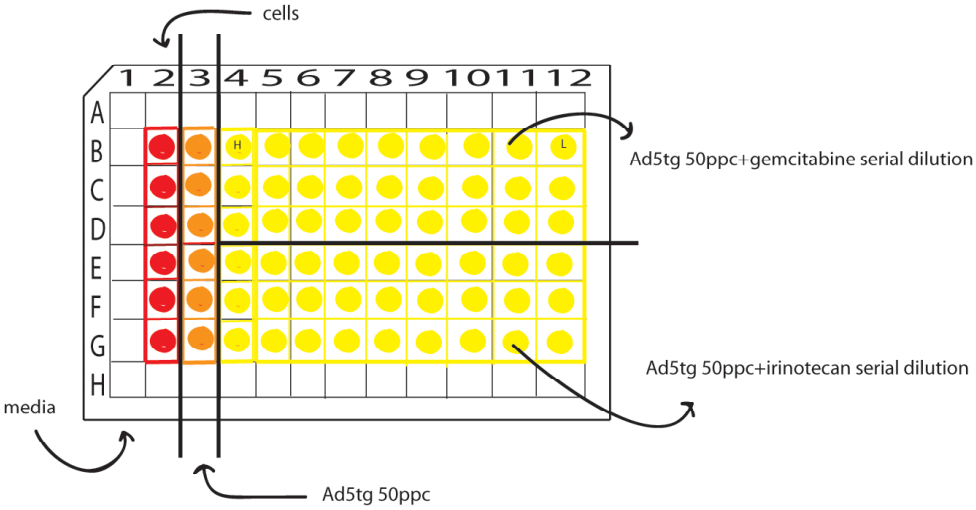


Fig. 8 A. Organisation of combination treatment with constant virus and serial dilution of chemodrug on the 96-well plate. **B**. Example of Ad5tg 50ppc and serial dilutions of gemcitabine and irinotecan respectively, in triplicates.

72 hours post infection, media was discarded and 100μl MTS solution/well was added (80% DMEM media 0% FCS, 20% MTS, 1% PMS). The plates were read in an ELISA microplate reader at a wavelength of 490nm (Opsys MR, Dynex Technologies). The values were transferred to the EXCEL graphing program for calculations.

CALCULATION OF CELL DEATH IN PERCENTAGE:

100 – (((sample – average background) / average control) * 100)

CALCULATION OF CELL DEATH IN PERCENTAGE FOR COMBINATION TREATMENTS:

Combination treatment with constant concentration of drug
100 – (((sample – average background) / (average drug alone – average background) * 100)

Combination treatment with constant virus
100 – (((sample – average background) / (average virus alone – average background) * 100)

Infection for Western Blotting and Flow Cytometry Assay

Cell line analysed: MIA-PaCa-2

24 hours before infection, $1x10^6$ cells/plate were plated in 10cm ∅ plates with 10ml of 10% FCS DMEM/plate. 3 plates per condition were plated, one for each time point: 24, 48 and 72 hours, plus one plate needed for cell counting before infection (Table 2).

Mock
Ad5tg 100 PPC
Ad5ΔΔ 100 PPC
Gem 40nM
Ad5tg 100 PPC + Gem 40nM
Ad5ΔΔ 100 PPC + Gem 40nM
Iri 15μM
Ad5tg 100 PPC + Iri 15μM
Ad5ΔΔ 100PPC + Iri 15μM

Table 2 Conditions analysed for western blotting and FACS.

The doses of viruses and chemotherapeutic drugs were chosen according to results from MTS assays described above. These suboptimal doses of gemcitabine and irinotecan resulted in <20% cell death on its own, but in increased cell death when combined with Ad5ΔΔ.

On the day of infection one
plate was trypsinised to count the cells. Viral dilutions of 100 ppc were prepared in 0% FCS DMEM, 2ml for each plate. The media was aspired and 2ml of the respective viral dilution was added to the plates, whereas 2ml of 0% FCS DMEM was added to the control plates (*e.g* mock infected cells). The cells were harvested 24, 48 and 72 hours after this time point (=infection). All plates were incubated at 37˚C for 2 hours, the optimal time for virus transduction of cells in culture. After 2 hours the media from all plates was aspired and 6ml of the chemotherapeutic drug dilutions in 10% FCS DMEM was added to the plates, whereas 6ml of 10% FCS DMEM was added to the drug free plates. All plates were then incubated at 37˚C until harvesting.

The media was removed and the cells were trypsinised at 24, 48 and 72 hours after infection,. Media and cells were combined and centrifuged, the supernatant removed and the pellet was resuspended in 2ml of PBS. 1ml was transferred to Eppendorf tubes for preparation of protein lysate for Western blotting and 1ml was transferred to FACS tubes. Both tubes were centrifuged again and the supernatants removed.

For Western blotting: The pellets were resuspended in RIPA buffer (50mM Tris HCl pH 8.0, 150mM NaCl, 1 mM PMSF, 1 mM EDTA, 1% (w/v) Igepal CA-630 (NP-40), 0.1% (w/v) SDS, 1 tablet of protease inhibitor (Roche) per 10 ml RIPA buffer, kept at 4˚C for 30 minutes and stored at -20˚C.
For Flow Cytometry Assay: The pellets were resuspended in 1ml 70% EtOH 4˚C, vortexed and stored at 4˚C (if analysed within 2 weeks, otherwise stored in –20°C).

Western Blotting. Cells were infected with viruses/chemotherapeutic drugs, harvested after 24, 48 and 72 h as described above. The proteins in each

sample were quantified by the Bradford assay (Bio Rad) and then diluted in RIPA buffer to a final concentration of 2μg protein/μl per sample. After diluting the samples, the proteins were quantified again to calculate the final protein concentration. A 10% SDS/PAGE acrylamide gels were prepared. The percentage of the gel was chosen according to the molecular weights of the proteins of interest. Each gel was loaded with 14 samples plus ladder (250-11kD). 20μg of protein was loaded per well. The proteins were separated on the gel and transferred to a polyvinyl-idene fluoride (PVDF) transfer membrane (GE Healthcare). For E1A and adenoviral capsid proteins, the membrane was cut at 55kD and incubated with primary antibodies (ab) separately since the antibodies needed different secondary antibodies (anti-mouse respective anti-rabbit). The membranes were incubated with the primary antibodies at 4˚C over night (Table 3).

	Dilution	Animal	Storage	Mol. weight	Supplier	Anti-ab Dilution
E1A	1:1000	Mouse	+4˚C	35-46kD	Santa Cruz	1:2000
Ad-Capsid	1:10000	Rabbit	+4˚C	55-130kD	Abcam	1:2000
Aurora-A Kinase	1:500	Mouse	-20˚C	46kD	BD Biosciences	1:2000
Aurora-B Kinase	1:500	Mouse	-20˚C	41kD	BD Biosciences	1:2000
MAD 2	1:2000	Mouse	-20˚C	24kD	BD Biosciences	1:2000
Vinculine	1:10000	Mouse	+4˚C	130kD	Abcam	1:2000

Table 3 The primary antibodies to the proteins E1A, adenoviral capsid proteins, Aurora A- and B-kinase and MAD-2

After washing, they were incubated with the secondary antibody for 2 hours in room temperature or 4 hours at 4˚C. Anti-mouse was used as secondary antibody for E1A, Aurora-A Kinase, Aurora-B Kinase and MAD-2, and anti-rabbit for adenoviral capsid proteins. After washing, the membranes were covered with a solution of detection reagent and the film developed in the dark room. The membranes were washed again and re-incubated over night

with antibodies against Vinculine. Vinculine was used as loading control for all membranes. For further details see Western Blot Protocol, *Appendix 1*.

Flow Cytometry Analysis. Cells were infected with viruses/chemotherapeutic drugs, harvested after 24, 48 and 72 h as described above. After fixation in 70% EtOH for at least 24h the cells were centrifuged and washed with PBS, supernatant was discarded and the cell pellets were treated with 100 μg/ml RNAse and 50μg/ml Popidium Iodide (PI). PI is commonly used to quantitatively assess DNA content. All samples were analysed by flow cytometry FACScalibur (Becton Dickinson, Immunocytometry Systems, Belgium) with CellQuest Pro Software version 4.0.2, acquiring 10000 cells/sample. During the process, adjustments were made so that the G_1 peak was positioned at 200 FL3-H/Pulse Width to allow more accurate comparison between samples. It was essential to exclude cell doublets, but to include polyploid cells, this was performed by gating which cells to take into account (Fig. 9).

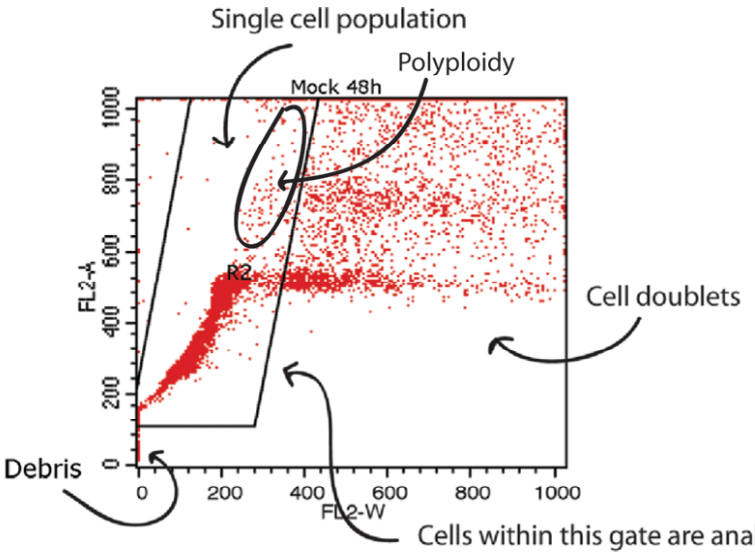


Fig. 9 Description of how the gates were adjusted for mock at each time point, 24, 48 and 72h.

Once the single cell population was identified the percentage of cells in G_1, S-phase and G_2/M could be estimated by subjectively applied markers for G1,

S-phase and G_2/M. The markers were set based on the profile of untreated cells (mock) at each time point (24, 48 and 72 hours), and applied for samples at the relevant time point (Cancer Research UK).

Infection for quantitative polymerase chain reaction (qPCR) and half maximum tissue culture infective dose ($TCID_{50}$)

Cells analysed: HUVEC

500000 cells/well were seeded in 6-wells plates in 3ml of endothelial cell basal medium-2 (EBM-2 Lonza, Clonetic®). The media was changed every 48 hours until the cells were confluent/growth arrested, 10 days after plating. Each sample was prepared in duplicates and 1 extra well/virus was plated to collect viruses 4 hours post infection, as control for viral uptake. One well was also needed for cell counting on the day of infection (Table 4). When the cells had reached confluence/growth arrest the infection was according to the procedure described for Western blotting and FACS. See above. The only difference was that cells were also harvested 4 hours post infection.

Mock
Ad5tg 100 PPC
Ad5ΔΔ 100 PPC
Ad5Δ19K 100 PPC

Table 4 Conditions analysed for qPCR and $TCID_{50}$.

Cells were harvested at 4, 24, 48 and 72 h post infection. 1ml of the media was collected, attached cells were trypsinised and media and cells were combined. After mixing, 500μl was transferred to cryovial tubes and stored at –80°C (for $TCID_{50}$). The remaining 500μl was centrifuged and washed with PBS. The pellet was resuspended in 200μl PBS and stored at –20°C (for qPCR).

Quantitative polymerase chain reaction. DNA was extracted from the samples using the QIAGEN kit (QIAGEN®) according to QIAamp® DNA

Mini and Blood Mini Handbook Spin protocol for Blood and Body Fluids. DNA concentrations were determined by Nanodrop (ThermoScientific) and adjusted to 4ng/μL with autoclaved ddH_20. 5μL/well was added to a 96-well plate. Forward (100nM) and reverse (100nM) primers for E2A and GAPDH were prepared with the Power SYBR Green Master Mix (Applied Biosystems) and added to each well. The reactions were run on qPCR (7500 Real Time PCR System; Applied Biosystems) and analyzed by the System SDS software. Each sample was made in duplicates, ie 2 reactions for GAPDH (glyceraldehydes 3-phosphate dehydrogenase) (normalisation reaction which should always produce a PCR product) and 2 reactions for E2A (a viral specific PCR product to evaluate viral DNA content as part of total DNA). Sterile water was used as a negative control for both GAPDH and E2A. The cycle threshold (CT) value for GAPHD was subtracted from the CT value for E2A and all duplicate values averaged. Calculations for average fold change relative to Ad5tg 24h was made in EXCEL using the $2^{-\Delta\Delta^{CT}}$ method: dCT= (CT E2A-CT GAPDH), ddCT= (dCT-dCT for Ad5tg 24h), fold change= POWER (2, -ddCT) (Tichopad, A. *et al*. 2003).

The experiment was repeated 3 times, using samples that derived from the same infection/collection. The first 2 repeats contained samples from the same aliquots, whereas the third repeat contained newly prepared dilution.

Adenovirus replication assay. $TCID_{50}$ was used for analysing viral replication in HUVEC. The viruses analysed were: Ad5tg, Ad5ΔΔ, Ad5Δ19K at 24, 48 and 72 hours post infection. Each sample was prepared in duplicates. An internal Ad5 (batch 290409) with known activity was included as control and analysed in triplicates. For the samples at 24 hours the dilutions used were $1x10^{-5}$ - 10^{-11} and for 48 and 72h, including the control, $1x10^{-7}$ - 10^{-13}. The last row on the plate had no virus (negative control). After 10 days the plates were inspected, well by well, to determine the cytopathic effect (CPE) of each sample. Infectious units per cell were calculated for each sample according to Spearman-Karber's formula ((Karber, G. 1931) & (Wang, Y *et al*. 2003)) and expressed as plaque forming units (pfu)/cell. For more details see $TCID_{50}$ protocol, *Appendix 2*.

Statistics. Statistical significance between EC_{50}-values was calculated using unpaired t-test with 95% confidence intervals in GraphPad Prism® software.

Pictures were made in Adobe Illustrator and Adobe Photoshop.

RESULTS

Cell-killing assays

To determine if the pancreatic cancer cell lines MIA-PaCa-2 and AsPC-1 were sensitive to the adenoviral mutants Ad5tg and Ad5ΔΔ, cell-killing assays were performed with serial dilutions of virus.

MIA-PaCa-2 and AsPC-1 are sensitive to both wildtype adenovirus type 5 (Ad5tg) and to the replication-selective E1ACR2- and E1B19K-deleted adenoviral mutant (AdΔΔ). Dose-dependent cell death was observed in MIA-PaCa-2 cells (Fig. 10 A) and AsPC-1 cells (Fig. 11 A) 72 hours after infection with Ad5tg and Ad5ΔΔ. The highest dose of both viruses, $1x10^5$ ppc, resulted in 95-100% cell death in MIA-PaCa-2. AsPC-1 was more resistant; more than 70% cell killing could not be achieved with $1x10^5$ ppc for either virus. There were no differences between Ad5tg and Ad5ΔΔ at the maximum concentration of virus ($1x10^5$ ppc). However, the cytotoxic effect of Ad5tg was significantly higher than Ad5ΔΔ, at the effective concentration killing 50% of cells (EC_{50}) in both cell lines (Fig. 10 B and 11 B).

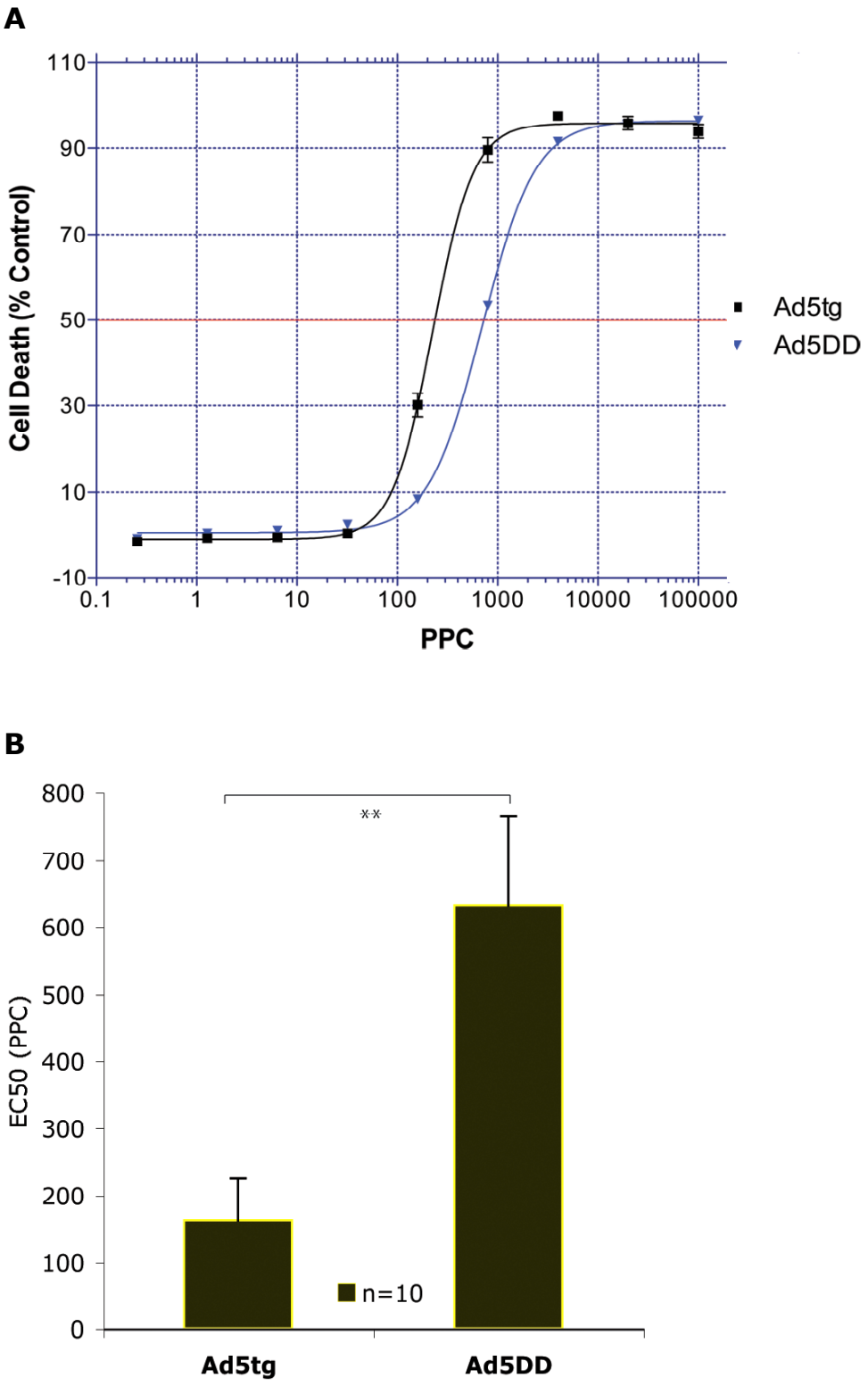


Fig. 10 MIA-PaCa-2 cells are sensitive to both Ad5tg and Ad5ΔΔ. **A**. Dose-response curves in MIA-PaCa-2 cells 72 h after infection with Ad5tg and Ad5ΔΔ. One representative experiment out of 10. **B**. Comparison of EC_{50}-values for Ad5tg and Ad5ΔΔ, averages ± SD, n=10, **p<0.01

AsPC-1

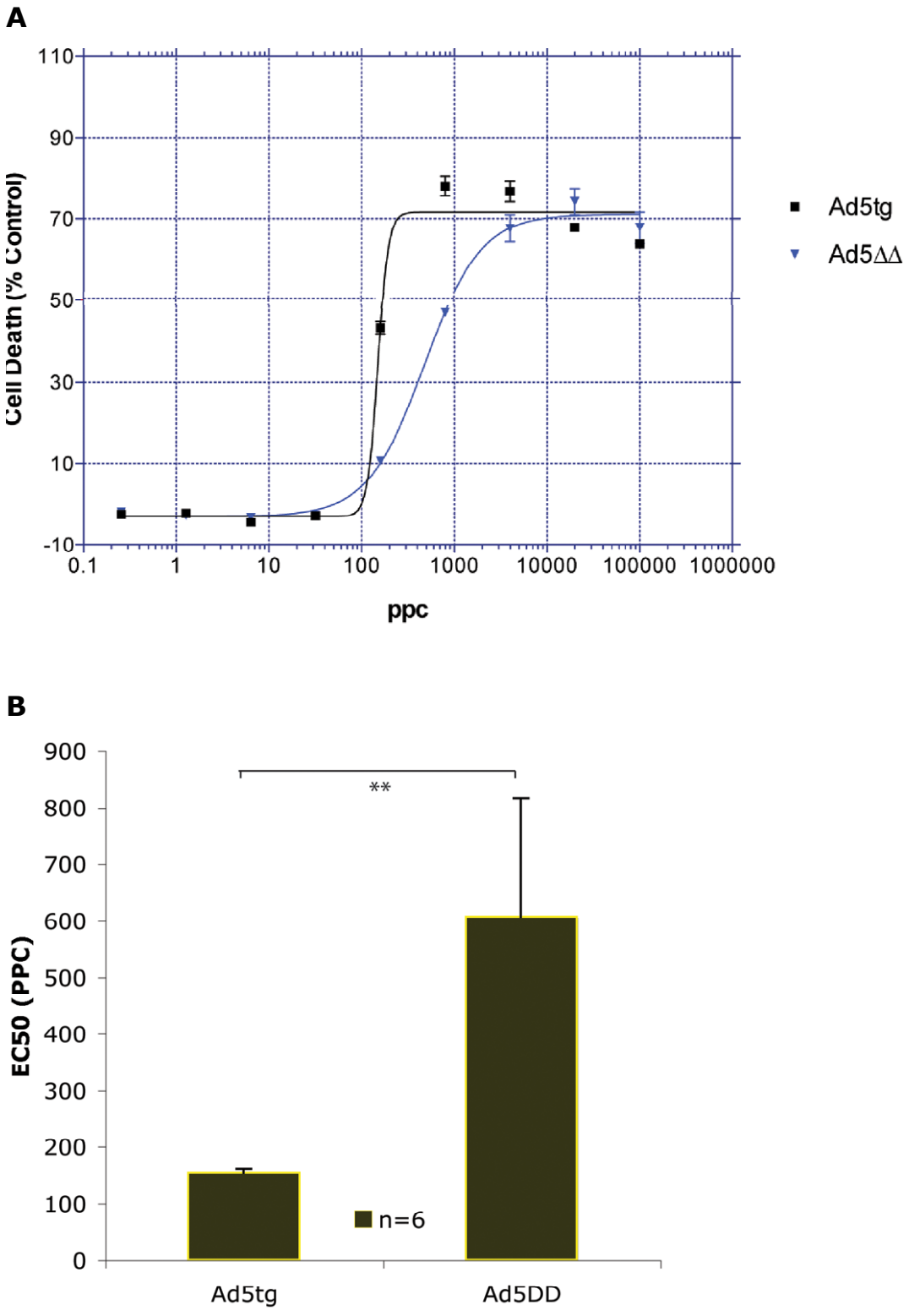


Fig. 11 AsPC-1 cells are sensitive to both Ad5tg and Ad5ΔΔ. **A**. Dose-response curve in AsPC-1 72 h after infection with Ad5tg and Ad5ΔΔ. One representative experiment out of 6. **B**. Comparison of EC_{50}-values for Ad5tg and Ad5ΔΔ, averages ± SD, n=6, **p<0.01

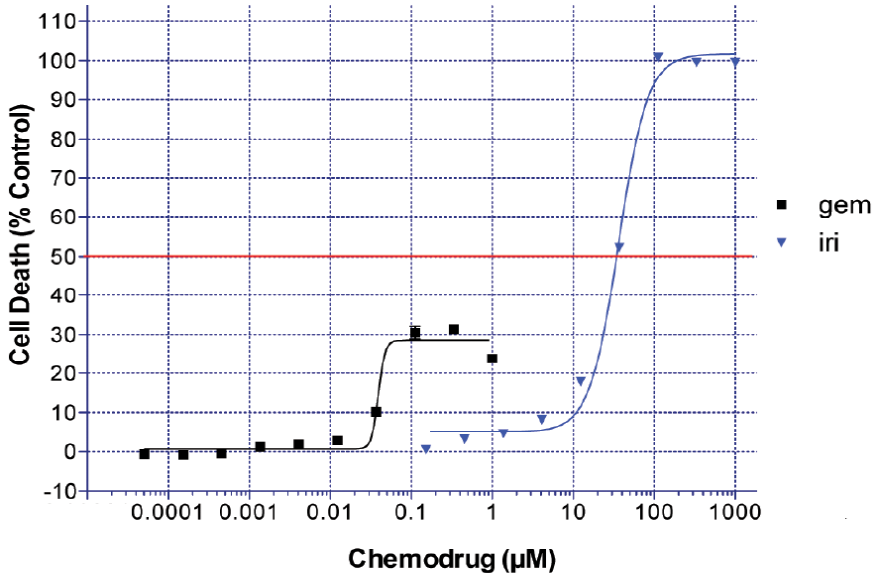
To determine if the pancreatic cancer cell lines MIA-PaCa-2 and AsPC-1 were sensitive to the chemotherapeutic drugs gemcitabine and irinotecan, cell-killing assays were performed with serial dilutions of chemodrugs.

MIA-PaCa-2 and AsPC-1 are sensitive to gemcitabine and irinotecan. A dose-related cell death was observed 72 h after treatment with irinotecan in both MIA-PaCa-2 and AsPC-1 (Fig. 12 A and B). Doses of 1mM irinotecan resulted in 95-100% cell death in MIA-PaCa-2 and <80% cell death in AsPC-1. Maximum dose of irinotecan, 34mM, did not result in an increase in cell death in AsPC-1 (data not shown). The average EC_{50} for irinotecan (serial dilution starting at 1mM) was 25μM (SD= 6, n= 6) in MIA-PaCa-2, and 38μM (SD=3.4, n=4) in AsPC-1.

Treatment with gemcitabine caused <50% cell death in both cell lines. AsPC-1 showed more resistance to gemcitabine than MIA-PaCa-2. 100μM gemcitabine resulted in <40% cell death in AsPC-1 whereas 1μM gemcitabine induced 30-40% cell death in MIA-PaCa-2. Higher doses (40μM, 100μM, 1mM) did not cause more than 50% cell death in MIA-PaCa-2. Since gemcitabine treatment resulted in less than 50% cell death in both cell lines, no EC_{50}-value could be calculated. The average gemcitabine concentration causing 20% cell death was 39.5nM (SD= 2.7, n=6) in MIA-PaCa-2 and 200nM (SD= 64, n= 6) in AsPC-1 cells.

MIA-PaCa-2

A



AsPC-1

B

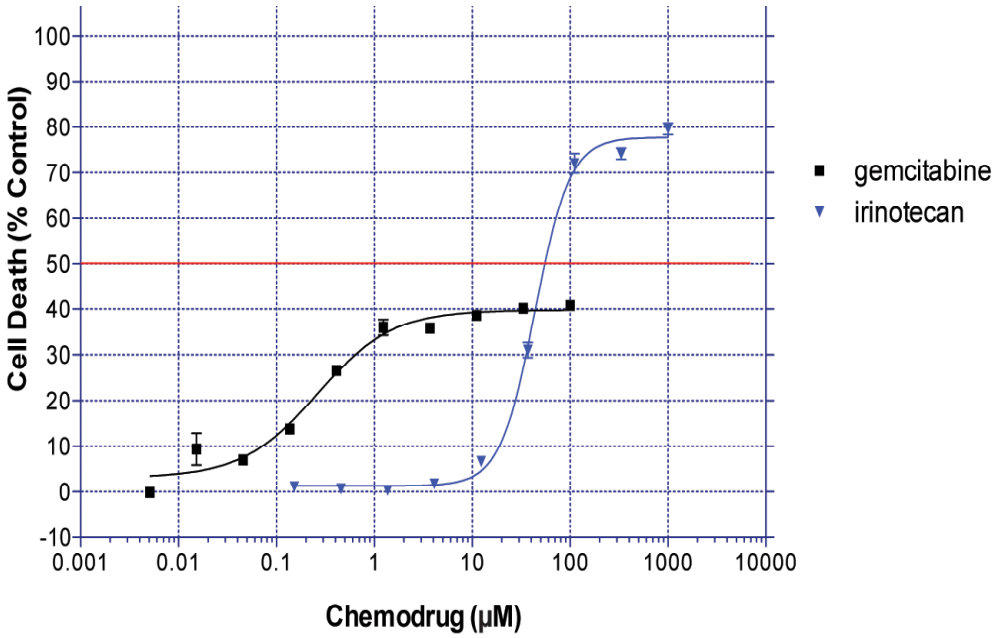


Fig. 12 Dose-response curves to gemcitabine and irinotecan. **A.** Dose-response curves in MIA-PaCa-2 cells. The graph is representative of 10 separate experiments. **B** Dose-response curves in AsPC-1 cells. The graph is representative of 6 separate experiments.

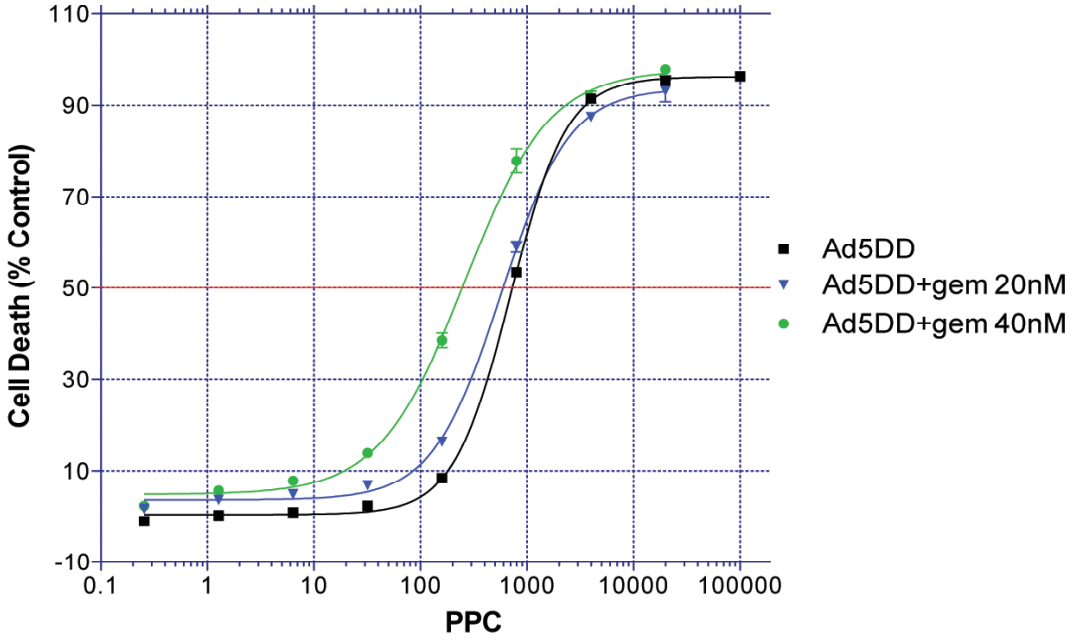
To determine whether the adenoviral mutants Ad5tg and Ad5ΔΔ could enhance cell-killing in combination with suboptimal doses of gemcitabine and irinotecan, cell-killing assays with combination treatments were performed in both cell lines. The selected drug doses caused less than 20% cell death alone and had been previously identified in dose-response curves to drug (Fig. 12 A and B).

Cell-killing in MIA-PaCa-2 cells is enhanced by combining Ad5ΔΔ with gemcitabine or irinotecan. The Ad5ΔΔ mutant in combination with either gemcitabine or irinotecan resulted in increased cell death compared to Ad5ΔΔ alone (Fig. 13 A and 14). The cell-killing was dose-dependent and significant for Ad5ΔΔ with gemcitabine ($p<0.05$) as for Ad5ΔΔ with irinotecan ($p<0.01$). The enhanced cell-killing in response to combination treatments with Ad5ΔΔ was observed for treatments with: constant concentration of chemotherapeutic drug and serial dilution of virus (Fig. 14 A), and constant concentration of virus and serial dilution of chemotherapeutic drug (Fig. 14 B).

Ad5tg in combination with suboptimal doses of irinotecan resulted in equal or increased cell death compared to Ad5tg alone. However, Ad5tg in combination with suboptimal doses of gemcitabine showed decreased cell-killing compared to Ad5tg alone (Fig. 13B). Similar additive or antagonistic effects has been previously observed in another cell line, PANC-1, in earlier studies (Dr. Cherubini & Kallin, unpublished work).

Average EC_{-50} values calculated on dose-response curves to virus in combination with different concentrations of drug relative to virus alone in are shown in Fig. 15.

A



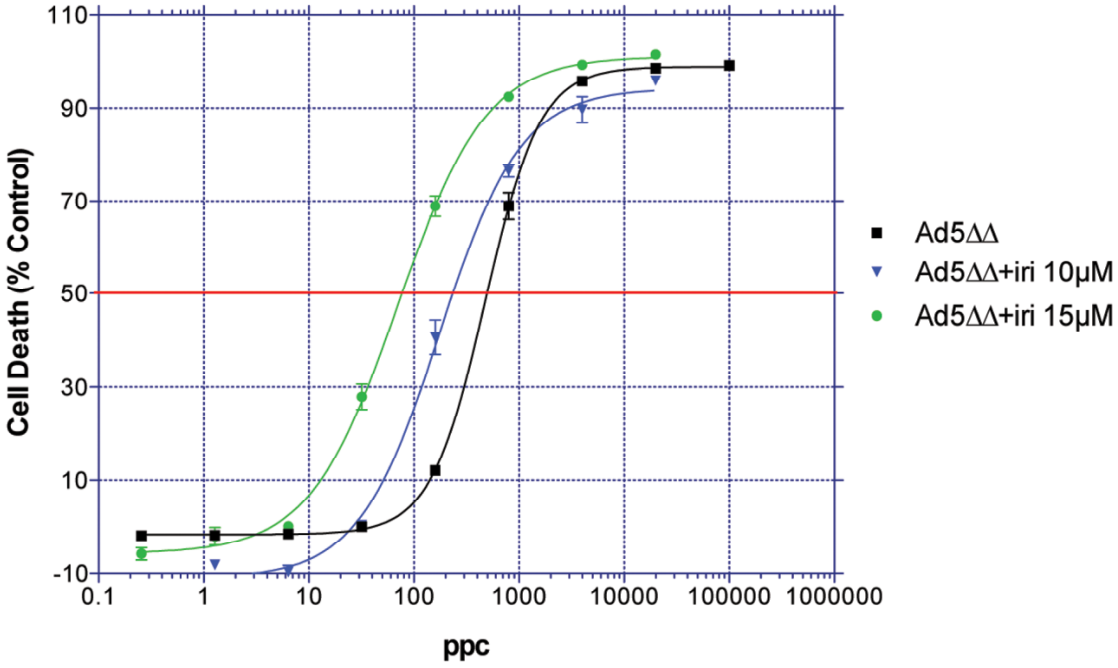


Fig. 13 Dose-response curves to virus and chemodrug in MIA-PaCa-2. **A**. Ad5ΔΔ in combination with gemcitabine and irinotecan. Selected graphs are representative for experiments with same concentrations of virus and drug. EC_{50}-values from dose-response curves with same concentrations generated an average EC_{50}, which allowed comparison between virus alone and combination treatments (Fig. 14). The values are corrected for cell death by drug alone.

B

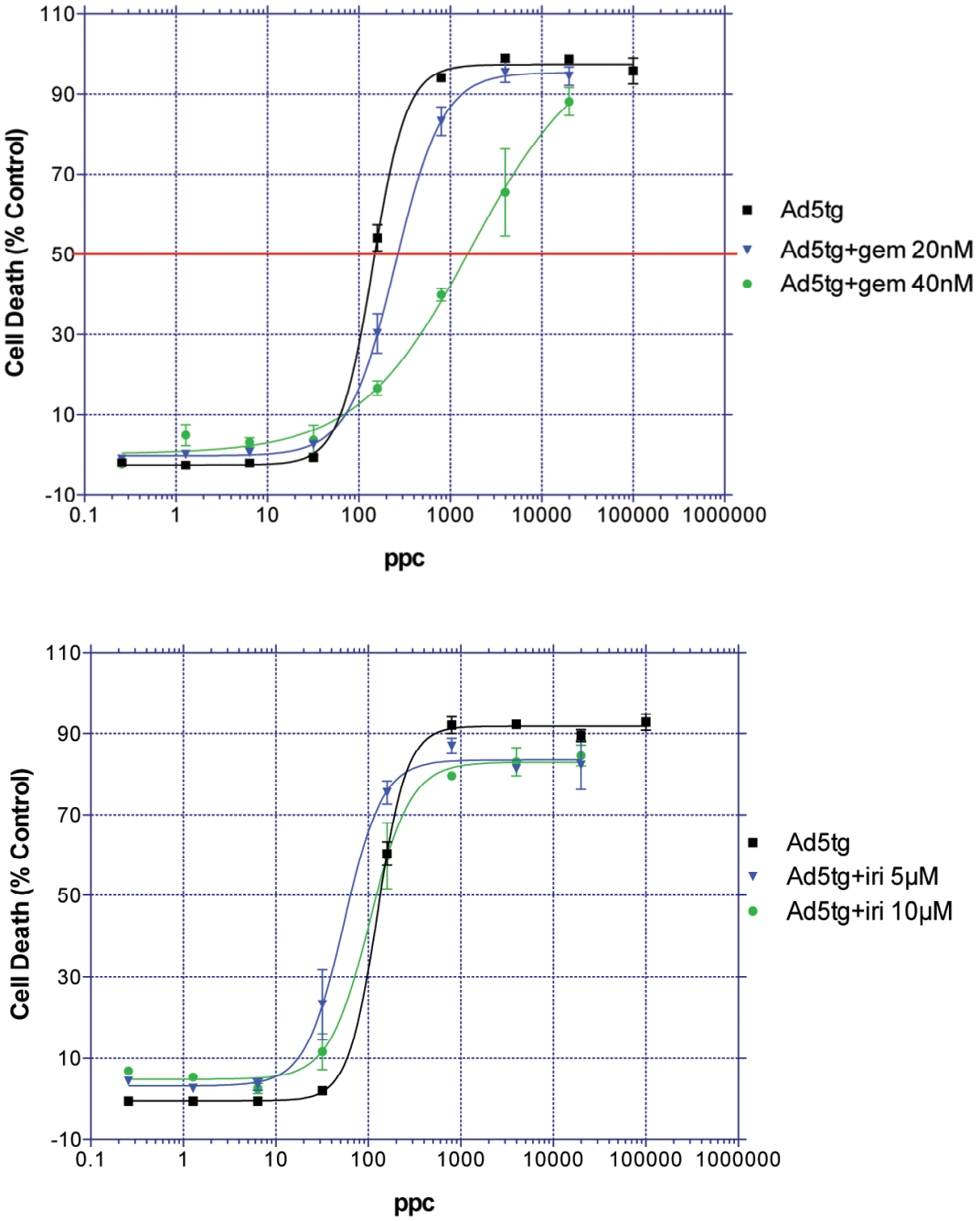


Fig. 13 Dose-response curves to virus and chemodrug in MIA-PaCa-2. **B.** Ad5tg in combination with gemcitabine and irinotecan. Selected graphs are representative for experiments with same concentrations of virus and drug. EC_{50}-values from dose-response curves with same concentrations generated an average EC_{50}, which allowed comparison between virus alone and combination treatments (Fig. 14). The values are corrected for cell death by drug alone.

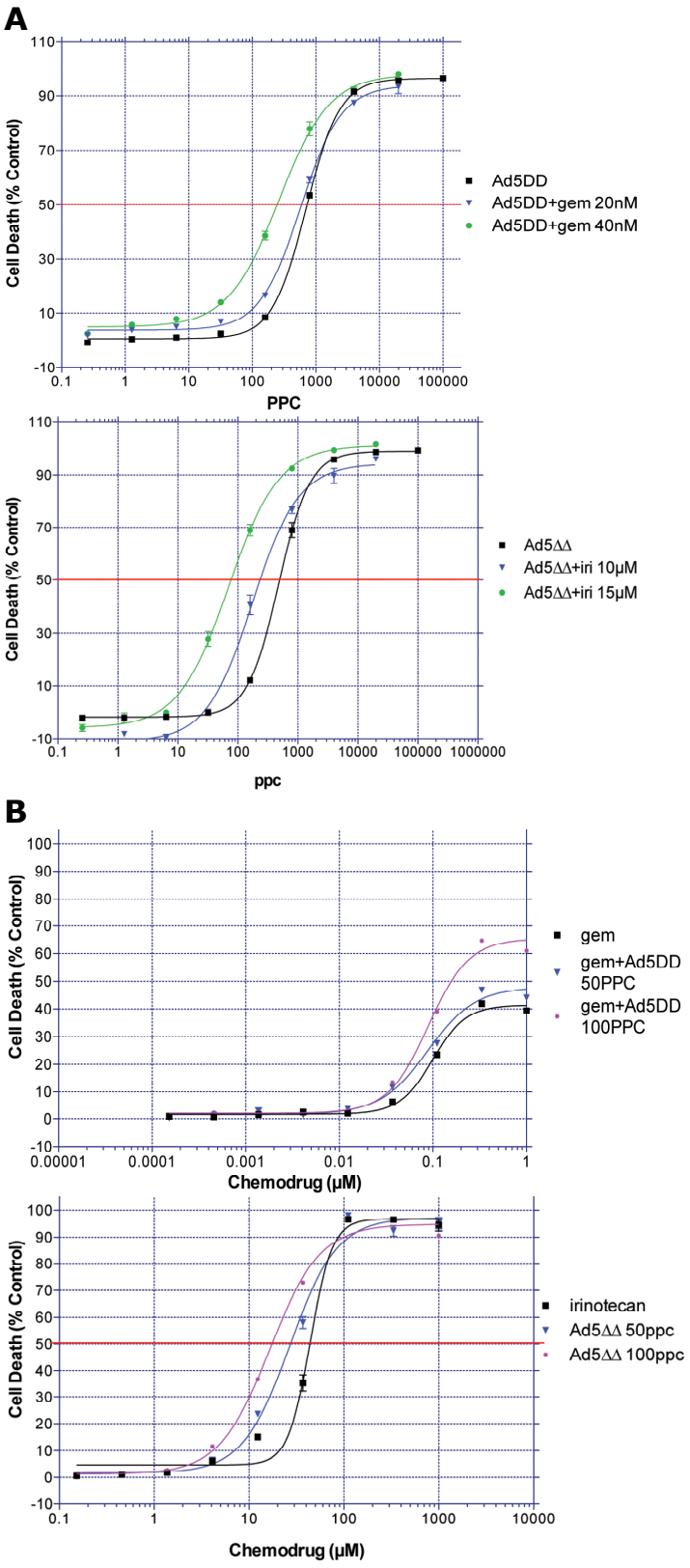


Fig. 14 Enhanced dose-dependent cell-killing by Ad5ΔΔ+chemodrug. **A.** Dose-response curves to combination treatments with constants concentration of gemcitabine or irinotecan and serial dilutions of Ad5ΔΔ. The values are corrected for cell death by drug alone. **B.** Dose-response curves to combination treatments with constant concentrations of Ad5ΔΔ and serial dilutions of gemcitabine or irinotecan. The values are corrected for cell death by virus alone.

MIA-PaCa-2

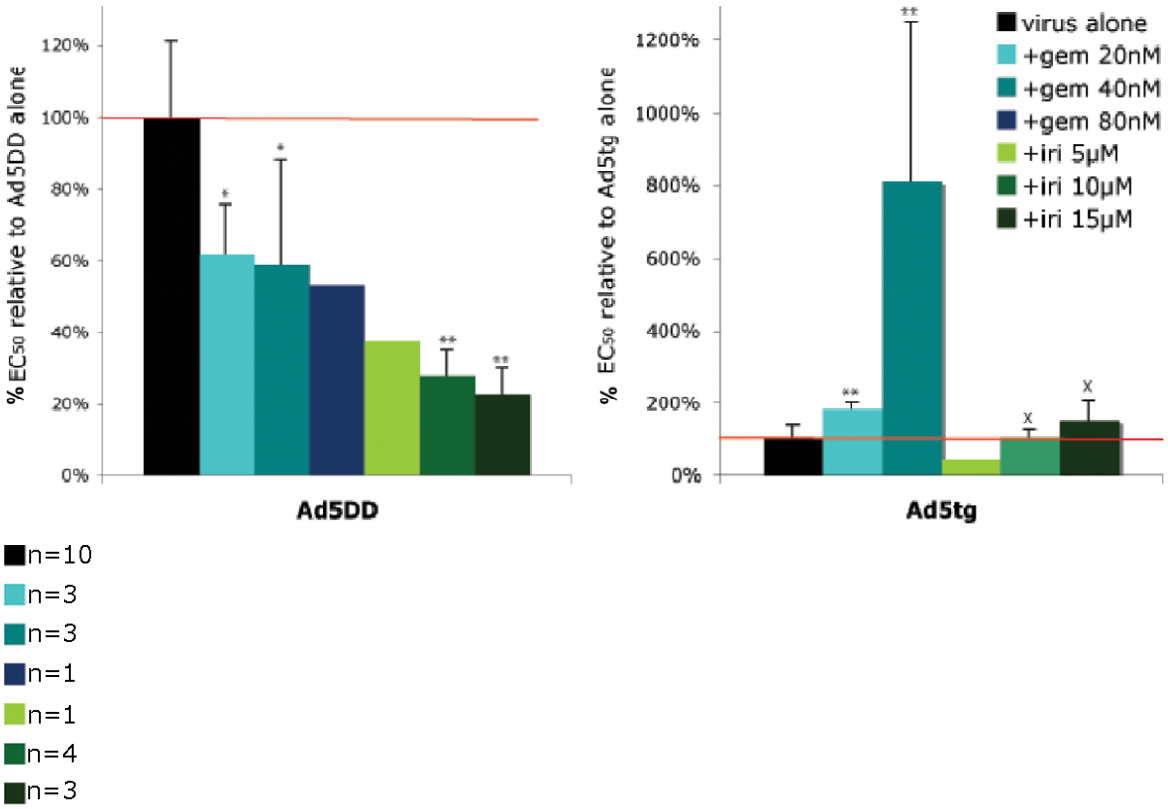


Fig. 15 Combination treatments with constant concentrations of chemodrugs and serial dilutions of viruses in MIA-PaCa-2 cells. Average ±SD calculated from EC_{50}-values for Ad5ΔΔ+gem, Ad5ΔΔ+iri, Ad5tg+gem and Ad5tg+iri relative to averages of EC_{50}-values for each virus alone. Ad5tg+gem 80nM gave too high values to fit graph. *p<0.05 **<p0.01 x= no significance

Cell-killing in ASPC-1 cells is enhanced by virus in combination with suboptimal doses of irinotecan. Combination treatment of both the Ad5tg and Ad5ΔΔ viruses with suboptimal doses of irinotecan (<20% cell death by drug alone) resulted in enhanced cell-killing compared to virus alone. Combination treatment with virus and suboptimal doses of gemcitabine showed no significant differences in cell-killing compared to virus alone. Average EC_{-50} values form dose-response curves (Fig. 15 A and B) allowed comparison between different combination treatments and virus alone (Fig. 16).

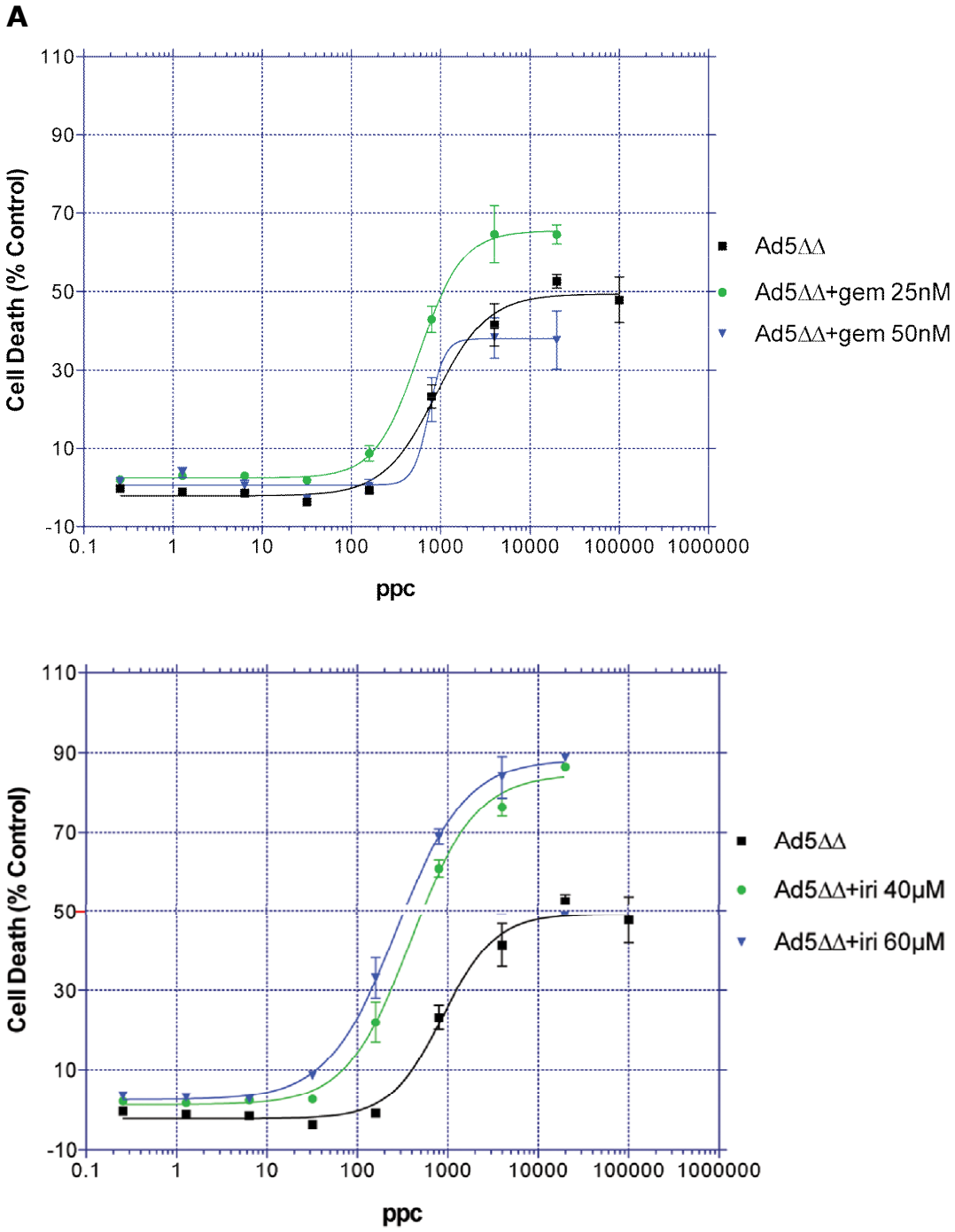


Fig. 15 Dose-response curves to virus and chemodrug in AsPC-1. **A.** Ad5ΔΔ in combination with gemcitabine and irinotecan. Selected graphs are representative for experiments with same concentrations of virus and drug. EC_{50}-values from dose-response curves with same concentrations generated an average EC_{50}, which allowed comparison between virus alone and combination treatments (Fig. 16). The values are corrected for cell death by drug alone.

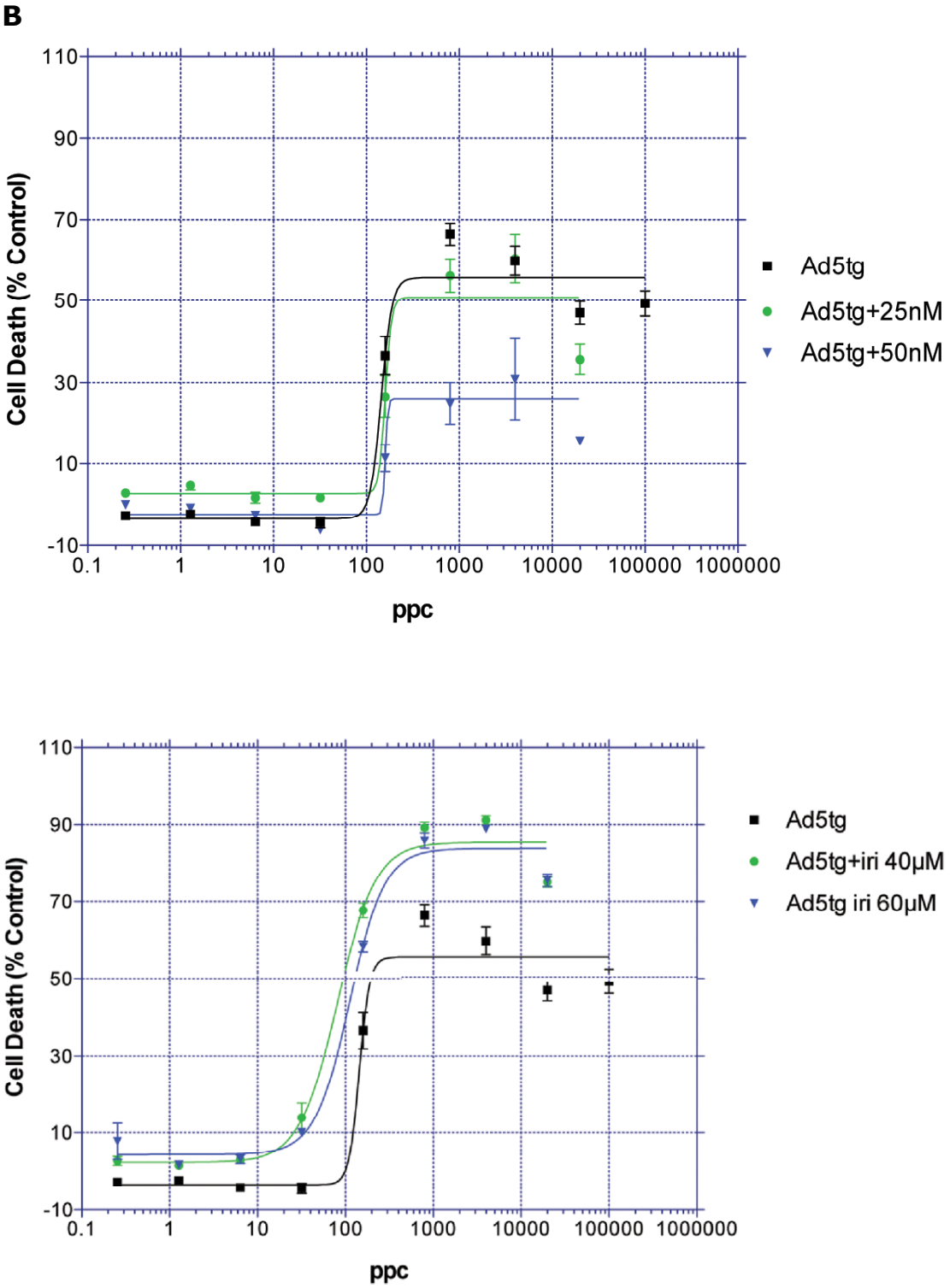


Fig. 15 Dose-response curves to virus and chemodrug in AsPC-1. **B.** Ad5tg in combination with gemcitabine and irinotecan. Selected graphs are representative for experiments with same concentrations of virus and drug. EC_{50}-values from dose-response curves with same concentrations generated an average EC_{50}, which allowed comparison between virus alone and combination treatments (Fig. 16) The values are corrected for cell death by drug alone.

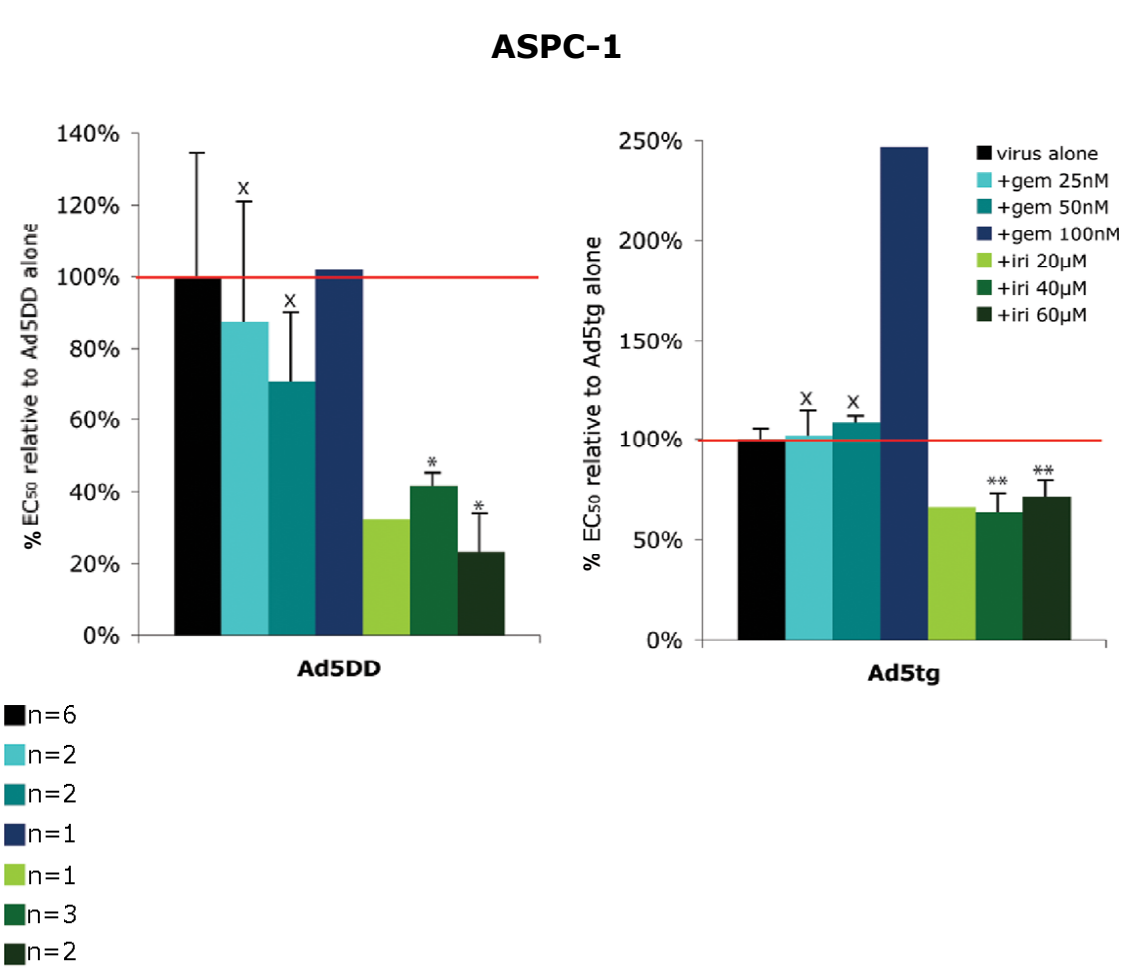


Fig. 16 Combination treatments with constant concentrations of chemodrugs and serial dilutions of virus in AsPC-1 cells. Average ±SD calculated from EC_{50}-values for Ad5ΔΔ+gem, Ad5ΔΔ+iri, Ad5tg+gem and Ad5tg+iri relative to average on EC_{50} for virus alone. *p<0.05 **<p0.01 x= no significance

Adenoviral gene expression in the presence of chemotherapeutic drugs

To determine whether the chemotherapeutic drugs gemcitabine and irinotecan could affect the viral life cycle, cell lysates were analysed for changes in viral gene expression focusing on E1A and adenoviral capsid proteins. E1A is expressed early in infection while capsid proteins are expressed in late stage of infection.

Adenoviral capsid proteins were expressed 48 hours after infection both for virus alone and in combination with gemcitabine (Fig. 17 A). However, after treatment with irinotecan, no expression could be detected after 48 hours (Fig. 17 B). Combination treatment with virus and irinotecan showed instead an increase in expression of E1A. This suggests that the presence of irinotecan caused an increase in early viral gene expression but a decrease, or delay, of the formation of the viral capsid, which occurs in a late stage of the viral life cycle.

Cells infected with Ad5ΔΔ, showed an increase in E1A expression compared to cells infected with Ad5tg, the difference was obvious especially after 24 h (Fig. 17 A and B).

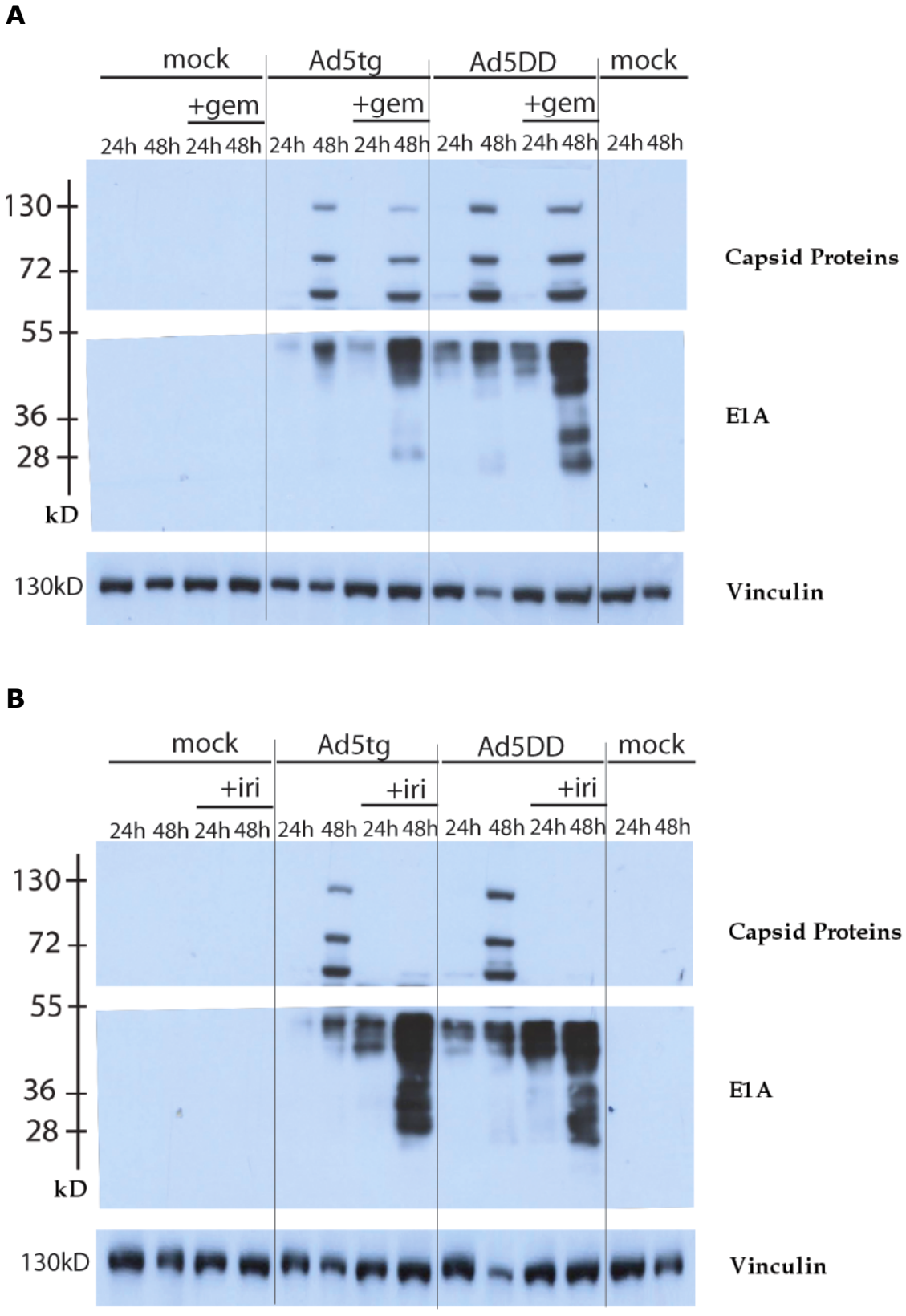


Fig. 17 Western blots of viral proteins in MIA-PaCa-2 cells treated with viruses in combination with **A**. suboptimal dose (40nM) of gemcitabine and **B**. suboptimal dose (15μM) of irinotecan.

Cell cycle analysis

Parallel with the quantification of viral proteins, cell cycle analysis was performed. The cell cycle was analysed to determine whether cell cycle changes could be involved in the enhanced cell killing in response to combination treatments with Ad5ΔΔ. MIA-PaCa-2 cells were analysed by FACS at 24, 48 and 72 hours after treatment.

Increase in the polyploid cell population in cells infected with Ad5ΔΔ. As described earlier, the viral mutant Ad5ΔΔ caused enhanced cell-killing in combination with gemcitabine, while Ad5tg combined with gemcitabine caused a decrease in cell-killing. A difference in polyploid cells was observed when the cell cycle profile for these two viruses was compared. Ad5ΔΔ caused an increase in polyploidy over time (Fig. 22), which could not be detected for cells treated with Ad5tg. This polyploidy, only observed in cells treated with Ad5ΔΔ, was present also in combination with chemotherapeutic drugs. Polyploidy might be one of the mechanisms behind the demonstrated synergy of Ad5ΔΔ in combination with gemcitabine. Ad5ΔΔ in combination with irinotecan also resulted in increased polyploidy compared to similar combinations with Ad5tg. The presence of chemotherapeutic drugs did not seem to affect the induction of polyploidy caused by Ad5ΔΔ.

Increase in the sub G_1 fraction in cells infected with Ad5ΔΔ in combination with irinotecan. Irinotecan caused an increase in the G_2/M-phase over time, but in combination with Ad5ΔΔ the increase in G_2/M was absent and instead, polyploidy was observed together with an increase in sub G_1 (Fig. 21). In combination with Ad5tg, the increase in G_2/M still occurred, polyploidy did not appreciably increase and sub G_1 was similar to irinotecan alone.

The adenoviral mutant, Ad5ΔΔ, seemed to cause aberrations in the normal cell cycle progression, which were not detectable in cells treated with Ad5tg. Gemcitabine treatment resulted in an increased cell population in S-phase after 48 hours (Fig. 18 and 19) while irinotecan treatment resulted in an increased cell population in S-phase at 24 hours after treatment followed by increased G_2/M at 48 and 72 hours after treatment (Fig. 20 and 21).

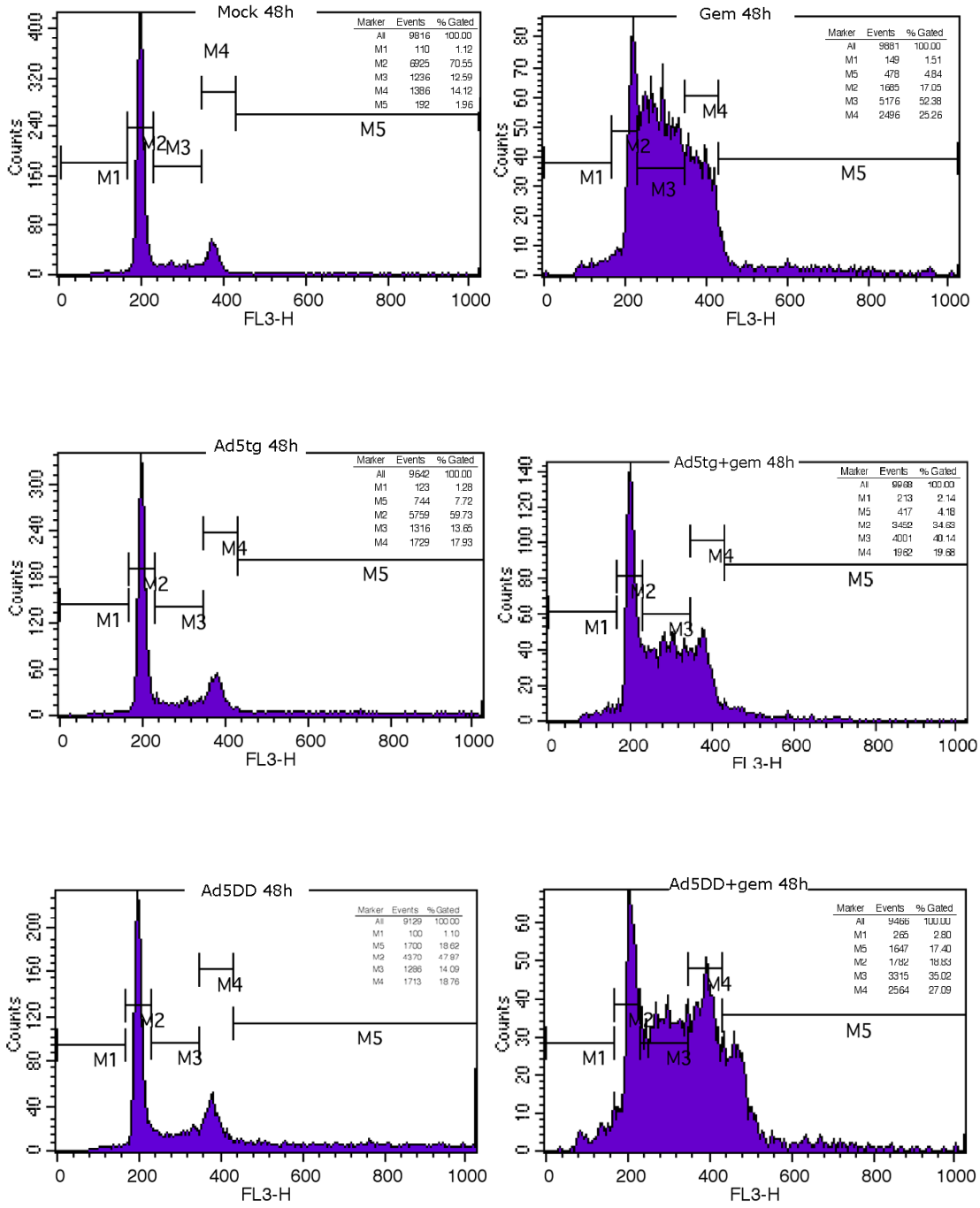


Fig. 18 Cell cycle profiles in cells treated with suboptimal doses of gemcitabine (40nM) in combination with Ad5tg or Ad5ΔΔ after 48 hours. M1= sub G_1, M2= G_1, M3= S-phase, M4= G_2/M and M_5= polyploid cells. (Please note that M5 comes after M1 in the graph legend.)

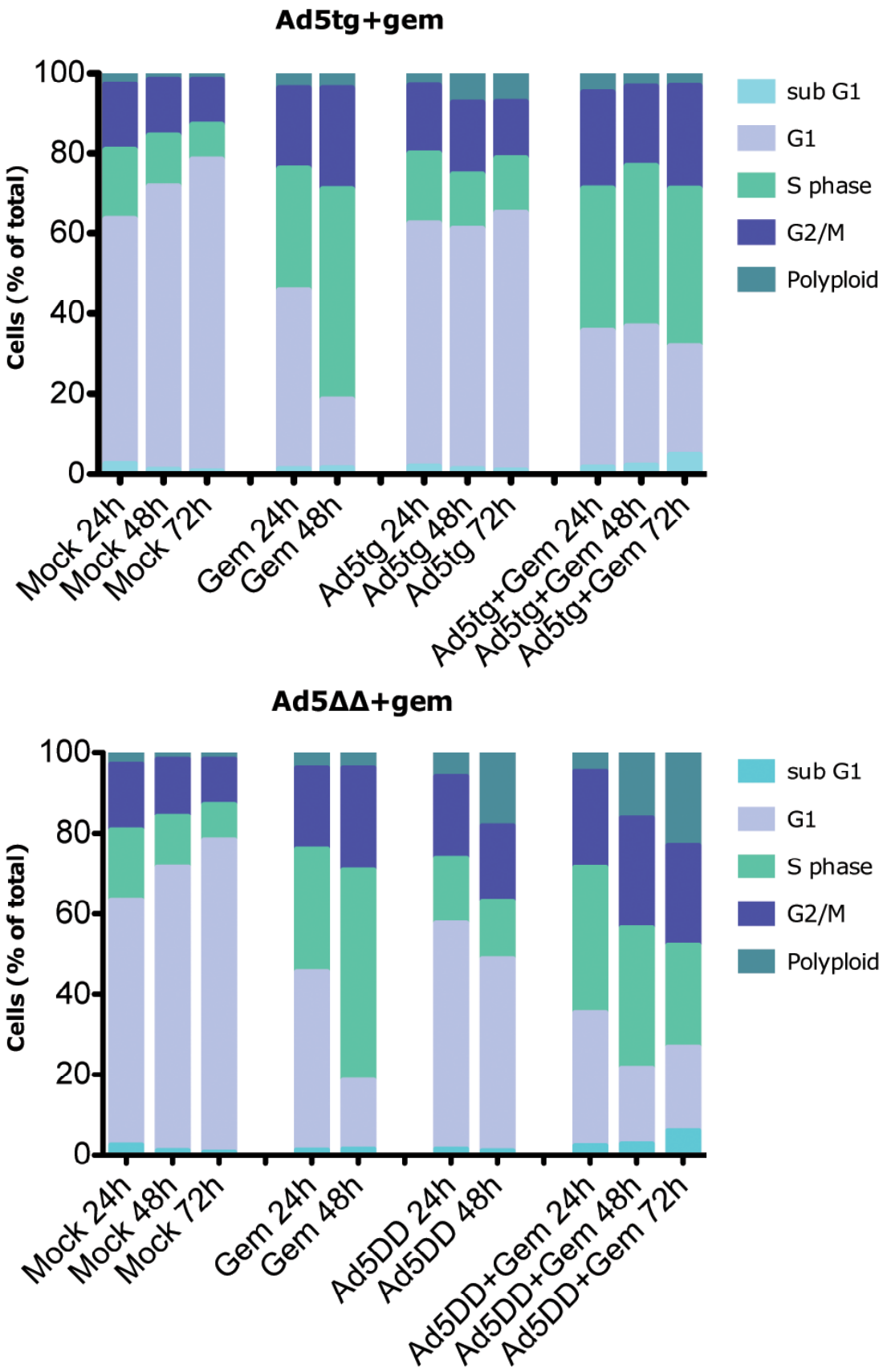


Fig. 19 The percentage of cells in different phases of the cell cycle. Ad5tg and Ad5ΔΔ in combination with gemcitabine at 24, 48 and 72 hours. The graphs show the percentage of cells in each stage of the cell cycle. (The sample for gem 72h and Ad5ΔΔ 72 hours were contaminated and cannot be shown.)

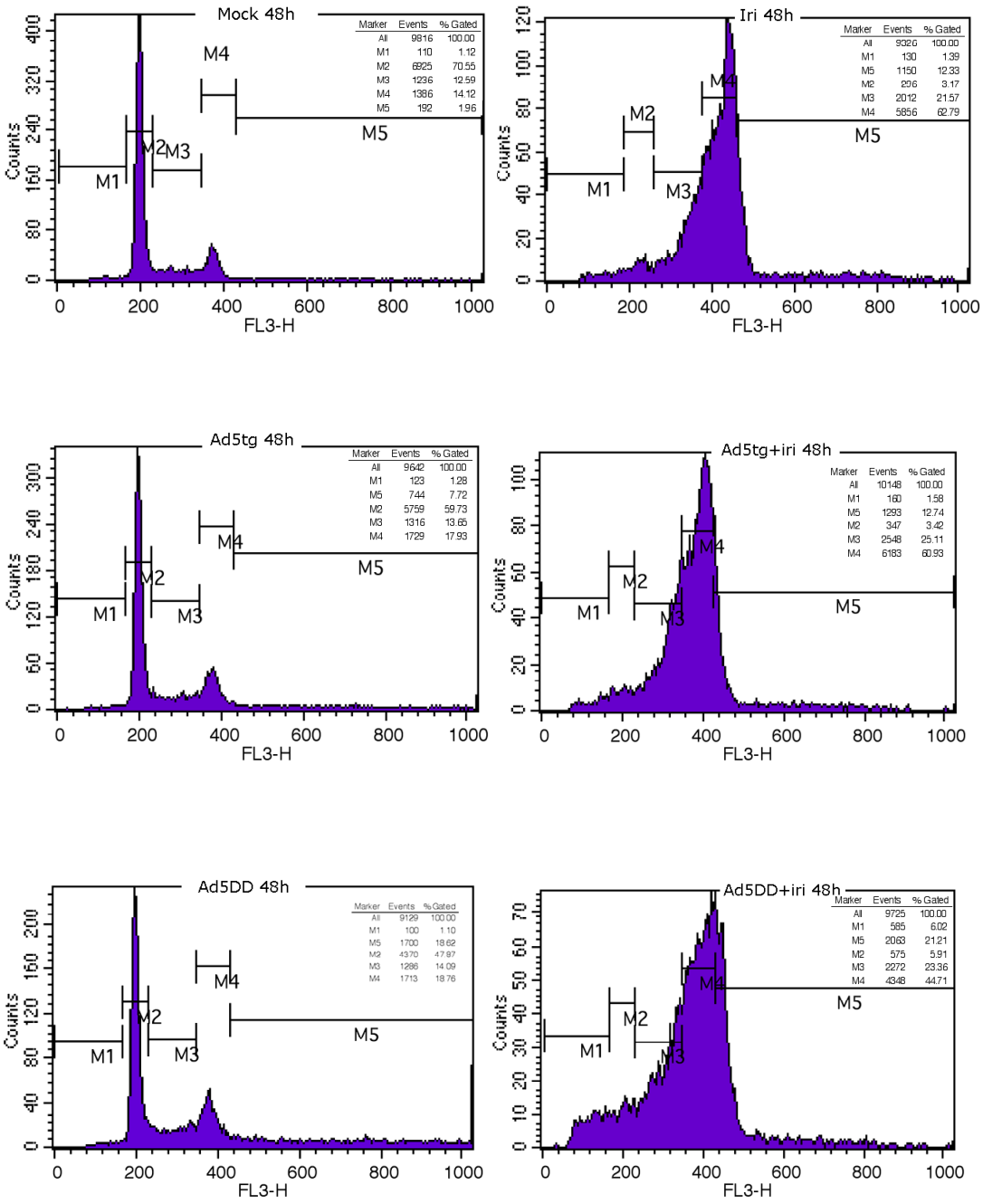


Fig. 20 Cell cycle profiles in cells treated with suboptimal doses of irinotecan (15μM) in combination with Ad5tg or Ad5ΔΔ after 48 hours. M1= sub G_1, M2= G_1, M3= S-phase, M4= G_2/M and M_5= polyploid cells. (Please note that M5 comes after M1 in the graph legend.)

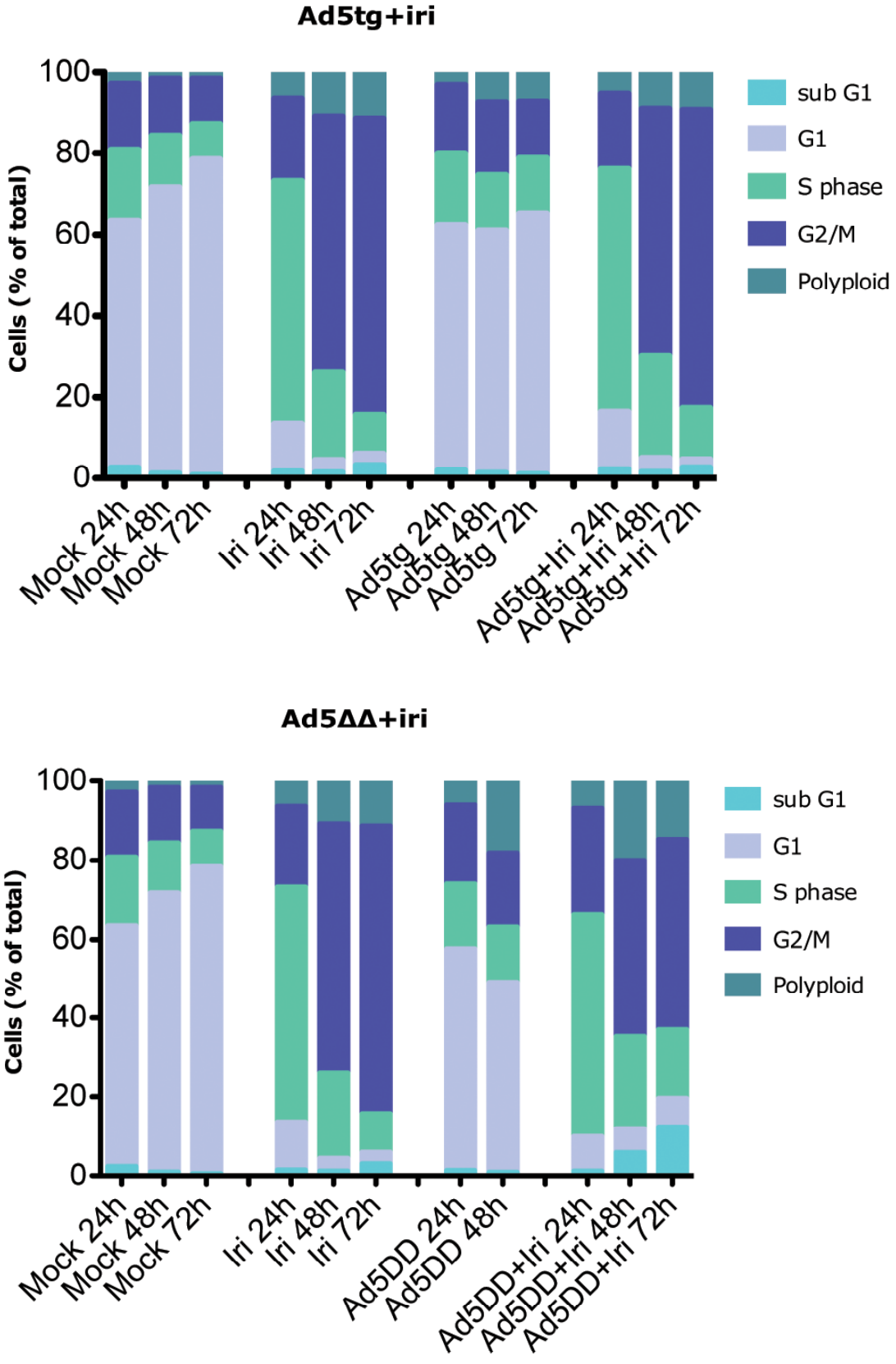


Fig. 21 Percentage of cells in the different phases of cell cycle. Ad5tg and Ad5ΔΔ in combination with irinotecan at 24, 48 and 72 hours. The graphs show the percentage of cells in each stage of the cell cycle. (The sample for Ad5ΔΔ 72 hours was contaminated and cannot be shown.)

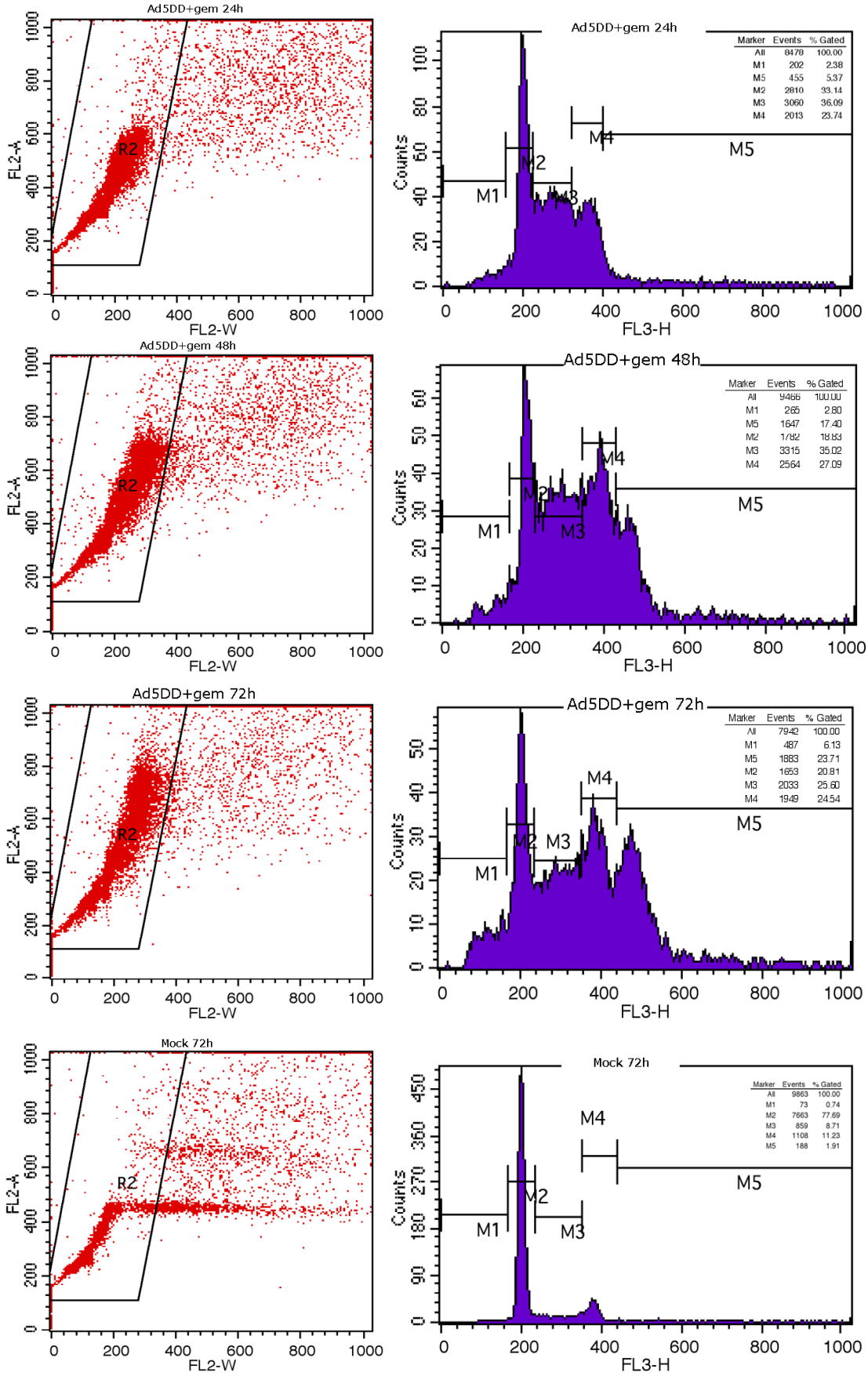


Fig. 22 Increased polyploid cell population over time in cells treated with Ad5ΔΔ and suboptimal doses (40nM) of gemcitabine compared to mock.

Expression of proteins involved in mitosis in the presence of virus and chemotherapeutic drugs

The changes in cell cycle progression that were demonstrated for treated cells above, especially the polyploidy caused by Ad5ΔΔ, led to questions about induction of mitosis. To determine whether virus and/or chemotherapeutic drugs affected the expression of proteins essential for mitosis, cell lysates were analysed for expression of the following proteins: Aurora A, Aurora B and Mad 2.

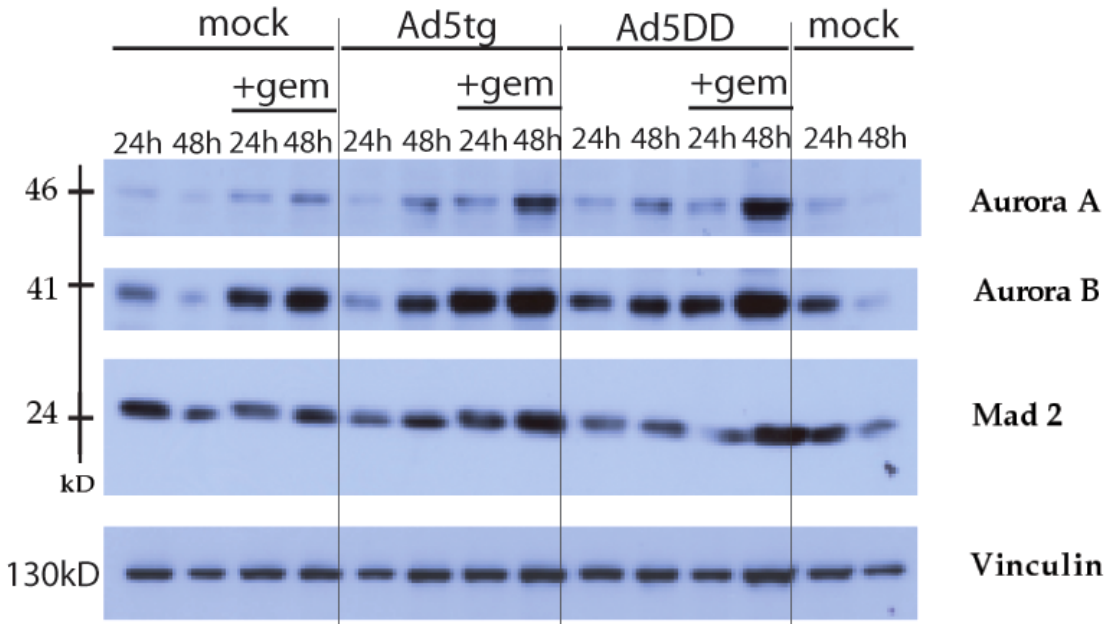
Virus and chemotherapeutics increase the expression of mitotic proteins. Aurora A-kinase expression was increased at both 24 and 48 hours in cells treated with irinotecan (Fig. 23 B) and gemcitabine (Fig. 23 B) compared to mock. The chemotherapeutic drugs in combination with viruses seemed to further increase the expression of Aurora A-kinase. A minor increase compared to mock could be detected in cells treated with Ad5tg and Ad5ΔΔ alone.

Aurora B was expressed to a greater extent in Ad5ΔΔ alone than in Ad5tg alone at both 24 and 48 hours. But the most visible increase was for both chemotherapeutic drugs in combination with both viruses, especially after 48 hours. Since irinotecan induced expression on its own, it was hard to evaluate if the combination with virus caused any further increase.

In untreated cells, the expression of Aurora B-kinase, and in some extent MAD-2, declined after 48 hours. In contrast, the expression in virus infected cells increased (Fig. 23 A and B).

Taken together, the expression of proteins involved in mitosis declined over time for untreated cells in contrast to treated cells. Gemcitabine and irinotecan induced expression of mitotic proteins, which would be compatible with the results from previous cell cycle analysis. Both viruses showed a tendency to induce expression as well.

A



B

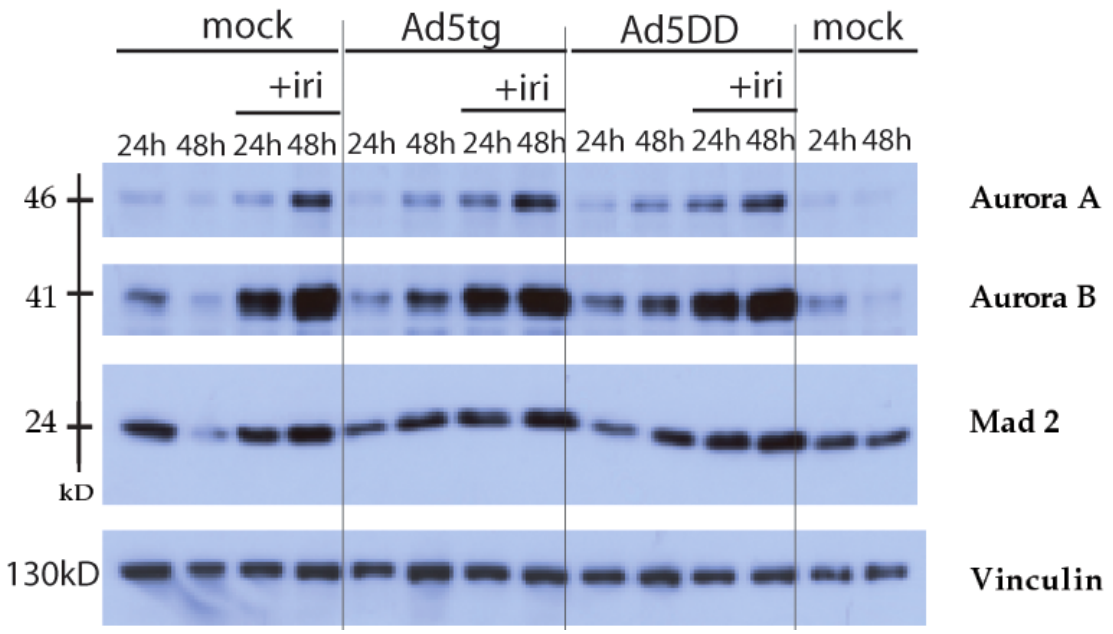


Fig. 23 Western blots of proteins involved in mitosis in MIA-PaCa-2 treated with virus in combination with **A.** suboptimal dose (40nM) of gemcitabine and **B.** suboptimal dose (15μM) of irinotecan.

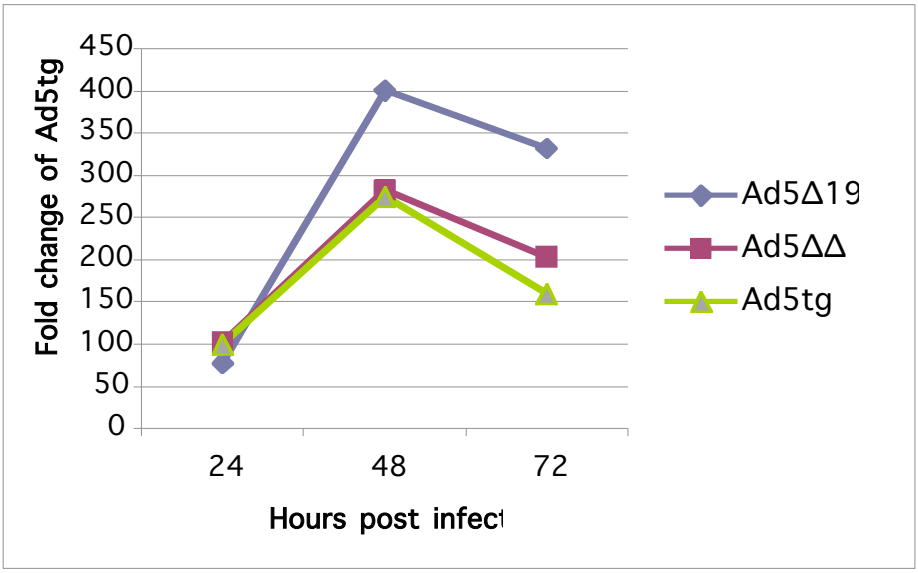
Adenoviral replication in human umbilical-vein endothelial cells

To determine whether the viral mutant Ad5ΔΔ replicates less than Ad5tg in normal human umbilical-vein endothelial cells, quantitative polymerase chain reaction and half maximum tissue culture infective dose were performed.

Ad5ΔΔ replicates in the same extent as Ad5tg in HUVEC . The results for qPCR (Fig. 24 A) and $TCID_{50}$ (Fig. 24 B) showed that Ad5ΔΔ replicated in HUVEC in the same extent as Ad5tg. Even though the 3 repeats of the qPCR showed different CT-values (data not shown), the results showed the same trend. The results from $TCID_{50}$, confirmed the result qPCR results. The replication was greatest for Ad5Δ19K. The replication increased over time for all viruses but the viral DNA declined at 72 hours after infection when cell death increased. Previous work (unpublished work by Dr. Cherubini) has shown that Ad5ΔΔ replicated less than Ad5tg in normal human bronchoepithelial cells (NHBE).

BrdU incorporation showed that HUVEC was not growth arrested (performed by Dr. Cherubini). Taken this into account, these results showed that Ad5ΔΔ replicated in proliferating HUVEC.

A



B

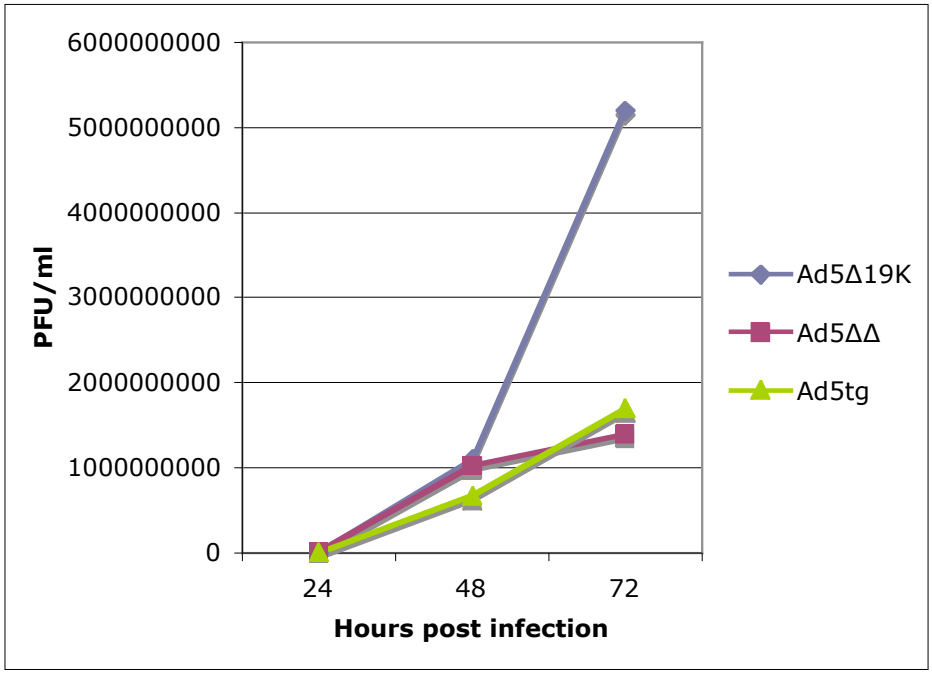


Fig. 24 Adenoviral replication in human umbilical-vein endothelial cells **A**. Viral DNA-quantification of Ad5Δ19K, Ad5ΔΔ and Ad5tg in HUVEC at 24, 48 and 72 hours after infection. Expressed as fold change to Ad5tg at 24 hours. Viral uptake control after 4 hours showed similar CT-value for all viruses. 1 out of 3 replicates shown. **B**. Viral replication of Ad5Δ19K, Ad5ΔΔ and Ad5tg in HUVEC at 24, 48 and 72 hours after infection. Expressed as plaque forming units (PFU)/ml.

DISCUSSION

The aims of this study were to investigate whether the E1ACR2- and E1B19K-deleted virus, Ad5ΔΔ, in combination with chemotherapeutic drugs could enhance cell-killing of pancreatic cancer cells and if so, investigate mechanisms behind the enhancement. In this report I demonstrate that Ad5ΔΔ lyses pancreatic cancer cells *in vitro*, enhances cell-killing in combination with chemotherapeutic drugs and induces polyploidy in infected cells. In addition, I show that the presence of chemotherapeutic drugs has an impact on viral gene expression and increases expression of proteins involved in mitosis.

To achieve the replication-selectivity by deletions in E1A and E1B, some of the viral cytotoxity might be compromised. The cytotoxic effect was significantly higher for wild type Ad5 (Ad5tg) than for the mutant Ad5ΔΔ in both MIA-PaCa-2 and AsPC-1 cells. The level of efficacy for Ad5ΔΔ may not be sufficient on its own. Previous studies have shown that Ad5Δ19K (Leitner, S. *et al.* 2009) and Ad5ΔΔ (Oberg, D. *et al.* 2010) mutants can enhance cell-killing in combination with the chemotherapeutic drug gemcitabine. The sensitization by Ad5Δ19K has been demonstrated to be specific to cancer cells (Leitner, S. *et al.* 2009) and Ad5ΔΔ was demonstrated to synergistically increase docetaxel-induced cell-killing in prostate cancer cells (Oberg, D. *et al.* 2010). Enhanced cell-killing was also demonstrated for Ad5ΔΔ in combination with the chemotherapeutic drug irinotecan (Dr. Cherubini & Kallin, unpublished work).

I also found that, combining Ad5ΔΔ with low doses of either gemcitabine or irinotecan resulted in enhanced cell-killing in MIA-PaCa-2 cells. In AsPC-1 cells, cell-killing was enhanced with irinotecan but not with gemcitabine. The different outcomes of combining Ad5ΔΔ with gemcitabine in the two cell lines could be due to differences in genetic aberrations in the two cell lines. In contrast to MIA-PaCa-2, AsPC-1 cells are mutated in SMAD4. SMAD4 is

involved in the tumour growth factor-beta (TGF-B) pathway and regulate cell cycle progression from G_1 to S-phase through suppressing Cyclin D. CDK2A, mutated in both cell lines, is also suppressing Cyclin D. It has been demonstrated that gemcitabine can prevent virus induction of S-phase cyclins and cyclin D has been shown to decrease in the presence of gemcitabine (Leitner, S. *et al.* 2009). This suggests that levels of cyclins might play a role in the cancer cell's response to gemcitabine. Previous studies have shown antagonistic effect of Ad5ΔΔ combined with gemcitabine in PANC-1 cells (Dr. Cherubini & Kallin, unpublished work), which are not mutated in SMAD4 (Sipos, B. *et al.* 2003), which suggest that the mechanisms of sensitivity and resistance are more complex than just alteration of one gene. Chemo-resistance to gemcitabine in pancreatic cancer has been demonstrated to depend on for example the expression of Bcl2/adenovirus E1B19K interacting protein (BNIP3). Drug-resistant cell lines expressed BNIP3 at lower levels (Akada, M *et al*. 2005). According to this resistance-classification, both MIA-PaCa-2 and AsPC-1 are intermediate sensitive to gemcitabine. Antagonistic effects were demonstrated when Ad5tg was combined with gemcitabine, in both cell lines. One theory, which might explain this, is that gemcitabine inhibits viral replication (Leitner, S. *et al.* 2009). Relative to the combination treatments, Ad5tg alone showed greater efficacy. This suggests that gemcitabine might repress the replication of Ad5tg. However, no antagonistic effect was observed when combining Ad5tg with irinotecan. In addition, combination treatments with irinotecan caused a significantly higher enhanced cell-killing than gemcitabine for both viruses. Enhanced cell-killing by virus in combination with irinotecan was also demonstrated in a previous study (Dr. Cherubini & Kallin, unpublished work). This suggests that irinotecan could be a potential candidate for pancreatic cancer treatment in combination with Ad5ΔΔ. This would be valuable in cancers that are resistant to gemcitabine. For all cell viability assays in this project, virus and drug were delivered to the cells at the same time point. Previous studies on MIA-PaCa-2 cells demonstrated synergistic effect when gemcitabine were delivered prior to the virus, but antagonistic effects when the virus was delivered prior to gemcitabine (Nelson, R. A. *et al.* 2009). This suggests that the order of delivery could affect the efficacy and antagonism might be prevented.

Gemcitabine is specifically targeting cells in S-phase causing premature chain termination during DNA-synthesis. Irinotecan acts mainly during S-phase but also in G_2, by inhibiting Topoisomerase I, essential for religation of single strand breaks during DNA-replication. E1A induces S-phase and is essential for viral replication. The presence of either irinotecan or gemcitabine caused increased E1A-expression for both Ad5tg and Ad5ΔΔ at 24 and 48 hours. These results indicate that both gemcitabine and irinotecan promote viral E1A expression. One factor behind this could be increased viral uptake in response to the drugs. My results are in contrast to previous studies, which demonstrated that gemcitabine decreased viral E1A expression at 48 hours for Ad5tg and Ad5Δ19K (Leitner, S. *et al*. 2009). Interestingly, the expression of adenoviral capsid proteins, which reached detectable levels after 48 hours for both viruses, was inhibited in the presence of irinotecan but not with gemcitabine. Taken together, irinotecan induced expression of early viral genes whilst it inhibited, or delayed, the expression of capsid proteins, essential for formation of the capsid. Gemcitabine had no detectable effect on late viral proteins under these conditions.

In untreated cells there was a decline in S-phase over time, which suggest that as cells get more confluent, cell proliferation decreases. This decline was absent in cells treated with chemotherapeutic drugs. Gemcitabine caused mainly an increase in S-phase over time, but also in G_2/M compared to untreated cells. Treatment with irinotecan caused increased cell population in S-phase at 24 hours followed by increased G_2/M-phase at 48 and 72 hours. When combined with virus, both drugs showed a decrease in S-phase but instead an increase in G_2/M. Interestingly, cells infected with Ad5ΔΔ caused polyploidy, which increased over time. The presence of drugs did not seem to affect the levels of polyploidy. In addition, Ad5ΔΔ in combination with irinotecan caused increased sub G_1 fraction. These both phenomena could not be observed for Ad5tg. Ad5tg combined with gemcitabine, which resulted in an antagonistic effect on cell death showed no increase in polyploidy. Polyploidy caused by Ad5ΔΔ could be one factor enabling enhanced cell-killing with drugs. Thus, Ad5ΔΔ alone caused the same increase in polyploidy after 48 hours, suggesting that polyploidy alone is not proportional to cell

death. However, sub G_1 was increased in the presence of drug compared to virus alone.

Since the population of cells in G_2/M increased with all combinations, further investigations of whether Ad5tg, Ad5ΔΔ, gemcitabine and irinotecan had an impact on mitosis, expression of Aurora A-kinase, Aurora B-kinase and MAD-2 were quantified. These proteins are essential for mitosis. In untreated cells, the expression of Aurora B-kinase, and MAD-2, declined after 48 hours, which supports previous results where S-phase and G_2/M decreased over time in untreated cells. In contrast, the expression of all three proteins increased over time in virus-infected cells. This suggests that viruses could affect mitosis, which might contribute to the polyploidy. Both chemodrugs increased the expression of Aurora A-kinase, which is involved in early mitosis. Irinotecan greatly increased the expression of Aurora A-kinase and Aurora B-kinase, further increases in combination with virus could not be detected under these conditions. Gemcitabine, especially together with Ad5ΔΔ, seemed to increase the expression of Aurora A-kinase after 48 hours and Aurora B-kinase at both 24 and 48 hours. Overall, these results suggest that both irinotecan and gemcitabine induce mitosis and a tendency that both viruses could stimulate mitotic protein expression to keep the cells cycling.

Ad5ΔΔ was shown to lyse cancer cells and enhance cancer cell-killing in combination with chemotherapeutic drugs. To determine whether Ad5ΔΔ replicates less than Ad5tg in normal HUVEC cells, viral replication assays were performed. Surprisingly, Ad5ΔΔ replicated to the same extent as Ad5tg in the HUVEC cells. This suggested that the cells were not growth arrested on the day of infection that was later verified by BrdU-uptake (Dr. Cherubini). Proliferating cells provide similar intra-cellular environments as cancer cells, therefore replication is less inhibited than in quiescent cells. Previous studies using normal human bronchial endothelial cells (NHBE) demonstrated that Ad5ΔΔ replicated less than Ad5tg both in proliferating and arrested cells (Dr. Cherubini & Kallin, unpublished work). It would be valuable to study the replication of Ad5ΔΔ in other normal cells, and also repeat the replication assay in HUVEC using other methods to reach growth arrest.

Technical considerations

Many factors in an experiment affect the outcome of the results. The qPCR results for viral DNA in the HUVEC cells were not entirely reproducible and should be repeated to get more solid result. However, the trend was the same in all experiments, and it answered the question whether Ad5ΔΔ replicated in HUVEC. The differences between experiments were possibly due to unintentional loading of different amount of DNA. Even though the same sample aliquots were used in the two first experiments, and the same volume loaded, the CT-values were different. When pipetting very small volumes, a small variation can cause big difference in the final concentration, such as an undetected tiny air bubble in the tip for example. Inaccurate pipettes can also be a problem, especially multichannel pipettes, where it is meant to be exactly the same volume in each tip.

Inconsistent results of experiment-repeats occurred for some of the cell-killing assays, even though the experiments were aimed to be exactly the same. When the dilutions of chemodrugs and virus were prepared, here also, very small volumes were used and small alterations in the actual concentration might have occurred. Gemcitabine gave more inconsistent results in AsPC-1 than in MIA-PaCa-2. This could be due to the fact that AsPC-1 showed greater resistance than MIA-PaCa-2, but it is plausible that alterations in cell-metabolism affect the results. Cell metabolism differs between different cancer cell lines, but also day to day due to the fact that they are living cells. Passage number or different cell concentration could influence cell metabolism. When the cells were plated for experiments, cell-counting was performed manually under a light microscope. Even though the cells were mixed prior to counting, the distribution could be uneven which could generate a misleading number. Too many, or too few cells would then be plated and the viral particles per cell you think you add, is actually not true. Depending on the cell number, the distribution and uptake of drug and virus might also alter. Over time I got to know my cells, suddenly odd cell-counts were always re-counted to confirm the first number.

Bacterial contamination was a problem in tissue culture for a while. The samples for cell cycle analysis, Ad5ΔΔ and gemcitabine at 72 hours were contaminated and could therefore not be used. To have a complete cell cycle analysis, the experiment for these conditions should be repeated. A low grade contamination would have been worse, if the contamination is not noticed, it will affect the results.

Future work

The viral replication of Ad5ΔΔ should be analysed in MIA-PaCa-2 to complete the studies of the virus in this cell line. When the *in vitro* studies are completed, *in vivo* studies using MIA-PaCa-2 xenografts in immuno-deficient mice should be performed: to investigate if the cell-death observed using Ad5ΔΔ combined with chemodrugs *in vitro*, will result in tumour regression *in vivo*. Furthermore, toxicology studies are important to do *in vivo*, to investigate if Ad5ΔΔ is safe for normal cells, alone and in combination with chemodrugs. It would be interesting to investigate the distribution of the virus in the tumour and surrounding tissues after intratumoural and intravenous injections.

Conclusions

Chemotherapeutics show low efficacy in pancreatic cancer since the majority of cancers are resistant to drugs. In addition, the side effects of chemotherapeutics are extensive. The use of oncolytic replication-selective adenoviruses in combination with low doses of chemotherapeutics is a novel and promising approach to target pancreatic cancer.

This study suggests that the adenoviral mutant Ad5ΔΔ in combination with either gemcitabine or irinotecan is a potential treatment for pancreatic cancer. Combination treatments with irinotecan could be an alternative to gemcitabine in gemcitabine-resistant cancers.

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Appendix

1. Western Blot Protocol (Dr. Gioia Cherubini)

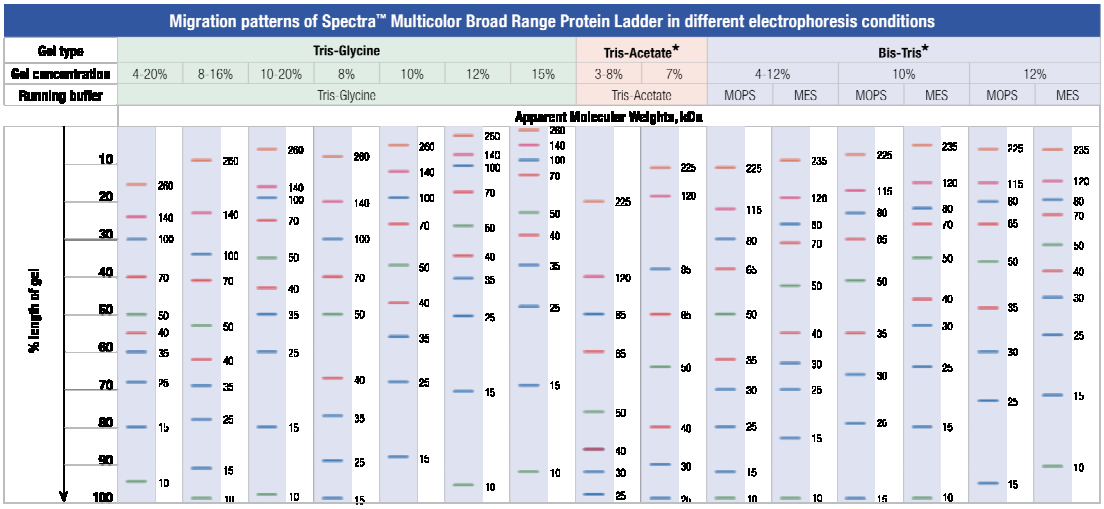
WESTERN BLOT PROTOCOL

A. PREPARATION OF CELL LYSATES

- Resuspend cell pellet in RIPA buffer
- 4° for at least 15-30'
- Store at -20° or procede to complete lysates preparation
- @ 10' 13000rpm (or max speed) 4°
- Bradford assay to quantify soluble proteins
- Add Laemmli buffer 2X
- Sonicate ~15'' at max amplitude
- @ 10' 13000rpm (or max speed)
- Store at -20° or proceed for western

B. PREPARATION OF GEL

- Choose the % of gel that you want on the basis of the molecular weight of the proteins you want to resolve (check on the outline, considering that you'll do Trys-Glycine gels)



* Migration patterns were determined using respective NuPAGE® precast gels.

Note. The apparent molecular weight of each protein (kDa) has been determined by calibration against an unstained protein ladder in each electrophoresis condition.

- Assemble the glass plates and spacers (1.5 mm thick)
- Pour the running gel to about 0.1 cm below the wells of the comb (<10ml).
- Seal with isopropanol.
- When gel has set, pour off the isopropanol and absorb the leftover isopropanol with 3mm paper
- Pour the stacking gel (~5 ml) and insert the comb immediately.
- When the stacking gel has set, place in gel rig and immerse in buffer.
- Prior to running the gel, flush the wells out thoroughly with running buffer.

C. PREPARE THE SAMPLES FOR THE RUN

- Calculate the amount of sample you need to load ~30μg.
- Boil for 5 min.
- Cool at RT for 5 min.
- Flash spin to bring down condensation prior to loading gel.

D. RUNNING THE GEL

- After flash spinning the samples, load into the wells.
- Be sure to use markers.
- Run at 150 V.
- Usual running time is about 1.5 hr.

E. MEMBRANE TRANSFER

- Assemble "sandwich" for Bio-Rad's semi-dry Transblot.

- Prewet the extra-thick blotting paper, the membrane and the gel in 1x Transfer buffer.
- Transfer for 1 hr at 20V.

F. CHECKING THE EFFICIENCY OF TRANSFER

- After transfer, wash the membrane with PBS to wash away the alcohol
- Pour the Red Ponceau solution on the membrane and let it rock for a couple of min.
- Recover the Ponceau solution.
- Wash the membrane with a solution of TCA 0.5% for a couple of min.
- Scan the membrane.

G. ANTIBODIES INCUBATION

- Prepare the blocking buffer (PBS/0.1% TWEEN/5% MILK) and block for 1 hour RT (room temperature).
- Incubate with primary antibody diluted in Blocking buffer over night at 4°.
- Wash 4 x 5 min with 0.1% Tween 20 in PBS.
- Incubate with secondary antibody diluted in blocking buffer for 1hr at RT or 2 hr at 4°.
- Wash 5-6 x 5 min with 0.1% Tween 20 in PBS.

H. DETECTION

- Prepare a solution of the detection reagent with a mixture of 1:1 (consider ~2.5 ml per membrane).
- Pour the reagent drop to drop on the membrane to cover it completely and leave it 1min.
- Put the membrane into a film cassette inside a plastic sheet, after removing the eccess of reagent.

I. REVELATION

- Put 2 films on top of each other and then on top of the membrane.
- Close the cassette and wait 1 min (be careful to not move the films or you'll get a blurred image).
- Put into the developer

2. Protocol for $TCID_{50}$ (Heike Muller)

The TCID 50 procedure is part of the virus characterisation process; in particular it is used to identify the infection rate of a virus. The cells used are always JH293 cells. The cells are first seeded in a 96 well plate, left over night and then infected with the relevant virus in a dilution series. Cells have to be counted before they are seeded, to ensure 10000 cells per well are plated out. The cells previously grown in a T175 flask shout be 60-80% confluent and look healthy (have a little haylo). Characterisation is always carried out in triplicates, at least two viruses are used: a known control and the one to be characterised. Make fresh medium for seeding!!!

Day 1

TCID50 is performed in triplicate for each virus i.e. 3 plates per virus. Usually TCID50 plates are set up for two viruses at a time. Include an additional 3 plates for the control. As TCID50 can be read between 10 and 14 days if plates have been set up a couple of days beforehand with a control included, an additional control does not have to be incorporated in the second set-up.

Grow JH293 cells in T125 flasks in 10% FBS-E4 until 60-80% confluent. Use a cell passage number between 5-25. Three T175 flasks will yield approximately 8.9xE7 cells. Grow more cells than are required

Disperse cells (per flask) by the addition of 5 ml trypsin/versene solution for 5 min at 37°C. Once the cells are coming off the flak surface always add 5 ml (per flask) of E4+10% FCS to stop the trypsin reaction. Disrupt cell layers into single cells by pipetting up and down several times, transfer dispersed cells to 50 ml tissue culture tube and centrifuge at 1200-1500 rpm for 4 min in a table top swinging-bucket rotor. If more than one flask is used combine all cell suspensions is one 50 ml tube.

Aspirate media and re-suspend cells in 10 ml 10% FBS-E4 (per flask). If only one flask is used, suspend in 8ml only so to not over-dilute. Add 9ul of the cell suspension to a haemocytometer. Count cells by haemocytometer and calculate cells/ml. Over-dilute the cells with 10% FBS-E4 to aid counting. For 3 flasks – have a total cell stock volume of 50ml. Calculate total number of cells in cell stock by multiplying by the volume.

Estimate total number of cells needed for assay e.g. one 96-well plate of 10000 cells/well/0.2ml = 960000 cells in 19.2 ml. Make excess: 1xE6 cells in 20 ml per 96-well plate. Dilute in culture media (10%FBS-E4). (10000 cells per well are required)

Total cells required = 1xE6 x no. of plates

Total volume of media = 20ml x no. of plates

Eg: total number of plates required = 15, needed are $15x10^6$ cells

Volume of media needed = 15x20 = 300 ml

Eg: cells counted in 4x4 square (x2 average) = 266

Makes 266 x 10^4 cells per ml = $2.66x10^6$ cells/ml

$15x10^6$ / $2.66x10^6$ = 5.64 ml

Needed are 5.64 ml of cell suspension in 300 ml 10% E4

For each sample to be tested assay in triplicate 96-well plates and add another set of three plates for the internal control = minimal assay is 6 plates for one sample.

Transfer diluted stock solution of cells (now at 50000cells/ml) into pipette trough. Use multichannel (12 channels) pipettor to transfer 200μl of cell suspension to each well in the 96-well plates.

Place cells in 37°C incubator (5% CO_2) for 24 h.

Day 2

Just prior to sample additions (24h after plating cells) prepare the internal control dilutions. The control is the Ad5 wild type CsCl-purified stock virus. This standard is in the range of 1xE11 –1xE12 pt/ml and is diluted serially in culture tubes as follows: 10μl in 10ml ($1/1000=10^{-3}$), take 10μl of this dilution into 10ml ($1/1000=10^{-6}$), and finally take 500μl of this dilution into 5ml ($1/10=10^{-7}$), all dilutions are in serum free E4 (E4 +0% FCS). The final dilution factor of control virus is 1xE-7.

Transfer diluted control (10^{-7}) into pipetting trough. Is done in triplicates. Use multichannel (12 channels) pipettor to transfer 20μl of diluted stock virus solution (diluted 1xE-7) to each well in the first row of plate 1+2+3. Pipette up and down 3 times and transfer 22μl to the second row (row 2-7) in each of the plates. Repeat the serial 10-fold dilutions from row 1 to 7, resulting in dilutions from 1xE-7 to 1xE-13. The last row on the plate is the negative control with uninfected control cells without virus. (From one row to another each 1/10 dilution)

(Repeat for plate 2 and 3 so that 3 plates with control virus are infected, do all at the same time to save tips).
Dilute the viral sample to be tested. Start with a low dilution initially 1xE-2 dilution and continue by infecting 3 plates as described previously by serial dilutions from 1xE-2 –1xE-8 in the plate. For unknown samples (never tested before) another set of 3 plates are set up starting at dilution 1xE-5 to 1xE-11 or if samples is very confluent and virus has been used before dilutes to 10^{-7}, same as control)
Place the infected cells in 37°C CO_2-incubator for 10-14 days, monitoring plates every second day.
Score plates on day 10 under light microscope. Count wells displaying cytopathic effect (CPE) in first row of plate, continue throughout the plate, until every well has been recorded
Enter number of infected wells for each dilution and each plate into Excel worksheet and calculate TCID50 data after first verifying that the worksheet is correct and valid

ACCEPTANCE CRITERIA

If any sign of CPE is visible in a well it is counted as CPE. To score a well as not showing CPE there must no sign of CPE in any area of the well

If the variability of the internal control (standard Ad5 wild type) is within ± 1 log unit in pfu/ml the assay is valid (compared to previous titration values).

If the variation of the internal control virus is greater than 1 log unit, or if the uninfected row of cells demonstrates CPE the assay is not valid and must be repeated.

In each assay the number of replicates has to be 3 for each dilution and each sample and include the standard virus and uninfected cells also in triplicates.