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Screening of pancreatic cancer cell lines with oncolytic adenovirus Ad5ΔE1ACR2ΔE1B19k (Ad5ΔΔ) in combination with chemotherapeutics

Cecilia Kallin

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Cecilia Kallin

Handledare: Prof. Stina Ekman, Institutionen för Biomedicin och Veterinär Folkhälsa, SLU

Biträdande handledare: Dr. Gunnel Halldén och Dr. Gioia Cherubini, Centre for Molecular Oncology and Imaging, Barts and the London School of Medicine and Dentistry, London

Examinator: Prof. Leif Norrgren, Institutionen för Biomedicin och Veterinär Folkhälsa, SLU

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SUMMARY

Pancreatic cancer is one of the most aggressive and lethal forms of cancer, with high mortality and short survival. Chemotherapy using Gemcitabine is the most common treatment used but tumours frequently show resistance to the drug, necessitating the development of new and more potent therapy options. The use of replication-selective oncolytic adenoviruses constitutes a novel and promising way of combating cancer with proven efficacy and safety, as well as potentially synergistic effects when combined with chemotherapy. Previous work by Leitner *et al* (2009) has shown that combining Gemcitabine with an adenoviral mutant deleted in the anti-apoptotic E1B19K gene (Ad5Δ19K) increases the cytotoxic effect of the treatment *in vitro* and *in vivo*. Based on that work, an adenoviral mutant, Ad5ΔΔ, was created as a potential new candidate for treatment of pancreatic cancer. It has a deletion of the pRb-binding E1ACR2 region in addition to the E1B19K deletion, abolishing its ability to induce S-phase and to prevent apoptosis. It is hypothesised that, due to the double deletions, the mutant will show increased selectivity to cancer cells, making it safer than its single deleted counterpart (Ad5Δ19K) but still retaining efficacy. Ad5ΔΔ has already showed promising results in pancreatic cancer cell line PT45 and normal immortalised cells. In this project, the cytotoxic and replicative ability of Ad5ΔΔ is tested on a wider range of pancreatic cancer cells as well as its capacity to sensitise pancreatic cancer cells to Gemcitabine and Irinotecan, drugs used in treatment of pancreatic cancer. The results show efficient replication of the viral mutant in all cell lines and moderate cytotoxicity of viral single treatment. An antagonistic effect was observed between viral and Gemcitabine treatment in cell lines insensitive to chemotherapy. However, cells that showed sensitivity to single treatment with drugs could be significantly sensitised by combining Ad5ΔΔ infection with chemotherapy.

SAMMANFATTNING

Bukspottkörtelcancer är en av de mest dödliga cancerformer som finns, mortaliteten är i princip hundraprocentig. Gemcitabine är förstahandsval i cellgiftsbehandlingen av sjukdomen. Tyvärr försvåras behandlingen ofta av resistensutveckling mot de kemoterapeutiska preparat som finns att tillgå. Det dåliga svaret på befintlig terapi gör det nödvändigt att utveckla nya och mer framgångsrika behandlingsalternativ. Selektivt replikerande onkolytiska adenovirus utgör en ny och lovande plattform för framtida cancerbehandling, oftast i kombination med befintlig cytostatikaterapi. Tidigare försök av Leitner *et al* (2009) har visat att man kan uppnå ökad cytotoxicitet hos cancerceller såväl *in vitro* som *in vivo* genom att kombinera Gemcitabine-behandling med en adenovirusmutant Ad5Δ19K. Ad5Δ19K besitter en deletion i den anti-apoptotiska genen E1B19K och saknar därmed förmågan att motverka programmerad celldöd. Baserat på dessa fynd har en ny mutant, Ad5ΔΔ, skapats med förhoppning om att denna kan bli en ny läkemedelskandidat inom behandling mot bukspottkörtelcancer. Utöver samma E1B19k-deletion som Ad5Δ19K, har Ad5ΔΔ även en deletion i den pRb-bindande E1ACR2-regionen. Den senare omöjliggör för viruset att framkalla övergång från G1- till S-fas i värdcellen. Förhoppningen med Ad5ΔΔ är att det genom sina dubbla deletioner ska vara ett säkrare virus att använda samtidigt som det visar effektiv cytotoxicitet i cancerceller. Viruset har redan visat lovande resultat i cancercellinjen PT45 samt i normala, immortaliserade cellinjer. I detta projekt utvärderas Ad5ΔΔs replikativa och cytotoxiska egenskaper på en större panel bukspottkörtelcancerceller samt dess förmåga att sensitisera cancerceller för Gemcitabine och Irinotecan, cytostatika som används i behandlingen av bukspottkörtelcancer. Resultaten visar effektiv virusreplikation i alla cellinjer samt måttlig cytotoxicitet. I cellinjer som är okänsliga för cytotstatika sågs antagonism mellan virus och läkemedelsbehandling, medan cellinjer som är känsliga för cellgifter kunde sensitiseras genom samtidig viral behandling.

INTRODUCTION

Viral gene therapy against cancer

Gene therapy can be defined as "a technique for correcting defective genes responsible for disease development" (Oak Ridge National Laboratory, 2009). Its aim is to restore the normal state of the target cell by expressing an inserted gene and producing a functional gene product. Cancer is the disease most commonly targeted by gene therapy in clinical trials; over 60% of all gene therapy trials globally are conducted to test a new treatment against cancer (Fig. 1). However, the approach in cancer gene therapy differs from gene therapy used against other genetic diseases; instead of restoring the normal state of the cell, the aim is to kill it. In the majority of clinical cancer trials this is achieved by using viruses as vectors (Fig. 2).

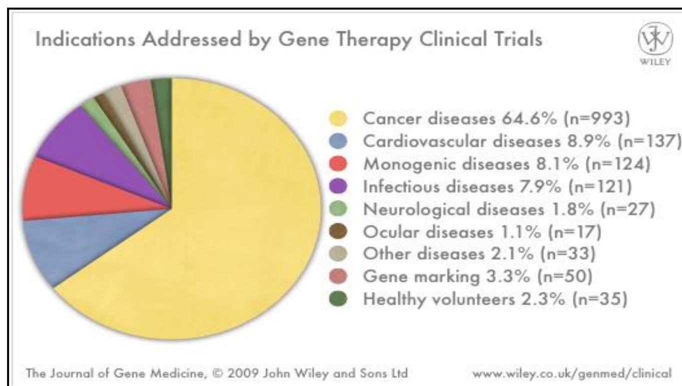


Fig. 1: Gene therapy in clinical trials.

http://www.wiley.co.uk/genmed/clinical/ (2009-05-08)

Viruses have evolved to efficiently transfer their genome into host cells and enable replication of viral genes within the cell (Walther and Stein, 2000). It is this ability that makes them suitable as delivery systems for therapeutic genes. Both RNA as well as DNA viruses can be used as vectors (Mancheño-Corvo and Martín-Duque, 2006) but the employment of DNA viruses is more wide spread. The most frequently used viral vectors derive from retroviruses, adenoviruses, adeno-associated viruses (AAV), poxviruses and herpesviruses, accounting for over 60% of the vectors used in clinical trials worldwide (Fig. 2).

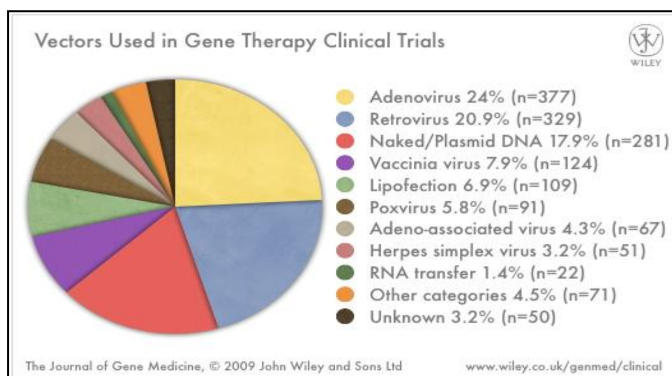


Fig. 2: Vectors used in gene therapy.

http://www.wiley.co.uk/genmed/clinical/ (2009-05-08)

There are basically three different methods by which viral vectors can target cancer cells (Reid *et al*, 2002, Ahn *et al*, 2009):

1. Gene delivery by non-replicating viruses
2. Oncolysis through replicating viruses (oncolytic virotherapy)
3. Combination of replication-induced oncolysis and expression of therapeutic genes

Oncolytic virotherapy

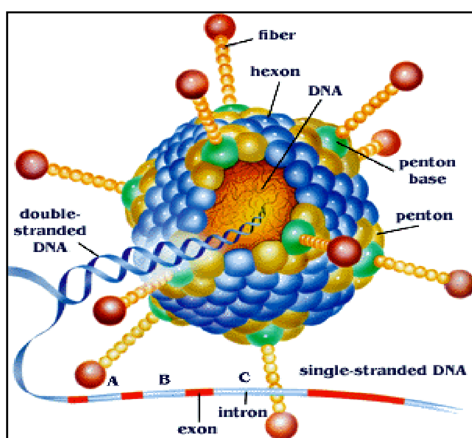
Oncolytic virotherapy means using the virus as a form of therapy causing oncolysis (lysing of tumour cells). Upon infection, the virus replicates within the cancer cell, giving rise to progeny viral particles that at the end of the viral life cycle burst the host cell, thereby killing it. Oncolytic viruses are viruses with either an inherent ability to infect and kill cancer cells or viruses that have been genetically engineered to do so, so called viral mutants (Parato *et al*, 2005).

In comparison to conventional cancer therapies, there are some noteworthy benefits with oncolytic vectors (Parato *et al*, 2005). Through recombinant DNA technology, they can be genetically engineered for optimal effects in the target cells. Genes can be deleted or added, increasing selectivity and potency. Viruses are naturally immune-stimulatory agents and can induce a host immune response against cancer cells, promoting tumour-specific inflammation (Väha-Koskela *et al*, 2007). Through the addition of immunogenic genes into the viral genome the immune response against the tumour can be potentiated. Their ability to self-replicate within the cancer cell and spread to neighbouring cells enables treatment to be self-perpetuating. It also means that for the virus to be safe, its replication has to be restricted to tumour cells only. There are many ways by which viral selectivity for cancer cells can be achieved (Parato *et al*, 2005). The two main approaches used today are tumour/tissue specific promoter regulated transcription of viral genes and mutational complementation (Wang *et al*, 2005). Viral uptake can be increased by targeting tumour specific antigens (Parato *et al*, 2005) and cancer cell killing can be further optimised through the combination of oncolysis with the delivery of toxic genes (Ahn *et al*, 2009).

Adenoviruses

Adenoviruses are the most commonly used oncolytic viral vectors (Fig. 2). They were first isolated and characterised in the 1950s (Shenk, 2001). The virus was isolated from human adenoid tissue (pharyngeal tonsils) from patients with respiratory disease. It is that original tissue that has given rise to the name of the virus. Over 51 serotypes of human adenoviruses have been identified (Russel, 2009). The serotypes are further divided into 6 subgroups, A-F, based on hemagglutination ability (Shenk, 2001). Adenoviruses used for gene therapy are mainly derived from subgroup C (Aghi and Martuza, 2005). Viral tropism differs with serotype but, in general, human adenoviruses show a tropism for epithelium and cause mainly respiratory disease (Shenk, 2001). Infection is normally subclinical but can be severe in immunosuppressed individuals (Quinn *et al,* 2002).

Adenovirus structure and genome organisation



Adenovirus is a non-enveloped, double-stranded DNA virus with a linear genome contained in an icosahedral protein shell called the viral capsid (Shenk, 2001). Particles range in size from 70nm to 100nm. The capsid is composed of structural subunits, including the hexon and penton capsomeres (Fig. 3).

Fig. 3: Structure of the adenovirus.

http://nobelprize.org/nobel_prizes/medicine/laureates/1993/illpres/big-adenovirus-v3.gif (2009-06-02)

All human adenoviruses have the same genome organisation, which means that the gene location along the genome does not differ between serotypes (Shenk, 2001). The viral genome is divided into early (E) and late (L) transcription units, with multiple gene products synthesised from each unit (Fig. 4).

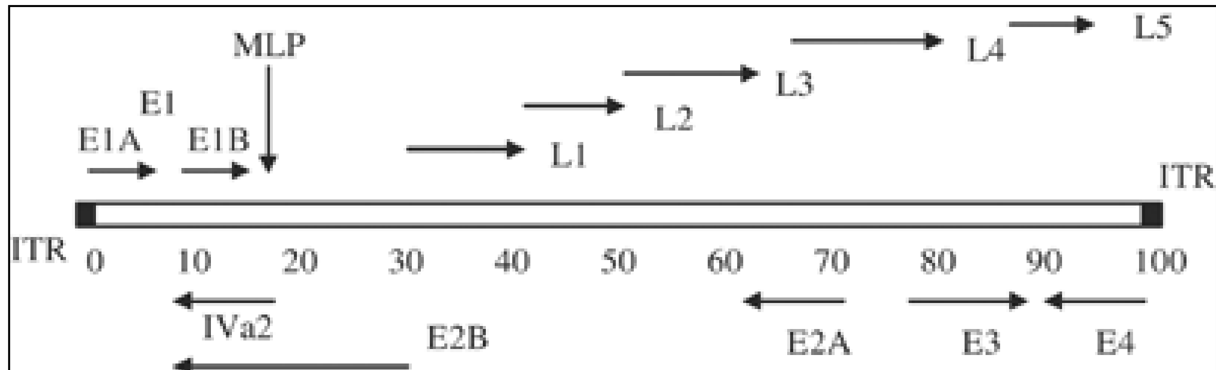


Fig. 4: Schematic representation of the adenoviral genome organisation.

Early proteins are labelled E and late proteins L. MLP Major Late Promoter (Ferreira *et al*, 2005).

Adenovirus lifecycle

The adenoviral life cycle can roughly be divided into three phases:

1) Attachment and entry
2) Viral gene transcription and replication
3) Virion assembly and release from the cell

The entire cycle lasts about 24 hours (Shenk, 2001). Most gene therapy studies are based on the closely related serotypes Ad2 and Ad5 of subgroup C (Aghi and Martuza, 2005).

Attachment and entry

The virus first attaches to the cell through interactions between the viral fiber protein and cell surface structures. First, the fibre knob of the viral capsule binds to the cell surface protein coxsackie and adenovirus receptor (CAR) followed by attachment of the viral pentonbase to cellular integrins $\alpha V\beta 1$ and $\alpha V\beta 5$ (Shenk, 2001). After endosome mediated internalisation, the virus migrates through the cytoplasm, sheds its protein shell and enters the nucleus through membrane pores. Once inside the nucleus, expression of early viral genes is initiated.

Viral gene transcription and replication

The first gene to be expressed after viral infection is E1A, whose main function it is to push the cell into S-phase, creating an environment which is favourable for replication of the viral genome (Shenk, 2001). E1A encodes two major proteins, 13s and 12s, whose conserved regions (CR) bind to and influence cellular transcription factors and gene regulatory proteins. This in turn leads to increased transcriptional activity in the cell and subsequent expression of viral genes necessary for the survival and replication of the virus within the host. The virus utilises mainly two pathways to provoke the quiescent cell into unscheduled cell cycle progression: inactivation of retinoblastoma tumour suppressor protein (pRb) and blocking of E1A binding protein p300 (EP300) in complex with CREB-binding protein (CBP). pRb normally binds to and inactivates the transcription factor E2F, preventing the transcription of

genes that promote progression from G1- into S-phase. The CR2 domain of the E1A proteins binds to and inactivates pRb which releases E2F, allowing cell cycle progression including replication of both cellular as well as viral DNA by the host replication machinery. p300 and CBP serve as co-activators for a number of transcription factors, including tumour suppressor p53. By inhibiting their activity through the CR1 domain of E1A, the virus can manipulate the expression and function of host genes.

Five to eight hours after infection, intranuclear viral DNA replication commences and continues until the cell dies (Shenk, 2001). The E2 genes encode proteins needed for viral DNA synthesis. Some cellular proteins, such as nuclear factors (NF) I, II and III, are also used in the viral replication process. Once viral DNA replication starts, late viral genes encoding structural proteins are expressed, enabling assembly of virions. The late genes are transcribed as a single, large primary mRNA molecule and turned into multiple, smaller transcripts (L1-L5) through splicing. The major late promoter controls the expression of the late viral proteins and is activated at the onset of replication.

Numerous defence mechanisms have been developed by the host to counteract the propagation of virus within the cell. The virus, in turn, has developed ways to avoid these antiviral defence mechanisms. The main aim of the cellular defence is to induce programmed cell death of infected cells, elicited through two major pathways: the intrinsic and the extrinsic apoptotic pathways. Unscheduled progress into S-phase induced by the adenovirus, elicits up-regulation of the tumour suppressor protein p53. p53 in turn activates effector proteins of the apoptosis pathway (Bax, PUMA) which promote downstream activation of a caspase cascade leading ultimately to cell death and disintegration. To prevent this from happening, the virus encodes two proteins, E1B55K and E4orf6, which are transcribed shortly after E1A expression. E1B55K and E4orf6 together bind to p53 and promote the proteosomal degradation of the tumour suppressor, preventing premature cell and subsequently viral death. Adenoviral infection can also induce a response through the extrinsic apoptotic pathway, involving mainly the cytokine TNFα and signalling through the death receptor Fas (Liu *et al*, 2005). Fas ligands, like TNFα, bind to the Fas receptor and activate caspases via Fas-associated protein with death domain, FADD. To counteract the action of TNFα-induced cell death, adenoviruses have developed the E1B19K protein; a functional homologue to the cellular anti-apoptotic protein Bcl2 (B-Cell Lymphoma 2). E1B19K binds to and blocks downstream effectors of apoptosis, thereby preventing premature cell death.

Virion assembly and release from cell

Viral transcripts are initially translated together with cellular equivalents (Shenk, 2001). As the infection progresses, the transport of cellular mRNAs to the cytoplasm is blocked by viral E1B and E4 proteins. This promotes the accumulation of late viral transcripts in the cytoplasm. In the cytosol, the viral mRNAs are translated into either structural proteins like components of the viral capsid or auxiliary proteins for virion assembly. A special packing sequence in the viral genome mediates DNA-capsid recognition and initiates the assembly of the capsid. Once an intact viral shell has been assembled, the viral DNA is added. Through processing of the capsid components by the L3 protein, the formed virion is rendered infectious. Two viral systems are responsible for killing the host cell. Firstly, L3 promotes breakdown of the cytoskeleton, enabling release of the viral particles by lysing the cell. Secondly, an E3-encoded protein adenovirus death protein (ADP) accumulates in the cytoplasm during the later stages of infection. The mechanism by which cell death is induced by ADP is still not entirely understood.

Conditionally replicating oncolytic adenoviruses

Adenoviruses are suitable as oncolytic viral vectors for a number of reasons. The genes responsible for infection, replication and pathogenesis are well known and the viral genome is easily manipulated (Shenk, 2001). In contrast to retroviruses, adenoviral genes are not incorporated into the host genome, abolishing the risk of insertional mutagenesis. Adenoviruses can infect both proliferating and non-growing cells (Horwitz, 2001), allowing the use of oncolytic mutants against slow growing tumours like prostate cancer. Also, viral replication and cellular carcinogenesis require inactivation of the same defence mechanisms/tumour suppressor pathways, enabling the selective targeting of cancer cells through genetic engineering of the virus (O'Shea, 2005).

Use of engineered adenoviral vectors have shown promising results in clinical trials, producing few and mild side-effects (Reid *et al*, 2002) with proven selectivity for cancer cells (Liu *et al*, 2004; Liu *et al*, 2005; Leitner *et al*, 2009). Tumour-selectivity of adenoviruses has mainly been achieved through mutational complementation or tumour/tissue specific promoter regulation of viral replication (Liu *et al*, 2005).

Regulation of viral transcription by tumour/tissue specific promoters

By placing viral genes essential for replication, for example E1A, under the control of promoters or enhancer elements that are up-regulated in cancer cells, the replication and subsequently oncolytic activity of the virus can be restricted to tumour cells (Ahn *et al*, 2009). This approach has been widely used for the targeting of prostate cancer, placing viral replication under the control of androgen receptor (AR) response elements. AR is a hormone dependent transcription factor that is frequently mutated and over-expressed in late stage prostate cancers.

Mutational complementation

Mutational complementation takes advantage of the "evolutionary convergence between the processes of carcinogenesis and adenoviral replication" (Liu *et al*, 2005). Both tumourigenesis and viral replication within a host depend on uncoupling cellular growth and proliferation processes from the safety and control mechanisms governed by multi-purpose tumour suppressor genes. For instance, many cancer cells have mutations blocking apoptotic pathways, like loss-of-function mutations of p53 (O'Shea, 2005) or abnormal and non-functional TNF-α signalling (Liu *et al*, 2005). Viruses on the other hand, have developed proteins like E1B55K/E4orf6 and E1B19K to block the induction of apoptosis in infected cells (Shenk, 2001). By deleting anti-apoptotic genes of a viral mutant, its replication can be restricted to cells in which cell death pathways are already non-functional, i.e. cancer cells. The virus would still be able to infect a normal cell, but as the apoptotic pathways are intact, the cell will undergo virally induced apoptosis and the infection will not spread (Fig. 5).

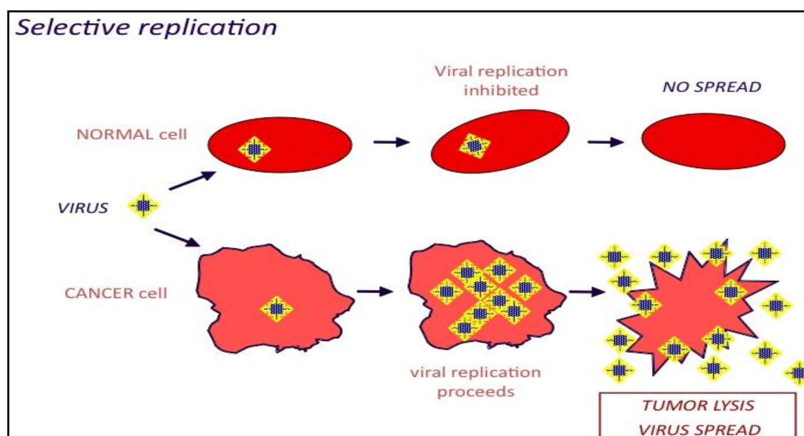


Fig. 5: Restriction of viral replication to cancer cells through selective replication.

Courtesy of Dr. Gunnel Halldén

Mutation of tumour suppressor p53 is the most commonly found genetic alteration in cancer cells. Several adenoviral mutants targeting this particular feature have been developed. One of which is ONYX-015 (*dl*-1520), the first adenoviral mutant used in clinical trials, designed to replicate selectively in p53-deficient cells (Ganly *et al*, 2000). It is deleted in the E1B55K region, making it unable to mediate destruction of p53 and thereby avoid p53-induced apoptosis. ONYX-015 has given rise to the first adenoviral mutant licensed for cancer therapy, Shanghai Sunway Biotech's H101, which is used in China to treat patients with head and neck cancer (Garber, 2006). Despite being proven safe and cancer cell specific in numerous trials, the efficacy of ONYX-015 as a single treatment is poor. However, the cytotoxic activity of the mutant can be markedly increased through combination with chemotherapy (Khuri *et al*, 2000).

Another aberration commonly found in cancer cells is inactivation of pRb and subsequently a defective G1-S-phase checkpoint. This main regulator of cell cycle progression is also targeted by adenoviral mutants, one of which is *dl*922-947 (Heise *et al*, 200). *dl*922-947 is deleted in the pRb binding E1ACR2 domain, a mutation that abolishes the viral ability to inactive pRb and push the cell into S-phase. Due to this loss of function, viral replication and survival is restricted to cancer cells with non-functional pRb pathways. *dl*922-947 shows good cytotoxic potential, efficiently killing a broad range of cancer cells, but is less safe than previous mutants like ONYX-015. The virus is namely to some extent capable to replicate in normal but proliferating cells because these, like cancer cells, have inactivated cell cycle checkpoints.

So whereas ONYX-015 shows promising safety properties but inefficient cytotoxic potential, *dl*922-947 possesses good cell killing qualities but is lacking in safety. One major problem with conditionally replicating viruses is that "gene deletions conferring selectivity also frequently result in reduced potency of the virus in tumours" (Heise *et al*, 2000). The focus in cancer gene therapy today therefore lies in producing a mutant that is able to kill cancer cells efficiently but unable to replicate in normal cells, both quiescent and proliferating.

Pancreatic cancer

Pancreatic cancer is the 11th most common form of cancer in the UK, with 6500-7000 new cases each year (CRUK CancerStats, 2006). The number of people affected does not differ between men and women but the incidence rate is slightly higher in men, especially with increased age. With roughly 7000 deaths a year in the UK, pancreatic cancer is the sixth most common cause of cancer related death. Symptoms associated with pancreatic cancer are vague and slow in onset, leading to patients often being diagnosed at a late stage. In the US, the average age at diagnosis is 72 (American Cancer Society, 2008). Usually time between diagnosis and death never reaches more than six months; the five year survival rate is only 2-3% in the UK and it has not improved in the last 40 years (CRUK CancerStats, 2006).

The main risk factors of pancreatic cancer are old age, smoking, obesity, diabetes mellitus type II (insulin-independent diabetes) and hereditary chronic pancreatitis (CRUK CancerStats, 2006). Smoking is the only established preventable factor. Other factors that influence the risk of developing pancreatic cancer include exercise, diet, exposure to chemicals and radiation and certain genetic disorders.

The absolute majority of pancreatic tumours originate from the exocrine pancreas, 95% of these are malignant adenocarcinomas (CRUK CancerStats, 2006). Mutations of the proto-oncogene k-Ras, tumour suppressor gene p53 and p16 are the most frequently observed genetic alterations in ductal adenocarcinomas (Moore *et al*, 2001).

The treatment options for pancreatic cancer are the same as for most forms of cancer: surgery (curative or palliative), radiotherapy, chemotherapy or combinations of two or more of the alternatives. At the time of diagnosis most cancers will have spread, making surgical resection impossible (Mulvihill *et al*, 2001). The first line of treatment after surgery is the chemotherapeutic drug Gemcitabine (Gemzar®). Gemcitabine (2', 2'-difluoro 2'-deoxycytidine, dFdC) is a cytidine analogue which is incorporated into the cellular genome, leading to premature DNA chain termination, cell cycle arrest and subsequent apoptosis (Mini *et al*, 2006). Despite being the standard chemotherapy for pancreatic cancer, Gemcitabine only prolongs the average patient survival by about 6 months (O'Reilly, 2009). Other drugs used in pancreatic cancer treatment, mostly in combination with Gemcitabine, include cisplatin, irinotecan (Campto®), paclitaxel (Taxol®) and docetaxel (Taxotere®), (American Cancer Society, 2008). Irinotecan (camptothecin) is a topoisomerase I inhibitor and functions like Gemcitabine as a DNA damaging agent. Topoisomerase I is an ubiquitous and essential enzyme that is responsible for the uncoiling of the nucleotide strands during DNA replication (Rothenberg, 1997). During uncoiling, single strand breaks are created in the DNA, enabling unwinding of the helix without torsional stress. Once the strands are uncoiled, the breaks are religated, allowing replication to occur. This process is catalysed by topoisomerase I. Camptothecin binds to topoisomerase I and blocks the religation step, leaving the DNA strand cleaved, which in turn brings the replication to a halt.

Despite availability of advanced medical and surgical treatment the prognosis of pancreatic cancer remains very poor. One important explanation for this is the fact that most pancreatic tumours are resistant or insensitive to available chemotherapeutic drugs (Mulvihill *et al*, 2001). The mechanisms behind drug resistance in pancreatic cancer are not fully understood but a partial explanation is the poor blood perfusion of pancreatic tumours creating a hypoxic environment within the mass. The unsatisfying results of current available treatment options necessitate the development of novel therapies such as oncolytic virotherapy.

Oncolytic virotherapy against pancreatic cancer

Leitner *et al* (2009) developed an E1B19K deleted Ad5 mutant (Ad5Δ19K) that in combination with Gemcitabine showed enhanced cytotoxicity. The virus was tested on the pancreatic cancer cell lines Suit-2 and PT45 and both cell lines showed significant sensitisation to Gemcitabine induced cell death in response to the combination treatment. Promising results were also observed *in vivo* using PT45 xenografts in athymic mice, which showed inhibition of tumour growth and prolonged survival. One interesting finding was that the synergistic effect on cell death was caused by enhancement of Gemcitabine-induced apoptosis, possibly explained by the pro-apoptotic functions of the E1A protein that could not be prevented in the absence of E1B19K gene expression. To further improve on viral potency, selectivity and to elucidate the mechanisms involved in the synergy, another viral mutant was constructed by the Viral Gene Therapy (VGT) group at the Centre for Molecular Oncology and Imaging at Barts and the London School of Medicine and Dentistry. This mutant, Ad5ΔE1ACR2ΔE1B19K (Ad5ΔΔ), is deleted in both the E1B19K region and the pRb binding CR2 domain of the E1A gene (Öberg *et al*, manuscript in preparation). It is hypothesised that the virus, through the double deletion, will prove to be safer and more selective than previous mutants because of the inability to bind pRb and induce S-phase in

normal cells. It is also hypothesised that through the defective E1A protein, the induction of apoptosis might be reduced or delayed, potentially resulting in higher viral replication in tumour cells even in the absence of the anti-apoptotic E1B19K protein. Dr. Cherubini of the VGT group has been testing the Ad5ΔΔ mutant in combination with Gemcitabine in PT45 cells and observed sensitisation to the drug (Dr. Cherubini, personal communication). She has also conducted replication studies with Ad5ΔΔ in primary cells, confirming the hypothesis that this mutant is unable to replicate in normal cells (unpublished data). The primary cells used in these studies were normal human bronchial epithelial (NHBE) cells (Dr. Cherubini, personal communication).

Project aims

The aim of this project was to screen Ad5ΔΔ in three different pancreatic cancer cell lines, evaluating replication and cytotoxicity. There were three main questions to be answered in the course of this work:

- Does Ad5ΔΔ replicate efficiently in pancreatic cancer cells?
- Does Ad5ΔΔ sensitise pancreatic cancer cells to chemotherapy?
- Does Ad5ΔΔ retain the ability to selectively kill cancer cells, as seen in the single deleted counterpart Ad5Δ19K?

The cell lines chosen were PANC-1, Suit-2 and BxPc-3, all pancreatic cancer cells lines with different origin and/or mutations. PANC-1 and BxPc-3 cells derive from primary adenocarcinomas, whereas Suit-2 cells originate from liver metastases. The majority of pancreatic cancer cells are ductal adenocarcinomas with mutations of the oncogene kRas (Moore *et al*, 2001). Most of these cells are kRas dependent, i.e. if the kRas pathway is successfully blocked, the cells will die. However, within the population of kRas mutated pancreatic cancer cells, there are cells which are independent of the activity of the kRas oncogene (Singh *et al*, 2009). To reflect the diversity in mutations found in pancreatic cancer cells, we chose two cell lines that are kRas mutated (PANC-1 and Suit-2), one kRas independent (PANC-1) and one kRas dependent cell type (Suit-2). BxPc-3 cells carry the kRas wild type. Apart from mutations in kRas, all cell lines used in the screening have mutations in tumour suppressor genes p53 and p16, another common feature of most pancreatic cancer cells (Moore *et al*, 2001).

If Ad5ΔΔ can show ability to efficiently replicate in pancreatic cancer cells and sensitise cells to chemotherapy in both *in vitro* and *in vivo* studies, the virus can be considered as a candidate for clinical trials. In the screening, Ad5ΔΔ was compared to three other adenoviruses: a wild type (wt) control Ad5tg and the single-deleted mutants Ad5Δ19K and ONYX-015 (*dl*1520). In some replication studies, a replication-defective adenovirus Ad5GFP was used as a negative control. The level of replication and subsequent cell killing was considered efficient if it approximated or exceeded that of the wt control. ONYX-015 was included in the study as it has been so frequently used in oncolytic research and offers a great deal of reference data. The drugs used to evaluate the sensitisation potential of the viral mutants were Gemcitabine (Gemzar®) and Irinotecan (Compto®), both currently used in the treatment of pancreatic cancer.

MATERIAL AND METHODS

Cells and cell culture

The cell lines used in the screening are PANC-1, Suit-2 and BxPc-3. These were obtained from the American Type Culture Collection (ATCC; VA, USA) and Cancer Research UK Cell Services (Clare Hall, CRUK, London). JH293 cells used for $TCID_{50}$ assays were also provided from the Cancer Research UK Cell Services.

Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with high 4.5 g/L glucose (PAA Laboratories GmbH, Pasching, Austria) containing L-glutamine, sodium pyruvate, and supplemented with 10% foetal calf serum (FCS) (PAA) and 1% penicillin/streptomycin (100 x) (PAA). The cells were passaged every 3-4 days using phosphate buffered saline (PBS) to wash the cells and trypsin (PAA) to detach the monolayer. The cells were incubated in a humidified atmosphere with 5% CO_2 and a temperature of 37°C. All reagents needed for cell culture were supplied by Cancer Research UK Cell Services.

Viruses and chemotherapeutic drugs

The viruses used in the project were: Ad5tg (wild type adenovirus type 5), Ad5Δ19K (Adtg deleted in the E1B19K-gene), Ad5ΔΔ (Adtg deleted in the E1ACR2-region and the E1B19K-gene) and ONYX-015 (*dl*1520; deleted in the E1B55K-gene) and Ad5GFP (non-replicating deleted in E1-genes). All viruses derive from the Ad5 serotype C and are replication-competent with the exception of Ad5GFP. Ad5tg is generated from the Ad5 plasmid pTG3602 containing the Ad5 wild type genome (Leitner *et al*, 2009; Öberg *et al*, manuscript in preparation). All mutants were generated by homologous recombination of the pTG3602 plasmid and plasmids containing the various deletions. Ad5GFP is a non-replicating virus deleted in E1 which has been replaced by CMV-GFP cassette. All viruses were constructed for in house use by the Viral Gene Therapy Group at the Centre for Molecular Oncology and Imaging at QMUL.

The drugs used in the cytotoxicity assays were Gemcitabine HCl (Gemzar®, Eli Lilly, Basingstoke, UK) and Irinotecan (Compto®, Pfizer, Sandwich, Kent, UK). Gemcitabine was dissolved in PBS and stored at -20°C at a concentration of 100mM. Irinotecan was bought already in solution at a concentration of 34mM and kept at room temperature.

MTS assays – cytotoxicity of chemotherapeutics and virus

The sensitivity of the cell lines to Gemcitabine, Irinotecan, viruses as well as combination treatments was determined by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) viability assays. Cells were seeded in DMEM media with 10% FCS on sterile 96 well plates at a cell density of 5000 cells/well in 100µl media. One row of wells contained only media and was used as a blank for background absorbance. The following day the media was replaced by 2% DMEM and cells were infected or treated with serial dilutions of virus and drug respectively, in a final volume of 100µl. Each infection and drug treatment was done in triplicate wells. One row of cells on each plate was left untreated and used as a control. In combination treatment assays, the controls were cells exposed only to the drug but not virus.

The serial dilutions of drugs and virus were done on a separate 96 well plate. Viruses were diluted 5-fold and drugs 3-fold. The starting dose, the multiplicity of infection (MOI), of each virus was $1x10^5$ ppc. For the drug treatment, the equivalent row of wells contained concentrations of 40µM and 1mM for Gemcitabine and Irinotecan respectively. The MOI as well as drug concentrations were established in previous work in the pancreatic cancer cell lines PT45 and Suit-2 (Dr. Cherubini, personal communication). In cases where resistance to the drug was observed in a cell line, the drug concentration was increased to a maximum of 400µM for Gemcitabine and 3.4mM for Irinotecan. When combination treatments were performed, 2% media with the calculated drug concentration was prepared separately and then added to the cells (90µl/well). Following this, serial dilutions of virus were added to the well in a volume of 10µl, keeping the total volume per well to 100µl throughout the assay.

Three days after infection, the plates were read using the MTS reagent according to the manufacturer's instructions (Promega, Madison, WI, USA). In live, metabolically active cells, the tetrazolium salt MTS is converted to a soluble formazan product by mitochondrial dehydrogenase. The product absorbs light at 490nm and its synthesis can therefore be quantified by reading the plates in an ELISA microplate reader (Opsys MR, Dynex Technologies) at a wavelength of 490nm. The reaction product is directly proportional to the amount of viable cells on the plate. From the number of live cells, a measurement of the amount of cell death in each well could then be calculated. The absorbance values for treated or infected wells were corrected against the corresponding blank wells containing only media (background) and compared to the untreated/uninfected control wells. The amount of cell death for each serial dilution step was calculated according to the following formula:

100 - [((average sample - average background)/average control - average background) * 100]

The results of each triplicate experiment were averaged and expressed as percentages of cell death in comparison to the untreated controls. In combination treatments the controls used were cells treated with drug alone. The data analysis was performed using the GraphPad Prism graphics software and dose response curves were generated for each agent alone and as well as combination of virus and drugs. This generated sigmoid curves which enabled the determination of the effective drug and/or virus concentrations killing 50% (EC_{50} values) of cells. Examples of viral dose response curves in PANC-1 cells and drug dose response curves in Suit-2 cells can be seen in Fig. 6. It was noted that 100% cell death was never achieved with Gemcitabine after only three days of incubation and the EC_{50} values for single treatment with Gemcitabine were therefore extrapolated from available data points; the maximum achieved cell death was set to 100%, the minimum to 0% and value in between the set constraints was used as the EC_{50} value.

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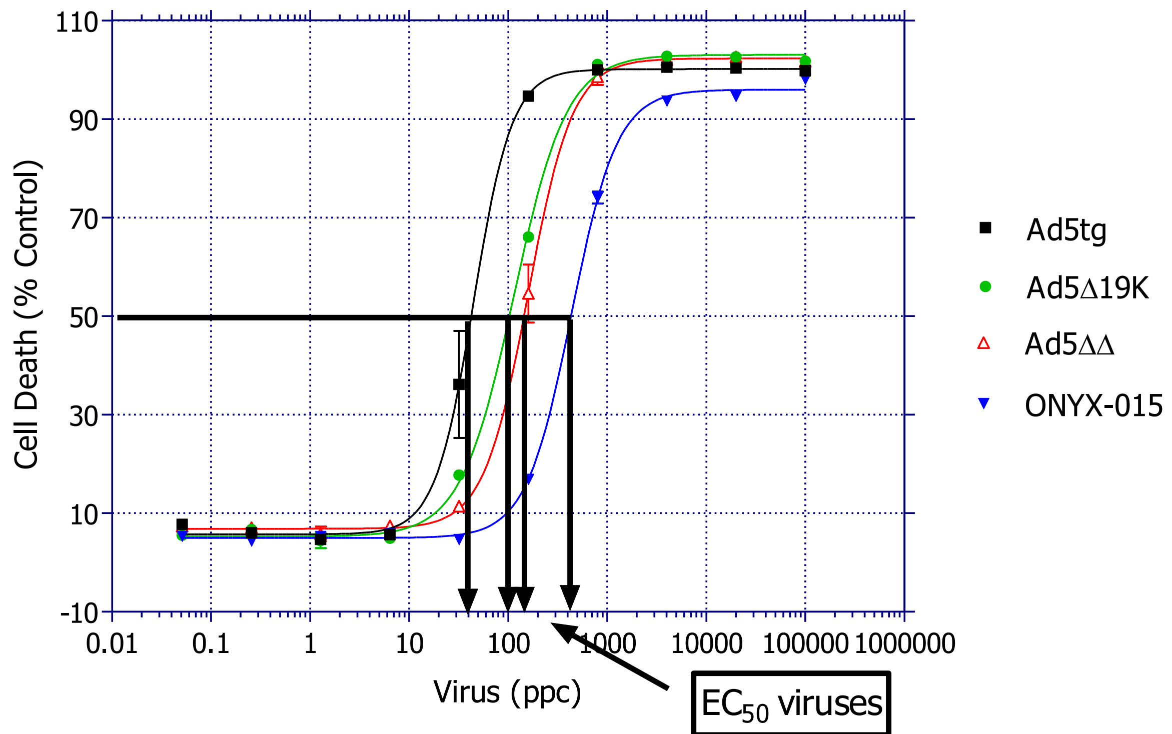

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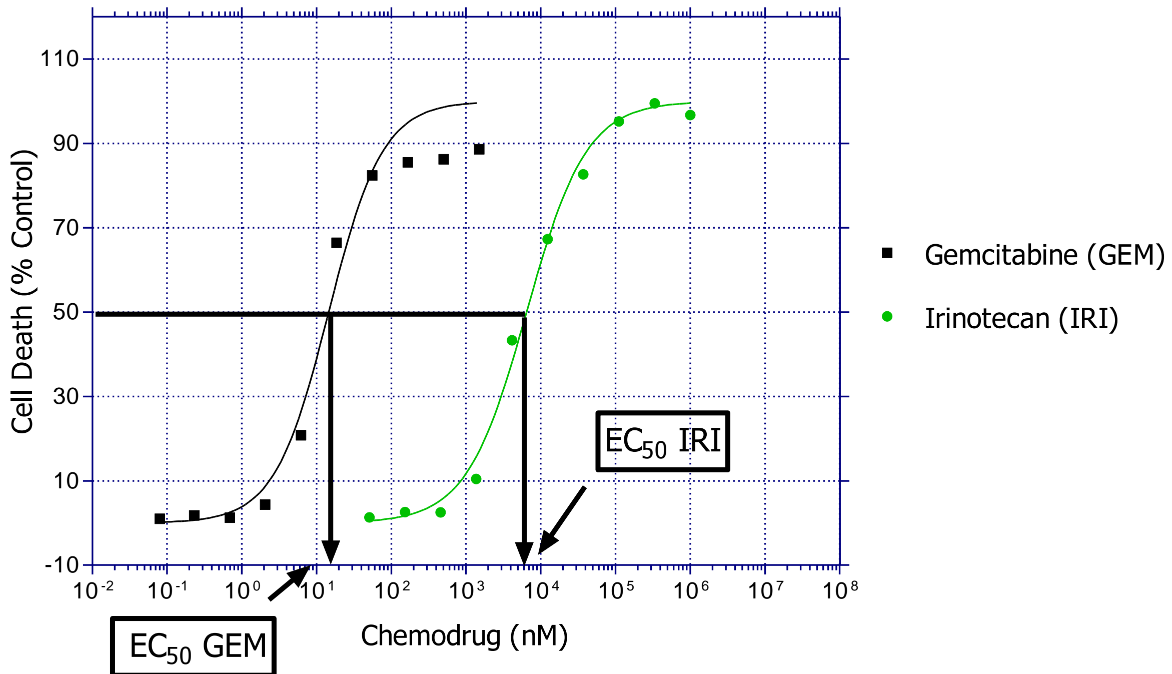

Fig. 6: Dose response curves to serial dilutions of virus (A) and drug (B) in PANC-1 and Suit-2 cells respectively.

Arrows indicate EC_{50} values of each individual treatment. Each infection and treatment was done in triplicates and the results averaged ± SD

Viral replication assays – $TCID_{50}$ and quantitative PCR

Viral replication and genome amplification in the cell lines was measured using a limiting dilution assay ($TCID_{50}$; tissue culture infective dose at 50%) and quantitative polymerase chain reaction (qPCR) respectively. $TCID_{50}$ measures the production of a virus in cells by quantifying the intact, infective viral particles released from and present in the infected cells. The results are determined as plaque forming units (PFU) per cell, a value that is calculated based on the cytopathic effect of the virions produced in the titre cell line (JH293 cells). In contrast to the biological titration of a $TCID_{50}$ assay, qPCR constitutes a physical method to estimate viral genome amplification. It quantifies the intracellular content of viral DNA by amplifying samples extracted from infected cells.

Infection

In the viral burst assay, samples for $TCID_{50}$ assays and qPCR were created simultaneously by infecting pancreatic cancer cells and harvesting them at three given time points. From these cell samples, intact viral particles were extracted for further infection of JH293 cells in the $TCID_{50}$ assay. From the qPCR samples, viral DNA was extracted and quantified.

Pancreatic cancer cells were seeded in 10% DMEM in 6 well-plates at a cell density of 100 000 cells/well for Suit-2 cells and 200 000 cells/well for PANC-1 and BxPc-3 cells. The total volume of each well was 2.0ml. After overnight incubation, the cells of one well were detached and counted. Based on this cell count, a virus dose correlating to an MOI of 100 ppc was calculated and cells infected accordingly. This value of 100 ppc was established in previous studies (Dr. Cherubini, personal communication). Viruses used for burst assays were Ad5tg and Ad5ΔΔ for $TCID_{50}$ samples and Ad5tg, Ad5ΔΔ and Ad5GFP for qPCR samples. All infections were done in duplicates. The cells were infected in serum-free medium 1.0ml/well for two hours after which the medium was removed and replaced with fresh 10% DMEM at a volume of 2.0ml/well. Cells were harvested at three time points after infection, 24 hrs, 48 hrs and 72 hrs. Both cells and media were collected for the $TCID_{50}$ assays (cells were scraped off the plates). Harvesting of qPCR samples was done by discarding the culture media and detaching the cells with trypsin. After inactivation of trypsin through addition of fresh DMEM, cells were centrifugated (5 min at 1500 rpm) and the pellet re-suspended in 200µl PBS. $TCID_{50}$ samples were stored at -80ºC and qPCR samples at -20ºC.

$TCID_{50}$

JH293 cells, a subclone of the human embryonic kidney (HEK) cells, were seeded on sterile 96 well plates at a cell density of 10 000 cells/well in a total volume of 200µl/well 10% DMEM. The following day burst assay samples were freeze-thawed with liquid nitrogen three times in order to break the cells and release intracellular viral particles. After centrifugation (5 min at 1500 rpm) samples were diluted 1:1000 and 20µl of the diluted solution was added to the top row of duplicate plates. The viral samples were serially diluted across the plates to a final dilution of $1{:}10^9$. The last row of cells on each plate was left uninfected and used as control. Three plates were infected with a control adenovirus (Ad5) which was used as to verify the accuracy of the assay. After 10 days of incubation at 37°C the plates were inspected and any sign of virally induced cytopathic effect (CPE) registered. Based on the CPE the titre of each burst assay sample was calculated using the Kärber formula (Kärber, 1931).

Quantitative PCR

Burst assay samples for qPCR were produced simultaneously as samples for $TCID_{50}$, using the non-replicating virus Ad5GFP in addition to Ad5tg and Ad5ΔΔ. Ad5GFP functioned as a negative control of replication. DNA was extracted from the samples according to QIAamp® DNA Mini and Blood Mini Handbook Spin protocol for Blood and Body Fluids (QIAGEN®). qPCR (7500 Real Time PCR System; Applied Biosystems) was done with the Power SYBR Green Master Mix (Applied Biosystems) and analysed by the System SDS software. Each individual sample was amplified in triplicates for each primer used: GAPDH (glyceraldehyde 3-phosphate dehydrogenase), a cellular protein whose expression functions as a standard for cellular DNA quantity, and hexon, a viral structural protein, used as a measure of viral DNA production. All triplicate values were averaged and the viral DNA results normalised against the cellular DNA values. Cycle threshold (Ct) values were exported into EXCEL worksheets for analysis and relative quantification was made using the $2^{-\Delta\Delta Ct}$ method (Tichopad *et al*, 2003). Relative quantification of DNA content meant that the qPCR value of each sample and timepoint was compared to the Ad5tg sample of the 24 hrs timepoint and expressed as a fold change of that sample allowing a comparison of replication of Ad5ΔΔ against that of wt Ad5tg.

Statistics

Each experiment was done at least in triplicate. Statistical analysis of data was done using Prism Software. The performed tests were one-way ANOVA (nonparametric) column analysis including Dunnett's post test. This test was used to compare EC_{50} data of combination cytotoxity assays (virus alone vs virus + drug).

RESULTS

Replication of viral mutant Ad5ΔΔ in pancreatic cancer cell lines PANC-1, Suit-2 and BxPc-3

In previous studies, Ad5ΔΔ was shown to be a relatively safe mutant with greatly attenuated replication in normal, non-cancerous cells (Dr. Cherubini, personal communication). However, its oncolytic potency had only been investigated in one cell line (PT45) that supported replication to levels similar to wild type virus. To further determine efficacy in pancreatic cancer cells, replication assays were performed in the additional three cell lines, PANC-1, Suit-2 and BxPc-3. The corresponding single deleted mutant Ad5Δ19K showed promising levels of replication in pancreatic cancer cell lines (PT45 and Suit-2; Leitner *et al*, 2009). As the two mutants have the same E1B19K deletion, this data supported the expectation that the double-deleted Ad5ΔΔ mutant would also replicate efficiently in cancer cells.

Viral DNA amplification was determined by qPCR in all three cell lines. The PANC-1 samples were also analysed for viral replication by $TCID_{50}$ assay. The qPCR data was a measure of the amount of viral DNA synthesised, while the $TCID_{50}$ analysis enabled quantification of the number of intact, and therefore infective, viral particles produced in pancreatic cells after infection. In the qPCR analysis the viral DNA content of all samples were compared to the corresponding content in Ad5tg infected cells at the 24 hrs time point. As described in Materials and Methods, the resulting relative levels of viral DNA were expressed as fold change of the 24 hrs Ad5tg sample.

Both qPCR and $TCID_{50}$ analysis showed that the double deleted mutant Ad5ΔΔ replicates efficiently in all pancreatic cancer cell lines, reaching levels comparable to or higher than Ad5tg (Fig. 7). When comparing qPCR results, a difference in viral DNA content between cell lines was noted. At 72 hrs after infection, the relative content of Ad5ΔΔ and Ad5tg DNA in PANC-1 and BxPc-3 cells was almost ten times higher than in Suit-2 (Fig. 7C). These differences could be related to variable permissiveness to replication between cell lines, however, the same pattern of viral DNA amplification was observed in all cell types. In each cell line the viral DNA content increased the most from 24 hrs to 48 hrs after which it reached a plateau. This was observed with both qPCR and $TCID_{50}$ assays. A slight difference in qPCR and $TCID_{50}$ data was seen in PANC-1 samples; according to the $TCID_{50}$ results the wt virus replicated more than Ad5ΔΔ, both at 48 hrs and 72 hrs, indicating that the amount of intracellular viral DNA did not correlate to the numbers of infective virions. Despite the differences, both analyses showed the same trend in viral replication in the cell line. In conclusion, the results of the viral replication assays show that Ad5ΔΔ can replicate efficiently in all cell lines tested.

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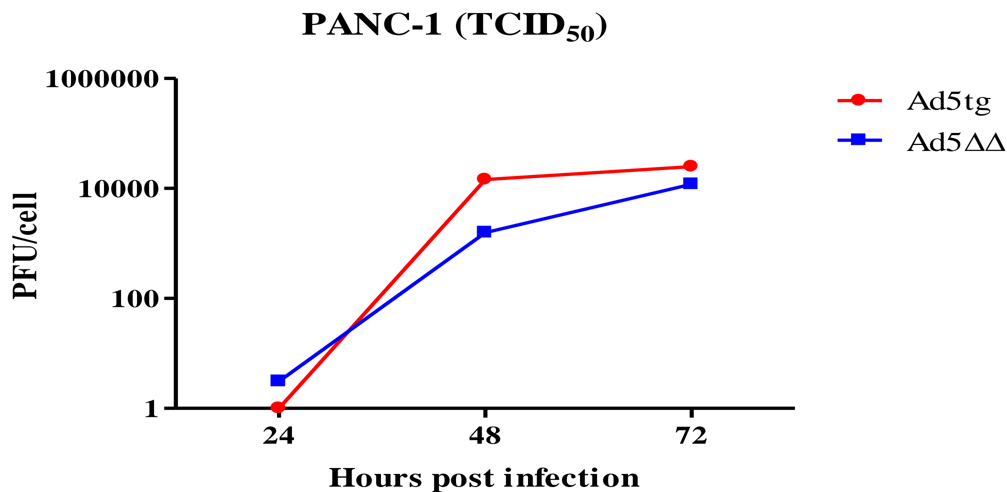

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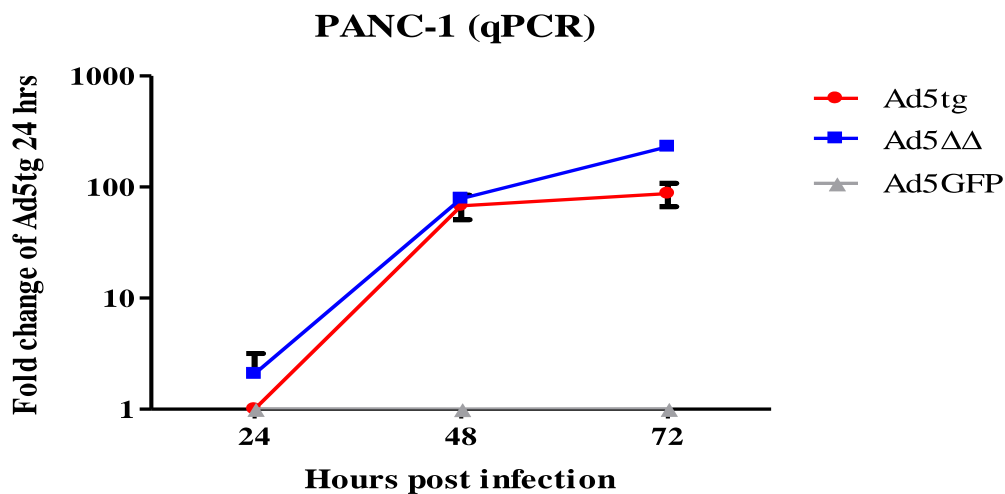

Fig.7: Adenoviral mutant Ad5ΔΔ replicates efficiently in pancreatic cancer cell lines PANC-1, Suit-2 and BxPc-3.

Replication of Ad5ΔΔ and Ad5tg in pancreatic cancer cell lines PANC-1 (A+B), Suit-2 (C) and BxPc-3 (D), measured by $TCID_{50}$ (A) and qPCR (B/C/D). **A:** Replication quantified by $TCID_{50}$ and expressed as increase in PFU/cell over time. All infections were done in duplicates and samples were analysed in duplicates in the $TCID_{50}$ assay. **B/C/D:** Replication measured by qPCR and displayed as increase in viral DNA content relative to the Ad5tg sample 24hrs post infection. A replication defective virus Ad5GFP was added as negative control. All infections were done in duplicates and each duplicate was assessed in triplicates qPCR-samples that were averaged. Results expressed as average of qPCR data ± SD.

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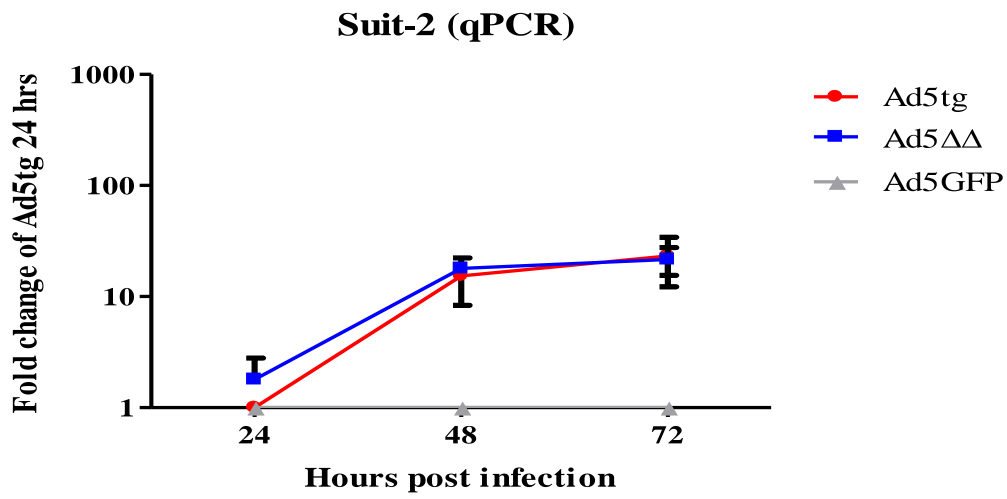

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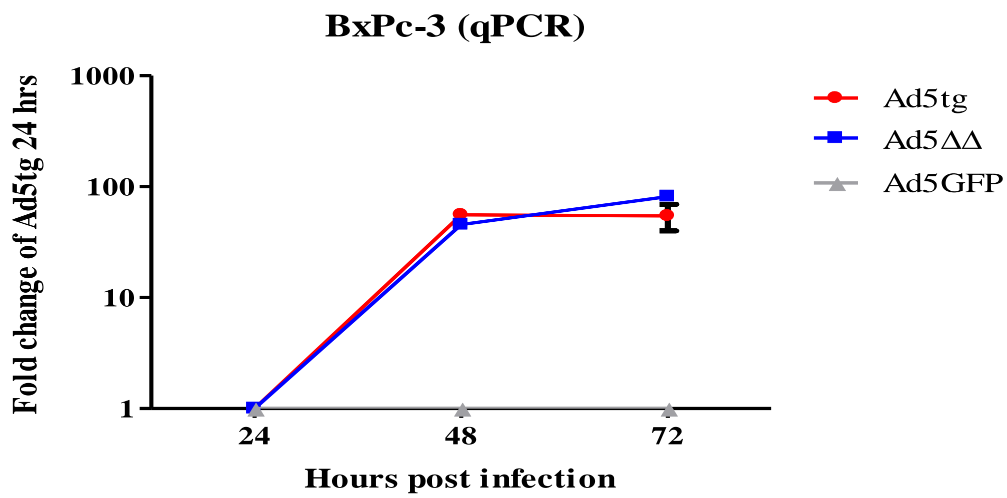

Cytotoxic effect of viral mutants and chemotherapeutic drugs on pancreatic cancer cell lines PANC-1, Suit-2 and BxPc-3

The second task of the project was to establish whether Ad5ΔΔ could sensitise cells to the cytotoxic actions of Gemcitabine (GEM) and Irinotecan (IRI). Before the cytotoxic potency of viral treatment in combination with chemotherapeutics could be evaluated, the effect of viruses and drugs on their own had to be investigated. This was done through the generation of dose response curves in cell death assays in which pancreatic cancer cells were treated with serial dilutions of virus and drug respectively. Three days after infection or treatment, the cells were analysed and the EC_{50} values calculated as described in Materials and Methods.

The viral cytotoxicity data showed that Suit-2 was the least sensitive cell line with average EC_{50} values of 182 ± 44 ppc for Ad5tg, 540 ± 36 for Ad5Δ19K, 993 ± 121 for Ad5ΔΔ and 2437 ± 603 for ONYX-015 (Fig. 8A). PANC-1 consistently showed the highest levels of sensitivity to the viruses with average EC_{50} values of 34 ± 13 ppc for Ad5tg, 55 ± 9 for Ad5Δ19K, 164 ± 4 for Ad5ΔΔ and 445 ± 25 for ONYX-015. In BxPc-3 cells EC_{50} values similar to the ones of PANC-1 cells were observed: 57 ± 19 ppc for Ad5tg, 180 ± 54 for Ad5Δ19K, 204 ± 59 for Ad5ΔΔ and 618 ± 120 for ONYX-015. Despite varying levels of sensitivity to virally induced cell death, the relationship between the viruses was preserved in all cell lines (Fig. 8A). Ad5tg was consistently the most potent virus, followed by, in descending order, Ad5Δ19K, Ad5ΔΔ and ONYX-015.

The drug dose response results showed varying sensitivity to Gemcitabine and Irinotecan in the three cell lines (Fig. 8B). With average EC_{50} values of 14.6 ± 4 nM for Gemcitabine and 6.7 ± 1.9 µM for Irinotecan, Suit-2 proved to be the most sensitive cell line to drug-induced cell death. The corresponding EC_{50} values in the other cell lines were 10.4 ± 8.8 µM (PANC-1) and 2.5 ± 1.6 µM (BxPc-3) for Gemcitabine and 44.1 ± 12.4 µM (PANC-1) and 31.4 ± 8.5 µM (BxPc-3) for Irinotecan. Despite the fact that the experiments were repeated 3-5 times, the overall variation between assays is remarkably large, especially in the cells showing insensitivity to the drugs.

The results from the single treatments suggest an inverted correlation between sensitivity to viral infection and chemodrugs. PANC-1 cells were the most sensitive to viral treatment but the most insensitive to chemodrugs. Suit-2, on the other hand, was the most sensitive cell line to drug treatment but showed the highest resistance to the viruses. By comparing the viral replication data (Fig. 7) with the cytotoxicity assays, it became clear that the sensitivity of a cell line to viral treatment corresponded well with its permissiveness to viral replication. Viral replication was lowest in Suit-2 cells, which also showed the highest resistance to virally induced cytotoxicity. Viruses replicated the most in PANC-1 cells, which showed the highest levels of sensitivity to viral infection.

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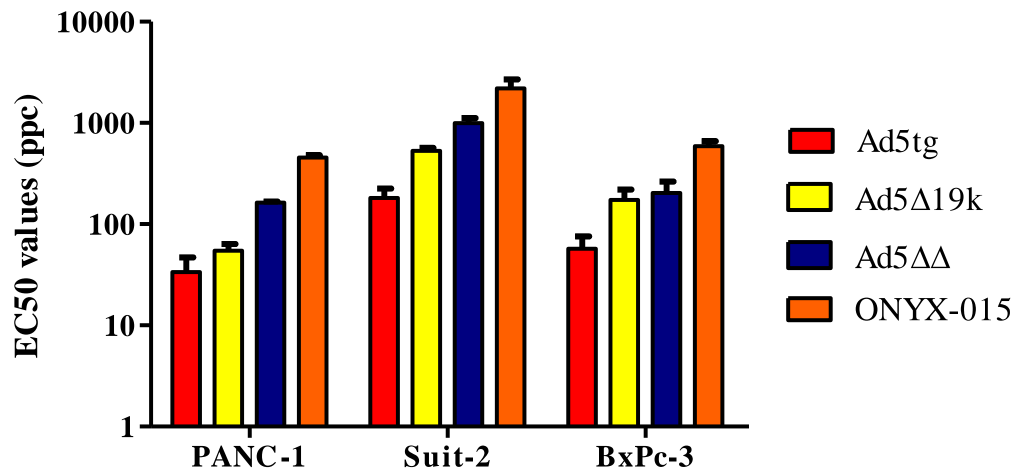

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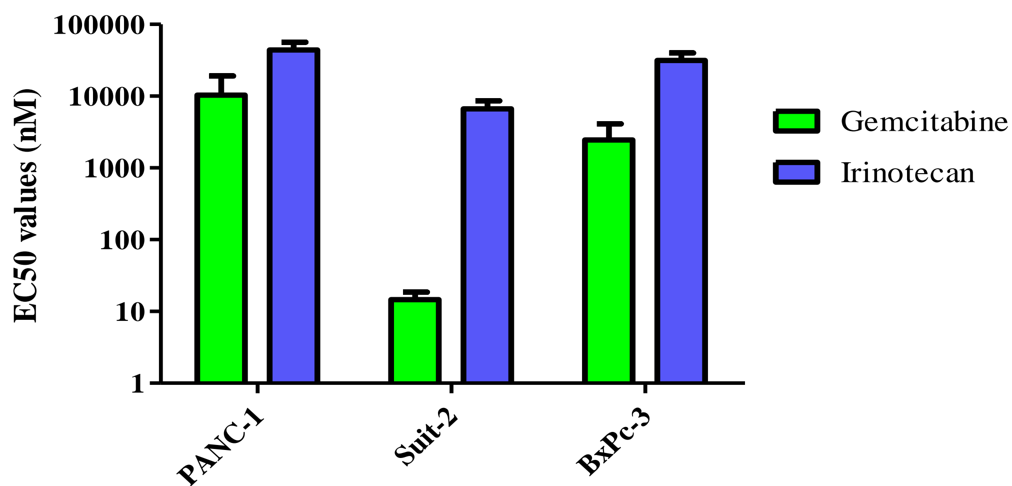

Fig. 8: Sensitivity to viral mutants and chemotherapeutics in pancreatic cell lines PANC-1, Suit-2 and BxPc-3.

All infections and treatments were done in triplicates and repeated at least twice; data represent average EC_{50} values ± SD. **A:** Single treatment dose-response cytotoxicity assays using wild type Ad5tg and viral mutants Ad5Δ19K, Ad5ΔΔ and ONYX-015. **B:** Dose response assays using serial dilutions of chemotherapeutic drugs Gemcitabine and Irinotecan.

Combination of viral mutants and chemodrugs in pancreatic cancer cell lines PANC-1, Suit-2 and BxPc-3

Once the dose response relationship of viruses and drugs had been established in each cell line, combination treatment cytotoxicity assays using both viral mutants and cytotoxic drugs were performed. This was done in order to answer the third question of the project: Would the double deleted Ad5ΔΔ retain the ability of its single deleted counterpart Ad5Δ19K to sensitise pancreatic cancer cells to chemotherapeutics? Combining viral mutants with cytotoxic drugs could be an effective way to increase the potency of the virus and/or the drug. If Ad5ΔΔ showed an ability to sensitise pancreatic cancer cells to cytotoxic drugs, combination therapies could prove to be a way of optimising the therapeutic effects in future clinical settings. By combining viral and drug treatment, the doses of both components can potentially be lowered, decreasing side effects.

Because it is the drug of choice in the treatment of pancreatic cancer, Gemcitabine was the drug primarily chosen for the combination studies. However, many pancreatic tumours often prove insensitive or resistant to the drug (Akada *et al*, 2005). We therefore included a second drug, Irinotecan, which is also currently used in the treatment of pancreatic cancer, but only in combination with Gemcitabine. As described in the introduction, both drugs inhibit DNA replication, but target different mechanisms.

In previous work by Dr. Cherubini, the double deleted mutant Ad5ΔΔ was found to sensitise pancreatic cancer cell line PT45 to Gemcitabine, achieving greatly increased levels of cell killing (unpublished data). The Ad5Δ19K mutant has also shown ability to significantly enhance Gemcitabine-induced cell death in PT45 and Suit-2 cells (Leitner *et al*, 2009). It was therefore tested if the combination of Ad5ΔΔ and chemodrugs could lead to sensitisation in a broader panel of pancreatic cancer cell lines (PANC-1, Suit-2 and BxPc-3).

In order to evaluate if sensitisation between two treatments occurs, the level of cell death induced by each component alone had to be low, otherwise any effects of the combination might have been masked. Drug concentrations inducing levels of 20% and 30% cell death as single treatment were used in combination treatment assays. For each experiment, the chemodrug doses were chosen from the single treatment dose response curves generated for both drugs in that particular cell line. The chosen doses for combination treatments never induced more than 35% cell death in any of the cell lines tested.

Combination of viral mutants and Gemcitabine shows dose-dependent antagonism in PANC-1 cells

From the single treatment dose response experiments performed in PANC-1 cells, it was concluded that the cells were highly insensitive to Gemcitabine with average EC_{50} values of 10.4 µM (± 8.8 µM) (Fig. 8B). The Gemcitabine concentrations for the initial combination treatment assay were chosen from the dose response data to 100nM and 200nM. The two doses induced as single treatments only 24% and 35% cell death respectively. In combination with Ad5tg and viral mutant Ad5Δ19K, high levels of antagonism was observed, with over a 150-fold increase in EC_{50} values for Ad5tg and almost a 100-fold in Ad5Δ19K (Fig. 9). The experiment was repeated with decreasing doses of Gemcitabine; in total seven different concentrations were tested, ranging from 5nM to 200nM. No cell death was observed by drug alone at doses < 100nM. The double deleted mutant Ad5ΔΔ was tested with a smaller range of drug doses (5nM, 10nM and 100nM) and showed dose dependent antagonism in a similar fashion to Ad5Δ19K (Fig. 9). Because both mutants showed the same behaviour in PANC-1 cells, the full range of drug concentrations was only tested on Ad5Δ19K and Ad5tg.

Interestingly, the level of antagonism when Ad5tg was used in the combination treatment was consistently higher than with both deletion mutants.

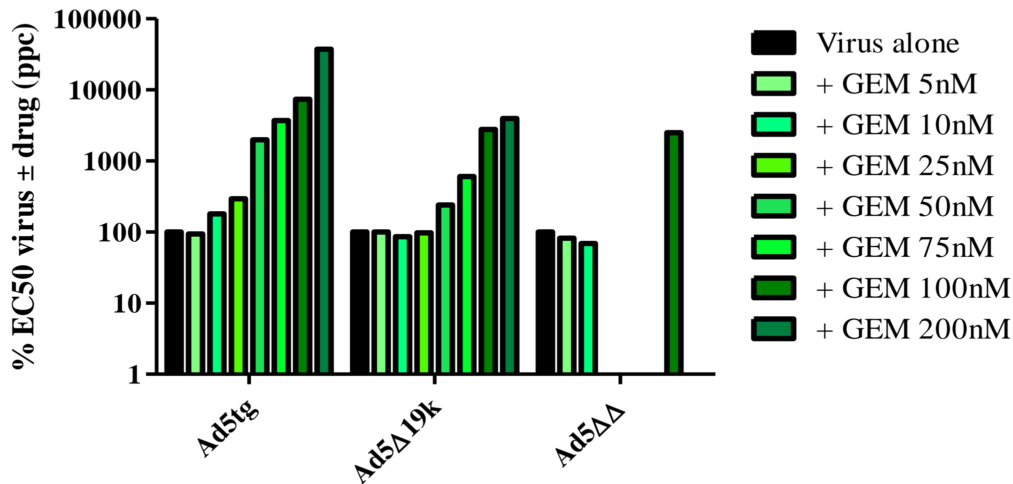

Fig. 9: Combination treatments with Gemcitabine (GEM) in PANC-1 cells.

Cytotoxicity assays performed with serial dilutions of viral mutants in combination with fixed, suboptimal doses of drug. Cells were infected and treated simultaneously with virus and drug 24 hrs after seeding (5000 cells/well). The analysis was performed 72 hrs after infection. Drug doses ranged from 5nM to 200nM, drug induced cell death never exceeded 35%. Infections were done in triplicates; for each combination one experiment was performed. Results are displayed as percentages of cell death induced by the virus alone. Values higher than that of single treatment imply antagonism; lower values suggest sensitisation to the drug by the virus.

No effect of combining viral mutants with Irinotecan in PANC-1 cells

Following the observation that cytotoxicity was decreased when combining Gemcitabine with viral therapy, we wanted to see if the antagonistic effect was present when using Irinotecan. Combination treatments with suboptimal doses of Irinotecan and serial dilutions of viral mutants were therefore performed. The drug doses were chosen from single treatment dose response curves, with the intention to use drug concentrations that produced around 20% and 30% cell death. 5µM and 10µM were chosen to be the most suitable for combination treatment, inducing on average < 25% cell death alone. Repeats were performed for experiments with Ad5tg and Ad5ΔΔ but not for Ad5Δ19K. No statistical analysis could subsequently be performed on the data for Ad5Δ19K.

The EC_{50} values of the combination treatments were on average marginally lower than the value of virus alone with all mutants tested, except for Ad5tg combined with 5µM Irinotecan (Fig. 10). However, no statistically significant difference could be observed when comparing combination treatments with virotherapy (Ad5tg and Ad5ΔΔ) alone. The results suggest that combining viruses with Irinotecan neither increases nor counteracts cytotoxicity in PANC-1 cells. However, additional experiments with improved reproducibility are necessary to clearly determine the response to combination treatments using Irinotecan.

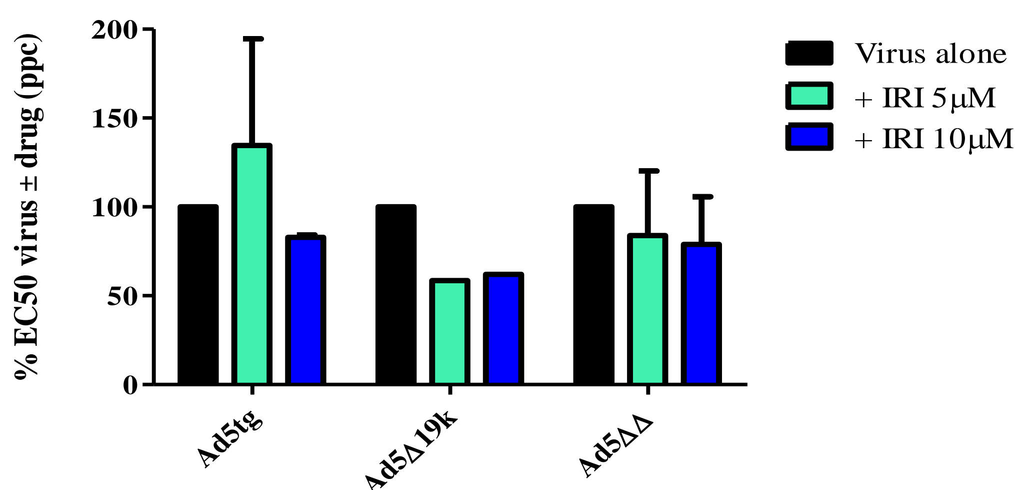

Fig. 10: Combination treatments with Irinotecan (IRI) in PANC-1 cells.

Cytotoxicity assays performed with combination of virus and with two fixed, suboptimal doses of drug. Cells were infected and treated simultaneously with virus and drug 24 hrs after seeding (5000 cells/well). The analysis was performed 72 hrs after infection. Drug induced cell death never exceeded 25%. Infections were done in triplicates; combination treatments using Ad5tg and Ad5ΔΔ were performed twice. Results are displayed as average percentages of cell death induced by the virus alone ± SD.

Viral mutants show sensitisation to Gemcitabine and Irinotecan in Suit-2 cells

Following the results in kRas independent PANC-1, combination treatment cytotoxicity assays were performed on kRas dependent Suit-2 cells with two fixed doses of Gemcitabine and Irinotecan (Fig. 11). The drug doses chosen from dose response data were doses of low cytotoxic effect; the average percentage of cell death induced by the drug alone were 18% and 20% for 5nM and 6nM of Gemcitabine respectively and 10% and 35% for Irinotecan 1.5μM and 3μM respectively.

All viruses showed ability to sensitise Suit-2 cells to both Gemcitabine and Irinotecan, including the wildtype Ad5tg (Fig. 9). Because none of the chemodrug doses induced substantial levels of cell death on their own, the effects of the combinations of viral and drug treatment were due to sensitisation. Statistically significant decrease in EC_{50} values was observed in all viruses when combined with Gemcitabine (* $p < 0.05$); especially when using Ad5Δ19K and Ad5ΔΔ. No significant increase in cell death was reached in combination with the lowest Irinotecan dose (1.5μM) but with the higher one (3μM). This was only observed for Ad5tg, Ad5Δ19K and Ad5ΔΔ but not for ONYX-015. The latter achieved no sensitisation of Suit-2 cells to Irinotecan. Overall, the sensitisation effect appeared to be dose dependent, with increasing drug concentrations leading to a proportional decrease in EC_{50} values, and Ad5ΔΔ in combination with both chemodrugs achieved the highest levels of sensitisation.

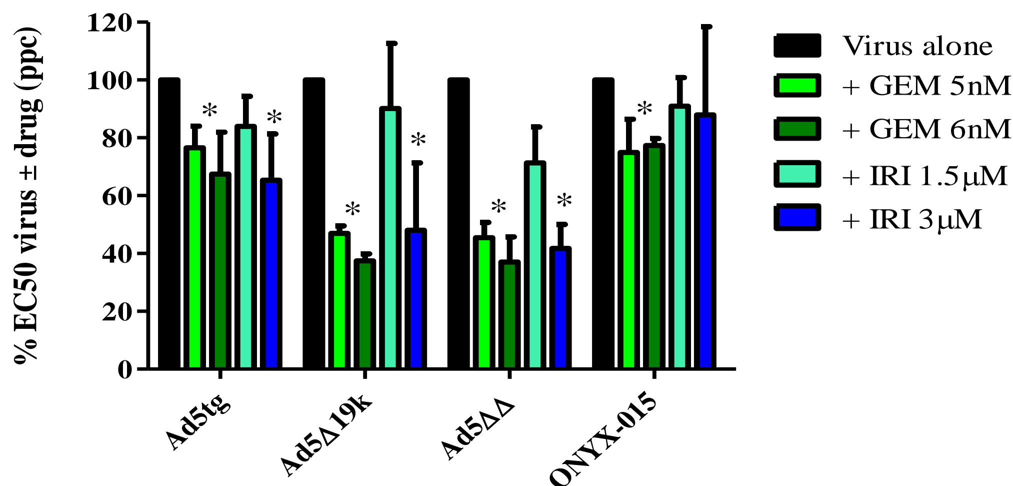

Fig. 11: Viral mutants show sensitisation to chemotherapy in Suit-2 cells.

Combination treatment cytotoxicity assays using serial dilutions of virus and fixed, suboptimal concentrations of Gemcitabine (GEM) and Irinotecan (IRI). Cells were infected 24 hrs after seeding (5000 cells/well) and plates were read 72hrs after infection. Infections and drug treatments were done simultaneously. All infections were done in triplicates and the experiment repeated up to three times for all mutants. Data represent EC_{50} averages ± SD; * $p < 0.05$.

Antagonistic effect of combining viral mutants with Gemcitabine in BxPc-3 cells

Once the effect of combination therapy had been established in the kRas mutated cell lines PANC-1 and Suit-2, a pancreatic cancer cell line with the kRas wild type was tested in similar combination assays. Combinations with Gemcitabine and viral mutants were tested first. After testing several doses of Gemcitabine, two suboptimal drug doses were chosen from single treatment dose response curves: Gemcitabine 20nM and 30nM producing on average 6% and 10% cell death respectively as single treatments.

In the combination treatment assay of BxPc-3 cells a slight tendency towards antagonism between Gemcitabine and the viral mutants was observed (Fig. 12). Combination with the higher drug dose (30nM) showed significant increases in EC_{50} values of all viruses (* $p < 0.05$). Similar to the results in PANC-1, Ad5tg showed the most prominent antagonistic effect in combination with the drug, whereas Ad5ΔΔ and Ad5Δ19k induced the smallest increase in EC_{50} values.

No effect of combining viral mutants with Irinotecan in BxPc-3 cells

In view of the fact that no sensitisation was observed in combination treatments of BxPc-3 cells with Gemcitabine, but rather antagonism, cytotoxicity assays were repeated using Irinotecan. From dose response data drug doses of low toxicity were chosen to 6μM and 8μM. As single treatments these concentrations induced on average 16% and 20% cell death respectively.

All viral mutants, except ONYX-015, showed a trend towards sensitisation when combined with Irinotecan (Fig. 12). However, no statistically significance of the decrease in EC_{50} values could be established. The results of the Irinotecan combination assay as well as the experiments performed with Gemcitabine, show similar behaviour in BxPc-3 cells as seen in PANC-1 cells.

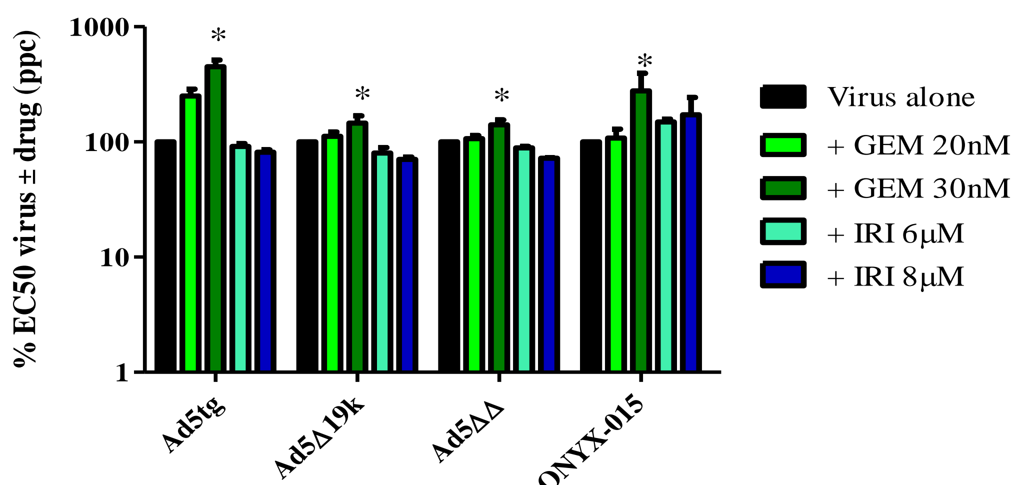

Fig. 12: Combination treatments with viral mutants and chemodrugs Gemcitabine (GEM) and Irinotecan (IRI) in BxPc-3 cells.

Average EC_{50} values of the combination assays expressed as percentage of cell death induced by virus alone. All infections were done in triplicates and repeated 2-5 times. Data represent average EC_{50} values ± SD, * $p < 0.05$. Viral mutants show antagonism when combined with Gemcitabine in BxPc-3 cells; no statistically significant effect was detected in combination assays using Irinotecan.

DISCUSSION

From the results of this project it was concluded that Ad5ΔΔ replicates efficiently in pancreatic cancer cells and that it has the potential to sensitise some cells to chemotherapy. However, sensitisation to Gemcitabine or Irinotecan seemed only possible in cell lines that already possessed some sensitivity to the drugs. Cells like PANC-1, and to a certain degree BxPc-3, that were insensitive to cytotoxic chemicals could not be sensitised by any of the viral mutants tested. Instead, dose dependent antagonism was observed in these cells. In cells that could be sensitised, i.e. Suit-2, Ad5ΔΔ produced the highest level of sensitisation together with Ad5Δ19K. However, in drug resistant cell lines, Ad5ΔΔ induced the lowest level of antagonism. True sensitisation to Irinotecan as well as Gemcitabine was only achieved in Suit-2 cells when combined with the action of Ad5ΔΔ. In the other two cell lines, combination with Irinotecan only produced a trend towards sensitisation but no statistically significant change to the virus alone. Gemcitabine, however, showed significant antagonism to the virotherapy. These two findings should be taken into account in future work; before Ad5ΔΔ can be used in combination with chemotherapy in clinical trials, more research has to be done to explore and elucidate the properties of the cancer cells potentially responsible for antagonism when combined with viral therapy.

One difficulty with the combination treatment cytotoxicity assays, especially in Suit-2 and BxPc-3, was to choose doses of low cytotoxicity from the dose response curves. The curves had in general a very steep incline and an uneven distribution of values with the majority around 100% and 0% cell death. This left very few data points in the middle range from which the doses generally were chosen. In this area of the dose response curve, marginal increases of the drug doses resulted in large changes in cell death observed. In the first MTS assay performed in BxPc-3 the dose response curve indicated that doses of around 50nM and 150nM would result in only 20%-30% cell death. When the combination treatment was performed using doses of 55nM and 150nM, the cell death by drug alone exceeded 50% and 70% respectively and the data could not be used. It is unclear what causes this behaviour in the cells. It may be due to changes in metabolism or environmental factors. It may also be due to differences in distributing the drug to the cells in single and combination treatments.

In the data from the viral replication assays, some discrepancy between the PANC-1 $TCID_{50}$ and qPCR results was noticed. According to the qPCR data, replication of Ad5ΔΔ exceeded that of the wt control whereas the $TCID_{50}$ results showed the opposite. A possible explanation for this phenomenon may be weakening of the virus due to repeated freeze-thawing during the $TCID_{50}$ assay. The process of freeze-thawing, done in order to release intracellular viral particles, constitutes a physical stress to the virus and can impair its infectivity (Dr. D. Öberg, personal communication). As the first $TCID_{50}$ assay using PANC-1 samples had to be performed twice, the repeated stress to the virus may have inhibited the viral infectivity. It is however remarkable that no decrease was seen in the effect of the wt control. This may suggest that Ad5ΔΔ is more sensitive to physical stress than its wt counterpart, potentially due to its double deletions.

One possible explanation for the antagonism to Gemcitabine is its inhibition of viral replication. Gemcitabine is a nucleotide analogue, blocking DNA-synthesis through premature chain termination. It is plausible that Gemcitabine is incorporated into the viral genome, inhibiting further replication of virus. Once the viral replication is blocked, the cytotoxic ability of the virus is lost as it relies on lysis by progeny virions to kill the cell. In accordance with this theory, higher levels of replication would also lead to higher levels of antagonism, something which was observed in PANC-1 cells. The suggestion that

Gemcitabine inhibits viral replication is supported by the work of Leitner *et al* (2009) and Raki *et al* (2005). Both groups showed Gemcitabine-induced delay of adenoviral replication in pancreatic and ovarian cancer cells respectively. Surprisingly, in both studies, the viral mutants retained ability to sensitise cells despite decrease in replication. To investigate if Gemcitabine has an inhibiting effect on viral replication in the pancreatic cancer cell lines used in this project, the replication studies need to be repeated in the presence of the drug.

It can prove harder to explain what property of the cell lines may contribute to the observed antagonism between chemotherapy and virotherapy. No pattern connected to kRas status of the cells was observed. As Gemcitabine is converted to an active metabolite by cellular kinases, differences in metabolic activity and enzyme systems between the cell lines could potentially explain the variation in drug sensitivity. Further studies analysing the pathways responsible for metabolism of Gemcitabine could be undertaken to investigate the matter.

In previous work on NHBE cells, Dr. Cherubini (manuscript in preparation) found that the Ad5ΔΔ was not cytotoxic to non-cancerous cells and could not replicate in normal cells. The data from this work in combination with previous work on PT45 cells (Leitner *et al*, 2009; Dr. Cherubini, personal communication) also suggest that the viral mutant is sufficiently cytotoxic to pancreatic cancer cells. It would nonetheless be beneficial, in future studies, to include additional pancreatic cancer cells lines with other mutations. The cell lines used in this study and previous work within the lab have, according to Akada *et al* (2005), been classified as sensitive (PT45, Suit-2) and intermediate sensitive (PANC-1, BxPc-3). It would be interesting to include a resistant cell line in future work with Ad5ΔΔ. By screening cells of all categories of drug sensitivity, more detailed information can be gathered about the behaviour of the virus in different cells. Through increased knowledge, the experimental setting can be tailored to resemble a clinical situation better.

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