Enhanced Cellular Uptake of Virus by Increased Expression Levels of the Coxsackie- and Adenovirus Receptor

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ABSTRACT

Replication–selective oncolytic adenoviruses have shown great promise as novel anti-cancer agents. These mutant viruses act through different mechanisms than traditional anti-cancer therapies and consequently do not develop cross-resistance with these drugs. A limiting factor for success with cancer treatment using oncolytic adenoviruses is the delivery and entry of virus to the correct target cells. An important step in adenoviral adsorption to host cells is the interaction between cellular CAR and viral fibre domains. Reports have indicated a down-regulation of CAR- levels in various cancer cell lines and clinical specimens. Enhancing the expression of CAR could potentially increase the efficacy of treatment with oncolytic adenoviruses. In this project, prostate cancer cell lines have been evaluated for their sensitivity to adenoviral infection, with and without treatment with the MEK-inhibitors PD98059, U0126 and the histone deacetylase inhibitor Trichostatin A. Trichostatin A was found to be a potent enhancer of sensitivity to viral-induced cytopathic effect in prostate cancer cell lines. Furthermore, prostate cancer cell lines have been evaluated for their surface expression of CAR, uptake of adenovirus and adenoviral replication with and without treatment with Trichostatin A. Trichostatin A was found to enhance CAR- expression and adenoviral uptake in some prostate cancer cell lines but did not have any significant effects on adenoviral replication. In conclusion, Trichostatin A could be used to improve the effects of oncolytic adenoviruses in treatment of prostate cancer through enhanced expression of CAR.

SVENSK SAMMANFATTNING

Onkolytiska virus är en ny behandlingsmetod för cancer, som utnyttjar helt andra verkningsmekanismer än gängse läkemedel och behandlingsformer. Adenovirus är små DNA-virus som infekterar epitelceller, replikerar, spränger sin värdcell och sprider sig i vävnaden. Onkolytiska adenovirus är modifierade på sådant sätt att de selektivt angriper tumörceller och lämnar friska celler oskadda. För att adherera till värdcellerna binder adenovirus med sitt ”fibre”-protein till CAR (Coxsackie-adenovirus receptor) och tar sig sedan in genom cellmembranet genom att utnyttja integriner på cellytan. Mycket litet är känt om CA-receptorns fysiologiska funktioner, den tros vara en celladhesionsmolekyl, men kan också vara inblandad i kommunikation celler emellan. Mängden CAR varierar mellan olika celltyper och är genomgående nedreglerad i flera typer av avancerade tumörer. Att kunna uppreglera CAR skulle potentiellt kunna förbättra cancerbehandling med onkolytiska adenovirus. I detta projekt har cellinjer från prostatatumörer screenats för känslighet mot adenovirusinfektion, med och utan behandling av MEK-hämmarna PD98059, U0126 och histone deacetylase-hämmaren Trichostatin A. Trichostatin A visades sig kunna förstärka känsligheten mot virus hos de använda cellinjerna. Därefter undersöktes samma cellinjer för att se om deras uttryck av CAR på cellytan, upptag av virus och virusreplikation förändrades efter behandling med Trichostatin A. Efter behandling ökade både uttrycket av CAR och upptaget av virus i de flesta cellinjer, däremot sågs inga förändringar av virusreplikationen. Sammantaget visade denna studie att Trichostatin A skulle kunna användas för att förbättra behandling av prostatacancer med onkolytiska adenovirus genom att öka uttrycket av CAR hos värdcellerna.

ABBREVIATIONS

Ad5	adenovirus serotype 5
CAR	Coxsackie and Adenovirus receptor
DAB	diaminobenzidine
DMSO	dimethyl-sulphoxide
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence- activated cell scanning
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
g	gram
GFP	green fluorescent protein
HDAC	histone deacetylase
H_2O_2	hydrogen peroxide
M	molar
MEK	mitogen activated protein kinase kinase
μ	micro- (suffix)
m	milli- (suffix)
μl	microlitre
MTS	3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) 2H-tetrazolium, inner salt)
RGD	arginine- glycine- aspartic acid
PBS	phosphate buffered saline
pH	$-\log[H^+]$
PI	propidium iodide
PMS	phenazine methosulphate
PrEC	prostate epithelial cells
PSA	prostate specific antigen
TCID50	tissue culture infectious dose 50%

INTRODUCTION

Treatment of tumours, particularly advanced and metastatic cancers, still proves to be a daunting task for the clinician. Problems of late diagnosis, drug resistance and a low therapeutic index, exert pressure on scientists to find mechanisms responsible for tumour growth and test novel approaches to therapy. The rapidly expanding knowledge of molecular biology and the discovery of new techniques have enabled scientists to develop targeted therapies, utilizing viruses as anticancer agents.

For a new cancer treatment to be effective in a clinical setting, it is of importance that tumour cells are selectively killed, and normal cells are left unharmed (Hawkins and Kirn, 2002). The treatment should have a novel mechanism of action, avoiding interference or cross-resistance with existing therapies (Hawkins and Kirn, 2002). The new treatment should be safe for the patient and complement existing therapies. Novel viral therapies have shown to be synergistic with current ones (Heise et al, 1997). Over the last ten years, gene therapy and replication selective oncolytic viruses have emerged, and may fulfil these criteria. Although setbacks (Stephensson et al, 2001) have dampened the initial enthusiasm, some of these methods provide a novel approach to cancer therapy (Bauerschmitz GJ et al., 2002).

1.1 Viruses in gene therapy and as oncolytic agents

Viruses have been used to create novel cancer therapeutics in two different ways, as vectors for gene therapy and as oncolytic agents (Vorburger SA and Hunt KK, 2002). When used as a vector for cancer gene therapy, the virus is the transport vessel that may deliver genes replacing defective tumour-suppressor genes, genes encoding prodrug- converting enzymes or genes with immuno-stimulatory functions (Russell WC, 2000). Cancer is the most common disease targeted with gene therapy (http://www.wiley.co.uk/genmed/clinical/). In parallel with the use of adenoviruses, retroviruses, adeno-associated viruses and others, non- viral delivery systems have also been developed (Bauerschmitz GJ et al., 2002). An adenovirus delivering the tumour suppressor gene *p53*, has recently been approved as a drug in China (Pearson et al, 2004).

The research conducted on viruses as vectors for gene therapy has promoted increased knowledge in the properties of viruses making them suitable also as oncolytic agents. Conditionally replicating oncolytic viruses are modified in such a way that viral replication and the subsequent death of the host cell is possible only in tumour cells (Alemany et al, 2000). Deletion of entire viral genes or functional gene elements, insertion of tissue specific promoters, viral coat modifications and use of inherently tumour selective viruses can all result in tumour specific replication (Hawkins and Kirn, 2002). Adeno, herpes, vaccinia, reo, polio and vesicular stomatitis viruses have all been tested using one or more of these approaches (Hawkins and Kirn, 2002). Oncolytic adenoviruses are being evaluated in clinical trials, and some have shown promising results in phase I and II (Vorburger SA and Hunt KK, 2002). An oncolytic adenovirus is currently under evaluation in phase III in China (Pearson et al 2004). So which are the

common denominators between the viruses and tumour cells that allow the construction of tumour selective viruses? Adenoviruses will serve as a specific example below.

1.2 Adenoviruses and their properties, suitability as oncolytic agents and different adenoviral mutants

The adenoviruses are a family of viruses with double-stranded linear DNA enclosed in an icosahedral capsid (Shenk, 2001). The protein capsid consists of three main subunits, 240 hexons, 12 pentons and fibre proteins, but also several other proteins (Russel, 2000; Shenk, 2001). The hexons and pentons together form the protein coat around the viral DNA, with the fibre proteins projecting from the penton bases (Shenk, 2001). The human adenoviruses cause several different clinical syndromes, most commonly acute febrile respiratory disease but also conjunctivitis and gasteroenteritis (Shenk, 2001). Viral serotype is determined through different antisera's ability to neutralise hexon and fibre protein. There are 49 serotypes of human adenoviruses currently described (Shenk, 2001).

In order for the virus to replicate DNA it has to enter a living cell and hijack the replicative machinery for DNA-replication and assembly of new viral particles. The adenoviral lifecycle consists of several, partially overlapping, steps resulting in the production of new virions and the lysis of host cells (Russell, 2000).

Infection is initiated by virus attachment to receptors (coxsackie and adenovirus receptor (CAR) and $\alpha v\beta 3/\alpha v\beta 5$ integrins) on the host cell membrane (Shenk, 2001). The receptor-virus complexes move on the cell surface to clathrin-coated pits and are internalised through receptor-mediated endocytosis (Shenk, 2001; Russel, 2000; Wang et al 1998). Disassembly of the virion is initiated, the virus escapes the endosome (Shenk, 2001), is transported to the nuclear membrane and the viral DNA enters the nucleus (Russell, 2000).

Immediately following host cell infection, early viral genes are transcribed, starting with the E1A gene (Shenk, 2001). The E1A gene products have several functions: they activate the transcription of the other viral genes and facilitate viral DNA replication by the induction of S-phase (Ben- Israel, 2002). This modulation of the cell cycle is achieved through the binding of E1A-proteins to host cell proteins involved in cell cycle regulation, the most important of these being the tumour suppressor protein pRb (Ben-Israel, 2002). Deregulation of the pRB checkpoint is mediated through the binding of E1A to pRB- causing release of the transcription factor E2F, affecting the transcription of genes regulating the cell cycle (Nevins, 1992). E1A proteins will also induce apoptosis in host cells through their cell cycle regulatory actions (Teodoro, 1995). Different E1B-proteins will counteract this induction of apoptosis through interaction with p53 and bcl-2 (Shenk 2002). The virus initiates and proceeds with replication of viral DNA, trying to evade the immune response of the host through the expression of E3B genes and finally transcribe and export late viral mRNAs to the cytosol (Shenk, 2001). The mRNAs have to be translated into proteins and the viral genome has to be packaged into the new capsid (Shenk, 2001). Virions accumulate and the host cell rupture cause spread of progeny virus (Shenk, 2001).

These processes are regulated by the transcription of viral genes, and executed by their different gene products (Russell 2002).

Using adenoviruses in an experimental setting is favourable for several reasons. The viral genome, replicative cycle and interaction with the host cell are well known and can be manipulated. It is possible to achieve a high–titer production in vitro (Shenk, 2001). The adenoviruses' relative genetic stability and the mild clinical symptoms of wild type infection improve the safety (Hawkins and Kirn, 2002). Wide tropism for different cell types, ability to induce S-phase in quiescent cells and lyse cells at the end of the replicative cycle, are all factors that contribute to the benefits of adenoviruses as oncolytic agents (Russell, 2000)

There are a number of ways in which adenoviruses can be modified to achieve tumour specificity. Functional deficiencies of p53 are common in the majority of cancers (Sherr, 1996) and this principle was used to create a viral mutant (dl1520) targeting cells lacking p53 (Bischoff JR et al., 1996). This was achieved by deleting the E1B 55K gene, which normally inhibits p53 function, so that viral replication was possible only in p53 deficient cells and not in normal cells with intact p53 (Bischoff JR et al., 1996). Another example is the viral mutant dl922-947, with deletion of amino- acids in the E1A region critical for pRb- binding, resulting in a virus that can only replicate in cells with already disrupted G1-S checkpoint (Heise C et al., 2000). This is common since mutations in the pRB-p16 pathway are present in most cancers (Sherr, 1996).

Transcriptional targeting is another way of achieving tumour specificity. As transcription of the viral gene E1A controls all subsequent viral activity in the host cell, tissue specific promoters can be created to limit the E1A expression (Hallenbeck et al, 1999; Rodriguez et al, 1997; Yu et al, 1999A; Yu et al 1999B).

1.3 Prostate cancer as a target for adenoviral therapy

Prostate cancer is the third most common type of cancer in men (Deutsch et al, 2004). Early stages of prostate cancer can be managed with radiotherapy, surgery or chemotherapy, although these treatments are connected to considerable risks (Herman et al, 1999). Recurring, advanced and metastatic cancers can initially be kept under control with androgen ablation therapy, but will eventually develop to androgen-independent tumours (Deutsch et al, 2004). For androgen independent stages no effective therapy is available (Deutsch et al, 2004).

Prostate cancer is a popular target disease for development of different treatments involving gene therapy or oncolytic viruses. Delivery of the HSV- *tk*- gene (transcribing the prodrug-converting enzyme thymidine kinase) in an adenoviral vector, and subsequent administration of the prodrug ganclovir, to patients with recurrent prostate cancer showed to be relatively safe and had some clinical effect in a phase I trial (Herman et al, 1999).

Prostate specific, replication competent adenoviruses have been created using different promoters driving viral replication (Rodriguez et al, 1997; Yu et al, 1999A; Yu et al 1999B). CN706 was made prostate specific through the insertion of parts of the human promoter of the PSA- gene to control the transcription of

E1A (Rodriguez et al, 1997). This virus was shown to selectively replicate in PSA expressing tumour cells (Rodriguez et al, 1997) and a phase I clinical trial has been performed (De Weese et al, 2001). CV 764 is another prostate specific oncolytic adenovirus, combining a PSA-promoter driving the expression of E1A and a promoter/enhancer region of the human kallikrein 2-gene (another prostate cancer marker) driving the expression of E1B (Yu et al 1999A). Finally, CV 787, with E1A expression controlled by the prostate specific rat probasin promoter, E1B controlled by the human promoter of the PSA- gene, and an intact E3 region, was 10000-fold more effective in PSA-positive cells compared to PSA- negative cells (Yu et al, 1999B). CV787 was effective in *in vivo* models and could infect also androgen-ablated cells, suggesting that treatment with CV787 could be combined with androgen ablation therapy (Yu et al, 1999B).

1.4 The coxsackie- and adenovirus receptor as a limiting factor in successful virotherapy

Since no oncolytic adenovirus (or adenoviral vector) will function properly if it is not delivered to the site of action, much effort has been made to characterise the events leading to attachment of virus and entry into the host cells. A 46 kD cell surface protein, the coxsackie and adenovirus receptor (CAR) has been isolated as the receptor for human adenoviruses subgroup C and the group B coxsackieviruses, (Bergelsson et al, 1997; Tomko et al, 1997). It comprises an extracellular immunoglobulin-like domain, a transmembrane part and an intracellular domain, and is a member of the immunoglobulin superfamily (Bergelsson et al, 1999, Tomko et al 1999). Cells insensitive to adenoviral infection could, if transfected with cDNA of CAR, take up Ad5 (Tomko et al, 1997). The cellular CAR interacts with the knob- domain of the adenoviral fibre protein. A region on the fibre protein has shown high degrees of homology between different adenoviral subgroups and an altered ability to mediate adenoviral infection when mutated, implicating this region as the site of interaction between the virus and CAR (Roelvink et al 1999). The CAR- fibre binding is believed to facilitate the interaction between RGD-motifs on the penton base and cell surface integrins (types $\alpha v\beta 3/\alpha v\beta 5$) leading to internalisation of virus (Wickham T, 1993). It seems that the CAR-fibre interaction is responsible for the viral attachment whereas the integrins are essential for the internalisation of virus (Wickham T, 1993). Supporting this theory is the fact that although the transmembrane and cytoplasmatic domains of CAR were deleted, the extra cellular domain can still support viral attachment but not internalisation (Wang and Bergelson, 1999)

The possibility to confer tumour selectivity in adenoviruses by means of transductional targeting has been explored in a number of publications (Dmitriev I et al., 1998; Roelvink PW et al., 1999; Wickham TJ et al., 1997; Wickham TJ et al., 1996a; Wickham TJ et al., 1996b; Michael SI et al., 1995). Alterations of different parts of the CAR-binding fibre can both block the binding to CAR, and redirect the virus to other cellular receptors. Not only can this be used to enhance specificity, but has also been evaluated for their potential to reduce the uptake in non-target organs (Martin et al, 2003, Nakamura 2003). Bispecific antibodies (Korn et al, 2004) and soluble CAR-ligand systems (Dmitriev, 2000) are yet other applications used to create tumour specificity.

The physiological functions of CAR are largely unknown. It has been suggested that CAR is a component of tight junctions (Cohen et al 2001). CAR has been shown to aggregate at tight junctions and associate with the tight junction protein ZO-1 (Cohen et al 2001). This CAR aggregation seems to be the result of interaction between CAR extracellular domains on adjacent cells, since deletion of transmembrane or cytoplasmatic domains did not affect this process (Cohen et al 2001). The aggregation of CAR at tight junctions also seems to impede viral infection (Cohen et al 2001).

CAR expression has been found down regulated in various tumour tissues and cell lines. The CAR mRNA levels in different bladder cancer cell lines vary and sensitivity to an adenoviral vector seems to correlate with levels of CAR-expression (Li et al, 1999A). The same applies for clinical specimens of bladder cancer (Okegawa et al 2001). Also prostate carcinoma cell lines (Okegawa et al 2000), paraffin-embedded prostate specimens (Rauen et al, 2002), melanoma (Hemmi et al, 1998), glioma (Miller et al, 1998), lung, pancreatic (Pearson 1999) and head and neck carcinoma cell lines (Li et al 1999 B) have been reported to show a similar pattern.

Not only does the degree of CAR-expression affect the probability of adenoviral infection, it seems that the level of CAR expression correlates with the degree of malignancy, less differentiated and malignant cells showing low levels of CAR (Rauen et al 1999, Okegawa et al 2000 and Okegawa et al 2001). This connection suggests an antitumoral biological mechanism of CAR (Okegawa et al 2000). Growth of PC3 (prostate carcinoma cells) transfected with CAR was inhibited in a dose dependent manner both *in vivo* and *in vitro* (Okegawa et al 2000). Both the extracellular and transmebrane domains were necessary for this tumour growth inhibition (Okegawa et al 2000). Also, expression of extracellular and transmebrane domains and a few amino acids of the cytoplasmatic domain of CAR in bladder cancer cells were required for growth inhibition (Okegawa et al 2001). It is suggested that CAR can function as a signalling protein, creating a cascade modulating cell growth (Okegawa et al 2001). The presence of functional CAR and slow growth were associated with high levels of the growth-inhibitory proteins p21 and phosphorylated pRB, which could be reversed administering an anti-CAR antibody (Okegawa et al 2001).

The similarities between the expression patterns of CAR and the tumour inhibiting cell adhesion and signalling glycoprotein E-cadherin in prostate carcinoma specimens is another indication of the anti-tumour effects of CAR (Rauen et al, 2002). As for E-cadherins, CAR is down regulated in initial stages of prostate cancer, only to reappear in metastatic disease (Rauen et al, 2002). The difference in CAR function between different cancer cell lines seems to be dependent on a differences in CAR- mRNA levels or post-translational modification rather than mutation of the CAR gene (Li et al 1999; Anders et al 2003).

1.5 MEK and HDAC inhibitors and their capacity to alter CAR-expression

The efficiency of treatment with oncolytic adenoviruses and adenoviral vectors is often low in target cells (Anders et al, 2003), possibly because the down-regulation of CAR. Different families of compounds have been evaluated as potential enhancers of adenoviral uptake or CAR expression (Anders et al 2003, Hemmiki et al 2003, Sachs et al 2004).

The signal transduction pathway Ras/Raf/MEK/ERK relays information from the cell surface to the nucleus, alters the degree of phosphorylation of transcription factors and participates in regulation of cell proliferation and differentiation and MEK- inhibitors have been evaluated as cancer drug (Sebolt- Leopold, 2004). The loss of expression of the cell adhesion molecules ZO-1 and E-cadherin in cancer seems to be correlated to oncogenic signalling via the Ras/Raf/MEK/ERK pathway and can be restored after treatment with MEK-inhibitors (Chen, 2000). The similarities between CAR and ZO-1 and E-cadherins suggested that MEK-inhibitors also could restore CAR expression. Indeed, both CAR-mRNA levels and cell surface expression could be increased in colorectal cancer cell lines, after treatment with the MEK-inhibitors U0126 and PD184352 (Anders et al, 2003). Interestingly, the localisation of CAR was also altered after MEK-inhibitor treatment, showing CAR-staining at cell-cell contacts rather than in the cytoplasm (Anders et al, 2003). Furthermore, CAR- mediated adenoviral uptake and cell killing by oncolytic adenoviruses was enhanced (Anders et al, 2003). Other investigators have stated an increase in adenovirus-mediated transgene expression when treating different ovarian cancer cell lines with the MEK- inhibitor PD98059 (Hemmiki et al, 2003).

HDAC (histone deacetylase)- inhibitors form another group of agents, which are reported to alter CAR-expression in cancer cells (Kitazono et al, 2001, Hemmiki et al, 2003, Sachs et al, 2004). HDAC-inhibitors promote acetylation of histones, resulting in uncoiling of DNA and transcription of parts of the genome (Rosato and Grant, 2003). Genes, with transcription at least partly regulated by the degree of histone acetylation, are often involved in control of cell cycle progression, differentiation or apoptosis (Rosato and Grant, 2003). The HDAC inhibitors have shown antiproliferative, apoptotic and differentiating effects in neoplastic cells, and are emerging as a novel type of cancer treatment (Rosato and Grant, 2003; Vigushin et al 2001). FR901228 is an HDAC- inhibitor shown to enhance both CAR- and integrin mRNA expression and adenovirus mediated transgene expression in different carcinoma cell lines (Kitazono et al, 2001). Trichostatin A (another, fungus-derived HDAC inhibitor) could both increase cell surface CAR levels, adenoviral uptake and transgene expression in ovarian cancer cell lines, but no significant change in CAR mRNA levels could be detected (Hemmiki et al, 2003). Recent data (Sachs et al, 2004) suggests that Trichostatin A-treatment can enhance both surface expression of CAR and the level of CAR mRNA in bladder cancer cell lines.

AIMS AND OBJECTIVES

The aim of this project is to determine whether viral potency can be enhanced by stimulation of CAR-expression in different prostate cancer cell lines.

The objectives are to:

- Screen different cell lines with viral mutants and Ad 5 wild- type, to identify a model system relatively insensitive to viral infection.
- Establish dose-response to several compounds known to enhance CAR-expression and select highest dose not having cytotoxic effect in vitro.
- Determine whether treatment of cells in vitro with compounds under the established conditions can increase viral infectivity.
- Monitor the changes in expression of CAR after treatment with compound.
- Monitor the changes in viral uptake after treatment with compound.
- Investigate whether treatment with compound can lead to an increase in viral replication.

MATERIAL AND METHODS

2.1 General tissue culture and cell lines

The human non small-cell lung carcinoma cell line H460, the HEK293 cells (human kidney cells transformed with adenovirus 5 E1A- gene), LNCaP (2003) and DU145 (human prostate carcinoma cell lines) were obtained from ATCC (VA, USA). LNCaP (2001) and PC3 (both human prostate carcinoma) and CMT64 (murine lung carcinoma) cell lines Cell Services, Cancer Research UK. These cell lines were maintained in DMEM- Dulbecco's Modification of Eagle's Media (Cell Services, Cancer Research UK) supplemented with 10% FCS- Fetal Calf Serum (Sigma-Aldrich Chemie, Germany) at 37 ° C and kept in a humidified atmosphere of 5% CO2. Cells used in the studies were of relatively low passage number (below passage number 25).
Normal prostate epithelial cells (PrEC) were purchased from Clonetics, USA and maintained according to instructions of the manufacturer.

2.2 Viruses and compounds

Features of viruses used in experiments were:
Ad5- wildtype adenovirus, serotype 5
dl 922-947- mutations in the pRB-binding domain of E1A(CR2), E3B-deleted
dl 312- E1A-E3B- deleted, replication incompetent
dl 337- missense mutation in E1B; E1B19K and E3B deleted.
dl 309- E3B deleted
AdGFP- replication- incompetent adenovirus expressing green fluorescent protein (GFP inserted in E1A- gene under control of CMV-promoter)

The MEK-inhibitors U0126 and PD 98059 and the HDAC inhibitor Trichostatin A (all from Calbiochem, US) were reconstituted in DMSO, and kept as aliquots at –20° C at a stock concentration of 5mM.

2.3. MTS assay for assessment of sensitivity to viral mutants

In order to determine the sensitivity to cytopathic effect induced by wildtype adenovirus (Ad5) and different deletion mutants in various cell lines the MTS (cell proliferation-cell killing) assay kit Cell Titre 96 Aqueous, One Solution Cell Proliferation Assay (Promega, WI, USA) was used.

The MTS assay is a method for determining the proportion of viable cells. MTS is a tetrazolium compound which, in the presence of metabolically active cells become converted by mitochondrial dehydrogenase enzymes, to a formazan product that is soluble in tissue culture medium. The degree of formation of this formazan product and resulting change in absorbance, as measured at 490 nm, is directly proportional to the metabolic activity of live cells. This value was used as the inverse measurement of the proportion of dead cells (Hallden et al, 2003).

The MTS-assay was performed according to previously described methods (Hallden et al , 2003). Briefly; cells grown in T175- flasks were trypsinated and counted, diluted to a concentration of 10^5 cells per ml and 100 µl of this cell

suspension was added to each well on 96-well plates, resulting in a final number of 10^4 cells per well. The plates were incubated for 24 hours at 37 ° C and kept in an atmosphere of 5% CO2. After 24 hours old media was removed and replaced with 90 µl DMEM containing 2% FCS in order to facilitate viral infection (Hallden et al 2003)

Virus aliquots were kept at –80° C. Immediately before use, the virus was thawed, kept on ice and diluted in 2% FCS media. A tenfold dilution series was prepared with viral concentrations starting at 10^{11} particles per ml. When 10 µl of this initial stock series was added to wells, it resulted in a tenfold fold lower viral concentration with a final volume of 100 µl per well. See below for complete dilution series.

Viral concentration, no	Particles per cell, 10^4 cells per well	Particles per well	Particles per ml in wells	Particles per ml in stock
V1	10^5	10^9	10^{10}	10^{11}
V2	10^4	10^8	10^9	10^{10}
V3	10^3	10^7	10^8	10^9
V4	10^2	10^6	10^7	10^8
V5	10^1	10^5	10^6	10^7
V6	1	10^4	10^5	10^6
V7	10^{-1}	10^3	10^4	10^5
V8	10^{-2}	10^2	10^3	10^4
V9	10^{-3}	10^1	10^2	10^3
V10	10^{-4}	1	10^1	10^2

Each viral concentration was assayed in triplicates. Untreated cells served as control for the number of total live cells and wells with media only served as blanks. Viral mutants investigated in this experiment were *Ad*5, *dl*922-947, *dl*337, *dl*309 and *dl*312. During the course of the experiment cell morphology and growth were assessed daily using light microscopy.
A typical assay layout could look like this:

Cell no	Cells only	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10
Cell no	Cells only	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10
Cell no	Cells only	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10
Cell no	Cells only										
Cell no	Cells only	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10
Cell no	Cells only	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10
Cell no	Cells only	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10
Cell no	Cells only										

Seven days after seeding of cells, assays were analysed using the MTS assay kit. MTS tetrazolium reagent and PMS electron coupling reagent were kept protected from light at –20° in aliquots according to the manufacturers instruction. The reagents were thawed just before use and diluted in serum free media to a concentration of 20% MTS, 1% PMS. Old media was removed and replaced with 100 µl of the above solution. This method is used because reports have stated that FCS can interfere with the assay and cause very high absorbance values for media alone. Plates were then returned to an atmosphere of 37° C and allowed to incubate for 2 hours (Hallden et al, 2003)

Absorbance was measured in an OpsysMR platereader Reader (Dynex Technologies Inc, Chantilly, US) at a wavelength of 490 nm and the optical density values were transferred to Microsoft Excel.

In order to determine the cell death at different viral concentrations the following formulas were used: **Live cells (z) =** (y-bl)/(c-bl)

Percentage of dead cells (w)= (1-z) x 100

Where z is the percentage of live cells in infected wells,
y is the optical density value for the infected well,
bl is the optical density for wells with media only
c is the optical density value for control wells containing cells only
(Hallden et al, 2003)

The cell death data was transferred to GraphPad Prism software to create graphs and calculate an EC_{50} value (a value of the viral concentration needed to kill 50% of cells) for each virus and each cell line.

2.4 Combination of compounds U0126, Trichostatin A or PD98059 and wildtype adenovirus in MTS assay

In these experiments cell lines H460, CMT64, LNCaP 2001, LNCap 2003, DU145 and PC3 were included. Cells were kept under similar conditions as previously described and seeded into 96-well plates at the same cell number (10^4 cells per well). 24 hours after seeding cells media was removed and replaced with 90 µl DMEM supplemented with 10% FCS per well.

The compounds U0126, Trichostatin A and PD98059 were diluted in DMEM containing 10% FCS, respectively, to final concentrations tenfold higher than the desired concentrations in wells. Each compound was tested at 2-3 different concentrations after an initial toxicity screening. 10 µl of these concentrated solutions were administered to wells resulting in final concentrations as shown in table below. Not all concentrations were analysed in all cell lines.

Final concentration of compounds tested:

Trichostatin A	1.0 µM	0.1µM	0.05µM
UO126	1.0 µM	0.1µM	-
PD98059	5.0 µM	0.5µM	-

48 hours after addition of compound, cells were infected with virus. Media containing compound was removed and replaced with 90µl DMEM including 2% FCS. Infection with Ad5 followed according to pattern described in 2.3. Each viral concentration in combination with each concentration of compound was assayed in triplicates. As well as blanks and controls as described for previous experiment, each concentration of virus only and compound only, served as additional controls to the experiment.

The experiment was terminated five days after addition of virus. Media was replaced with MTS reagent as described above, plates read in the OpsysMR platereader at 490 nm and subsequently analysed.

2.5 Flow cytometry staining for coxsackie and- adenovirus receptor (CAR)

An initial screening was performed to determine the normal expression of CAR in different cell lines, harvesting cells 72 h after seeding. When trying to determine CAR levels after treatment with Trichostatin A, media was exchanged 24 hours after seeding for DMEM 10% FCS containing 0.05-0.1 µM Trichostatin A.

Particular care was taken to create a single cell suspension-achieved by pipetting the cell suspension up and down several times after trypsin treatment and controlling that >90% of cells were single cells in the microscope. The suspension was then moved to 15 ml falcon-tubes and centrifuged for 4 minutes at a speed of 1000 rpm (ALC-centrifuge PK-130, T535) The supernatant was removed and the cells were resuspended in 1 ml of a flow cytometry buffer containing 2% FCS and 0.1% 0.5 mM EDTA pH 8.0 in PBS holding +4 C. From this step onwards samples were kept on ice. The same buffer was used throughout the experiment.

Samples were transferred to eppendorf tubes and centrifuged at 2000 rpm for 3 minutes (Sorwall Fresco, 3328, Hereaus). The original buffer was removed and replaced with 200 µl of buffer containing 5% normal rabbit serum (DAKO A/S Denmark) and 1/500 monoclonal mouse- anti-CAR antibody (RmcB, ATCC, US).

Samples were incubated on ice for 90 minutes and gently shaken every 20 minutes. Cells were again centrifuged at 2000 rpm for 3 minutes, antibody-containing supernatant removed. The samples were then resuspended in 200µl buffer and placed on ice for five minutes. This wash step was repeated three times.

After the last wash cells were incubated at room temperature for 30 minutes with 200µl secondary antibody (rabbit-antimouse-immunoglobulins-FITC (DAKO A/S Denmark)) 1/30 in flow cytometry buffer. Samples were again washed three times according to description above and finally suspended in 400µl of buffer containing 0.625µg/ml of propidium iodide.

Cytometric analysis ensued, using a FACSCalibur (BD Biosciences) cytometry machine, and data was analysed using CellQuestPro software. 10000 live cells were counted per sample for the initial CAR-screening and 25000 for the

Trichostatin- experiment. Live cells (negative for PI) were gated and a marker was set to include 1% of the right hand tail population of negative control cells. Different markers were set for all the different cell lines and these markers were applied in the analysis to determine the percentage of CAR- expressing cells in positive samples. Controls included in the Trichostatin-experiment were a) cells not treated with Trichostatin A and b) samples with the primary antibody omitted (for both treated and non-treated cells).

2.6 Immunocytochemistry staining for CAR.

Cells were seeded in chamberslides and allowed to attach over night. Wells were emptied from media and cells were washed with PBS holding +4 °C for 2x5 minutes. Cells were fixed in 4% formaldehyde for 30 min. at room temperature. Cell were washed twice in PBS and permeabilised with 0.2 % TritonX-100 in PBS. Cells were again washed twice in PBS. Cells were blocked with 5% normal rabbit serum (DAKO A/S Denmark) in PBS for 40 minutes in room temperature. Cells were then incubated with 1/300 monoclonal mouse- anti-CAR antibody (RmcB, from ATCC, US) in PBS at 4 °C overnight in a humidified chamber.

For immuno-fluorescence staining, cells were washed 3x5 min. in PBS, treated with rabbit-antimouse-immunoglobulins- FITC (DAKO A/S Denmark)) 1/30 in PBS for 30 minutes, washed again and mounted with Vectashield containing propidium iodide. Slides were viewed with an OlympusBX-51 fluorescence microscope.

Peroxidase-stained cells were washed 3x5 min in PBS and incubated with 1/300 secondary rabbit-antimouse IgG-biotin (DAKO A/S Denmark) for 30 min. Cells were again washed, incubated with 1/300 streptavidin-HRP (DAKO A/S Denmark) in PBS for 30 minutes. The peroxidase reaction was developed using DAB (1mg/ml) (DAKO A/S Denmark), 0.03% hydrogen peroxide in PBS for 3-5 min in room temperature. Cells were counterstained in hematoxylin, dehydrated in raising concentrations of ethanol and xylene and mounted.

2.7 Flow cytometry for AdGFP uptake after treatment with Trichostatin A

Cells were treated with 0.05-0.1µM Trichostatin A in DMEM including 10% FCS for 48 h. Cells were then infected with 10 particles per cell of Ad5GFP, in serum free media, for 2 h. After another incubation of 24 h in DMEM including 10% FCS, cells were harvested, transferred to 15 ml falcon-tubes and centrifuged at 1500 rpm for 5 min (ALC- PK130, T535). Supernatants were removed, the resulting pellets were resuspended in 1 ml PBS and transferred to eppendorf-tubes. Cells were again centrifuged at 1500 rpm (IEC Micromax-851, IEC) for 5 min and the supernatant removed. Pellets were suspended in 0.5 ml fixation buffer (IC-fixation buffer, eBiosciences) and kept at 4°C for 1 h. Samples were centrifuged as above, resuspended in PBS and samples run in the FACSCalibur cytometry machine. 25.000 cells per sample were counted. Controls included in this experiment where: untreated non-infected cells and cells infected with

AdGFP without previous treatment with Trichostatin A. A marker was set on the negative control-population to include1% of cells and this marker was applied to samples to determine the percentage of GFP- positive cells.

2.8 Measurement of the adenovirus replication after treatment with Trichostatin A, using the limiting dilution method

At day 0 cells were seeded in 60-mm culture dishes at a number of 10^6 cells per dish in DMEM 10 % FCS. After 24 hours media was removed and replaced with DMEM 10% FCS containing Trichostatin A in concentrations ranging between 0.1-0.05µM depending on cell line. As control, dishes were left untreated and assessed with light microscopy.

Cells were returned to the incubator and were after 48 hours infected with Ad5. Media was removed and replaced with 1ml serum free media per plate and cells were infected with a number of 100 viral particles per cell for 2 hours. Again media was removed and replaced with 4ml DMEM including 10 %FCS.

24 hours after viral infection the cells were harvested using a cell scraper (BD Labware, NJ, US) forcing the cells to detach from the dishes. The cells and media were transferred to falcon tubes and exposed to three subsequent freeze- thaw cycles (liquid nitrogen - 37° C water bath) to create a cell lysate with free virus.

HEK 293 cell- a cell line supporting viral replication- was then seeded into 96 well plates in 200 µl media at a number of 10^4 cells per well. The lysates obtained from the freeze-thaw cycles were diluted in serum free media to an initial concentration of 1/10 to 1/1000 depending on cell line. 22µl/well of the initial dilutions was applied all across the top row of the 96 well- plates of HEK 293 cells. After mixing contents in wells a tenfold dilution series was created on the plate, taking 22 µl from the first row- mixing with media in the second row etc. Row H was left uninfected. A control consisting of Ad5 only at a starting concentration of 10^7 particles per ml was included. Plates were returned to incubator and kept at 37° C 5 % CO_2. After seven days, the plates were visually examined for cytopathic effect, indicating productive virus infection, typical signs being cells rounding up or forming plaques. The number of wells in each row exhibiting cytopathic effect was counted and a $TCID_{50}$ value (value of 50% tissue culture infectious dose) was calculated using a Microsoft Excel template. Results are shown as pfu/ml (plaque forming units per ml) and pfu/cell and are an average of duplicate samples each assayed on duplicate plates of HEK 293 cells.

RESULTS

3.1 Screening of tumour cell lines for sensitivity to viral infection.

An initial screening was performed to evaluate sensitivity to wild- type Ad5 and the oncolytic adenoviruses dl922-947, dl 337 and dl 309 in the prostate cancer cell lines PC3, DU145, LNCaP 2001 and LNCaP 2003, the murine lung carcinoma cell line CMT 64 and the human lung carcinoma cell line H460. The replication incompetent mutant dl312 was included as a negative control. Cytopathic effect and cell death was determined with the MTS- assay as described in 2.3. A dose-response curve was generated for each virus in each cell line as shown in fig 1. The EC50 values corresponding to these graphs are shown in table 1. The relative order of potency of the different viral mutants varied in the different cell lines (Fig 2). All cell lines showed higher sensitivity to dl 337 and dl 309 as compared to Ad5. None of the cell lines infected with dl312 developed cytopathic effects even at the highest concentrations tested. The sensitivity towards dl 922-947 varied between cell lines, an increased sensitivity compared to Ad5 was seen only in LNCaP2003, while lower sensitivity was seen in H460, DU145, CMT64, LNCaP2001 and PC3. PC3 cells were relatively insensitive to infection with Ad5, whereas DU145 and H460 cell lines were highly susceptible to both Ad5 and different viral mutants (Table 2, fig. 3). All cell lines were used in future experiments, since they covered a range of sensitivities from insensitive (PC3) to highly susceptible (H460, DU145) with LNCap and CMT 64 cells displaying average sensitivities to adenovirus. H460 was included as a control, known from the literature to be susceptible to adenoviral cytopathic effect and support replication in vitro and CMT 64 as a murine control cell line, similar to H460 but with different time-pattern for viral replication. Ad5 was used as model virus in following studies since all cell lines were least sensitive to this virus compared to the viral mutants. Note that the dl 922-947 mutant used in this experiment was from a batch later determined to have lower potency than previously determined.

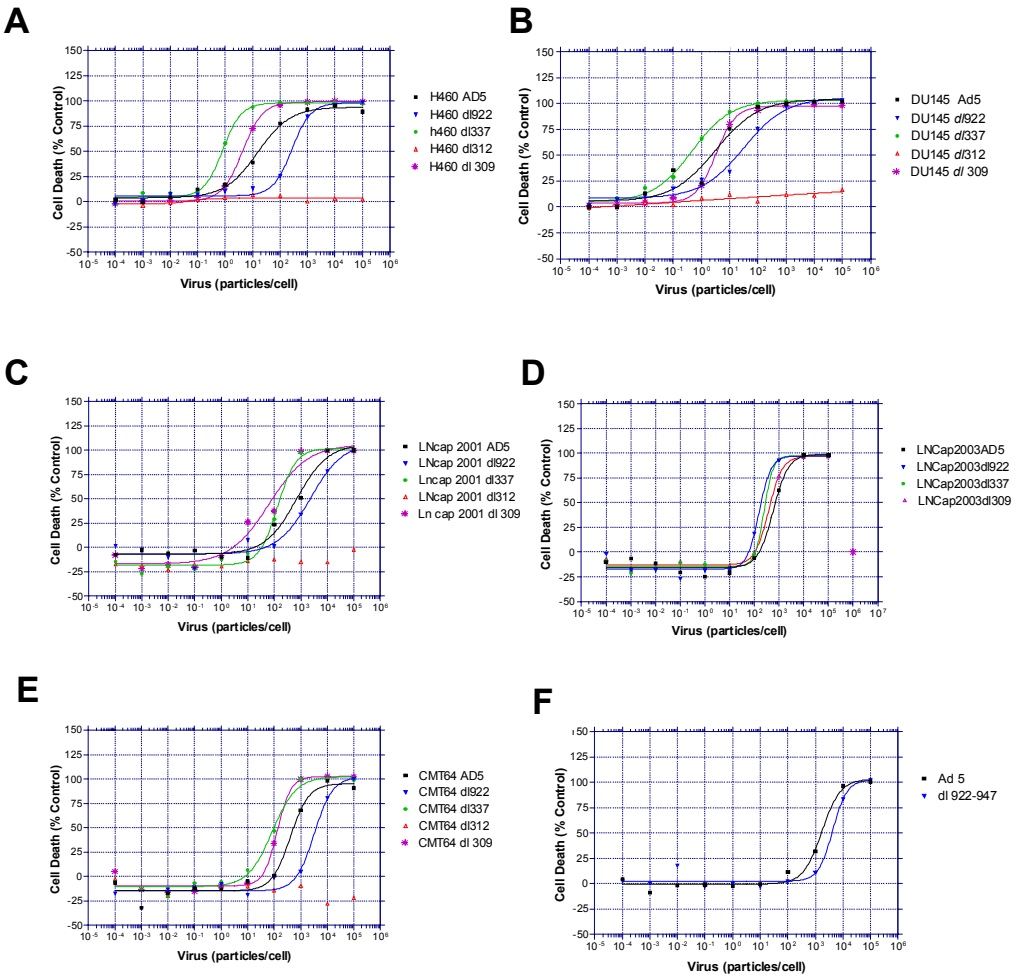

Figure 1. Dose response curves of Ad 5 and deletion mutants in the different cell lines (A) H460, (B) DU 145, (C)LNCap 2001,(D) LNCap 2003, (E) CMT 64, (F) PC3.

Each viral concentration in each cell line was assayed in triplicate and the development of cytopathic effect was assessed 6 days after infection using the MTS- assay.

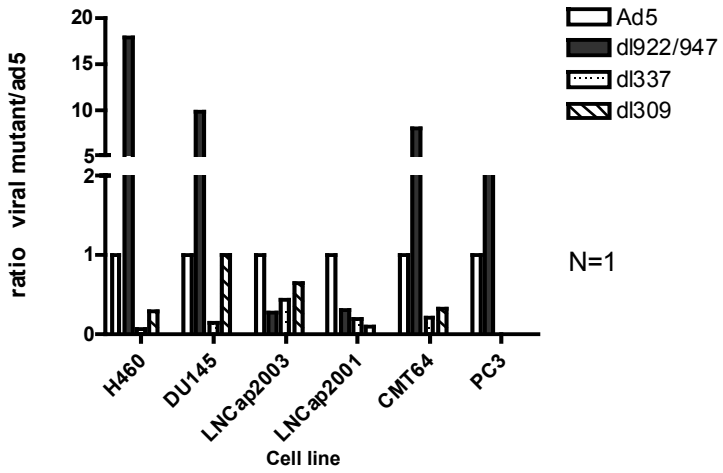

Figure 2. Relative sensitivity to different viral mutants as compared to Ad 5 wildtype.

EC50 values (ppc) for each viral mutant in each cell line was compared to the EC50 value of Ad5 in the cell line. Values >1 indicate a higher EC50 value for mutants than for Ad5 and hence a lower sensitivity to the viral mutant.

EC50 values,particles per cell

Cell line	Ad5	dl922/947	dl337	dl309	dl 312
H460	15	271	1	4	>100000
DU145	3	32	1	3	>100000
LNCap2003	582	156	249	379	>100000
LNCap2001	692	2306	133	63	>100000
CMT64	404	3202	84	131	>100000
PC3	1715	4243	-	-	-

N=1

Table 1. EC-50 values for different viral mutants.

The EC-50 value indicate the concentration of the different viruses required to kill 50% of cells.

3.2 Screening of cell lines with various compounds for cytotoxic effects

Trichostatin A, PD98059 and UO126 were screened for their induction of cytopathic effects in several concentrations between 0.05-50 μM in the MTS assay (data not shown). Concentrations of 1 μM or higher Trichostatin A caused some degree (5-40%) of cytopathic effect in all cell lines. LNCaP 2003 and CMT 64 also showed cytopathic effect at higher concentrations of U0126 (>1 μM) and PD 98059 (>1 μM) while other cell lines were sensitive at concentrations higher than 10 μM U0126 and 25 μM PD98059. Treatment with Trichostatin A at concentrations 0.1μM -1 μM affected the cell growth pattern as observed by light microscopy (Fig. 3). Cell lines usually growing in clusters (H460, CMT 64) changed to a more dispersed growth pattern when treated with 1.0 μM and 0.1μM Trichostatin A respectively. LNCap 2001 cells increased their cytoplasm/ nucleus ratio after treatment with Trichostatin A at 0.1 μM and higher. PC3 cells became more elongated after treatment with Trichostatin A at 1.0 μM and higher. Cells were not counted after treatment but a small fraction of the population appeared to detach and die at the concentrations used in fig. 3. In additional experiments using lower concentrations of each agent it was determined that the following concentrations of Trichostatin A did not affect cell growth pattern or did not appear to kill cells; H460 (0.1μM), PC3 (0.1μM), DU145 (0.1μM), LNCaP 2001(0.05μM), LNCaP 2003(0.05μM), CMT64 (0.05μM). Therefore, in following studies these dose levels were included for in depth evaluation of combination treatments with Ad5, CAR expression, viral uptake and replication studies.

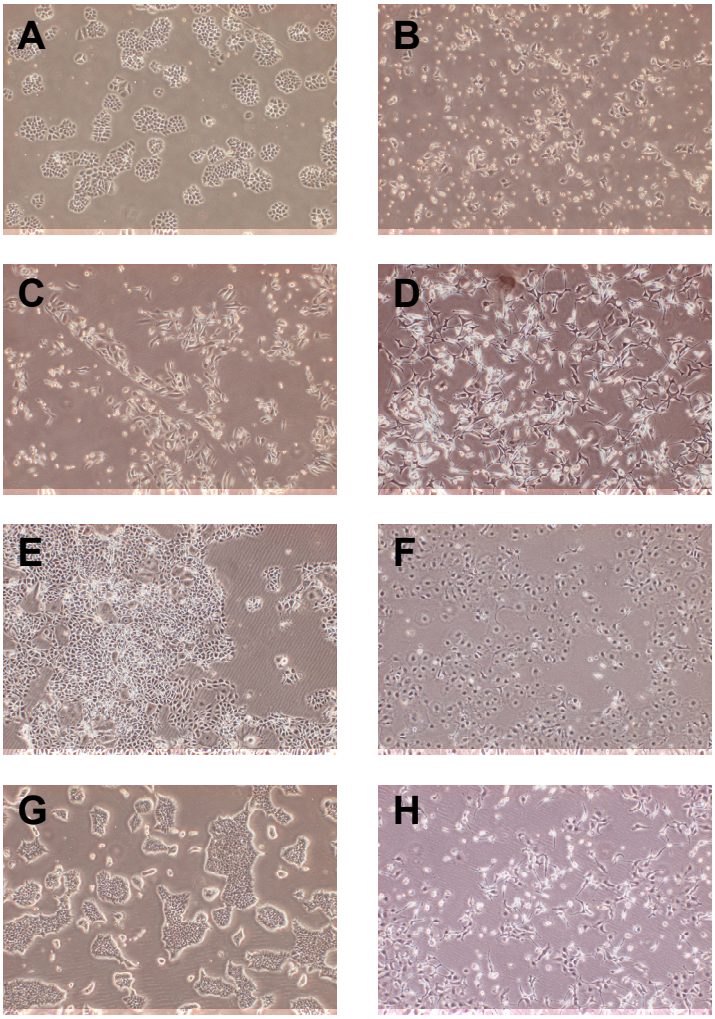

Figure 3. Changes in cell appearance and growth pattern seen 48 h after administration of Trichostatin A (TA) using light microscopy (40X)

(A)H460 control; (B) H460 1μM TA; (C)PC3 control; (D) PC3 1μM TA; (E)LNCap2001 control; (F) LNCap 2001 0.1μM TA; (G) CMT 64 control (H) CMT64 0.1μM TA.

3.3 Effects on sensitivity to adenoviral infection after treatment with Trichostatin A, PD98059 and U0126.

The cell lines H460, PC3, DU145, LNCaP 2003, LNCaP 2001 and CMT 64 were investigated for changes in sensitivity to infection with Ad 5 after treatment with the MEK- inhibitors PD98059, UO126 and the HDAC- inhibitor Trichostatin A, using the MTS assay. Full dose response curves to Ad5 for all cell lines with and without compounds are shown in fig 4 and 5. In this experiment, treatment with Trichostatin A enhanced the sensitivity to infection with Ad 5 in all cell lines except for PC3 cells (fig 6a, table 2). LNCaP 2003 cells were tenfold more sensitive to infection after treatment with 0.1 μM Trichostatin A, as was the murine control cell line CMT 64. Treatment with PD98059 and U0126 did not show as uniform result. PD98059 caused an increase in viral cell killing in cell lines H460, DU145, LNCaP 2001 but caused a decrease in cell lines PC3, LNCaP 2003, CMT64 (fig 6b, table 2). U0126 (fig 4c, table 2) showed the same pattern as described for PD 98059 above.

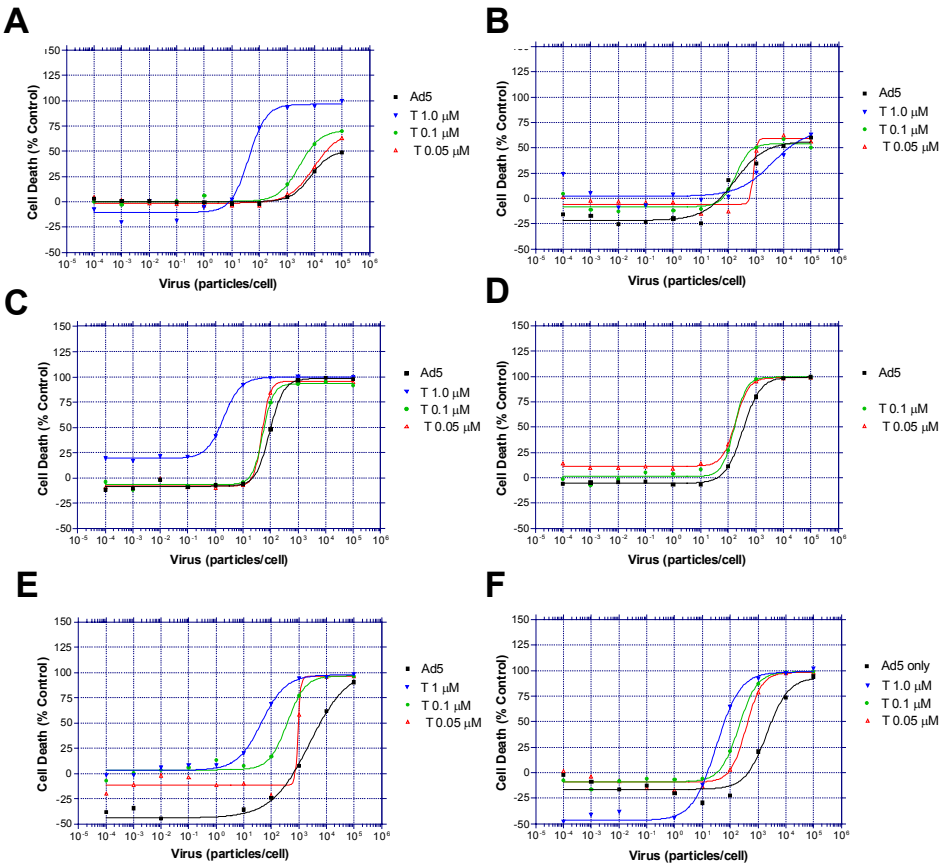

Figure. 4. Dose-response curves to Ad 5 after treatment with Trichostatin A (TA).

(A) H460, (B) PC3, (C)DU145, (D) LNCap 2001, (E) LnCap 2003, (F) CMT64. Cells were treated with TA for 48 h and were then infected with virus. Assays were read 5 days after infection. Each concentration of virus was assayed in triplicate with the MTS assay. The control for treatment with virus+ compound was compound alone, and for virus alone untreated cells.

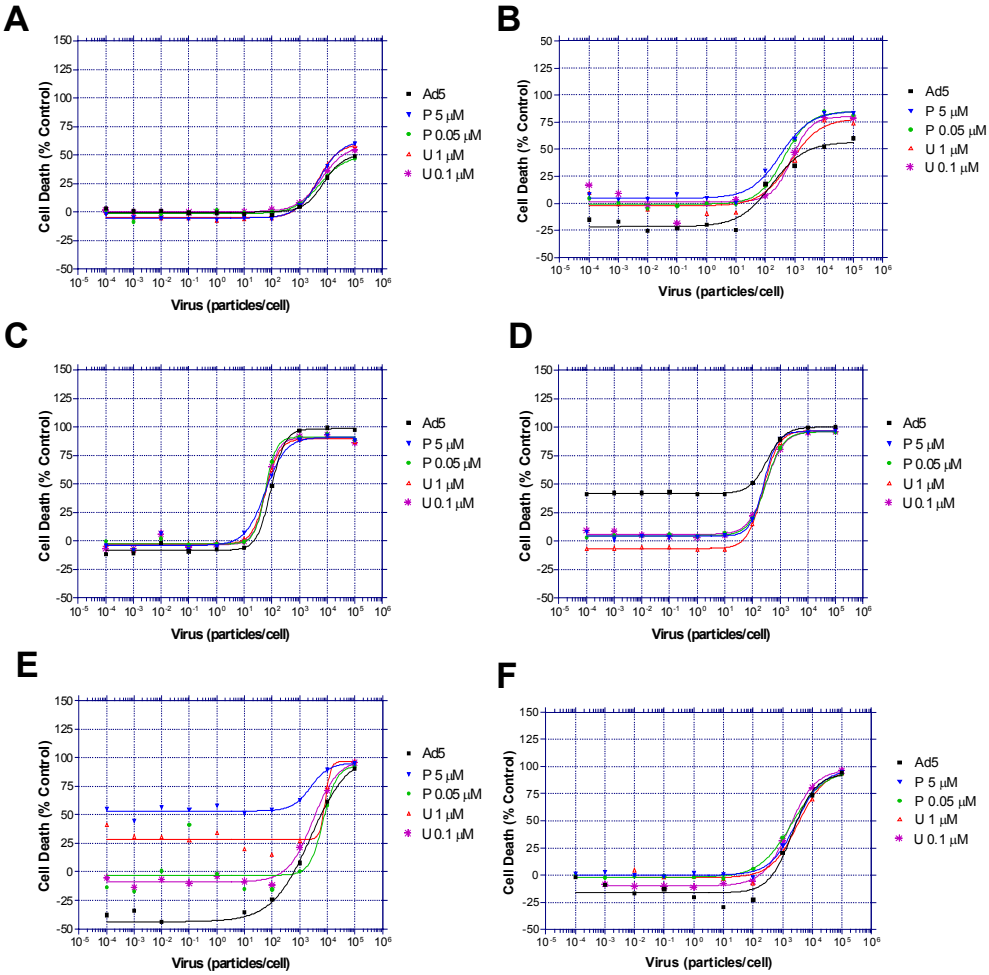

Figure. 5. Dose-response curves to Ad 5 after treatment with PD98059 and UO126.

(A) H460, (B) PC3, (C) DU145, (D) LNCap 2001, (E) LnCap 2003, (F) CMT64. Cells were treated with compound for 48 h and were then infected with virus. Assays were read 5 days after infection. Each concentration of virus was assayed in triplicate. The control for treatment with virus+ compound was compound alone, and for virus alone untreated cells.

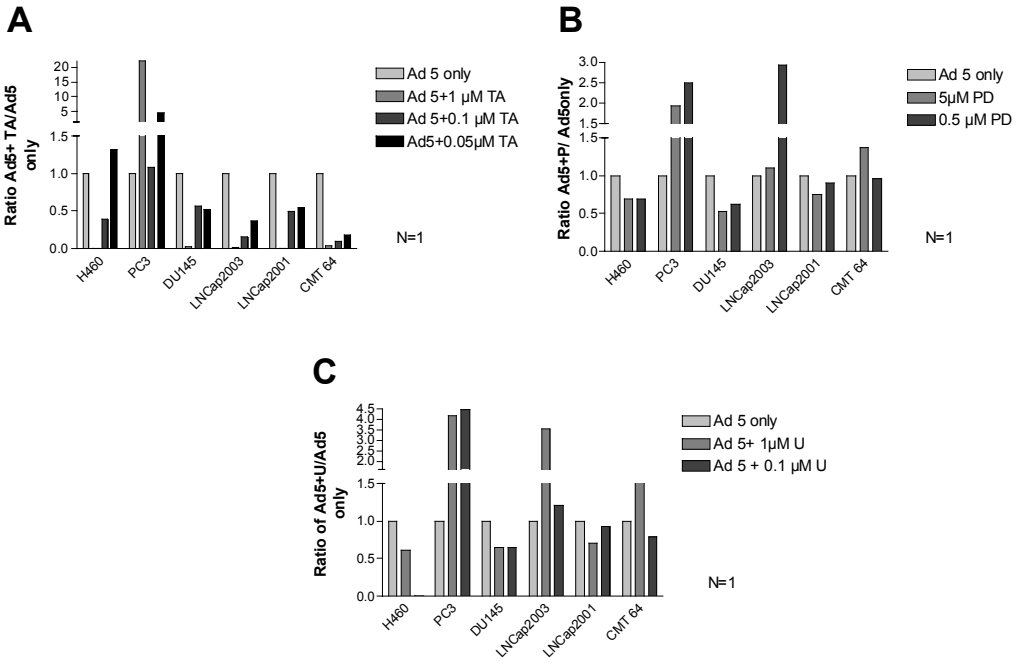

Figure 6.Treatment with compounds affect viral cell killing.

Relative sensitivity to Ad 5 after treatment with compounds (A) Trichostatin A, (B) PD98059, (C) U0126. Values in these graphs were derived from data shown in Fig. 5 and Table 2. EC 50-values for virus+compound was compared to EC 50 values for virus alone. Values <1 indicate a lower EC50 value for combination treatment than for virus alone and hence a higher sensitivity to the compound+virus combination.

Cell line	Ad 5 only	Ad 5+1 µM TA	Ad 5+0.1µM TA	Ad5+0.05µM TA	0.5 µM PD	Ad 5+1µM U	Ad 5+0.1µM U
H460	7245	41	2838	9625	5042	4458	
PC3	170	3830	184	757	423	707	772
DU145	93	2	53	49	58	61	60
LNCap2003	2463	44	381	889	7202	8686	2973
LNCap2001	339		167	184	308	239	313
CMT 64	2103	73	203	376	2020	3270	1668

Table 2. EC50 values (particles per cell) for Ad5 alone and in combination with drugs.

The EC-50 value indicate the concentration of Ad5 combined different compounds Trichostatin A, PD98059 and U0126 required to kill 50% of cells. Values in this table were derived from data shown in Fig. 5.

3.4 Effects on sensitivity to adenoviral infection after treatment with Trichostatin A, PD98059 and U0126 in normal prostate epithelial cells.

When treating normal prostate epithelial cells (PrEC) with the MEK- inhibitors PD98059, UO126 and the HDAC- inhibitor Trichostatin A, a different pattern was seen compared to the prostate cancer cell lines. Treatment with Trichostatin A caused a tenfold decrease in sensitivity to Ad5 in PrEC cells, U0126 a sixfold decrease and PD98059 a 34 % increase in sensitivity. On the other hand, treatment with Trichostatin A (1μM) caused approximately a tenfold increase in sensitivity to Ad 5 in all prostate cancer cell lines. A high degree of cell death was observed in normal prostate epithelial cells treated with Trichostatin A (40-60 %). PD98059 caused in the prostate cancer cell lines PC3 a 37% decrease, DU 145 a 39% decrease in sensitivity and in LNCaP 2003 no change. U0126 caused in the prostate cancer cell lines slight decreases in sensitivity to Ad5 (Fig. 7 and table 3)

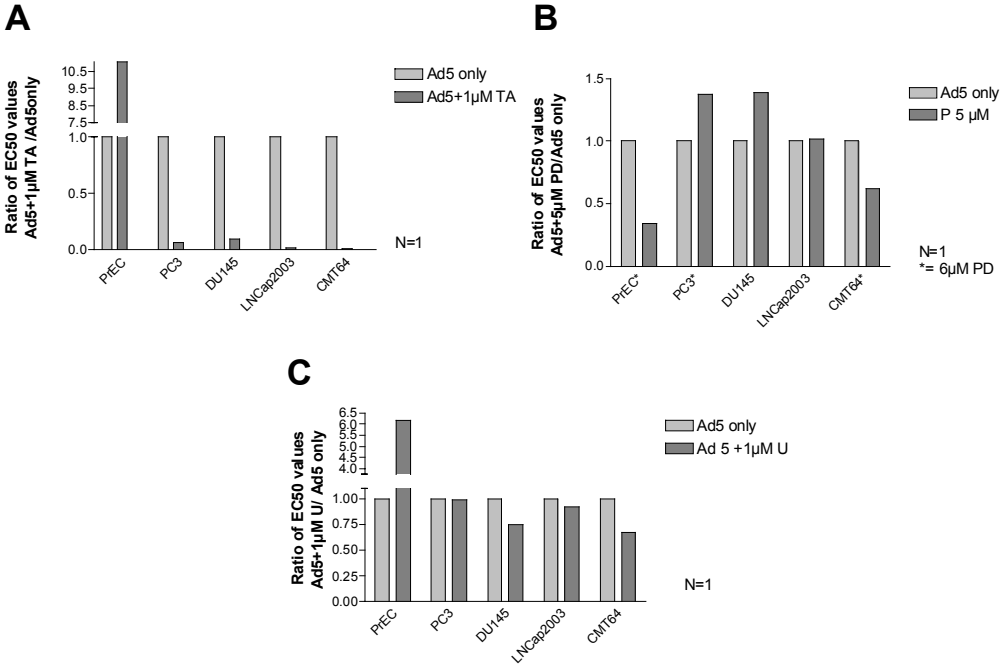

Figure 7. Comparison between normal prostate epithelial cells and prostate cancer cell lines.

Changes in relative sensitivity to Ad 5 after treatment with (A) 1µM Trichostatin A, (B) 5 µM PD98059 and (C) 1µM U0126. Cells were treated with compounds for 48 h and were then infected with virus. Assays were read 4 days after infection. Each concentration of virus was assayed in triplicate. The control for treatment with virus+ compound was compound alone, and for virus alone untreated cells. Data derived from table 3.

Cell line	Ad 5 only	Ad 5 +1µM TA	PD 5-6µ M	U 1µ M
PrEC*	1	8	2e-001	4
PC3*	3672	228	5055	3649
DU145	518	48	721	389
LNCap2003	2222	32	2507	2050
CMT64*	5078	47	3178	3415

Table 3. EC50 values (particles per cell) for Ad5 alone and in combination with drugs.

The EC-50 value indicate the concentration of Ad5 combined different compounds Trichostatin A, PD98059 and U0126 required to kill 50% of cells. *6 µM P

3.5 CAR expression in untreated cells

The cell lines H460, PC3, DU145, LNCap 2003, LNCap 2001 and CMT 64 were screened for expression of CAR using flow cytometry (FACS). H460 and DU145 showed high levels of CAR-positive cells- 65 and 72 % respectively, while PC3, LNCaP 2003 and CMT 64 had no detectable or low levels of CAR-expression. (Fig 8, Table 4).

This data was verified by immunohistochemistry using both peroxidase staining (fig 9) and fluorescent staining with FITC (fig 10) Results confirmed the flow cytometry data above with H460 and DU145 cells having CAR present at the cell surface at high levels (Fig 9 A,E) Faint membrane bound staining could also be observed in the LNCaP(2001)cells while no detectable CAR staining was present in other cell lines.

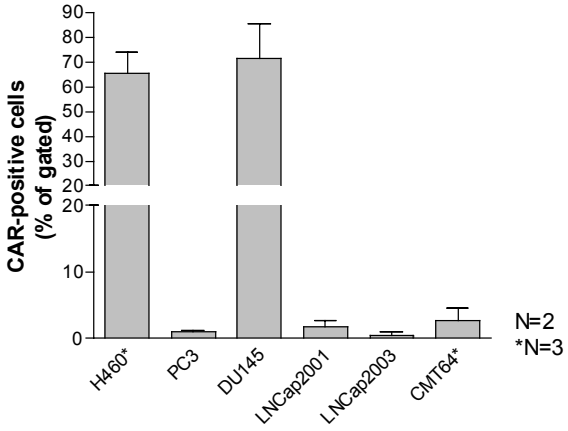

Figure. 8. Expression of CAR in the cell lines H460, PC3, DU145, LNCap(2001), LNCap (2003) and CMT 64 as measured with flow cytometry.

Graph shows the proportion of CAR positive cells in a population of 10000 live cell counts. The experiment was performed 2-3 times. Cells incubated with monoclonal mouse antiCAR antibody (RmcB, 1:500) and a secondary rabbit antimouse- FITC antibody as described in Material and Methods,2.5. A negative control with omitted primary antibody for each cell line was included.

Cell line	CAR +	SEM	
H460 *	65	8.7	
PC3	1	0.1	
DU145	72	13.7	
LNCap 2003	5e-001	0.4	
CMT 64 *	3	1.8	**N=2**

Table 4. Standard error of the mean of percentage of CAR-positive cells as detected by flow cytometry. *N=3.

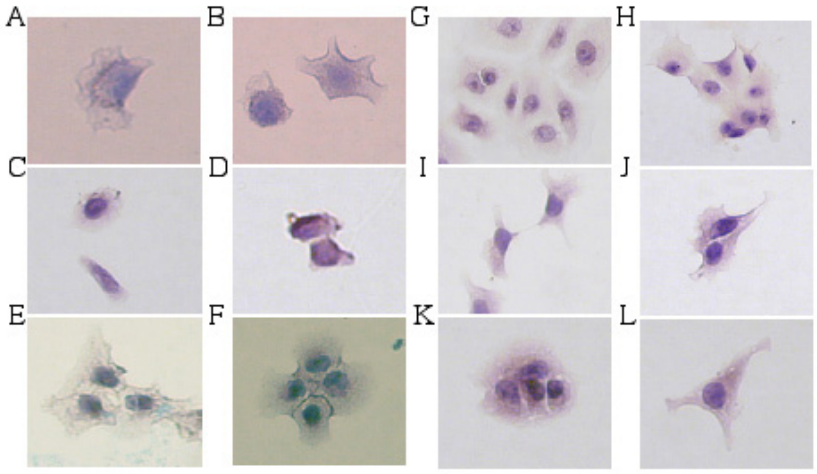

Figure 9. Cell surface expression of CAR as detected with immunocytochemistry.

(A) H460 control, (B) H460 CAR, (C) PC3 control, (D) PC3 CAR, (E) DU145 control, (F) DU145 CAR, (G) LNCap 2001 control, (H) LNCap 2001 CAR, (I) LNCap 2003 control, (J) LNCap 2003 CAR, (K) CMT 64 control, (L) CMT 64 CAR. Primary antibody RmcB (monclonal mouse antiCAR antibody) used at a dilution of 1:300 and detected with peroxidase staining as described in Material and Methods 2.6. Images viewed at 200x magnifiction, light microscopy.

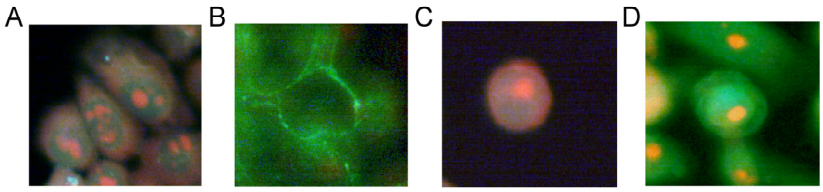

Figure 10. Expression of CAR as detected with immunocytochemistry.

(A) H460 control, (B) H460 CAR, (C) PC3 control, (D) PC3 CAR. Cells stained with primary antibody RmcB (monclonal mouse antiCAR antibody) used at a dilution of 1:300. Secondary antibody conjugated with FITC, cells counterstained with PI. Images viewed at 200x normal magnification, fluorescent microscopy.

3.6 Changes in CAR expression after treatment with Trichostatin A

To establish whether treatment with Trichostatin A could enhance the levels of CAR-expressing cells in different cancer cell lines, cells were exposed to Trichostatin A 48h before harvest and flow cytometric analysis was performed as described in section 3.5. Negative controls for both treated and untreated cells were included. A comparison was made between the proportion of CAR-positive cells in treated and untreated samples. The greatest increase in the number of CAR-positive cells was seen in the H460 (77%) and PC3 (8-fold) cell lines. However, DU145 (8%)and CMT64 (2-fold) cell lines showed only a slight increase and Trichostatin A- treatment did not increased the numbers of CAR-positive cells in LNCaP 2003-cells.

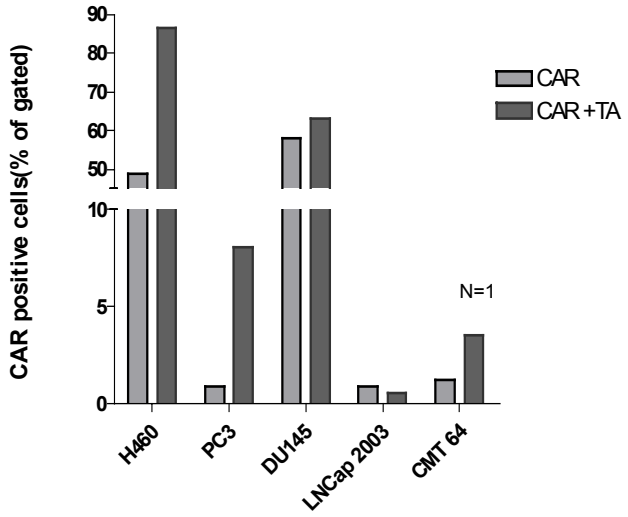

Figure. 11. Increase in the percentage of CAR- positive cells after treatment with Trichostatin A.

Cell lines H460, DU145 and PC3 were treated with 0.1 µM TA, LNCap2003 and CMT 64 0.05 µM TA 48h before harvest and flow cytometric analysis as described in Material and Methods 2.7.

Cell line	CAR	CAR +TA
H460	48.9	86.5
PC3	0.9	8.0
DU145	57.9	63.0
LNCap 2003	0.9	0.6
CMT 64	1.2	3.5

N=1

Table 5. Percentage of CAR- positive cells before and after treatment with Trichostatin A.

Data derived from Figure 11.

3.7 Changes in viral uptake after treatment with Trichostatin A

In order to determine whether treatment with Trichostatin A could increase the uptake of virus, cells were exposed to Trichostatin A 48h before infection with 10 particles per cell of AdGFP. Cells were harvested 24 h after viral infection and flow cytometry was performed. Non-treated, non- infected cells were included as controls. The proportion of AdGFP-positive cells in treated samples were compared to the proportion of AdGFP positive cells in untreated but infected samples.

3.5 The uptake of AdGFP in untreated cells was generally low, but showed an increase in all cell lines after treatment with Trichostatin A (Fig. 10). H460 cells showed the greatest increase in viral uptake after TA- treatment (>10 fold), followed by LNCaP 2003(6-fold), PC3 (3-fold) and CMT 64 (3fold) cells. DU145 showed the smallest increase after treatment with Trichostatin A (2-fold).

In conclusion, the increases in CAR-expression seen after treatment with Trichostatin A (Fig. 11-Table 5) correlates with enhanced viral uptake in all cell lines tested a part from LNCaP 2003 cells, where no increase in CAR-expression could be detected, but a 6-fold increase in adenoviral uptake could be seen. H460 cells showed a 77% increase in CAR-expressing cells and a >10fold increase in viral uptake. A 8-fold increase of CAR-expressing cells in PC3 and a 2-fold increase in CAR-expressing cells in CMT 64 corresponded to a 3-fold increase in viral uptake in both cell lines. DU145 cells showed only a modest increase in CAR-expression (8%) and a 2-fold increase in viral uptake.

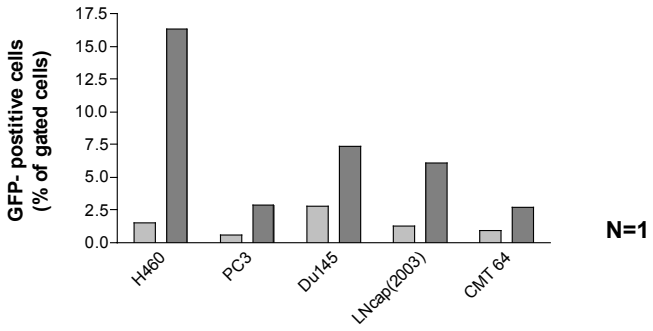

Figure. 12. Changes in viral uptake after treatment with Trichostatin A.

Graph shows the proportion of GFP- positive cells in populations infected with AdGFP with and without treatment with TA. Cell lines H460, DU145 and PC3 were treated with 0.1 µM TA, LNCap2003 and CMT 64 with 0.05µM TA 48 h before infection with AdGFP (10ppc) and were harvested and analyzed with flow cytometry. 25000 cells were counted per sample.

Cell line	AdGFP	AdGFP-TA
H460	2	16
PC3	1	3
Du145	3	7
LNcap(2003)	1	6
CMT 64	1	3

N=1

Table 6. Percentage of Ad-GFP positive cells after treatment with Trichostatin A. Cell lines H460, DU145 and PC3 were treated with 0.1 µM TA, LNCap2003 and CMT 64 with 0.05µM TA. Data derived from Fig 12.

3.8 Changes in adenoviral replication after treatment with Trichostatin A

Changes in adenoviral replication after treatment with Trichostatin A were determined with the $TCID_{50}$-limiting dilution method as described in 2.8. Treatment with Trichostatin enhanced the viral replication slightly in the PC3 and CMT 64 cell lines (Fig 13). No difference was seen in LNCap 2003-cells between treated and untreated samples. In the H460 cells a decrease in the amount of viral particles produced was seen after treatment with Trichostatin A. However, none of these changes were statistically significant, H460 p=0.27, PC3 p=0.22, LNCaP 2003 p=0.68 and CMT64 p=0.07. The DU145 cells were also included in the study, but viral replication (pfu/ cell) could not be determined; an additional assay would have to be performed at a lower starting dilution of these samples.

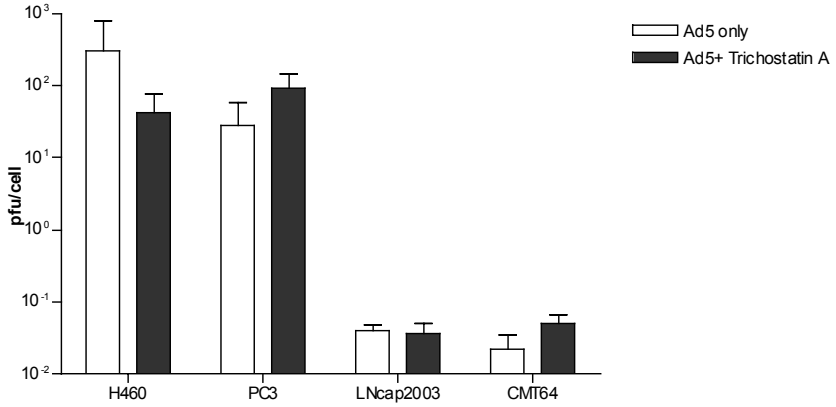

Figure. 13. Replication of Ad 5 alone and in combination with Trichostatin A.

Graphs show particle forming units/cell produced by the respective cell line 24 h after infection with 100 ppc Ad5. Cell lines H460, DU145 and PC3 were treated with 0.1 µM TA, LNCap2003 and CMT 64 with 0.05µM TA 48 h before infection with Ad5. Data is from 2 experiments assayed in duplicate samples by TCID50- limiting dilution method as described in Material and Methods 2.8.

DISCUSSION

4.1 Differences in sensitivity to viral infection in tumour cell lines

When screening cell lines for sensitivity to Ad5 and different replication specific oncolytic viruses, *dl*922-947 deletion mutants required high doses of virus to be able to infect the different cell lines. This result was later found to be caused by using a batch of *dl*922-947 with reduced activity. Similar studies performed by other members in the lab with other batches of virus and reported in the literature (Heise et al, 2000) have clearly demonstrated that a majority of cancer cell lines are more sensitive to the *dl*922 mutants than Ad5 wildtype virus. The experiment was performed only once, with each viral concentration assayed in triplicate and hence need to be repeated and verified. Several different cell lines were included in order to find both cell lines that were sensitive to virus and others that were not. These differences in sensitivity could be because of an increased viral uptake or an increase in replication. The mechanisms that were principally responsible for these differences between cell lines were determined in later experiments. All cell lines, when infected with Ad5, had a relatively low sensitivity to virus compared to most viral mutants. This makes Ad5 a suitable model for attempts to enhance viral infectivity in these cell lines.

4.2 Evaluation of cytotoxic effects of Trichostatin A

When evaluating the different degrees of cell killing after treatment with different concentrations of the MEK and HDAC inhibitors, treatment with low concentrations of Trichostatin A revealed changes in cell growth pattern and morphology. This observation corresponds with the fact that HDAC inhibitors are being evaluated as antitumoral agents and have been shown to cause tumour cells to differentiate; arrest in G_1 or G_2 or even induce apoptosis (Rosato et al). These effects seem to depend on what cell line is treated and at what concentration of Trichostatin A. In future studies it would be interesting to include more concentrations of Trichostatin A when evaluating CAR-surface expression, since the effects of compounds might be dependent on dose.

Trichostatin A have been well tolerated in in vivo studies and caused differentiation of advanced tumours, indicating that use of this compound is clinically applicable (Vigushin et al, 2001). Since HDAC inhibitors affect transcription of various cell cycle control proteins (Rosato et al, 2003), it would be interesting to know whether the cell lines treated with Trichostatin A were all arrested in a certain phase of the cell cycle, since this might affect adenoviral replication. In addition, if differentiation is induced by Trichostatin A treatment in the cell lines, this could be detected using Western blot analysis for differentiation marker proteins. The activity of caspase 3 could be detected by immunohistochemistry in order to determine if Trichostatin A induces apoptosis in these cell lines.

4.3 Trichostatin A increases the sensitivity to virus

The effects of treatment with Trichostatin A, PD98059 and U0126 on adenoviral cell killing were investigated. Treatment with Trichostatin A, even at low concentrations, showed an increase in viral cell killing in all cell lines a part from PC3. These cells did not show an increased sensitivity to virus after treatment with Trichostatin A in this experiment. These results need to be repeated and verified. Trichostatin A was the compound causing the strongest and most uniform change in sensitivity to viral infection in all cell lines. A synergy study should be performed to determine whether synergy exists between Trichostatin A and Ad5. An experiment should be performed investigating if treatment with Trichostatin A after viral infection might have an effect on sensitivity to virus, thus implying also other mechanisms than enhanced viral uptake behind the increases in sensitivity. Different replication selective oncolytic viruses should also be evaluated. Particularly interesting would be to see if treatment with Trichostatin A could enhance the effects of the prostate specific oncolytic viruses CN706, CV764 and CV787 in prostate cancer cell lines.

It would be ideal if treatment with a compound could protect normal cells from viral infection and enhance the effects of virus in tumour cells. Therefore, the effects of viral sensitivity after treatment with the MEK- inhibitors PD98059, U0126 and the HDAC- inhibitor Trichostatin A, were compared between primary prostate epithelial cells (PrECs) and prostate cancer cell lines. This experiment was performed before an extensive cytotoxicity screening and needs to be repeated at a lower dose, since Trichostatin A at the concentration used (1 μM) killed a large proportion of PREC control cells (40-60%). Data was still shown since the experiment compared cells treated with compound + virus to cells treated with compound alone Even if normal cells should show to be more sensitive to treatment with Trichostatin A than tumour cells, localised treatment with Trichostatin A could create a barrier of normal cells dying before virus could replicate and spread further in the tissues.

4.4 The degree of CAR-expression is related to the sensitivity to virus infection.

The expression of CAR in untreated cells as measured by flow cytometry varied between the cell-lines investigated in this project. The human prostate cancer cell line DU145 and the human lung carcinoma cell line H460 showed high proportions of CAR positive cells (65% and 72% percent, respectively). The data for DU145 is consistent with previously published data (Okegawa et al, 2000), where DU145 cells have shown high proportions of CAR-positive cells in flow cytometry (82%). On the other hand, LNCap 2001, LNCap 2003 and PC3 cell lines showed in this study very low proportions of CAR-positive cells, compared to data from the same author (Okegawa et al, 2000), where LNCap-cells showed 63% and PC3- cells 35% CAR-positive cells. CAR-expression might vary because of the time after seeding and the degree of confluency of cells at harvest before analysis. Also receptor internalisation caused by the staining process might affect the proportion of CAR- positive cells. Two versions of LNCaP cells were assayed but no clear differences in the proportion of CAR-positive cells could be seen. Results from immunocytochemistry experiments confirmed flow cytrometry

data, with H460 and DU145 cell lines clearly staining positive for CAR on the cell surface.

The relative sensitivity to Ad5- induced cytopathic effect in the different cell lines corresponded well with the degree of CAR- expression in most cell lines. Particularly DU145 cells showed in all experiments high sensitivity to Ad5 combined with high CAR-expression. Divergences in these patterns, as seen with H460 and PC3 cells in some assays, could be because of the time elapsed from viral infection to reading of assay and the different time-patterns of replication in different cell lines.

4.5 Treatment with Trichostatin A causes an increase in both CAR-expression and viral uptake.

An increase in the proportion of CAR-expressing cells could be seen in all cell lines a part from LNCaP2003 after treatment with Trichostatin A. Treatment with Trichostatin A enhanced viral uptake in all cell lines investigated. When performing assays determining viral uptake, a low proportion of AdGFP infected cells was seen overall. This was probably due to the early harvest of cells (24 h after viral infection) and the fact that the cell number was not determined on the day of infection, resulting in a low infection dose. Still, it is promising that even at this low grade infection, differences in infectivity could be seen between treated and non-treated cells.

Both the CAR expression experiment and the viral uptake experiment need to be repeated and verified, but the overall impression is that treatment with Trichostatin A causes an increase in CAR-expression correlated with an increase in viral uptake in prostate cancer cell lines. This principle was proven recently in bladder cancer cell lines (Sachs et al, 2004) The HDAC- inhibitor FR901228 has been shown to increase both expression of αv- integrins and CAR (Kitazono et al 2001). In future studies of Trichostatin A, it could be interesting to investigate whether integrin-expression also is enhanced, since this might provide an explanation as to why cell lines not showing a clear increase in CAR- expression (LNCaP, 2003, DU145) still shows an increase in viral uptake and sensitivity to virus after treatment with Trichostatin A. An assay ruling out other mechanisms than CAR-expression could be performed, letting virus compete with soluble viral fibre.

When investigating the effects of Trichostatin A- treatment on viral replication, no significant changes were seen, suggesting that the changes in CAR- expression and viral uptake are the principal mechanisms behind increased sensitivity to viral infection. The replication study should be repeated and verified and additional time points for harvest of virus should be included.

CONCLUSION

The use of oncolytic adenoviruses as single agent therapy for cancer has so far not proven efficacious in the clinic. Some anticancer agents have shown to increase the expression of CAR. In this study, the HDAC-inhibitor Trichostatin A has been shown to enhance the expression of CAR in H460, PC3, DU145 and CMT64 cell lines. This is consistent with observations of ovarian (Hemmiki et al, 2003) and bladder (Sachs et al, 2004) cancer cell lines. This increase in receptor expression was paralleled by enhancement of viral uptake and cytopathic effect in prostate cancer cells. Trichostatin A has potential as a novel combination treatment in combination with oncolytic adenoviruses but needs thorough pre clinical evaluation both in vitro and in vivo animal studies.

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